Safety, Compositional, and Nutritional Aspects of Bollgard II Cotton Event 15985

Conclusion Based on Studies and Information Evaluated According to FDA's Policy on Foods from New Plant Varieties

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Submitted by

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Abbreviations Used in this Summary of the Safety, Compositional and Nutritional Aspects of Bollgard II Cotton Event 15985

AA	Amino acids
APHIS	Animal and Plant Health Inspection Service
B.t. or *B.t.k.*	*Bacillus thuringiensis* organism
Bt	Protein derived from *Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
CFR	Code of Federal Regulations
CFSAN	Center for Food Safety and Nutrition
CFU	Colony-forming units
CPFA	Cyclopropenoid fatty acids; antinutrients in cotton
cry1Ac	Gene in Bollgard® Cotton encoding the Cry1Ac insecticidal protein
Cry1Ac	Insecticidal protein produced in Bollgard® cotton
Cry2Aa	Insecticidal protein produced by *Bacillus thuringiensis*
cry2Ab	Gene in Bollgard II Cotton encoding the Cry2Ab insecticidal protein
Cry2Ab, Cry2Ab2, IPP2	Insecticidal protein produced in Bollgard II cotton Event 15985
CTP	Chloroplast transit peptide
CVM	Center for Veterinary Medicine
DW	Dry weight
DP50B	Delta and PineLand Company cotton variety of Bollgard® cotton
E. coli	*Escherichia coli*
EG7699	Strain of *B.t.* altered to produce the Cry2Ab protein
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EPA	United States Environmental Protection Agency
FDA	United States Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FR	United States Federal Register
fwt	Fresh weight
GUS	β-glucuronidase protein
HPLC	High Performance Liquid Chromatography
IgEs	Immunoglobin subclass Epsilon (E)
*Kpn*I	Restriction endonuclease that cuts DNA at specific locations
LOD	Limit of detection
kDa	Kilodaltons
NCPA	National Cottonseed Products Association
NOEL	No Observed Effect Level
NOS 3'	Nopaline synthase 3' polyadenylation sequence

nptII or *kan*	Gene encoding for the enzyme neomycin phosphotransferase type II
NPTII or Kan	Neomycin phosphotransferase IIprotein
OECD	Organization for Economic and Co-operation and Development
PCR	Polymerase chain reaction
P-e35S or e-35S	Cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
PIR	Protein Information Resource Database
PV-GHBK11	Plasmid vector
PV-GHBK11L	Linear fragment of the plasmid vector used in transformation of Bollgarad II cotton
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOP	Standard operating procedure
uidA	Gene encoding the GUS protein
USDA	United States Department of Agriculture
WHO	World Health Organization

Note: Standard abbreviations, e.g., units of measure, are according to the format described in "Instructions to Authors" in the Journal of Biological Chemistry.

INFORMATION TO SUPPORT THE HUMAN FOOD AND ANIMAL FEED SAFETY OF BOLLGARD II COTTON EVENT 15985

I. Introduction

Bollgard® cotton, developed by Monsanto Company, has been adopted broadly by growers since its introduction in 1996 as it provides effective protection from the feeding of lepidopteran insect pests such as tobacco budworm, pink bollworm and cotton bollworm. Growers typically apply significantly less insecticide to control these pests, realize higher yields and achieve greater profitability using these improved Bollgard varieties, as compared to conventional insecticide products (Fernandez-Cornejo and McBride, 2000).

Monsanto Company has now developed a new genetically modified cotton event, Bollgard II, using particle acceleration plant transformation procedures to insert the *cry2Ab* insect control gene and the *uidA* scorable marker gene into the Bollgard cotton genome. This new event provides effective control of insect pests such as the cotton bollworm, tobacco budworm and pink bollworm, as well as armyworm.

A. Subject of the Request

Monsanto Company is filing this summary of the safety and nutritional assessment of this new insect-protected cotton event with the Food and Drug Administration (FDA) based on scientific data and information evaluated according to FDA's Policy on Foods from New Plant Varieties (FDA, 1992), as well guidance provided in the "Guidance on Consultation Procedures - Foods Derived from New Plant Varieties", October 1997. Data is presented on the safe history of use of the crop, the source of donor genes, the molecular characterization of the modified plant, the stability of the insertion of genetic elements, characterization of proteins produced in the modified plant and their levels, the composition of the modified cotton compared to conventional cotton and toxicology, digestibility and safety of the proteins produced.

These data establish that Bollgard II cotton event 15985 and all progeny derived from it by traditional plant breeding is as safe as other conventional cotton varieties.

B. Application of FDA Food Policy

The Food and Drug Administration has authority under the Federal Food, Drug, and Cosmetic Act (FFDCA) to ensure the safety and wholesomeness of most foods, including foods developed through modern biotechnology. FDA regulates foods and food ingredients developed using genetic engineering by the same provisions and regulations under the FFDCA that it regulates other food products. FDA's authority to ensure the safety of foods under the FFDCA is derived from Section 402(a)(1), the food adulteration provision and Section 409, the food additive provision.

In its May 29, 1992 statement of policy concerning "Foods Derived from New Plant Varieties," ("Food Policy" or the "Policy"), the Food and Drug Administration provided

guidance for determining whether a new plant variety developed with the aid of new genetic techniques is as safe and nutritious as its parental variety (FDA, 1992). The policy focuses on issues related to changes in food crops, whether intended or unexpected. The Policy is based on decision trees that are designed to establish whether the new plant variety is materially different in composition, safety or any relevant parameter from its parental variety. In the policy, the agency noted that consultations on new plant varieties are appropriate forums for developers of the products and the agency to discuss scientific and regulatory issues prior to market entry. The agency encouraged developers to consult early in the development phase of the product.

Monsanto Company initiated its consultation with the FDA by meeting with members of the Center for Food Safety and Applied Nutrition (CFSAN) and the Center for Veterinary Medicine (CVM) in March, 1995 to define and discuss the scientific data and information necessary to support the safety and nutritional assessment of insect-protected cotton varieties expressing a *Bacillus thuringiensis* protein. Since 1995, Monsanto has completed the consultation for three cotton events containing the Cry1Ac protein. Bollgard cotton event 531 has been commercially produced in the United States since 1996. In addition, from 1994 to 1999, Monsanto has completed the consultation process on a number of other insect-protected crops, including corn, potato, and tomato that express similar Bt proteins.

Monsanto has followed the guidance in the 1992 policy to assess whether cotton event 15985 is materially different from cotton currently being marketed in the United States.

C. The Safety and Nutritional Assessment of Bollgard II Cotton Event 15985

The dietary safety of Bollgard II cotton event 15985 is based on (1) extensive animal toxicity testing of Cry proteins; (2) a history of safety of Cry2A and GUS and; (3) results of *in vivo* and *in vitro* safety studies conducted with the proteins. These data, together with compositional data, support the conclusion that cotton event 15985 is as safe and nutritious as conventional cotton currently being marketed.

The concepts and approaches Monsanto has employed are derived from, and consistent with, the principles outlined by independent international bodies such as the Organization for Economic Co-operation and Development (OECD), the United Nations World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) (OECD, 1992; FAO, 1995; WHO 1996) and consistent with the guidance presented in the flow charts found in the FDA Food Policy (FDA, 1992). For each question, we have developed answers based on extensive studies or analyses. Our data and findings in every case have led us to the conclusion of "no concern", as described in the relevant sections of the following summary. Following the Agency's Food Policy, these data have provided us with a basis for concluding that Bollgard II cotton event 15985 is as safe and nutritious as the cotton varieties grown commercially today.

D. Coordination with Other U.S. Federal Agencies

Before commercializing Bollgard II cotton event 15985, Monsanto has taken or will take the following actions in the United States:

1. Bollgard II cotton event 15985 is within the scope of the FDA policy statement concerning regulation of products derived from new plant varieties, including genetically engineered varieties, published in the Federal Register on May 29, 1992. As a result of consultations on insect-protected cotton with the FDA since March, 1995, Monsanto is providing this summary of the food and feed safety and nutritional assessment of Bollgard II cotton event 15985 to the Agency prior to commercial distribution.

2. Under regulations administered by the Animal and Plant Health Inspection Service (APHIS) of USDA (7 CFR 340), Bollgard II cotton event 15985 is currently considered a "regulated article." Monsanto will request a determination of nonregulated status for this cotton event and all progenies derived from crosses between this line and other cotton lines.

3. Substances that are pesticides as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. section 136(u)) are subject to EPA's regulatory authority. A request for registration of Cry2Ab as a plant-incorporated protectant was submitted to EPA in April, 2000. Pursuant to section 408(d) of the Federal Food Drug and Cosmetic Act (FFDCA), requests for exemptions from the requirement of tolerances for Cry2Ab and GUS protein were submitted to EPA in the fall of 1999.

II. Rationale for the Development of Bollgard II Cotton

A. Rationale for the Development of Insect-Protected Cotton

Cotton is the leading plant fiber crop produced in the world and the most important in the United States. Cotton production in the United States is located primarily in the tier of 15 southern states stretching from California to North Carolina, with approximately 13 million acres grown annually. Lepidopteran insects are the main insect pest problem in cotton. During the growing season other insects (*e.g.*, cotton boll weevil, lygus bugs, fleahoppers, spider mites, thrips, and aphids) are also present. The primary lepidopteran pests infesting cotton are cotton bollworm, tobacco budworm and pink bollworm. These insect pests infest approximately 80% of the planted acres with approximately $180M spent annually for chemical control (Luttrell *et al.*, 1993).

Bollgard cotton, introduced commercially in the United States in 1996, produces the Cry1Ac protein and has been adopted broadly by growers because it provides effective protection from the feeding of lepidopteran insect pests, such as tobacco budworms, pink bollworms and cotton bollworms. Growers typically apply significantly less insecticide to control these pests, realize higher yields and achieve greater profitability using these Bollgard cotton varieties, as compared to conventional products. Gianessi and Carpenter

(1999) estimated that the planting of Bollgard cotton varieties reduced insecticide applications by two million pounds in 1998 alone, compared to 1995, which was the year prior to the introduction of Bollgard. For these and other reasons, more than 3.9 million acres of Bollgard varieties were planted in 1999, or more than 31% of the total cotton acreage (USDA, 1999).

Bollgard II cotton provides increased control of the major insect pests of cotton, such as the tobacco budworm, pink bollworm, and cotton bollworm, as well as armyworm. Combining the Cry2Ab protein with the Cry1Ac protein already in the marketplace, or using the Cry2Ab protein as a stand alone product, will provide an additional tool to delay the development of lepidopteran resistance to Bt protein in cotton, as Cry2 is a different Bt protein class. Bollgard II cotton, in combination with a refuge and the other components of Monsanto's resistance management plan, represents a substantial program to significantly delay the development of insect resistance to cotton containing the Cry1Ac protein.

B. Benefits of Insect-Protected Cotton

The primary benefits of Bollgard cotton, supported by data in the current literature, are reduced insecticide use, improved control of target pests, improved yield, reduced production costs, improved profitability, reduced farming risk and improved opportunity to grow cotton where pest pressure is severe, resulting in improved economics for cotton growers (Edge *et al.*, 2000). There also are a number of secondary benefits associated with the reduction in insecticide use, which include improved beneficial insect and wildlife populations, reduced runoff of insecticides, reduced air pollution, and improved safety for farm workers and neighbors.

III. Description of the Method of Transformation and the Molecular Biology of the Plant

Bollgard II cotton event 15985 was generated using the particle acceleration transformation system. The plasmid vector, PV-GHBK11 (Figure 1), contains two adjacent plant gene expression cassettes: the gene of interest, *cry2Ab,* and the scorable marker gene *uidA,* which encodes for the GUS protein. The vector inserted into the cotton genome was a linearized fragment of the plasmid, designated PV-GHBK11L.

A. The Vector PV-GHBK11L

The plasmid vector, PV-GHBK11, is an 8.7Kb high copy number pUC based plasmid. It contains well-characterized DNA elements for selection and replication of the plasmid in bacteria. The host for DNA cloning and vector construction was *E. coli* XL1Blue, a derivative of the common laboratory *E. coli* K-12 strain. The genetic elements in PV-GHBK11 are listed in Table 1; sizes listed here include non-functional DNA needed for the cloning. The ori-pUC is from the plasmid pUC19 (Vieira and Messing, 1987) and it provides the origin for replication and maintenance in *E. coli.* The *npt*II gene is for selection on kanamycin of bacteria containing the plasmid.

The chimeric gene cassette that produces the Cry2Ab protein consists of the enhanced 35S promoter (Odell *et al.*, 1985), the fully synthetic *cry2Ab* coding sequence, and the 3' nontranslated region of the nopaline synthase gene from *Agrobacterium tumifaciens* which provides the signal for mRNA polyadenylation. The *cry2Ab* gene cassette was transferred to an intermediate plasmid as a *Not*I fragment. This intermediate plasmid contained the following elements: enhanced 35S promoter, the *E. coli uidA* gene, the 3' nontranslated polyadenylation signal from the nopaline synthase gene of *Agrobacterium tumifaciens* and a multi-cloning site containing a *Not*I site. The plasmid, PV-GHBK11 is a result of the fusing of the *Not*I *cry2Ab* containing fragment into the *Not*I site of the intermediate plasmid.

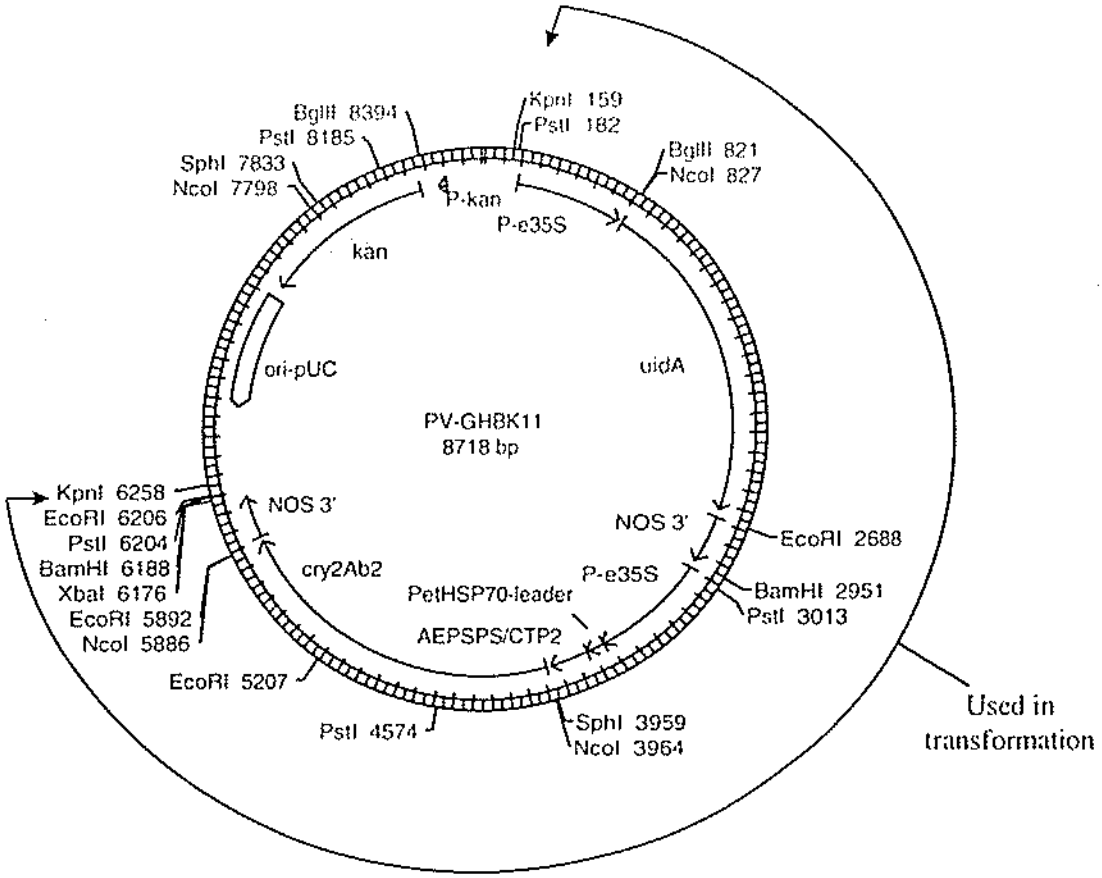
The HPLC-isolated linear restriction fragment of the plasmid vector, designated PV-GHBK11L, utilized for transformation of Bollgard II cotton event 15985, contains only the *cry2Ab* and *uidA* plant gene expression cassettes and does **not** contain the *nptII* selectable marker gene or origin of replication (Figure 2).

B. Method of Transformation

The plasmid containing the *cry2Ab* and *uidA* gene cassettes, PV-GHBK11, was propagated in *E. coli*, purified from bacterial suspensions using column purification. The gene of interest and the marker gene were purified away from the vector backbone by cutting with a restriction endonuclease *Kpn*I (Ausubel *et al.*, 1987) and subsequently separated and purified based on size differences by HPLC. This linear fragment is designated PV-GHBK11L. The purified linear DNA, PV-GHBK11L, was then precipitated onto gold particles using calcium chloride and spermidine, essentially as described by John (1997).

The cotton tissue that is the recipient of the introduced DNA, variety DP50B, is the Delta and Pine Land Company commercial variety containing the Bollgard *cry1Ac* gene. DNA was introduced into the cotton meristems by the particle acceleration method described by John (1997). Germline integration of DNA was detected by histochemical staining for GUS in vascular tissue. Nontransformed tissue was removed over time, thus promoting growth of meristems containing the introduced DNA. The resulting seed from these plants was then screened for the production of the Cry2Ab protein. The preparation of Bollgard II cotton event 15985 is described in Figure 3.

Figure 1. Plasmid Map of PV-GHBK11.



The *Kpn*I segment of PV-GHBK11 plasmid used to generate insect-protected cotton event 15985.

Figure 2. Linear Map of DNA Segment PV-GHBK11L.
The DNA segment, PV-GHBK11L used to generate insect-protected cotton event 15985 by particle acceleration technology.

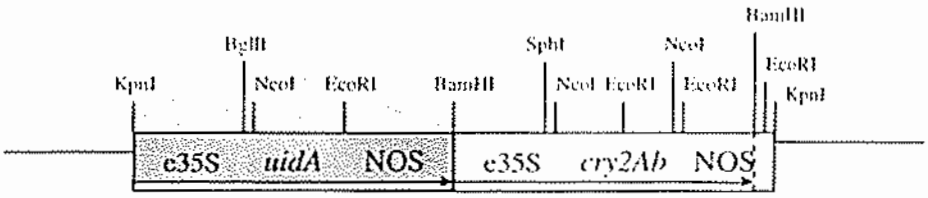


Figure 3. Development of Bollgard II Cotton Event 15985.

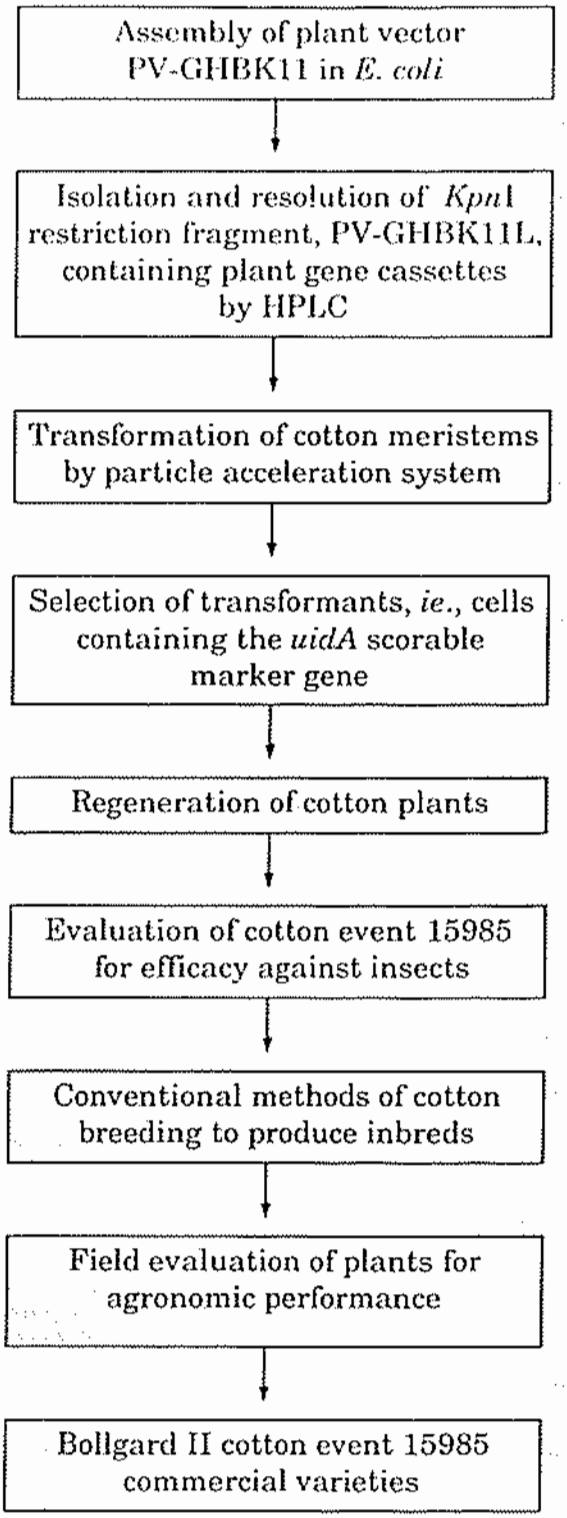


Table 1. Summary of DNA Components of the Plasmid PV-GHBK11.

Genetic Element	Range (bp)	Function (reference)
P-e35S	183-797	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with a duplicated enhancer region used to drive expression of the *uidA* gene.
Intervening Sequence	798-828	Synthetic sequence, polylinker.
uidA	829-2637	The *uidA* gene from *E. coli* plasmid pUC19 encoding a β-D-glucuronidase (GUS) protein (Gilissen *et al.*, 1998).
Intervening Sequence	2638-2692	Synthetic sequence, polylinker
NOS 3'	2693-2948	The 3' nontranslated region of the nopaline synthase (NOS) gene from *Agrobacterium tumifaciens* which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983).
Intervening Sequence	2949-3013	Synthetic sequence, polylinker.
P-e35S	3014-3627	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region used to drive expression of the *cry2Ab* gene.
PetHSP70-leader	3628-3727	Heat shock protein 70 gene 5' untranslated leader sequence from petunia.
AEPSPS/CTP2	3729-3959	The N-terminal chloroplast transit peptide from *Arabidopsis thaliana* EPSPS gene (Van den Broeck, et al.,1985).
Intervening Sequence	3960-3965	Synthetic linker sequences.
cry2Ab	3966-5873	The synthetic *cry2Ab* gene based on the sequence from *Bacillus thuringiensis* (Widner and Whiteley, 1990).
Intervening Sequence	5874-5896	Synthetic linker sequence.
NOS 3'	5897-6152	The 3' nontranslated region of the nopaline synthase (NOS) gene from *Agrobacterium tumifaciens* which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983).
Intervening Sequence	6153-6277	Synthetic linker sequence.
Backbone	6278-158	(Vieira and Messing, 1987).
lacZ	6278-6516	A partial lacI coding sequence, the promoter P-lac and a partial coding sequence for β-D-galactosidase or lacZ protein.
ori-pUC	6661-7315	A plasmid replication origin which permits propagation of DNA in bacterial hosts such as *E. coli*.
nptII (kan)	7396-8363	The gene for the enzyme neomycin phosphotransferase type II from Tn5, a transposon isolated from *Escherichia coli* (Beck *et al.*, 1982). The *nptII* gene also contains a 0.153 kb portion of the 0.378 kb *hle* gene from Tn5.
P-kan	8452-8501	Promoter for *nptII* gene obtained from Tn5.
Intervening Sequence	159-182	Synthetic linker sequence.

C. Inserted Genes and the Proteins Encoded

1. Nomenclature

Cry2Ab is a protein derived from *Bacillus thuringiensis* and has also been designated Cry2Ab2, CryIIB, CryB2 or CryIIAb (Liang and Dean, 1994; Widner and Whiteley, 1990; Crickmore, *et al.*, 1998) or the Monsanto designation Insect Protection Protein 2 (IPP2). In the current nomenclature scheme for Cry proteins, names are assigned according to amino acid similarity to established holotype proteins as defined by Crickmore *et al.* (1998). In this nomenclature, Cry proteins with similar amino acid sequences are grouped together. Cry proteins with the same Arabic numeral, *e.g.*, Cry2, share at least a 45% amino acid sequence identity. Those with the Arabic numeral and upper case letter, *e.g.*, Cry2A, share at least a 75% sequence identity. Finally, Cry proteins with the same Arabic numeral, upper case letter and lower case letter, *e.g.*, Cry2Ab, share a greater than 95% sequence identity.

2. The *cry2Ab* Gene

Bacillus thuringiensis (*B.t.*) is a gram-positive bacterium commonly present in soil and that has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). *Bacillus thuringiensis* subsp. *kurstaki*, present in commercial microbial pest control products such as DIPEL® and Crymax®, contains both the *cry2Aa* and *cry2Ab* genes. The *cry2Aa* gene is expressed in these commercial products; however, the *cry2Ab* gene is a pseudogene, which even though present is not expressed due to an inefficient *cry2Ab* promoter (Dankocsik *et al.*, 1990). Therefore, the Cry2Ab protein is not naturally produced in soil bacteria or sprayable microbial formulations (Widner and Whiteley, 1990; Crickmore, *et al.*, 1994). Both the *cry2Aa* and *cry2Ab* genes are located on the same 100 MDa plasmid (Donovan, *et al.*, 1988; 1989) and the sequence of the *cry2Ab* gene has been fully characterized (Widner and Whiteley, 1990).

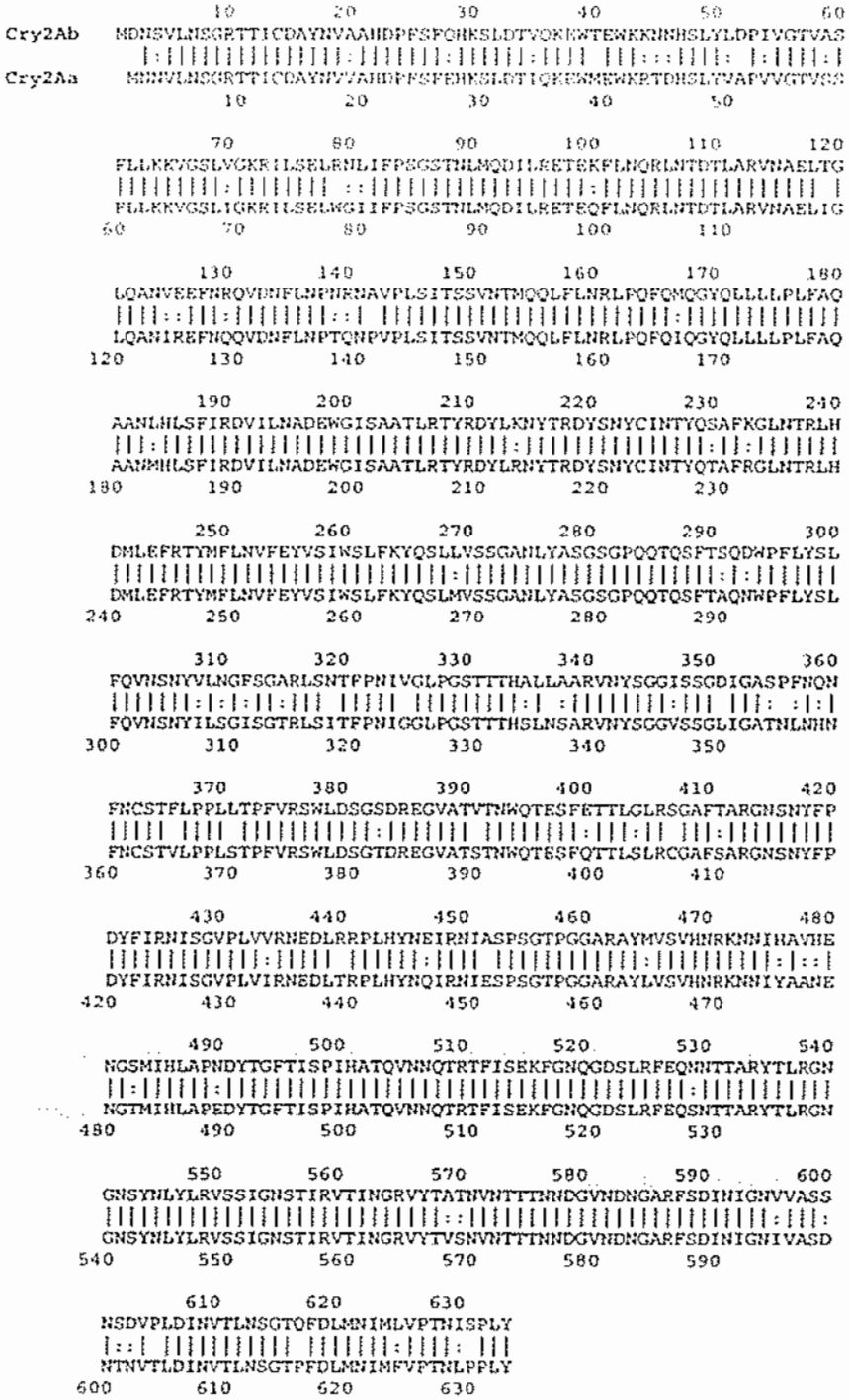
3. The Cry2Ab Protein

Assessment of the safety of the Cry2Ab protein produced in cotton plants required production of sufficient quantities of material to conduct safety tests. Due to the extremely low levels of Cry2Ab protein produced in event 15985 plants, it was necessary to produce Cry2Ab protein by bacterial fermentation to generate sufficient protein to conduct the safety studies.

Since the *cry2Ab* gene is not naturally expressed in *Bacillus thuringiensis* subsp. *kurstaki*, the *cry2Ab* pseudogene with the necessary promoter region was cloned into *Bacillus thuringiensis* strain EG7699. The *cry2Ab* gene expression product was then isolated and purified from the modified EG7699 bacterial strain. The Cry2Ab protein product (GenBank Accession No. X55416) is 633 amino acids in length, with an approximate mass of 71 kDa (Widner and Whiteley, 1990; Dankocsik *et al.*, 1990). The deduced amino acid sequence of the Cry2Ab protein introduced into plants is shown in Figure 4. An additional amino acid (position 2, Figure 4) was introduced to create a restriction enzyme cleavage site for cloning purposes. The coding region of the Cry2Ab protein is highly similar to the

Cry2Aa protein (Figure 4), sharing 88% amino acid sequence identity (Widner and Whiteley, 1990; Dankocsik *et al.*, 1990) and 97% amino acid similarity (amino acid identities and conservative amino acid substitutions). The Cry2Ab protein that is present as a stable protein product in transgenic cotton plants is predicted to contain an additional three amino acids due to processing of the chloroplast transit peptide (underlined positions 77-79, Figure 5).

Figure 4. Deduced Amino Acid Sequences of Cry2Ab and Cry2Aa Proteins.



Legend: Alignment of the deduced amino acid sequences of Cry2Ab and Cry2Aa proteins.

| = identical AA; : = AA conservative substitutions (similarities)

Figure 5. Deduced Cry2Ab Protein Sequence as Produced in Cotton. The sequence deduced from the DNA used to transform cotton. The chloroplast transit peptide is shown in italics (residues 1-79). The Cry2Ab protein corresponds to residues 80-713. The underlined amino acids (residues 77-79) correspond to the predicted portion of the chloroplast transit peptide remaining after processing. The amino acid at position 81 (D, aspartic acid) corresponds to the residue introduced for cloning purposes (position 2, Figure 4).

```
  1  MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51  LKKSGMTLIG SELRPLKVMS SVSTACMLAM DNSVLNSGRT TICDAYNVAA
101  HDPFSFQHKS LDTVQKEWTE WKKNNHSLYL DPIVGTVASF LLKKVGSLVG
151  KRILSELRNL IFPSGSTNLM QDILRETEKF LNQRLNTDTL ARVNAELTGL
201  QANVEEFNRQ VDNFLNPNRN AVPLSITSSV NTMQQLFLNR LPQFQMQGYQ
251  LLLLPLFAQA ANLHLSFIRD VILNADEWGI SAATLRTYRD YLKNYTRDYS
301  NYCINTYQSA FKGLNTRLHD MLEFRTYMFL NVFEYVSIWS LFKYQSLLVS
351  SGANLYASGS GPQQTQSFTS QDWPFLYSLF QVNSNYVLNG FSGARLSNTF
401  PNIVGLPGST TTHALLAARV NYSGGISSGD IGASPFNQNF NCSTFLPPLL
451  TPFVRSWLDS GSDREGVATV TNWQTESFET TLGLRSGAFT ARGNSNYFPD
501  YFIRNISGVP LVVRNEDLRR PLHYNEIRNI ASPSGTPGGA RAYMVSVHNR
551  KNNIHAVHEN GSMIHLAPND YTGFTISPIH ATQVNNQTRT FISEKFGNQG
601  DSLRFEQNNT TARYTLRGNG NSYNLYLRVS SIGNSTIRVT INGRVYTATN
651  VNTTTNNDGV NDNGARFSDI NIGNVVASSN SDVPLDINVT LNSGTQFDLM
701  NIMLVPTNIS PLY
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4. The β-Glucuronidase (*uidA*) Gene

The development of plant varieties containing useful new traits introduced by plant genetic engineering depends upon an effective means to select for the rare transformed plant cells containing the inserted gene(s) of interest from those plants cells that fail to take up or maintain the added DNA. Regenerating each cell from that transformation experiment to test for the presence of the gene of interest would be both impractical and prohibitory, as the frequency of transformation is as low as 1 in 10,000 to 100,000 of the treated cells (Fraley *et al.*, 1983). Therefore, a scorable marker is used to identify the cells to be carried forward through the regeneration process. The β-glucuronidase gene, *uidA*, also known as *gus* or *gusA* gene, is derived from *Escherichia coli* strain K12 (Jefferson, *et al.*, 1986). The sequence has been fully characterized and is available in GenBank (Jefferson, *et al.*, 1986; Schlaman *et al.*, 1994). This gene encodes for the enzyme β-D-glucuronidase (GUS).

5. The β-Glucuronidase (GUS) Protein

β-D-glucuronidase is an exohydrolase that catalyzes the hydrolysis of a range of the β-glucuronides into their corresponding acids and the aglycones (Oshima *et al.*, 1987), including the artificial substrate p-nitrophenyl-ß-D-glucuronide. Hydrolysis of this chromogenic compound releases a blue dye that functions as a visible scorable marker in plant transformation processes (Jefferson *et al.*, 1987). The biochemistry and catalytic activity of this protein have been thoroughly studied (Wang and Touster, 1972). The enzyme has a molecular weight of 68 kDa and does not require any cofactors for activity (Levvy and Marsh, 1959). β-glucuronidase functions within a pH range of 5.0 to 7.5 (Jefferson, 1993) and its heat stability has been determined, as a half-life of 15 minutes at 60°C (Jefferson and Wilson, 1991). The deduced amino acid sequence of the GUS protein as expressed in cotton event 15985 is presented in Figure 6.

GUS protein was originally isolated from *E. coli* (Stahl and Fishman, 1974). *E. coli* is ubiquitous in the digestive systems of vertebrates, including humans (Jefferson *et al.*, 1986), where primary glucuronidation activity occurs in the liver. Endogenous GUS activity is also observed in other tissues, such as kidney, spleen, breast milk, adrenal glands and the alimentary tract (Gilissen *et al.*, 1998). Glucuronide conjugation increases the water solubility and excretability of foreign substances from the body (Dutton, 1980). GUS activity is also observed in a large number of other bacteria, including other anaerobic digestive tract bacteria such as *Clostridium* and *Bacteroides* (Hawkesworth *et al.*, 1971), as well as many bacteria (Levvy and Marsh, 1959; Ritz *et al.*, 1994). GUS is also present in cattle and in a number of invertebrate species, including nematodes, molluses, snails, and insects (Gilissen *et al.*, 1998).

GUS activity has also been detected in over 50 plant species in various tissues, including embryo, fruit, seed coat and endosperm (Hu *et al.*, 1990). These species include a number of human food sources, including potato, apple, almond, rye, rhubarb, and sugar beet (Schulz and Weissenbock, 1987; Hodal *et al.*, 1992; Wozniak and Owens, 1994) and constitute an extensive history of safe exposure.

Figure 6. Deduced Amino Acid Sequence of Plant-Produced GUS Protein.
The sequence deduced from the DNA used to transform cotton.

1	MVRPVETPTR	EIKKLDGLWA	FSLDRENCGI	DQRWWESALQ	ESRAIAVPGS
51	FNDQFADADI	RNYAGNVWYQ	REVFIPKGWA	GQRIVLRFDA	VTHYGKVWVN
101	NQEVMEHQGG	YTPFEADVTP	YVIAGKSVRI	TVCVNNELNW	QTIPPGMVIT
151	DENGKKKQSY	FHDFFNYAGI	HRSVMLYTTP	NTWVDDITVV	THVAQDCNHA
201	SVDWQVVANG	DVSVELRDAD	QQVVATGQGT	SGTLQVVNPH	LWQPGEGYLY
251	ELCVTAKSQT	ECDIYPLRVG	IRSVAVKGEQ	FLINHKPFYF	TGFGRHEDAD
301	LRGKGFDNVL	MVHDHALMDW	IGANSYRTSH	YPYAEEMLDW	ADEHGIVVID
351	ETAAVGFNLS	LGIGFEAGNK	PKELYSEEAV	NGETQQAHLQ	AIKELIARDK
401	NHPSVVMWSI	ANEPDTRPQA	AREYFAPLAE	ATRKLDPTRP	ITCVNVMFCD
451	AHTDTISDLF	DVLCLNRYYG	WYVQSGDLET	AEKVLEKELL	AWQEKLHQPI
501	IITEYGVDTL	AGLHSMYTDM	WSEEYQCAWL	DMYHRVFDRV	SAVVGEQVWN
551	FADFATSQGI	LRVGGNKKGI	FTRDRKPKSA	AFLLQKRWTG	MNFGEKPQQG
601	GKQ				

D. Molecular Characterization of Bollgard II Cotton Event 15985

Molecular analysis was performed to characterize the inserted DNA in Bollgard II cotton event 15985. Southern blot analysis was used to determine the insert number (number of integration loci within the cotton genome), the copy number (the number of transgenes at a single locus), the intactness of the *cry2Ab* and *uidA* coding regions, the intactness of the *cry2Ab* and *uidA* cassettes, and to confirm the absence of plasmid backbone sequence derived from plasmid PV-GHBK11. Plasmid PV-GHBK11, the plasmid backbone, the *cry2Ab* and *uidA* coding regions, the enhanced CaMV 35S promoter, and the NOS 3' polyadenylation sequence were all used as probes. Additionally, the 5' and 3' insert-to-plant junctions were verified using the polymerase chain reaction (PCR).

The data show that Bollgard II cotton event 15985 contains one DNA insertion from PV-GHBK11 (Table 2). The insert contains one copy of both the *cry2Ab* and *uidA* cassettes. The *cry2Ab* coding region and cassette are complete, however the restriction site following the NOS 3' polyadenylation sequence in the cassette is no longer present. The *uidA* coding region and its NOS 3' polyadenylation sequence are also complete, however, 260 bp of the 5' end of the enhanced CaMV 35S promoter of the *uidA* cassette is not present in the inserted *uidA* gene cassette. The e35S promoter is still functional despite this truncation, as demonstrated by production of the GUS protein. This event does not

contain any detectable backbone sequence derived from plasmid PV-GHBK11. It is therefore concluded that full-length Cry2Ab and GUS proteins should be produced in event 15985 as a result of integration of the DNA segment derived from plasmid PV-GHBK11. Production of the full-length Cry2Ab and GUS proteins in cotton event 15985 have been confirmed by western blot analysis. A detailed discussion of the molecular characterization of Bollgard II cotton event 15985 is contained in Appendix 1.

Table 2. Summary of Molecular Characterization Data for Cotton Event 15985.

Cotton Event 15985	
# of new insertions	One
# of copies of *cry2Ab* and *uidA* cassettes	One of each

Genetic Element	
enhanced CaMV 35S promoter (*uidA*)	Missing 260 bp from 5' end (~40%)
uidA coding region	Intact
NOS 3' polyadenylation sequence (*uidA*)	Intact
enhanced CaMV 35S promoter (*cry2Ab*)	Intact
cry2Ab coding region	Intact
NOS 3' polyadenylation sequence (*cry2Ab*)	Intact
Backbone DNA	Not detected

E. Segregation Data and Stability of Gene Transfer of Bollgard II Cotton Event 15985

1. Segregation and Stability

To determine the stability of Bollgard II cotton event 15985 across generations, a series of progeny tests were conducted based on a qualitative Cry2Ab Enzyme-linked immunosorbent assay (ELISA). The results of four generations are reported below (Table 3). Statistical significance for the segregation data was determined using Chi square analysis.

All generations segregated as expected for a single insertion site. The R1 progeny of Bollgard II cotton event 15985 yielded the expected segregation ratio of 3:1 with respect to the detection of Cry2Ab protein. Progenies of event 15985 backcrossed to commercial cotton cultivars yielded the expected segregation ratio of approximately 1:1 with respect to the Cry2Ab protein. The Chi square analysis of the segregation results are consistent with a single active site of insertion into the genomic cotton DNA, segregating according to Mendelian genetics. These data confirm that the DNA insert in Bollgard II cotton event 15985 contains a DNA insert of a single locus that segregates according to Mendelian genetics and therefore remains stably integrated in the plant genome over selfed generations and over successive backcross generations.

Table 3. Segregation Data and Analysis of Progeny of Bollgard II Cotton Event 15985.

	Expected		Observed [1]		
Generation [2]	Positive	Negative	Positive	Negative	ChiSq
R1 (3:1)	202.5	67.5	210	60	1.11 [ns]
R2 (3:1)	45	15	43	17	0.356 [ns]
BC1F1 (1:1)	199	199	213	185	1.970 [ns]
BC2F2 (3:1)	568	189	549	208	2.477 [ns]

[1] Data expressed as number of positive and negative plants based on Cry2Ab qualitative ELISA.
[2] R1 seed was from the initial R0 transformant in a DP50B background.
R2 seed was pooled from heterozygous R1 plants in a DP50B background.
BC1F1 and BC2F2 plants were pooled from five different elite cultivar backgrounds.
[ns] not significant at p=0.05 (chi square = 3.84, 1 df).

2. Cotton Event 15985 Generation Stability: Southern Blot Analysis

The purpose of this study was to assess by Southern blot analysis the genetic stability of the additional DNA inserted to produce the Bollgard II cotton event 15985 across five plant breeding generations. Genomic DNA samples from the R1, R2, R3, R4 generations and two different second-generation lines of backcrossing (BC2F3) were digested, blotted, and probed with the entire *cry2Ab* coding region to assess the stability of the inserted DNA over time and breeding generations. The restriction enzyme *Sph*I was selected because it generates a unique Southern blot banding pattern fingerprint for event 15985 when probed with the *cry2Ab* coding region. The results are presented in Appendix 2. The non-transgenic control DNA and the parental control DNA produced no hybridization to *cry2Ab*, as expected. The data show that no differences in banding pattern were observed among DNA extracted from any of the five plant breeding generations. This demonstrates that the DNA insert is stable in the plant genome across five plant breeding generations.

IV. Safety of the New Cotton Event

The flowcharts presented in the FDA Food Policy (FDA, 1992) were utilized to organize the following summary of the studies conducted and other information which demonstrate the substantial equivalence of Bollgard II cotton event 15985 to the parental control line and other cotton varieties grown commercially.

A. Safety Assessment of New Varieties: The Host Plant, Cotton

The first component of the safety assessment under the FDA Food Policy regards the safety of the host organism, cotton (Figure 7). Cotton, *Gossypium hirsutum* L., is the leading plant fiber crop produced in the world. Both cottonseed oil and to a lesser extent cotton fiber, in the form of processed cotton linters, are routinely used for human food products and have a history of safe use that is well documented (NCPA, 1999a).

Additionally, cottonseed, meal, crude cottonseed oil, hulls and gin trash are used in animal feeds for cattle, sheep, goats, horses, poultry, swine, fish and shrimp (NCPA, 1999b). Food and feed uses of cotton are limited due to the natural toxicants present in the crop: gossypol and cyclopropenoid fatty acids (CPFA). These antinutritional components have been thoroughly studied and do not necessitate further analytical or toxicological testing.

1. Cotton Production

Cotton is grown worldwide, typically in arid regions of the tropical or sub-tropical areas (Niles and Feaster, 1984). It is grown primarily for the value of the fiber, with cottonseed being a by-product. Cotton production in the United States was 13.9 million acres planted in 1999 (USDA, 2000).

2. Cotton as a Food Source in the United States

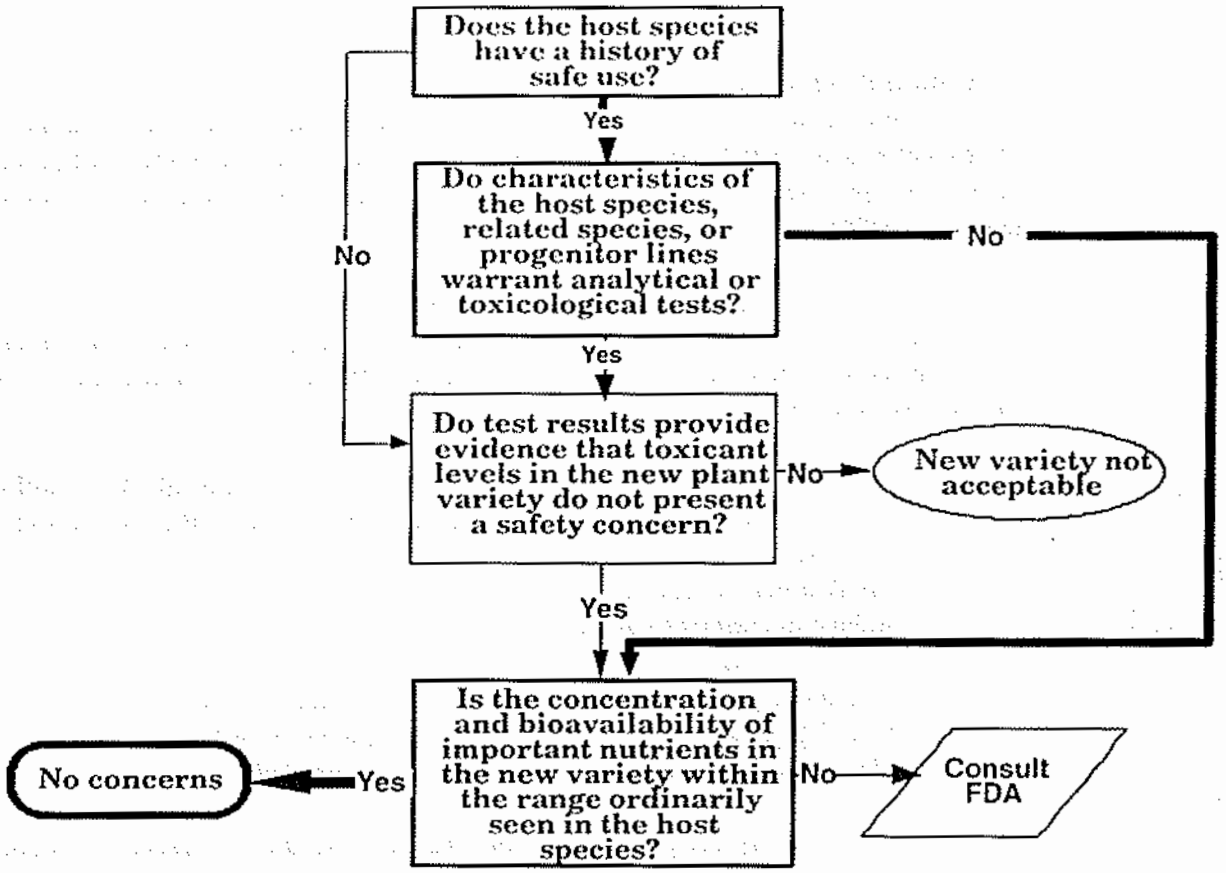
Cottonseed is processed into four major products: oil, meal, hulls, and linters. Processing of cottonseed typically yields (by weight): 16% oil, 45% meal, 26% hulls, and 9% linters, with 4% lost during processing (Cherry and Leffler, 1984).

Cottonseed is highly processed during the production of oil and meal. After hulling, the cottonseed is flaked by a rolling process to facilitate oil removal. Prior to oil extraction, the flakes are heated to:

(i) break down the cell walls
(ii) reduce the viscosity of the oil
(iii) coagulate the protein
(iv) inactivate proteins and kill any microbial contamination
(v) detoxify gossypol by the combination of heat and moisture
(vi) fix certain phosphatides in the meal to minimize refining losses.

After heating, oil is typically removed from the meal by direct solvent extraction with hexane. The material left after the extraction of the crude cottonseed oil is the cottonseed meal. The gossypol levels in the meal after extraction are reduced by approximately half. Crude cottonseed oil is further processed, depending on the end use of the product.

Figure 7. Safety Assessment of New Varieties: The Host Plant (taken from FDA Food Policy Figure 2). The pathway leading to "No concerns" for Bollgard II cotton event 15985 is highlighted with bold arrows.



Further processing (refining) for all the uses of cottonseed oil includes deodorization and bleaching. Deodorization greatly reduces the cyclopropenoid fatty acid content of the oil due to extreme pH and temperature conditions (NCPA, 1990). A winterization step is added to produce cooking oil, whereas for solid shortening an hydrogenation step is added to transform the liquid oil into a solid fat. The resulting oil contains no detectable protein (Fuchs *et al.*, 1993). Cottonseed oil is a premium quality oil that is used for a variety of food uses, including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil. Cottonseed meal is not currently used for human consumption in the United States (Morgan, 1990; NCPA, 1999a), but is principally sold as feed for livestock (NCPA, 1999a). The presence of gossypol and

cyclopropenoid fatty acids in cottonseed limits its use as a protein supplement in animal feed except for cattle, which are affected by these components at higher levels than other animals. According to the National Cottonseed Products Association, more than half of the cottonseed products, meal and hulls, go into animal feed. Inactivation or removal of these antinutritional components during processing enables the use of some cottonseed meal for catfish, poultry and swine, accounting for most of the remaining cottonseed meal.

The hull is the tough protective covering of the cottonseed removed prior to processing the seed for oil and meal. Hulls are used as a high fiber component of livestock feeds due to their high cellulose and lignin content (NCPA, 1990). Gin trash, the dried plant material cleaned from the fiber during ginning, is also another source of roughage for livestock feeds.

The short fibers on the cottonseed, or linters, consist primarily of cellulose (>95%) (Wakelyn *et al.*, 1998). After extensive processing at alkaline pH and high temperatures, the linters can be used as a high fiber dietary product. Food uses include casings for bologna, sausages, frankfurters, and to improve viscosity in products such as toothpaste, ice cream, and salad dressings (NCPA, 1990). Based on the composition of linters and the extensive processing undertaken prior to food use, cellulose used for food derived from cotton linters is not expected to contain any detectable protein (Sims *et al.*, 1996).

3. Characteristics of the Parental Variety

The cotton cultivar used as the parental variety for transformation was Delta and Pine Land Company variety 50B, (DP50B), derived from Bollgard cotton event 531. This cotton event was commercialized in the United States in 1996 and expresses the Cry1Ac insecticidal protein and the NPTII selectable marker protein. Cotton varieties derived from this event were grown on more than 3.9 million acres in the United States in 1999.

a. Compositional Analysis of Bollgard II Cotton Event 15985

The final assessment in the decision tree presented in Figure 7 regards the bioavailability and levels of nutrients in event 15985. Forty-eight different components of cottonseed were evaluated by Covance Laboratories, Inc. (Madison, WI) as part of the safety and nutritional assessment of this product. The level of components important for food and feed uses were assessed for insect-protected cotton event 15985 and compared to that of the parental control (DP50B), as well as to other commercial cotton varieties analyzed in the study. Field trials were conducted at eight U.S. locations within six states in 1998 (Texas, Arizona, Mississippi, South Carolina, Louisiana and Alabama). Compositional analyses of seed samples collected in 1998 U.S. trials were conducted to measure proximates (protein, fat, ash, carbohydrate, moisture, fiber, calories), amino acids, fatty acids, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), gossypol, cyclopropenoid fatty acids and aflatoxin content of seed. Seed collected from Bollgard II cotton event 15985, the parental line DP50B, and seed from the non-transgenic control line DP50 were analyzed. Compositional analyses of cottonseed oil (bleached and deodorized) and meal (toasted) samples processed at Texas

A&M were also conducted. For cottonseed oil, levels of fatty acids, vitamin E, gossypol and cyclopropenoid fatty acids were determined. For meal samples, gossypol levels were assessed. The composition data across all eight U.S. field sites are summarized in Tables 4-12.

Statistical evaluation of the composition data showed that in 48 comparisons from the eight U.S. trials, there were six instances where the mean values for event 15985 were statistically significantly different from the Bollgard (DP50B) parental line. All of these significantly different means were within the 95% confidence interval and within the range of analyses for commercial cotton. Furthermore, the statistically different means were not observed at all locations, demonstrating the impact of environmental conditions on variability.

Therefore, these statistically significant differences are not considered biologically relevant since 1) the means were within the 95% confidence interval and within the range of analyses for commercial cotton, and 2) the statistically different means were not observed at all locations. Compositional analyses of refined cottonseed oil from event 15985 were consistent with commercial cotton variety ranges for fatty acid levels, vitamin E and gossypol levels. The gossypol levels of toasted cottonseed meal samples derived from events 15985 were consistent with gossypol levels from commercial cotton varieties. Therefore, it is concluded that event 15985 is not materially different from other commercially available cotton varieties.

i) Materials and Methods

Seed samples were ginned, acid delinted and shipped under ambient conditions to Covance Laboratories, Inc. for compositional analyses. Seed samples, including hulls, were analyzed for proximates (protein, fat, ash, carbohydrate, moisture, fiber, calories), amino acids, fatty acids, cyclopropenoid fatty acids, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), total gossypol and aflatoxin content. Carbohydrate values in seed were determined by calculation. Cottonseed oil and meal samples were processed at Texas A&M and shipped frozen on dry ice to Covance Laboratories, Inc. for cyclopropenoid fatty acid, vitamin E and gossypol analyses. Cottonseed meal samples were analyzed for gossypol levels. The methods used by Covance Laboratories, Inc. are summarized in Appendix 3.

ii) Proximate Analyses

The levels of the major components of cottonseed (protein, fat, ash, moisture, carbohydrate, fiber and calories) were determined for seed from each field site and are reported as averages across sites (Table 4). There were no statistically significant differences in seed proximate levels between Bollgard II event 15985 and the parental control DP50B. All mean values fell within the reference ranges generated, as well as literature ranges available for protein (12 - 32%), fat (16.1 - 26.7%), ash (4.1 - 4.9%) and moisture (5.4 - 10.1%) (Berberich *et al.*, 1996).

iii) Amino Acid Composition

There were no amino acid parameters that were statistically significantly different from the parental control variety, DP50B (Table 5). Therefore, the amino acid composition of the seed from cotton event 15985 was equivalent to the composition of the seed from the parental DP50B control. All mean values fell within the reference ranges generated.

iv) Fatty Acid Composition

Fatty acid profiles were evaluated in cottonseed for event 15985 and there were no statistically significant differences for palmitic, palmitoleic, oleic, linolenic and gamma linoleic, arachidic, behenic or lignoceric acids compared to DP50B (Table 6). Small, but statistically significant differences were observed for myristic, stearic, and linoleic acids, between event 15985 and control. All significantly different mean values for event 15985 were within the nontransgenic and commercial cotton reference ranges, as well as within the 95% confidence intervals (Table 7) and ranges published in the literature (Berberich *et al.*, 1996). Therefore these differences were not considered biologically relevant.

v) Mineral Analyses

Mineral levels of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc were measured in cottonseed (Table 8). There were no statistically significant differences in any mineral levels obtained for the event 15985 and the means were all within the nontransgenic and commercial reference ranges.

vi) Toxicant Analyses

a) Gossypol Analyses

Gossypol is classified as a terpenoid aldehyde, and is one of a family of terpenoid compounds produced by genera in the plant tribe Gossypiae. Gossypol is produced in lysigenous glands of the seed, leaf, stem and root of the cotton plant, and provides insect protection to the plant.

Gossypol content of cottonseed varies with variety and environmental conditions that include root chilling, nematode and disease infections, toxic chemicals, temperature and moisture stress (Bell, 1991). Gossypol tends to be lower in cottonseed from plants grown at locations with higher than average temperatures, and at higher levels when plants are grown under increased rainfall conditions (Pons *et al.*, 1958). However, analysis of gossypol levels in different cotton genotypes grown in different geographical locations shows a strong genotype-environment interaction. Gossypol levels that are reported in the literature for different cotton varieties grown under various environmental conditions range from 0.39% to 1.7% dry weight of the cottonseed (Abou-Donia, 1976) or a greater range, 0.33 to 2.4% as determined in Indian and Russian samples tested (Berardi and Goldblatt, 1980).

When cottonseed is flaked and heated during processing to oil and meal, the lysigenous glands are ruptured and gossypol is released. Some of the gossypol binds to seed components, primarily to proteins through the free amino groups of lysine. The binding of

gossypol during processing is important because the free form of gossypol is considered toxic, whereas the bound form is unavailable and essentially inactive (Martin, 1990; Berardi and Goldblatt, 1980).

Total gossypol levels were measured in cottonseed from all test and control lines collected across all eight field test locations (Table 9). There were no statistically significant differences in the gossypol level obtained for event 15985 and the mean value was within the nontransgenic and commercial reference ranges.

b) Cyclopropenoid Fatty Acid Analyses

The cyclopropenoid fatty acids, sterculic and malvalic acid, are unique fatty acids common in cotton. Cyclopropenoid fatty acids (CPFA) are naturally present in cotton and are considered to be undesirable, anti-nutritional compounds of concern for food and feed safety. Refining of cottonseed oil includes deodorization and bleaching, which greatly reduces the CPFA content of the oil due to extreme pH and temperature conditions (NCPA, 1990). Sterculic and malvalic acids are 18 and 17 carbons long, respectively, and contain a double bond at the propene ring. The cyclopropenoid fatty acids inhibit the desaturation of stearic to oleic acid, which alters membrane permeability. The levels of cyclopropenoid fatty acids must be minimized due to this undesirable effect (Cherry and Leffler, 1984; Phelps *et. al.*, 1965).

Statistically significant differences were observed for the mean values of malvalic (0.45%), dihydrosterculic (0.18%)and sterculic (0.30%) between event 15985 and control DP50B (Table 9). The absolute magnitude of each difference as a percent of the control ranged from 15-22%. All significant mean differences for event 15985 were within the 95% confidence interval for each true mean difference and mean values were within the nontransgenic and commercial reference ranges (Table 7), as well as literature ranges (Berberich *et al.*, 1996). Additionally, only one of the four replicated field locations showed statistically significant differences between 15985 and the control. Therefore the differences were not considered biologically relevant.

c) Aflatoxin Analyses

Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* that may contaminate food and feed products (Jorgensen and Price, 1981). Cottonseed is one of the commodities most commonly contaminated by aflatoxins (Bagley, 1979). The aflatoxins are highly-substituted coumarins containing a fused dihydrofurofuran. There are four major aflatoxins produced in cotton by *Aspergillus*: B_1 and B_2 are designated because of their blue fluorescence, and G_1 and G_2 because of their green-yellow fluorescence (Wogan and Busby, 1980). The aflatoxins are potent animal toxins and carcinogens, and have been epidemiologically implicated as environmental carcinogens in humans. The most toxic and highly regulated aflatoxin is B_1 (Park and Stoloff, 1989; Stoloff *et al.*, 1991). Aflatoxin contamination of cottonseed is regulated under State and Federal feed adulterant provisions (Price *et al.*, 1993).

Cottonseed that is damaged by insect feeding on the cotton boll is susceptible to infection by the *Aspergillus* fungi. *Aspergillus* infection of cottonseed that results in aflatoxin production is generally initiated through insect damage in the field rather than during seed storage (NCPA, 1990). While normal refining procedures remove the toxin from the oil resulting from contaminated seed, the residual meal is still contaminated. Aflatoxin content of foods and animal feeds throughout most of the world are strictly regulated (Stoloff *et al.*, 1991). Contamination results in losses for the producers, processors, and animal feed industries that depend on cottonseed for feed (Park and Stoloff, 1989). The U.S. Food and Drug Administration has established a regulatory threshold for aflatoxin B_1 in cottonseed fed to dairy cows at 20 µg/kg or 20 ppb (Jorgensen and Price, 1981). Aflatoxins in cottonseed are transferred to milk in slightly modified form (Park *et al.*, 1988), and the U.S. regulations prohibit aflatoxin levels over 0.5 mg/kg in milk. Cottonseed containing less than 300 ppb aflatoxin B_1 may be fed to mature beef cattle, but levels above this limit may not be used as feed. Therefore, it is important to minimize conditions that favor growth of *A. flavus* or *A. parasiticus* on cottonseed and to monitor for its presence for health and economic reasons.

The levels of four primary aflatoxins (B1, B2, G1, G2) were undetected in the cottonseed for event 15985, control DP50B and the nontransgenic and transgenic commercial reference lines at a LOD of 0.1 mg/g (Table 9).

Table 4. Summary of Proximate Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials.

Component	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range [1]	Commercial Reference range [2]
Protein, %	26.13 (21.45-28.82)	26.06 (21.93-28.15)	25.96 (21.76-27.79)	21.76-27.79	21.76-28.15
Fat, %	20.52 (17.54-27.42)	20.37 (16.04-23.48)	19.74 (15.44-23.64)	15.44-23.64	15.44-23.83
Ash, %	4.36 (3.93-4.81)	4.38 (4.06-4.67)	4.34 (3.76-4.85)	3.76-4.85	3.76-4.85
Fiber, crude %	16.83 (14.93-17.95)	17.17 (15.42-19.69)	17.19 (15.38-19.31)	15.38-19.31	15.38-20.89
Carbohydrate, %	49.09 (42.97-52.69)	49.23 (46.85-51.93)	49.94 (45.64-52.44)	45.64-53.62	45.64-53.62
Calories/100g DW	485.33 (468.50-520.01)	484.45 (463.09-498.71)	481.57 (457.77-499.84)	457.77-499.84	457.77-500.49
Moisture, %	5.99 (4.34-7.59)	6.05 (4.22-7.28)	6.03 (3.97-7.26)	3.97-7.49	3.97-8.47

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.
[1] Range includes data from four commercially available cotton varieties.
[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 5. Summary of Amino Acid Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials.

Amino Acid (% total AA)	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
aspartic acid [3]	10.02 (9.74-10.49)	9.98 (9.76-10.39)	9.95 (9.78-10.45)	9.75-10.45	9.75-10.45
threonine	3.56 (3.37-3.77)	3.56 (3.40-3.90)	3.55 (3.38-3.73)	3.38-3.73	3.38-3.90
serine	4.77 (4.23-5.04)	4.77 (4.21-5.20)	4.78 (4.16-5.08)	4.16-5.08	4.16-5.20
glutamic acid [3]	20.82 (20.09-21.27)	20.95 (20.09-21.68)	20.93 (20.24-21.25)	20.24-21.25	20.09-21.68
proline	4.17 (4.03-4.46)	4.14 (4.00-4.50)	4.12 (3.93-4.38)	3.93-4.38	3.93-4.50
glycine	4.61 (4.51-4.72)	4.62 (4.51-4.88)	4.60 (4.54-4.68)	4.54-4.68	4.50-4.88
alanine	4.32 (4.20-4.48)	4.31 (4.18-4.60)	4.27 (4.15-4.41)	4.15-4.41	4.15-4.60
cystine	1.79 (1.68-2.03)	1.85 (1.46-2.12)	1.87 (1.67-1.99)	1.67-1.99	1.46-2.12

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

[3] Asparagine and glutamine are converted to aspartic acid and glutamic acid during the hydrolytic portion of the method.

Table 5. Summary of Amino Acid Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials (continued).

Amino Acid (% total AA)	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
valine	4.97 (4.77-5.34)	4.94 (4.72-5.34)	4.89 (4.72-5.22)	4.72-5.22	4.72-5.34
methionine	1.71 (1.55-1.97)	1.75 (1.46-2.03)	1.75 (1.49-1.98)	1.49-1.98	1.46-2.03
isoleucine	3.58 (3.47-3.79)	3.56 (3.45-3.78)	3.53 (3.38-3.71)	3.38-3.71	3.38-3.78
leucine	6.58 (6.45-6.86)	6.56 (6.44-6.94)	6.52 (6.43-6.65)	6.42-6.65	6.38-6.94
tyrosine	2.85 (2.73-2.91)	2.85 (2.66-3.05)	2.83 (2.72-2.96)	2.72-2.96	2.66-3.05
phenylalanine	5.68 (5.54-5.79)	5.70 (5.58-5.84)	5.66 (5.51-5.75)	5.51-5.75	5.51-5.84
lysine	5.10 (4.81-5.46)	5.08 (4.84-5.50)	5.11 (4.90-5.55)	4.88-5.55	4.83-5.55
histidine	3.07 (3.00-3.13)	3.09 (3.01-3.23)	3.09 (3.06-3.12)	3.06-3.12	3.01-3.23

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 5. Summary of Amino Acid Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials (continued).

Amino Acid (% total AA)	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
arginine	11.37 (10.69-11.95)	11.24 (6.88-11.96)	11.49 (10.98-11.80)	10.98-12.10	6.88-12.17
tryptophan	1.02 (0.95-1.23)	1.03 (0.93-1.20)	1.03 (0.94-1.22)	0.94-1.22	0.93-1.26

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 6. Summary of Fatty Acid Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials.

Fatty Acid (% total fatty acids)	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
myristic (14:0)	1.26 (0.88-2.94)	0.92 (0.74-1.91)	1.02 (0.77-2.15)	0.77-2.40	0.64-2.40
palmitic (16:0)	25.80 (24.50-27.90)	25.92 (24.90-27.60)	25.81 (24.30-28.10)	24.30-28.10	23.40-28.10
palmitoleic (16:1)	0.56 (0.33-0.65)	0.58 (0.43-0.68)	0.63 (0.43-0.98)	0.43-0.98	0.43-0.98
stearic (18:0)	2.63 (2.41-3.10)	2.38 (2.24-2.60)	2.30 (2.06-2.71)	2.06-3.11	2.06-3.11
oleic (18:1)	15.58 (13.60-18.10)	15.59 (13.30-18.10)	15.40 (12.90-17.40)	12.90-20.10	12.90-20.10
linoleic (18:2)	52.52 (47.70-55.50)	53.10 (49.00-55.80)	53.31 (49.50-57.10)	46.00-57.10	46.00-57.10
linolenic and gamma linoleic (18:3)	0.13 (0.050-0.29)	0.14 (0.05-0.55)	0.11 (0.05-0.31)	0.05-0.31	0.05-0.55

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 6. Summary of Fatty Acid Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials (continued).

Fatty Acid (% total fatty acids)	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
arachidic (20:0)	0.30 (0.25-0.43)	0.29 (0.25-0.36)	0.27 (0.24-0.34)	0.24-0.34	0.24-0.36
lignoceric (24:0)	0.14 (0.05-0.26)	0.12 (0.05-0.26)	0.14 (0.05-0.29)	0.05-0.29	0.05-0.29

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 7. Summary of Statistically Significant Differences in Composition for Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials.

Significant Parameter	15985 Mean	DP50B (Control) Mean	Mean Difference	Number of Sites with Significant Differences[1]	Commercial Range [2]	p Value	95% Confidence Interval	Difference as Percent of Control
myristic acid	1.26	0.92	0.33	2	0.64-2.40	0.004	0.11 - 0.56	36%
stearic acid	2.63	2.38	0.25	3	2.06-3.11	<0.001	0.18 - 0.32	11%
linoleic acid	52.52	53.1	-0.58	1	46-57.10	0.038	(-1.13) - (-0.035)	1%
malvalic acid	0.45	0.39	0.058	0	0.17-0.61	0.024	0.0084 - 0.11	15%
sterculic acid	0.30	0.25	0.054	0	0.13-0.66	0.034	0.0041 - 0.10	22%
dihydro-sterculic acid	0.18	0.15	0.036	3	0.11-0.22	<0.001	0.021 - 0.051	24%

[1] Data is from the four replicated sites.
[2] Range includes data from 10 commercial varieties of cotton.

Table 8. Summary of Mineral Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials

Mineral	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
calcium (% DW)	0.15 (0.13-0.19)	0.15 (0.13-0.20)	0.15 (0.12-0.20)	0.12-0.33	0.12-0.33
copper (mg/kg DW)	7.18 (4.27-10.12)	7.24 (4.39-9.51)	7.48 (4.39-10.35)	4.39-10.35	4.39-10.35
iron (mg/kg DW)	50.83 (43.92-57.56)	51.13 (41.84-60.76)	54.13 (42.57-72.15)	42.57-72.15	41.84-72.15
magnesium (% DW)	0.41 (0.37-0.47)	0.41 (0.37-0.49)	0.41 (0.37-0.47)	0.37-0.47	0.37-0.49
manganese (mg/kg DW)	14.11 (11.96-16.53)	14.10 (11.17-16.81)	14.11 (12.16-16.39)	12.16-18.31	11.17-18.31
phosphorus (% DW)	0.70 (0.58-0.83)	0.71 (0.61-0.88)	0.73 (0.63-0.86)	0.63-0.86	0.61-0.88
potassium (% DW)	1.16 (1.07-1.24)	1.15 (1.09-1.22)	1.15 (1.08-1.23)	1.08-1.24	1.08-1.25

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 8. Summary of Mineral Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials (continued).

Mineral	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
sodium (% DW)	0.14 (0.067-0.21)	0.15 (0.039-0.30)	0.14 (0.04-0.25)	0.0054-0.25	0.0054-0.30
zinc (mg/kg DW)	40.30 (27.70-52.50)	41.06 (27.39-51.20)	40.97 (31.66-48.62)	31.66-48.62	27.39-51.20

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

Table 9. Summary of Toxicant Analyses of Bollgard II Event 15985 Cottonseed Samples from the 1998 U.S. Field Trials

	15985	DP50B (parent)	DP50 (nontransgenic control)	Non-transgenic Reference range[1]	Commercial Reference range[2]
Total gossypol (% DW)	1.00 (0.79-1.29)	0.97 (0.78-1.24)	0.96 (0.72-1.23)	0.72-1.23	0.71-1.24
CPFA malvalic (C-17) (% total fatty acids)	<u>0.45</u> (0.26-0.71)	0.39 (0.22-0.51)	0.39 (0.17-0.61)	0.17-0.61	0.17-0.61
CPFA sterculic (C-18) (% total fatty acids)	<u>0.30</u> (0.21-0.58)	0.25 (0.16-0.44)	0.24 (0.13-0.43)	0.13-0.56	0.13-0.66
CPFA dihydrosterculic (C-19) (% total fatty acids)	<u>0.18</u> (0.12-0.22)	0.15 (0.11-0.17)	0.16 (0.12-0.19)	0.12-0.22	0.11-0.22
Aflatoxin B1 (ppb)	<1.00	<1.00	<1.00	<1.00	<1.00
Aflatoxin B2 (ppb)	<1.00	<1.00	<1.00	<1.00	<1.00
Aflatoxin G1 (ppb)	<1.00	<1.00	<1.00	<1.00	<1.00
Aflatoxin G2 (ppb)	<1.00	<1.00	<1.00	<1.00	<1.00

Underlined values are statistically significant relative to the DP50B control ($p \leq 0.05$). Values represent samples taken from 8 U.S. field sites in 1998.

[1] Range includes data from four commercially available cotton varieties.

[2] Range includes data from ten commercially available transgenic and nontransgenic cotton varieties.

vii. Compositional Analyses of Refined Cottonseed Oil

Cottonseed oil samples were generated by pooling cottonseed by line across the eight field sites for processing to a single oil sample per line. As only one sample was generated for each line, the results were not statistically analyzed. Compositional analyses were conducted on cottonseed oil samples for the following components: fatty acid composition, vitamin E content, gossypol content and cyclopropenoid fatty acid content.

a) **Fatty Acid Profiles.** Fatty acid profiles were evaluated in refined cottonseed oil for event 15985 and are listed in Table 10. The values generated show that the fatty acid levels of oil from event 15985 and control DP50B were consistent with nontransgenic and commercial reference ranges and there were no statistically significant differences in fatty acid levels between event 15985 and the parental control DP50B.

b) **Vitamin E (α-Tocopherol) Analyses.** Vitamin E levels measured in refined cottonseed oil prepared from pooled cottonseed samples from all eight sites were similar to levels reported from the commercial reference ranges (Table 11). The vitamin E level for cottonseed oil from event 15985 was 59.8mg/100g. Commercial cottonseed oil samples ranged from 45.1-58.5mg/100g for vitamin E levels. The vitamin E level for cottonseed oil from event 15985 and commercial oil samples fall within the range (10.2 - 66.0 mg/100g) reported in the literature by Dicks (1965). Therefore, these differences are not considered biologically relevant.

c) **Gossypol Analyses.** Gossypol levels were measured in refined cottonseed oil in samples pooled from eight field test locations (Table 11). Free and total gossypol levels measured in all test, control and reference oil samples were all below the limit of detection of the analytical method (<0.005 %FW).

d) **Cyclopropenoid Fatty Acid Analyses.** CPFA levels were measured in refined cottonseed oil prepared from pooled cottonseed samples. The mean values observed for sterculic (0.205%), dihydrosterculic (0.165%) and malvalic (0.378%) acids for event 15985 were similar to those of the control DP50B and were within the ranges determined for oil derived from commercial cotton varieties (Table 12).

viii. Compositional Analyses of Toasted Cottonseed Meal

Toasted cottonseed meal samples were generated by pooling cottonseed across eight field sites for processing into a single sample per line. As only one meal sample was generated for each line, the results were not statistically analyzed. The free gossypol level for toasted cottonseed meal from event 15985 was 0.037% fwt, which was similar to cottonseed meal from parental control DP50B (0.042%). Free gossypol levels in cottonseed meal samples generated from commercial cottonseed varieties ranged from 0.025 to 0.068%.

The total gossypol level for cottonseed meal from event 15985 was 0.968% fwt. Cottonseed meal from parental control DP50B was 1.05%. Total gossypol levels in meal samples generated from commercial cottonseed varieties ranged from 0.933 to 1.43%. Gossypol values for cottonseed meal from event 15985 are consistent with values from commercial varieties.

Table 10. Summary of Fatty Acid Analyses of Bollgard II Event 15985 Cottonseed Oil Samples from the 1998 U.S. Field Trials.

Fatty Acid (% total fatty acids)	15985	DP50B (parent)	DP50 (nontransgenic control)	Commercial Reference range[1]
myristic (14:0)	1.32	0.980	1.06	0.923-1.45
pentadecanoic (15:0)	<0.100	<0.100	<0.100	<0.100
palmitic (16:0)	23.9	25.2	25.3	22.7-26.3
palmitoleic (16:1)	0.832	0.735	0.78	0.735-0.954
heptadecanoic (17:0)	<0.100	<0.100	<0.100	<0.100
stearic (18:0)	2.04	2.34	2.04	1.98-2.34
oleic (18:1)	15.1	15.7	14.7	14.7-17.8
linoleic (18:2)	55.6	53.7	54.9	51-54.9
linolenic and gamma linoleic (18:3)	0.171	0.152	0.145	0.120-0.152
arachidic (20:0)	0.176	0.244	0.178	0.178-0.244
behenic (22:0)	<0.100	0.103	<0.100	<0.100-0.103

Values represent samples pooled from 8 U.S. field sites in 1998.
[1] Range includes data from five commercially available cotton varieties.

Table 10. Summary of Fatty Acid Analyses of Bollgard II Event 15985 Cottonseed Oil Samples from the 1998 U.S. Field Trials (continued).

Fatty Acid (% total fatty acids)	15985	DP50B (parent)	DP50 (nontransgenic control)	Commercial Reference range[1]
lignoceric (24:0)	<0.100	<0.100	<0.100	<0.100

Values represent samples pooled from 8 U.S. field sites in 1998.
[1] Range includes data from five commercially available cotton varieties.

Table 11. Summary of Analyses of Bollgard II Event 15985 Cottonseed Meal and Oil Samples from the 1998 U.S. Field Trials.

	15985	DP50B (parent)	DP50 (nontransgenic control)	Commercial Reference range[1]
OIL Free gossypol (%FW)	<0.005	<0.005	<0.005	<0.005
OIL Total gossypol (%FW)	<0.005	<0.005	<0.005	<0.005
OIL Vitamin E (mg/100g)	59.8	45.1	53.4	45.1-58.5
MEAL Free gossypol (%FW)	0.037	0.042	0.041	0.025-0.068
MEAL Total gossypol (%FW)	0.986	1.05	1.04	0.933 - 1.43

Values represent samples pooled from 8 U.S. field sites in 1998.
[1] Range includes data from five commercially available cotton varieties.

Table 12. **Summary of Cyclopropenoid Fatty Acid Analyses of Bollgard II Event 15985 Cottonseed Oil Samples from the 1998 U.S. Field Trials.**

Cyclopropenoid Fatty Acid (% total fatty acids)	15985	DP50B (parent)	DP50 (nontransgenic control)	Commercial Reference range[1]
malvalic (C-17)	0.378	0.384	0.377	0.294-0.405
sterculic (C-18)	0.205	0.227	0.217	0.216-0.289
dihydrosterculic (C-19)	0.165	0.169	0.146	0.146-0.202

Values represent samples pooled from 8 U.S. field sites in 1998.
[1] Range includes data from five commercially available cotton varieties.

ix. Conclusion

The FDA's Food Policy recommends that key nutritional and biochemical components of genetically modified plant varieties be assessed prior to commercial introduction. Monsanto has performed extensive analytical studies to compare the composition of Bollgard II cotton event 15985 to the parental control, commercially available cotton varieties and reported literature values where available. The compositional data demonstrate that cottonseed from event 15985 is substantially equivalent to the parental control cotton and other cotton varieties grown commercially. This point, together with the safe history of use of the host organism, cotton, as a source of animal feed and human food, leads to the overall conclusion of "no concern" in response to the questions posed by the FDA in Figure 7, "Safety Assessment of New Varieties: the Host Plant".

B. Safety Assessment of New Varieties: The Donor Organism(s)

The safety assessment of the new variety includes an assessment of the donor organisms, per the FDA Food Policy (Figure 8). The donor organisms for each genetic component of the linearized fragment of PV-GHBK11 is listed in Table 13. Each of the inserted genetic elements in Bollgard II cotton event 15985 is derived from a well-characterized source organism that is not commonly allergenic, nor warrants further analytical or toxicological testing. Further, only the specific sequenced genes were transferred to the host organism, cotton.

Table 13. Donor Organisms for Bollgard II Cotton Event 15985.

Genetic Element	Source
cry2Ab	*Bacillus thuringiensis* subsp. *kurstaki*
uidA	*Escherichia coli*
P-e35S	Cauliflower mosaic virus
NOS 3'	*Agrobacterium tumefaciens*
Pet HSP70 leader	*Petunia hybrida*
AEPSPS/CTP2	*Arabidopsis thaliana*

1. Donor Organism: *Bacillus thuringiensis* subsp. *kurstaki*

Bacillus thuringiensis (*B.t.*) is a gram-positive bacterium that is commonly found in soil and has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). Based on the available scientific data, EPA and other regulatory agencies have determined that use of registered *B.t.k.* products poses no significant risks to human health, non-target organisms or the environment (EPA, 1998). *Bacillus thuringiensis* has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding cotton (*Gossypium hirsutum*) (Monsanto, 1994) and corn (*Zea mays*) (Monsanto, 1996). The characteristics of this organism do not warrant additional analytical or toxicological testing.

2. **Donor Organism:** ***Escherichia coli*** **K12**
The β-glucuronidase gene, *uidA*, also known as the *gus* or *gusA* gene, is derived from *Escherichia coli* strain K12 (Jefferson *et al.*, 1986). *E. coli* is ubiquitous in the environment and in the digestive systems of vertebrates, including humans (Jefferson *et al.*, 1986). The same genetic element from the same donor organism is contained in Roundup Ready sugar beet line 77, previously reviewed by FDA (Monsanto, 1998). The characteristics of this organism do not warrant additional analytical or toxicological testing.

3. **Donor Organism: Cauliflower Mosaic Virus**
The cauliflower mosaic virus, which is the donor organism for the P-e35s promoter, is a plant virus commonly infecting many food crops; however, is not known to infect cotton. There is no sequence relationship between the P-e35s promoter sequence and any known mammalian virus. The cauliflower mosaic virus has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding soybean (*Glycine max*) (Monsanto, 1994), canola (*Brassica napus*) (Monsanto, 1995), cotton (*Gossypium hirsutum*) (Monsanto, 1995), corn (*Zea mays*) (Monsanto, 1996) and sugar beet (*Beta vulgaris*) (Monsanto, 1998). This virus is not known to be a human or animal pathogen, is not commonly allergenic, and does not warrant analytical or toxicological tests.

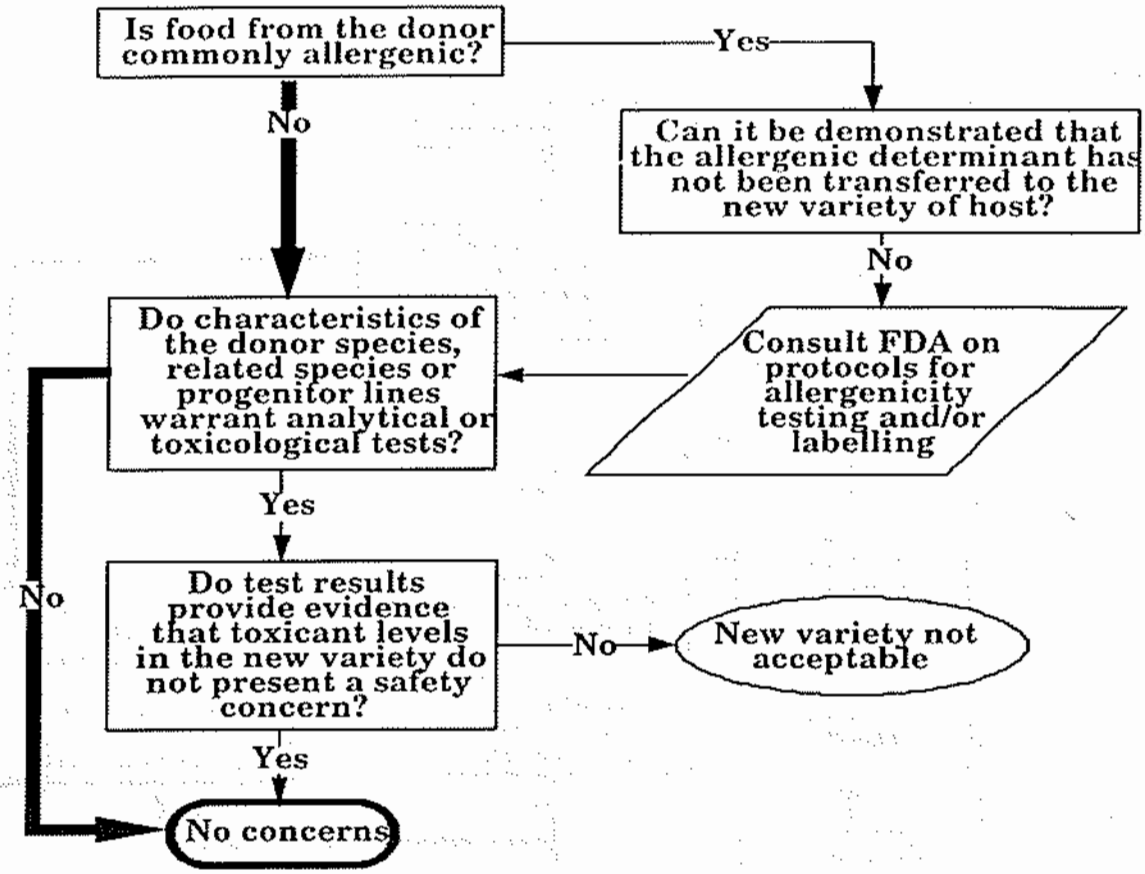
4. **Donor Organism:** ***Agrobacterium tumefaciens***
Agrobacterium is not known for human or animal pathogenicity, is not commonly allergenic and does not warrant additional analytical or toxicological tests. *Agrobacterium tumefaciens* has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding glyphosate tolerant soybean (*Glycine max*) (Monsanto, 1994),), cotton (*Gossypium hirsutum*) (Monsanto, 1994), canola (*Brassica napus*) (Monsanto, 1995), corn (*Zea mays*) (Monsanto, 1996) and sugar beet (*Beta vulgaris*) (Monsanto, 1998). The characteristics of this organism do not warrant additional analytical or toxicological testing.

5. **Donor Organism:** ***Petunia hybrida***
Petunia is not known for human or animal pathogenicity, is not commonly allergenic and does not warrant analytical or toxicological tests.

6. **Donor Organism:** ***Arabidopsis thaliana***
Arabidopsis thaliana is not known for human or animal pathogenicity, is not commonly allergenic and does not warrant analytical or toxicological tests.

These points lead to the conclusion of "no concern" for the sources of the donor gene as listed in Figure 8, due to the lack of allergenic potential or need for additional toxicological testing of the donor organisms.

Figure 8. Safety Assessment of New Varieties: The Donor(s) (taken from FDA Food Policy Figure 3). The pathway leading to "No concerns" for Bollgard II cotton event 15985 is highlighted with bold arrows.

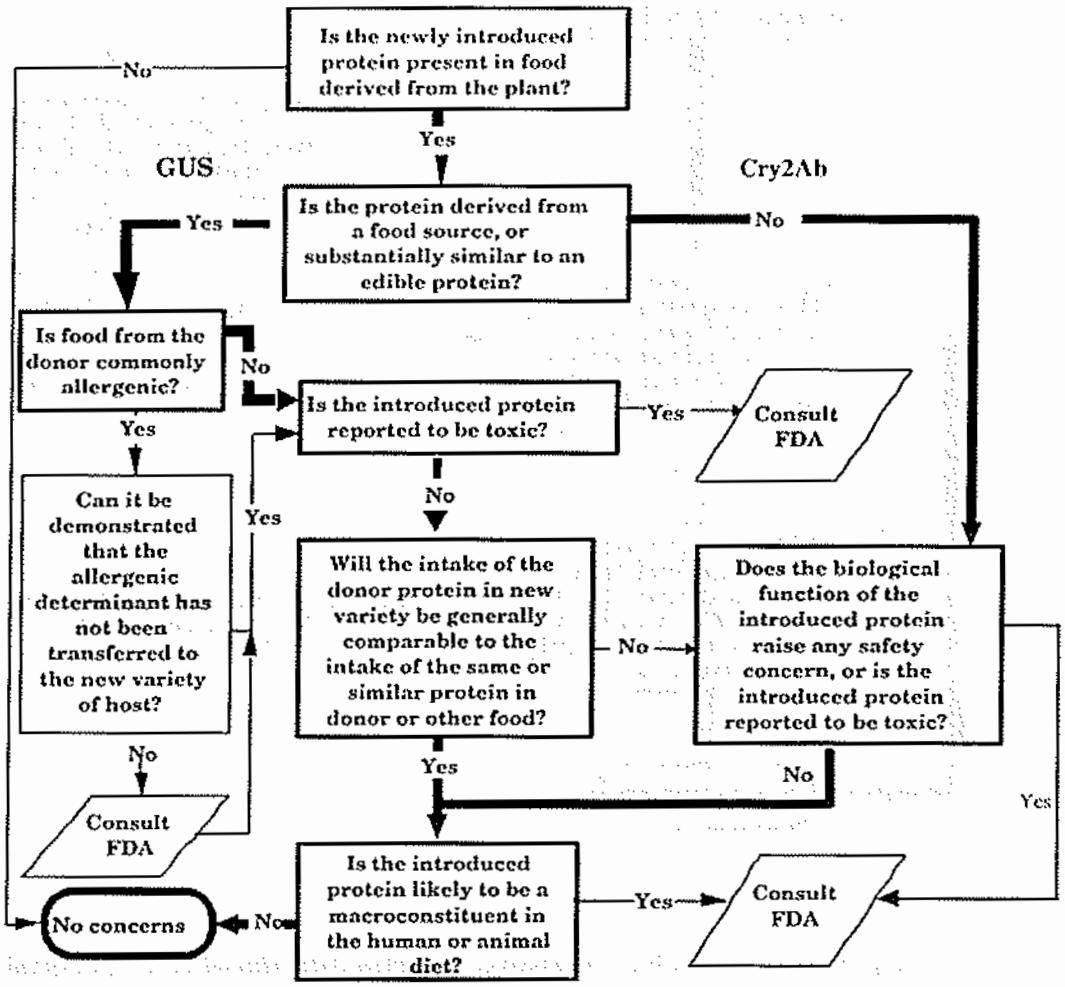


C. Safety Assessment of New Varieties: Proteins Introduced from Donor(s)

FDA's Statement of Policy "Foods Derived From New Plant Varieties", published in the Federal Register May 29, 1992 (FDA, 1992) and the proposed Plant Pesticide Policy of the Environmental Protection Agency (EPA, 1992), agree that under FIFRA the EPA has regulatory oversight for plant pesticides and selectable markers used in the plant transformation process for the purpose of identifying the cells containing the pesticidal gene. Monsanto has consulted with and submitted studies to the EPA supporting the registration of the Cry2Ab insect control protein as a plant pesticide in cotton and requested an exemption from the requirement of a tolerance for this protein.

The safety assessment of the new variety includes an assessment of the introduced proteins, Cry2Ab and GUS, per the FDA Food Policy (Figure 9).

Figure 9. Safety Assessment of New Varieties: Proteins Introduced from Donor(s) (taken from FDA Food Policy Figure 4). The pathways leading to "No concerns" for each introduced protein in Bollgard II cotton event 15985 are highlighted with bold arrows.



1. The Cry2Ab Protein

To address the FDA flowchart "Safety Assessment of New Varieties: Proteins Introduced from Donor(s)" (Figure 9), the protein expression levels from the introduced *cry2Ab* gene are provided, as well as an evaluation of the safety of the protein produced. A description of the *cry2Ab* gene and the encoded protein is provided in Section III.C.

a. Expression of the Cry2Ab Gene in Bollgard II Cotton Event 15985

Studies were conducted to characterize the protein produced and determine the levels of the protein in the selected food and feed components of cotton: cottonseed and whole

plant. Levels of the Cry2Ab protein were estimated in whole plant and cottonseed samples collected from eight field locations in 1998, which were representative of the major U.S. cotton production regions. Locations in Texas and Arizona represented 'plains' type cotton culture and locations in Mississippi, South Carolina, Louisiana and Alabama were chosen for typical southern and southeastern cotton environmental conditions. Samples collected from event 15985 and the parental control line, DP50B, were analyzed using an enzyme-linked immunosorbent assay (ELISA) to estimate the levels of Cry2Ab protein present. The limit of detection (LOD) for the Cry2Ab assay was determined to be 2.65 µg/g fwt in leaf tissue and 2.31 µg/g fwt in seed tissue.

Cry2Ab protein in cotton event 15985 was detected at low levels in various plant tissues at a number of times throughout the growing season. The levels of Cry2Ab protein in cottonseed tissue across all locations ranged from 31.8 to 50.7 µg/g fresh weight, with a mean of 43.2 ± 5.7 µg/g. In whole cotton plant tissues, the mean level of Cry2Ab protein ranged from 7.28 to 10.46 µg/g fresh weight, with a mean of 8.80 ± 1.20 µg/g. No Cry2Ab protein was detected in the control line DP50B.

b. Cry2Ab Human and Animal Health and Safety

Cry2Ab is a protein derived from *Bacillus thuringiensis*, which is not a food source, nor is the protein substantially similar to any known edible proteins. Further, there is a history of safe dietary exposure to *B.t.* residues in or on raw agricultural commodities.

The safety of the Cry2Ab protein is based on (1) the biological function of the protein; (2) extensive animal toxicity testing of Cry proteins, including the highly homologous Cry2A class; (3) a history of safe consumption of Cry proteins by humans and; (4) results of *in vivo* and *in vitro* safety studies conducted with the Cry2Ab protein. These results, described below, establish the absence of adverse effects in animals fed Cry proteins at exposures millions of times higher than estimated human dietary exposures.

As a consequence of the low mammalian toxicity of *B.t.*, all microbial *B.t.* products approved have been exempted from the requirement of a tolerance (EPA, 1998). EPA has since established separate tolerance exemptions by amendment for various *B.t.* proteins produced in transgenic plants, such as Cry1Ab, Cry1Ac, and Cry3Aa (EPA 1995a-d; 1996a,b; 1997).

i. Biological Function of the Cry2Ab Protein

The protein produced in Bollgard II cotton event 15985, Cry2Ab, is 88% amino acid sequence identical to the Cry2Aa protein produced by the *B.t.k.* bacterium. The *B.t.k.* strain controls insect pests by the production of crystalline insecticidal proteins known as delta-endotoxins. These proteins are produced as the bacterium enters the sporulation phase and can account for approximately one-third of the weight of the bacterial cell.

Cry proteins are insecticidal proteins that exhibit a complex, multicomponent mode of action (English and Slatin, 1992). Ultimately, the proteins bind to specific sites in the midgut epithelium cells of susceptible insects, opening cation-selective channels in the cell membrane. The cells swell due to an influx of ions and water, leading to cell lysis and ultimately the death of the insect (Höfte and Whitely, 1989).

Mammalian species are not susceptible to Cry proteins, including Cry2Ab. This may be explained in part by the fact that conditions required for the complex steps in the mode of action described by English and Slatin (1992) do not exist in mammals or most invertebrates. No receptors for Cry proteins have been identified on intestinal cells of mammals such as rats and rabbits (Sacchi *et al.*, 1986; Hofman *et al.*, 1988). The results of some of these studies have been published in scientific reviews (Ignoffo, 1973; Shadduck *et al.*, 1983; Siegel and Shadduck, 1989). These scientific considerations support the history of safe use of *B. thuringiensis* preparations. Based on the available scientific data, EPA and other regulatory scientists have determined that use of registered microbial *B. thuringiensis* products pose no significant risks to human health or non-target organisms.

ii. Animal Safety Testing of Cry Proteins in *B.t.* Microbial Formulations

The low mammalian toxicity of *B.t.* microbial insecticide mixtures containing Cry proteins has been demonstrated in numerous safety studies (EPA, 1998; Monsanto, 1997) conducted over the last 40 years. These include subchronic and chronic feeding studies and acute oral, dermal and inhalation studies in rats. Additionally, primary eye irritation, acute oral and acute dermal studies have been conducted in rabbits, and a subacute dietary study was conducted in humans. A number of these toxicology studies have been published (DeBarjac, *et al.*, 1980; Fisher and Rosner, 1959; Meeusen and Atallah, 1990; Shadduck, 1983; Siegel *et al.*, 1987). Extensive review of these studies by EPA, initially in 1982 and again in 1989, and the overall conclusion of lack of toxicity of Cry proteins, led EPA to focus the testing requirements for microbial-derived products on acute oral, pulmonary and intravenous toxicity studies (EPA, 1989; Sjoblad *et al.*, 1992). Again in 1998, following review of all applicable safety studies conducted with *B.t.* products containing Cry proteins, EPA concluded that "Toxicology studies submitted to the U.S. Environmental Protection Agency to support the registration of *B. thuringiensis* subspecies have failed to show any significant adverse effects in body weight gain, clinical observations, or upon necropsy." EPA also concluded that "The large volume of submitted toxicology data allows the conclusion that the tested subspecies are not toxic or pathogenic to mammals including humans" (EPA, 1998).

Cry2Aa protein exhibits a high degree of amino acid similarity (97%) to the Cry2Ab protein produced in cotton (Figure 4). Thus, safety studies conducted with microbial *B.t.* products containing Cry2A proteins are relevant to the safety assessment of Cry2Ab protein. As shown in Table 14, Cry2A protein, as a component of various *B.t.* microbial products, has been tested in acute, subchronic and chronic toxicity studies with rats, rabbits, sheep and humans. The highest doses administered to animals in these studies produced no observable effects (NOEL), consistent with the absence of toxicity of other Cry proteins when fed at high doses to mammals.

iii. History of Safe Consumption of *B.t.* Residues on Food Crops

There is a history of safe dietary exposure to *B.t.* residues in or on raw agricultural commodities. EPA has recognized the potential for dietary exposure to Cry proteins from use of microbial sprays on food crops: "The use patterns for *Bacillus thuringiensis* may

result in dietary exposure with possible residues of the bacterial spores on raw agricultural commodities. However, in the absence of any toxicological concerns, risk from the consumption of treated commodities is not expected for both the general population and infants and children" (EPA, 1998) and "*B.t.* has not been reported to cause adverse health effects on human health when present in drinking-water or food." (IPCS, 1999).

B.t. microbial formulations have been applied for decades to raw agricultural commodities that are consumed in unprocessed form by humans. These include berry crops, cabbage, grapes, tomatoes, celery, lettuce, and spinach (EPA, 1998). For certain crops, a significant percentage of the total amount grown in the United States has been treated with *B.t.* microbial preparations, *e.g.*, raspberries (30%), celery (46%), and cabbage (39%) (EPA, 1998). Residual levels of *B.t.* spores and microbes persist on foliage for several days following foliar application of microbial formulations (Leong *et al.*, 1980; Dynamac, 1986). Thus, if commodities such as celery are consumed within a few days of application, there could be dietary exposure to *B.t.* microbes and spores, as well as to *B.t.* Cry proteins.

There has been only limited sampling of raw agricultural commodities for *B.t.* residues; broccoli and cabbage leaves were reported to have mean residues of 10^6 to 10^7 *B.t kurstaki* (Dipel) spores/cm^2 leaf tissue (background *Bacillus* counts on unsprayed leaves were < 10^2 spores/cm^2) (Leong *et al.*, 1980). In separate *B.t.* residue trials, residual *B.t. kurstaki* levels expressed as spores/gram plant tissue from days 0-7 post treatment were: 10^6 for celery (background not reported), 10^6 for collard greens (background 10^3), 10^6 for kale (background 10^3) and 10^6 for lettuce (background not reported) (Dynamac, 1986). Exposure has also been shown to natural populations of *Bacillus thuringiensis* strains that contain crystal proteins active against lepidoptera in granaries in Korea (Kim *et al.*, 1998); they are also ubiquitous in soils (IPCS, 1999). Thus, there is a history of safe dietary and occupational exposure to Cry proteins, including those of the Cry2A class, which are highly similar to the Cry2Ab protein that is the subject of this request.

Table 14. No Observed Effect Levels for Microbial *B.t.* Preparations Containing Cry2A Proteins

Test Substance [1]	Animal Model	NOEL [2]	Reference
		Acute Oral Toxicity Studies	
Crymax	Rat	> 2.5-2.8 x 10^8 CFU/rat	Carter & Liggett, 1994
Crymax	Rat	> 5050 mg/kg	EPA, 1996b
Cutlass OF	Rat	> 10^8 CFU/rat	David, 1989
Dipel	Rat	> 2670 mg/kg	EPA, 1996b
Dipel	Rat	> 3.4 x 10^{11} spores/kg	EPA, 1986
Dipel	Rat	> 4.7 x 10^{11} CFU/kg	EPA, 1986
Dipel	Rat	> 5000 mg/kg	EPA, 1986
Dipel	Rabbit	> 2x10^9 spores/animal	EPA, 1986
Dipel	Rabbit	> 6.9 x 10^7 spores/kg	EPA, 1986
		Subchronic Oral Toxicity Studies	
Dipel	Rat	>8400 mg/kg/day/90 days	McClintock *et al.*, 1995
Dipel	Sheep	10^{12} spores/day/153 days	Hadley *et al.*, 1987
Dipel	Rat	1.3 x 10^9 spores/kg/day	McClintock *et al.*, 1995
		Chronic Oral Toxicity Study	
Dipel	Rat	8400 mg/kg/day/2 years	McClintock *et al.*, 1995
		Human Oral Toxicity Study	
Dipel	Human	1000 mg/day/3 days	McClintock, *et al.*, 1995; EPA, 1986

[1] Crymax contains Cry2A, Cry1Ac, Cry1C
Cutlass OF contains Cry2A, Cry1Aa, Cry1Ab, Cry1Ac
DIPEL contains Cry2A, Cry1Aa, Cry1Ab, Cry1Ac

[2] These NOELs represent the highest doses tested. Doses are expressed in various units for *B.t.* microbial technical grade materials, *e.g.*, milligrams technical ingredient per kilogram body weight, or more commonly CFU or spores per animal or kilogram body weight. It is not possible to directly compare doses on a milligram technical material per kilogram of body weight basis. This is due to the fact that colony-forming units (CFU) or spore count can range from approximately 10^8 to 10^{11} per gram of technical grade *B.t.* microbial material (McClintock *et al.*, 1995). Secondly, the Cry protein content in different *B.t.* microbial preparations may vary depending on the microorganism and fermentation conditions. Cry2A protein dosages administered to animals in the referenced studies range from milligrams to grams per kilogram of body weight.

iv. Safety Testing of Cry2Ab Protein

Assessment of the safety of the Cry2Ab protein to humans and animals includes information characterizing the biological and physicochemical properties of the introduced protein, and assessments of the digestibility, potential allergenicity and mammalian toxicity of the protein. Table 15 lists each of the confirmatory protein safety studies for Cry2Ab and the equivalent studies previously conducted by Monsanto for the Cry2Aa protein. The following sections summarize the results of numerous studies conducted by Monsanto demonstrating that Cry2Ab is not toxic to mammals and therefore presents an acceptable risk to human safety. Furthermore, the conclusions of safety studies for the Cry2Ab protein are in agreement with data confirming the safety of the Cry2Aa protein, which is highly similar to the Cry2Ab protein.

Table 15. Summary of Protein Safety Studies for Cry2Ab and Cry2Aa Proteins.

Assessment Study	Test Substance	Results
In vitro Digestive Fate	Cry2Ab	Half-Life <15 sec in SGF; digested to stable tryptic core in SIF [1]
	Cry2Aa	Half-Life <15 sec in SGF; digested to stable tryptic core in SIF [1]
Allergen Sequence Similarity	Cry2Ab	Not homologous to known protein allergens
	Cry2Aa	Not homologous to known protein allergens
Toxin Sequence Similarity	Cry2Ab	Not homologous to known protein toxins or other proteins of concern to human health
	Cry2Aa	Not homologous to known protein toxins or other proteins of concern to human health
Acute Mouse Oral Toxicity	Cry2Ab	No effects at highest dose tested, 1450 mg/kg
	Cry2Aa	No effects at highest dose tested, 4011 mg/kg

[1] SGF = Simulated Gastric Fluid; SIF = Simulated Intestinal Fluid

a) Characterization of the Introduced Cry2Ab Protein

Due to the extremely low levels of Cry2Ab protein produced in cotton, it was necessary to produce sufficient quantities of Cry2Ab protein by bacterial fermentation for the development of analytical methods (*e.g.*, ELISA) and to conduct safety studies. Cry2Ab protein was produced in and purified from *Bacillus thuringiensis* strain EG7699. Characterization of this *B.t.* protein preparation was done using analytical methods and functional tests specifically selected to assess the identity, concentration, strength in bioassay, purity and composition. In addition, solubility and storage stability studies were performed. The Cry2Ab protein produced by *Bacillus thuringiensis* strain EG7699 was shown to have equivalent molecular weight and immunoreactivity to the protein

expressed in cotton, to lack detectable post-translational modification (glycosylation), to have equivalent electrophoretic mobility and detection with specific antibodies, and to have similar functional activity. Thus, the Cry2Ab proteins derived from both bacterial fermentation and plant sources were established to be physicochemically and functionally equivalent.

b) Digestion of Cry2Ab Protein in Simulated Gastric and Intestinal Fluids

The purpose of this study was to assess the *in vitro* digestibility of Cry2Ab protein (apparent molecular weight of ≈63 kDa) using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) mammalian digestion models. The Cry2Ab protein was incubated in SGF and SIF at 37 °C for up to 2 hours and 24 hours, respectively. Protein stability was assessed at specific time points using SDS-PAGE (limit of detection, 10 ng; limit of resolution, ≥2 kDa) and/or immunoblotting (limit of detection, 5 ng; limit of resolution, ≥2 kDa) for each incubation. Moreover, a *Helicoverpa zea* insect bioassay (EC_{50}) was used to assess Cry2Ab protein functional activity remaining after selected incubation times.

SDS-PAGE analysis of SGF incubations showed that by 15 seconds greater than 98% of the Cry2Ab protein was digested and that no fragments ≥2 kDa of the parent protein were resolved. Immunoblot analysis of SIF incubations showed that a relatively stable Cry2Ab protein fragment (≈50 kDa) was produced within 1 minute and observed for at least 24 hours. Cry1, Cry2 and Cry3 class proteins yield stable, tryptic core fragments when incubated in SIF (Monsanto, 1997). These observations were corroborated by insect bioassays showing rapid loss of activity in SGF and stable activity in SIF.

This *in vitro* assessment of Cry2Ab protein digestibility indicates that the Cry2Ab protein will be readily digested in the mammalian stomach.

c) Allergenic Potential of the Cry2Ab Protein

Although large quantities of a range of proteins are consumed in human diets each day, rarely do any of these tens of thousands of proteins elicit an allergenic response (Taylor, 1992). Although there are no predictive bioassays available to assess the allergenic potential of proteins in humans (FDA, 1992), physicochemical and human exposure profiles of the protein provide a basis for assessing potential allergenicity relative to known protein allergens. Thus, important considerations contributing to the allergenicity of proteins ingested orally include exposure and an assessment of the factors that contribute to exposure, such as stability to digestion, prevalence in the food, and consumption pattern (amount) for the specific food (Metcalfe *et al.*, 1996; Kimber *et al.*, 1999).

A key parameter contributing to the systemic allergenicity of certain food proteins appears to be stability to gastrointestinal digestion, especially stability to acid proteases like pepsin found in the stomach (Astwood *et al.*, 1996; Astwood and Fuchs, 1996; Fuchs and Astwood, 1996; FAO, 1995; Kimber *et al.*, 1999). Important protein allergens tend to be stable to peptic digestion and the acidic conditions of the stomach if they are to reach the intestinal mucosa where an immune response can be initiated. As noted above,

the *in vitro* assessment of the Cry2Ab protein digestibility indicates that the protein will be readily digested.

Another significant factor contributing to the allergenicity of certain food proteins is their high concentrations in foods (Taylor, 1992; Taylor *et al.*, 1987; Fuchs and Astwood, 1996). Most allergens are present as major protein components in the specific food representing from 2-3% up to 80% of total protein (Fuchs and Astwood, 1996). This is true for the allergens in milk (Taylor *et al.*, 1987; Baldo, 1984; Lebenthal, 1975; Taylor, 1986), soybeans (Shibasaki *et al.*, 1980; Burks *et al.*, 1988; Pederson and Djurtoft, 1989), and peanuts (Barnett *et al.*, 1983; Sachs *et al.*, 1981; Barnett and Howden, 1986; Kemp, 1985). In contrast to this generality for common allergenic proteins, Cry2Ab protein is present at low levels in these plants (<0.004% seed on a dry weight basis).

It is also important to establish that the protein does not represent a previously described allergen, and further, does not share potentially immunologically relevant epitopes (amino acid sequences recognized by IgEs). *Bacillus thuringiensis* and its formulations used as microbial pesticides have not been described as sensitizing allergens, including through oral exposure (McClintock *et al.*, 1995). Thus there is no apparent history of allergy associated with crystal proteins from *B.t.* In addition, the amino acid sequence of the Cry2Ab protein was compared to protein sequences associated with allergenicity, as described below.

A database of protein sequences associated with allergy and coeliac disease was assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt) and from current literature. The keyword "allergen" was used to retrieve allergen sequences from the public domain databases. Additional unique allergens found only in current literature were appended, creating an updated database containing 567 unique protein sequences. The deduced amino acid sequence of the Cry2Ab protein was compared to these sequences using the sequence alignment tool FASTA. Cry2Ab shares no structurally-significant sequence similarity to sequences within the allergen database and does not share potential immunologically-relevant amino acid sequences greater than seven contiguous identical amino acids. Cry2Aa also shares no significant sequence similarity with known allergen sequences.

The data and analyses described above and summarized in Table 16 support the conclusion that the Cry2Ab protein does not pose a significant allergenic risk, as it is not derived from an allergenic source, does not possess immunologically relevant sequence similarity with known allergens and does not possess the characteristics of known protein allergens.

Table 16. Characteristics of Known Allergenic Proteins[a]

Characteristic	Allergens	Cry2Ab
Allergenic source of gene	yes	no
Stable to digestion	yes	no
Similar sequence to allergens	yes	no
Prevalent protein in food	yes	no

[a] As described in Taylor (1992) and Taylor *et al.* (1987)

d) Toxic Potential of the Cry2Ab Protein

Bioinformatics

The safety assessment of a protein expressed in genetically modified crops includes structural comparisons of the introduced protein with proteins associated with toxicity or other adverse health effects. Specifically, a biologically-relevant sequence similarity to a known toxin (*i.e.*, a sequence apparently derived from a common ancestor gene) may indicate that additional toxicological assessments be done.

A database of 4677 protein sequences associated with toxicity has been assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt). The deduced amino acid sequence of the Cry2Ab protein was compared to sequences in this toxin database using the FASTA sequence alignment tool. In addition, the deduced amino acid sequence of the Cry2Ab protein was compared to all protein sequences in publicly-available genetic databases to screen for structural similarity to pharmacologically-active proteins. Apart from expected similarities to other known crystal (Cry) proteins found in *Bacillus thuringiensis* and related species, no additional significant structural similarities were observed.

The results of these bioinformatics analyses indicate that the Cry2Ab protein is not similar to any toxin or other protein relevant to animal or human health. Likewise, the Cry2Aa protein shares no significant sequence similarity with protein toxins relevant to animal or human health.

Acute Oral Toxicity of Cry2Ab Protein in Mice

The low mammalian toxicity of *B.t.* microbial insecticide mixtures containing Cry2A protein has been demonstrated in numerous safety studies (Sjoblad *et al.*, 1992). Acute administration is considered appropriate to confirm the safety of Cry2Ab, because proteins that are toxic typically act via acute mechanisms (Sjoblad *et al.*, 1992; Pariza and Foster, 1983; Jones and Maryanski, 1991).

Three groups of ten male and ten female mice were given acute, oral dosages of Cry2Ab protein at 67.3, 359 or 1450 mg/kg body weight, respectively. A separate group of ten male and ten female protein control animals received bovine serum albumin at a dose of 1200 mg/kg. The doses administered were designed to evaluate the potential hazards of the Cry2Ab protein at the highest acute oral dose that could be delivered to mice.

There were no adverse effects attributed to the oral administration of Cry2Ab protein in male and female mice at doses of 67.3, 359, or 1450 mg/kg body weight. The No-Observed-Effect-Level (NOEL) for toxicity of Cry2Ab protein administered as an acute dose by gavage to mice was considered to be at least 1450 mg/kg, the highest tested dose. The highest dose administered represented the highest feasible dose based on test system capacity and protein solubility. This NOEL is comparable to those determined for other Cry proteins in the Cry2A class (Table 14).

e) Calculation of Exposure Margins for Consumption of Cry2Ab Protein in Food and Feed Derived from Bollgard II Cotton

The final question in the safety assessment of the newly introduced protein is whether or not it is likely to be a significant component in the human or animal diet (Figure 9). There will be negligible human or animal dietary exposure to the Cry2Ab protein present in transgenic cotton. The human consumable fractions of cotton are cottonseed oil and linters (NCPA 1990). Both are processed both chemically and thermally such that all proteins, including the *B.t.* protein, would be removed or denatured (Sims *et al.*, 1996; Sims and Berberich, 1996).

Although exposure to Cry2Ab protein is considered to be negligible, a dietary exposure margin was calculated based on the worst-case assumption that Cry2Ab protein could survive processing and be present at very low levels in cottonseed oil. A dietary exposure margin for dairy cow consumption of whole cottonseed was also calculated (Table 17).

In a two-year chronic rat feeding study with Dipel®, a *B.t.* microbial formulation containing Cry2A protein, the NOEL was considered to be 8400 mg/kg/day (Table 14). Even if Cry2A protein represented only 1% of the product tested, the daily Cry2A dose over most of the rats' lifetimes would have been 84 mg/kg/day, which is also several orders of magnitude higher than the worst-case human exposures to Cry2Ab protein from consumption of cotton-derived food products (Table 17). Based on these extremely large exposure margins and the absence of toxicity in animal safety studies, there would be no unreasonable risks to, or adverse effects expected in humans or farm animals, from consumption of food and feed products derived from Bollgard II cotton.

c. Cry2Ab Human Health and Safety Conclusions

The Cry2Ab protein has been shown to be safe for consumption by both humans and animals by the:

1. general recognition of the safety of Bt proteins, including those of the Cry2A class;
2. high degree of sequence similarity of the encoded proteins of the *cry2Ab* and the *cry2Aa* genes present in commercial *B.t.* formulations, which have a history of safe use;
3. rapid digestion of Cry2Ab in SGF and conversion to the expected tryptic core protein in SIF, as expected for Bt proteins;
4. lack of homology of Cry2Ab with known allergens;
5. lack of homology of Cry2Ab with any known protein toxins or other proteins associated with adverse mammalian or human health effects;
6. lack of acute toxicity of Cry2Ab to mammals, as demonstrated by a mouse acute oral gavage study; and
7. low dietary exposure to the Cry2Ab protein from consumption of cotton food products.

These facts support the conclusion of "No Concerns" as listed on Figure 9 for the introduced protein Cry2Ab in Bollgard II cotton event 15985.

Table 17. Calculated Dietary Exposure Margins for Cry2Ab Protein

A.		HUMANS		
Food/Feed	Cry2Ab Level in Food/Feed	Daily Food/ Feed Consumed	Dose (mg/kg)	Exposure Margin
Cottonseed oil	2.6×10^{-4} µg/gram	0.07 grams/kg	1.8×10^{-8}	8×10^{10}

- Assume cottonseed protein is present in refined cottonseed oil at the limit of detection of the assay (1.3 µg protein/ml) of oil since none was detected.
  Assume 1 ml oil = 1 g oil.
  Cry2Ab represents 0.02% of the total protein in cottonseed.
  Therefore, 1.3 µg/ml x 0.02% = **2.6×10^{-4} µg Cry2Ab/g oil**
- Total disappearance of cottonseed oil in food is estimated to be 9×10^8 lb/yr (personal communication, NCPA).
  The US population is 270×10^6 people.
  Assume average human weight of 60kg.
  Therefore, (9×10^8 lb/yr) / (270×10^6 people) = 3.3 lb per capita per year
  [(3.3 lb/capita/yr)/ 365 days] x 453.6 g/lb = 4.1 g per capita per day
  (4.1 g/capita/day)/ 60 kg = **0.07 g cottonseed oil/kg bodyweight**
- Dose = (0.07 g oil/kg) x (2.6×10^{-4} µg Cry2Ab/g oil) = **1.8×10^{-8} mg/kg**
- Exposure Margin = (NOEL from Cry2Ab mouse gavage study)/(Human Dose) =
  (1450 mg/kg) / (1.8×10^{-8} mg/kg) = **8×10^{10}**

B.		DAIRY COW		
Food/Feed	Cry2Ab Level in Food/Feed	Daily Food/ Feed Consumed	Dose (mg/kg)	Exposure Margin
Cottonseed	43.2 µg/gram	5.3 grams/kg	2.3×10^{-1}	6.3×10^3

- Assume average dairy cow weighs 600 kg (Hoard's Dairyman, 1984)
- Assume a dairy cow eats 7 lb of cottonseed per cow per day (Hoard's Dairyman, 1984)
  Therefore, (7 lb/cow/day) x (453.6 g/lb) = 3171 g cottonseed per cow per day
  (3171 g cottonseed/cow/day) / (600 kg/cow) = **5.3 g seed/kg/day**
- Dose = (5.3 g seed/kg/day) x (43.2 µgCry2Ab/g seed) = 229 µg Cry2Ab/kg/day
- Exposure Margin = (NOEL from Cry2Ab mouse gavage study)/(Cow Dose) =
  (1450 mg/kg) / (2.3×10^{-1} mg Cry2Ab/kg/day) = **6.3×10^3**

2. The GUS Protein

To address the FDA flowchart "Safety Assessment of New Varieties: Proteins Introduced from Donor(s)" (Figure 9), the protein expression levels from the introduced *uidA* gene are provided, as well as an evaluation of the safety of the expressed protein, GUS.

a. Expression of the GUS Protein in Bollgard II Cotton Event 15985

A validated Enzyme-Linked Immunosorbent Assay (ELISA) was performed to estimate the GUS protein levels in the plant leaf and seed tissue samples. Samples were collected from eight field locations in the United States during 1998 field trials as described previously for the compositional analyses and Cry2Ab expression. The mean level of GUS protein in Bollgard II cotton event 15985 was 58.8 µg/g in cottonseed, with a range of values from 37.2 - 82.3 µg/g fwt. The range of GUS expression in cotton leaf tissue was 51.7 - 176 µg/g fwt, with a mean value of 106 µg/g fwt.

b. History of Safe Consumption of the GUS Protein

The history of safe use of the GUS protein is extensive. Exposure of humans to the GUS protein is commonplace through intestinal epithelial cells and intestinal microflora, bacterial exposure and numerous foods containing the GUS protein with no known harmful effects (Gilissen, *et al.*, 1998). GUS activity has been detected in over 50 plant species in various tissues including embryo, fruit, seed coat and endosperm (Hu *et al.*, 1990). These species include a number of human food sources, including potato, apple, almond, rye, rhubarb, and sugar beet (Schulz and Weissenbock, 1987; Hodal *et al.*, 1992; Wozniak and Owens, 1994). GUS is also present in beef and in a number of invertebrate species, including nematodes, molluscs, snails, and insects (Gilissen *et al.*, 1998). Even when ingested in raw foods such as shellfish or apples, GUS is not known to cause harmful effects (Gilissen *et al.*, 1998). Likewise, the metabolites of *E. coli* GUS activity are non-toxic (Gilissen *et al.*, 1998).

The *E. coli*-derived GUS enzyme expressed by Bollgard II cotton event 15985 is 99.8% homologous and functionally equivalent to the GUS enzyme from *E. coli* naturally present in the human gut. The 0.2% non-homology is due to the addition of a restriction site at the beginning of the sequence for plant transformation purposes. Therefore, the GUS protein produced in Bollgard II cotton event 15985 is substantially similar to an edible protein, as noted on the FDA decision tree in Figure 9.

c. Allergenic Potential of the GUS Protein

The *uidA* gene was not obtained from a source known to be allergenic. GUS was obtained from *E. coli* (Jefferson, 1986), a bacteria prevalent in the gastrointestinal tract of man and other animal species. A database of protein sequences associated with allergy and coeliac disease was assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt) and from current literature. Additional unique allergens found only in current literature were appended creating a database containing 567 unique protein sequences. The amino acid sequence of the GUS protein was compared to these sequences using the sequence alignment tool FASTA. The GUS protein sequence did not share any structurally significant sequence similarity to sequences within the allergen database.

Another significant factor contributing to the allergenicity of proteins is their high concentrations in foods that elicit an allergenic response (Taylor, 1992; Taylor *et al.*, 1987; Taylor *et al.*, 1992; Fuchs and Astwood, 1996). Most allergens are present as

major protein components in the specific food representing from 2-3% up to 80% of total protein (Fuchs and Astwood, 1996). This is true for the allergens in milk (Taylor *et al.*, 1987; Baldo, 1984; Lebenthal, 1975; Taylor, 1986), soybeans (Shibasaki *et al.*, 1980; Burks *et al.*, 1988; Pederson and Djurtoft, 1989), and peanuts (Barnett *et al.*, 1983; Sachs *et al.*, 1981; Barnett and Howden, 1986; Kemp, 1985). In contrast to this generality for common allergenic proteins, GUS protein is present at low levels in these plants (<0.007% dry weight in the seed).

d. Digestion of GUS Protein in Simulated Gastric and Intestinal Fluids

A key parameter contributing to the allergenicity of food allergens appears to be stability to gastrointestinal digestion, especially stability to acid proteases like pepsin found in the stomach (Astwood and Fuchs, 1996; Fuchs and Astwood, 1996; FAO, 1995). Protein allergens must be stable to the peptic digestion and the acid conditions of the stomach system if they are to reach and pass through the intestinal mucosa where an immune response can be initiated.

The GUS protein degraded rapidly when added to simulated gastric and intestinal fluids (SGF and SIF), which simulate human digestion, as assessed by both western blot analysis and enzymatic activity assays. Within 15 seconds of exposure to SGF, there was no detectable GUS protein by western blot or enzymatic activity. After two hours in SIF, a very faint band was observed in the western blot and the protein had lost approximately 91% of its original enzymatic activity. Based on these results, it is concluded that the GUS protein, if ingested by humans, will readily degrade in the digestive tract (Fuchs and Astwood, 1996). However, any additional human exposure to this protein from cotton-derived food products would not be expected since the processing removes or denatures the protein.

e. Toxic Potential of the GUS Protein

A database of protein sequences associated with toxicity was also assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt). The keyword "toxin" was used to retrieve 4677 toxin sequences from the public domain databases. The amino acid sequence of the GUS protein was compared to protein sequences in the toxin database using the FASTA sequence alignment tool. In addition, the amino acid sequence of the GUS protein was compared to all protein sequences in the publicly available genetic databases to screen for structural similarity to other known proteins, including pharmacologically active proteins. The test sequence, GUS, shared sequence similarities to homologous *Escherichia coli* and other glucuronidase proteins, as expected. These proteins have not been described as toxins relevant to human health. No other structural homology was observed.

f. Acute Mouse Gavage Study with the GUS Protein

Acute administration was considered appropriate to assess the safety of GUS, since proteins that are toxic typically act via acute mechanisms (Sjoblad *et al.*, 1992; Pariza and Foster, 1983; Jones and Maryanski, 1991). The GUS protein used in this evaluation was over-produced and purified from *Escherichia coli*, characterized and administered by

gavage to mice in an acute toxicity test at target doses of 0, 1, 10, and 100 mg/kg body weight.

There were no treatment-related adverse effects in mice administered GUS protein by oral gavage at actual dosages up to 69 mg/kg, the highest dose tested. There were no statistically significant differences in body weight, cumulative body weight or food consumption between the vehicle or bovine serum albumin protein control groups and GUS protein-treated groups. Results demonstrated that the GUS protein is non-toxic to mice.

The highest dose tested represents a 4 x 10^8 safety margin relative to the average U.S. human consumption of cottonseed oil (4 g/day) and protein levels in the oil at the limit of detection of the assay (1.3 µg protein/ml of oil). Previous feeding studies with large doses of *Escherichia coli* strain K12 containing GUS in humans and animals have also demonstrated the safety of the GUS protein, since no adverse effects were observed (Flamm, 1993).

g. Calculation of Exposure Margins for Consumption of GUS Protein in Food and Feed Derived from Bollgard II Cotton

The final questions in the safety assessment of the GUS Protein (Figure 9) involve the exposure of humans and animals to the protein in the diet, specifically whether the protein will be consumed at similar levels in other foods or as a macroconstituent of the diet. There will be virtually no exposure to the GUS protein from genetically modified cotton. As described above in Section 2a, the GUS protein is present in the cottonseed at very low levels (<0.007% seed, dry weight). The only human consumable fractions of cotton are cottonseed oil and linters used as food additives (NCPA, 1990). Each of these fractions is processed chemically and with heat such that transgenic protein should be denatured (Sims *et al.*, 1996; Sims and Berberich, 1996). Additionally, the lack of stability of the GUS protein suggests that cooking or high temperature processing of foods would eliminate the protein activity (Jefferson and Wilson, 1991). Further, rapid gastric digestion of the protein would also limit the direct exposure to humans and animals. Therefore, the amount of additional GUS enzyme exposure to humans from Bollgard II cotton event 15985 is insignificant and, therefore, of no health concern.

h. Conclusions

The GUS protein has been shown to be safe for consumption by both humans and animals by the:

1. natural occurrence of the GUS protein in the human gut and other organisms, including foods, and a history of safe use in foods;
2. lack of allergenic potential of GUS;
3. rapid digestion of GUS in simulated gastric and intestinal fluids;
4. lack of homology of GUS with any known protein toxins;
5. lack of acute toxicity of GUS to mammals as determined by a mouse acute oral gavage study.

These facts support the conclusion of "No Concerns" as listed in Figure 9 for the introduced protein GUS in Bollgard II cotton event 15985.

V. Conclusion for the Safety Assessment of Bollgard II Cotton Event 15985

Monsanto Company has developed a new, genetically modified cotton event, Bollgard II, using particle acceleration plant transformation procedures to insert the *cry2Ab* insect control gene and the *uidA* scorable marker gene into the Bollgard cotton genome. This new event provides effective control of insect pests such as the cotton bollworm, tobacco budworm and pink bollworm, as well as armyworm. This product has potential use as an additional insect resistance management tool.

The host plant, cotton, has a long history of safe use. The results of extensive compositional analyses of the cottonseed, oil and meal demonstrate that the levels of the important nutritional and antinutritional components in event 15985 are comparable to the parental variety and are within established ranges for commercial cotton varieties. The two additional proteins in event 15985 are present at very low levels in cottonseed and are unlikely to remain in highly processed cotton food and feed products. The safety of the introduced proteins has been assessed through 1) history of safe food and feed use of the proteins or highly similar proteins, 2) determination of no allergenic potential of the introduced proteins, and 3) determination of no toxic potential of the introduced proteins.

These data lead to a conclusion of "no concerns" for every criterion in the flowcharts outlined in the FDA's Food Policy. Bollgard II cotton event 15985 is not materially different in composition, safety, or any relevant parameter from cotton now grown, marketed, and consumed. Sales and consumption of food and feed products derived from Bollgard II cotton event 15985 would be fully consistent with the Agency's Food Policy, the Federal Food, Drug, and Cosmetic Act, and current practices for the development and introduction of new cotton varieties.

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Appendix 1. Molecular Characterization of Bollgard II Cotton Event 15985

1. PURPOSE

The purpose of this study was to characterize the additional DNA inserted into Bollgard® cotton line DP50B to produce the Cry2Ab2 cotton event 15985. Genomic DNA was analyzed by Southern blotting to determine the number of insertion events, the copy number(s) of the inserted DNA, the integrity of the inserted promoters, coding regions, and polyadenylation sequences, and the presence or absence of plasmid backbone sequence. All analyses were performed with both the initial Bollgard® cotton line DP50B and with the newly produced 15985 event to characterize the newly inserted DNA. In addition, the flanking sequence of the 5' and 3' insert-to- plant junctions (previously determined by Genome Walking) were confirmed by PCR.

2. SUMMARY

Southern blot analysis was used to determine the insert number (number of integration loci within the cotton genome), the copy number (the number of transgenes at a single locus), the intactness of the *cry2Ab2* and *uidA* coding regions, the intactness of the *cry2Ab2* and *uidA* cassettes, and to confirm the absence of plasmid backbone sequence derived from plasmid PV-GHBK11. Plasmid PV-GHBK11, the plasmid backbone, the *cry2Ab2* and *uidA* coding regions, the enhanced CaMV 35S promoter, and the NOS 3' polyadenylation sequence were all used as probes. Additionally, the 5' and 3' insert → plant junctions were verified using the polymerase chain reaction (PCR).

The data show that Cry2Ab2 cotton event 15985 contains one DNA insertion, in addition to the insert present in the parental line DP50B. The insert contains one copy of both the *cry2Ab2* and the *uidA* cassettes; the *cry2Ab2* coding region and cassette are intact and the *uidA* coding region and its NOS 3' polyadenylation sequence are intact, however 260 bp of the 5' end of its enhanced CaMV 35S promoter are not present. This event does not contain any detectable backbone sequence derived from plasmid PV-GHBK11. It is therefore concluded that only full-length Cry2Ab2 and GUS proteins should be produced in 15985 as a result of integration of the DNA segment derived from plasmid PV-GHBK11.

Summary Tables of Molecular Characterization Data for Cotton Event 15985

Cotton Event 15985	
# of new Insertions	One
# of copies of *cry2Ab2* and *uidA* cassettes	One of each

Genetic Element	
enhanced CaMV 35S promoter (*uidA*)	Missing 260 bp from 5' end (~40%)
uidA coding region	Intact
NOS 3' polyadenylation sequence (*uidA*)	Intact
enhanced CaMV 35S promoter (*cry2Ab2*)	Intact
cry2Ab2 coding region	Intact
NOS 3' polyadenylation sequence (*cry2Ab2*)	Intact
Backbone DNA	Not detected

3. MATERIALS AND METHODS

3.1 Test substance.
The test substance for this study was the insect protected cotton event 15985.

3.2 Control substances.
The control substances for this study were cotton lines DP50 (non-transgenic) and DP50B (*cry1Ac* event 531, negative for *cry2Ab2*). Although the protocol specified the use of greenhouse grown leaf tissue for DP50, leaf tissue was obtained from other sources (*i.e.*, Production Plans 98-01-36-03, 98-32-36-31, and 99-01-36-03).

3.3 Reference substances.
Plasmid PV-GHBK11, the source plasmid, served as the primary reference substance in these analyses. The plasmid, mixed with DNA from the DP50 control substance, was used as a size indicator and a positive hybridization control in Southern blot analysis. Additionally, molecular size markers from Boehringer Mannheim [Molecular Weight Markers II (23.1 Kb-0.6 Kb) and IX (1.4 Kb-0.072 Kb), catalog #236 250 and #1449 460, respectively] and Gibco BRL [High Molecular Weight DNA Marker (48.5 Kb-8.3 Kb) and 100 bp ladder (2.1 Kb-0.1Kb), catalog #15618-010 and #15628-019, respectively] were used for size estimations.

3.4 Southern blot strategy.
Genomic DNA from insect protected cotton event 15985 was digested with a variety of restriction enzymes and subjected to Southern blot hybridization analysis to characterize the DNA encoding Cry2Ab2 and GUS integrated into the genome of DP50B. A map showing the linear DNA fragment, PV-GHBK11L, that was used to generate the 15985 transgenic cotton event, along with the locations of the restriction sites utilized for Southern analysis, is shown in Appendix 1, Figure 2. The Southern blot figures present in this report are representative of the data generated in the study. The methods listed are representative of those used to generate the data in this report.

3.5 DNA isolation.
DNA extracted from leaf tissue was used for all of the analyses in this report except for the nontransgenic sample on the *uidA* gene cassette intactness blot probed with the NOS 3' polyadenylation sequence probe which was isolated according to the method of Rogers and Bendich (1985). Leaf tissue was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Approximately 1 g of the ground leaf tissue was transferred to 13 ml centrifuge tube containing 6 ml of the extraction buffer [2.5 ml DNA extraction buffer (350 mM Sorbitol, 100 mM Tris pH 7.5, 5 mM EDTA), 2.5 ml Nuclei lysis buffer (200 mM Tris pH 7.5, 50 mM EDTA, 2 M NaCl, 2% CTAB), and 1 ml Sarkosyl (5% solution)]. The samples were incubated at 65°C for approximately 30 minutes with intermittent mixing. Four and a half milliliters of a mixture of chloroform:isoamyl alcohol (24:1) at room temperature was added to the samples. The suspension was mixed for 2 to 3 minutes, and the two phases separated by centrifugation for 15 minutes at ~1,000 x *g* at 4°C. The aqueous (top) layer was removed using a transfer pipet and placed into a 13 ml centrifuge tube. Five milliliters of 100% isopropanol

were added, and the tubes were mixed by inversion to precipitate the DNA. The precipitated DNA was pelleted by centrifuging at ~1,000 x *g* for 5 minutes at 4°C. The pellet was washed with approximately 1 ml of 70% ethanol and centrifuged for an additional 5 minutes at ~1,000 x *g* at 4°C . The DNA was allowed to dry at room temperature and re-dissolved in Tris-EDTA buffer at 4°C overnight.

3.6 DNA quantitation.
The purified genomic DNA was quantitated using a Hoefer DyNA Quant™ 200 Fluorometer (San Francisco, CA)(SOP BR-EQ-0065-01) with Boehringer Mannheim Molecular Weight Marker IX used as a calibration standard.

3.7 Restriction enzyme digestion.
Approximately 10 µg of genomic DNA from the test and control lines were used for the restriction enzyme digests. Overnight digests were performed at 37°C according to SOP GEN-PRO-010-01 in a total volume of 500 µl using 100 units of restriction enzyme. Some of the control digests were spiked with either 5 or 10 pg of PV-GHBK11. All restriction enzymes were purchased from Boehringer Mannheim. After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3M NaOAc and 2 volumes (~1 ml relative to the original digest volume) of 100% ethanol, followed by incubation at -20°C for at least one hour. The digested DNA was pelleted by centrifugation, washed with 70% ethanol, vacuum dried for 10-20 minutes, and re-dissolved at room temperature in either water or TE.

3.8 Agarose gel electrophoresis.
Digested DNAs were separated on 0.8% agarose gels in 1X TBE buffer according to SOP GEN-PRO-003-01. A 'long run' and a 'short run' were performed for each Southern blot analysis. The long run facilitated greater resolution of the higher molecular weight DNAs while the short run ensured that all smaller molecular weight DNAs were retained on the gel. The long run/short run involved a 4-6 hour electrophoresis at 80-85 V and an overnight (9-15 hour) run at 35-38 V. After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide for 20-30 minutes and photographed.

3.9 DNA probe preparation.
DNA from plasmid PV-GHBK11 was isolated from overnight *E. coli* cultures. Probe templates homologous to the *cry2Ab2* coding region, *uidA* coding region, the enhanced CaMV 35S promoter, the NOS 3' polyadenylation sequence, and the entire backbone region were prepared by PCR using PV-GHBK11 as the template.

Approximately 25 ng of each probe template, except the NOS 3' polyadenylation sequence, were labeled with ^{32}P-dCTP using the random priming method (RadPrime DNA Labeling System, Life Technologies). The NOS 3' polyadenylation sequence was labeled using PCR with NOS 3' template (15 ng), NOS 3' specific primers (0.25 µM each), 1.5 mM $MgCl_2$, 3 µM dATP, dGTP, and dTTP, 100 µCi of ^{32}P-dCTP and 2.5 Units of *Taq* DNA polymerase in a final volume of 20 µl. The cycling conditions were as follows: 1 cycle at 94°C for 3 minutes; 5 cycles at 94°C for 45 seconds, 55°C for 30

seconds, and 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. The radiolabeled probe was purified using a Sephadex G-50 column (Boehringer Mannheim).

3.10 Southern blot analysis.

Southern blot analyses (Southern, 1975) were performed according to SOP GEN-PRO-025-02. Following electrophoresis, the gel was incubated in depurination solution (0.125 N HCl) for ~ 10 minutes followed by denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes, and then neutralizing solution (0.5 M Tris-HCl pH 7, 1.5 M NaCl) for ~30 minutes. The DNA from the agarose gels was transferred to Hybond-N™ nylon membranes (Amersham) using a Turboblotter™ (Schleicher & Schuell). The DNA was allowed to transfer for 4 hours to overnight (in 20X SSC) and covalently cross-linked to the membrane with a UV Stratalinker™ 1800 (Stratagene) set to autocrosslink. The blots were prehybridized an average of 2 hours in an aqueous solution of 0.5 M sodium phosphate, 7% SDS (w/v), and 0.1 mg/ml *E. coli* tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 14-21 hours at approximately 65°C. Membranes were washed at least four times in an aqueous solution of 0.1% (w/v) SDS and 0.1X SSC for 15 minute intervals at 65°C. Multiple exposures of the blots were generated using Kodak Biomax MS™ film in conjunction with one Kodak Biomax MS™ intensifying screen. Blots were stripped according to SOP GEN-PRO-025-02 or by incubating the blot with boiling 0.1% (w/v) SDS and allowing it to cool to room temperature.

3.11 Insert number.

The insert number (the number of integration sites of newly introduced transgenic DNA in the cotton genome) was evaluated. The test and control DNAs were digested with the restriction enzyme *Sca*I, which does not cleave within the DNA segment used for transformation. This enzyme released a segment containing the inserted DNA and adjacent plant genomic DNA. The plasmid-spiked DP50 'short run' samples were also digested with *Xba*I to linearize the plasmid. The blot was probed with the reference plasmid PV-GHBK11.

3.12 Copy number.

The number of copies of the transformation cassette inserted into each locus was determined by digesting the test genomic DNA with the restriction enzyme *Sph*I, an enzyme that cuts only once in the linear DNA segment used to generate the event. The blot was probed with the reference plasmid PV-GHBK11.

3.13 *cry2Ab2* coding region intactness.

The integrity of the *cry2Ab2* coding region was determined by digestion with a restriction enzyme, *Nco*I, that cleaves at the 5' and 3' ends of the *cry2Ab2* coding region. The blot was probed with the full length *cry2Ab2* coding region.

3.14 *cry2Ab2* cassette intactness.

The integrity of the *cry2Ab2* cassette (enhanced CaMV 35S promoter, *cry2Ab2* coding region, and NOS 3' polyadenylation sequence) was assessed by digestion with the

restriction enzyme *Bam*HI, which cleaves at the 5' and 3' ends of the *cry2Ab2* cassette. The blot was sequentially probed with each element of the cassette.

3.15 *uidA* coding region intactness.
The integrity of the *uidA* coding region was determined by digestion with the restriction enzymes *Eco*RI and *Bgl*II, which cleave at the 5' and 3' ends of the *uidA* coding region, respectively. The blot was probed with the full length *uidA* coding region.

3.16 *uidA* cassette intactness.
The integrity of the *uidA* cassette (enhanced CaMV 35S promoter, *uidA* coding region, and NOS 3' polyadenylation sequence) was assessed by digestion with the restriction enzymes *Bam*HI and *Sph*I, which cleave at the 5' and 3' ends of the *uidA* cassette. The blot was sequentially probed with each element of the cassette.

3.17 Analysis for backbone fragments.
The backbone region of the plasmid is defined as the *Kpn*I restriction fragment of PV-GHBK11 that was not used to transform the plant. It consists of a bacterial origin of replication, ori-pUC, and the *nptII* gene under the control of a bacterial promoter. To confirm the absence of backbone, genomic DNA was digested with the restriction enzyme *Kpn*I and probed with the full-length backbone region.

3.18 Verification of 5' and 3' genomic flanking sequences.
The sequence of the 5' and 3' insert → plant genomic DNA junctions were determined previously using Clontech's Universal Genome Walker™ Kit. Primers were designed to verify these junctions by PCR. The 5' junction was verified using one primer designed to the 5' genomic flanking sequence paired with a second primer in the enhanced CaMV 35S promoter of the *uidA* gene. The 3' junction was verified using a primer designed to the 3' genomic flanking sequence with a second primer located in the *cry2Ab2* gene. The PCRs were conducted using 100 ng of leaf genomic DNA (1-2 µl) as a template, 10 pmol of each primer (1 µl each), and PCR Supermix (Gibco BRL cat no. 10572-014) in a 25 µl reaction volume. The amplification of the reactions was performed under the following cycling conditions: 1 cycle 94°C for 3 minutes; 30 cycles 94°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes; 1 cycle 72°C for 4 minutes. The PCR products were separated on a 1% agarose gel in 1X TAE and visualized by staining with ethidium bromide.

4. RESULTS AND DISCUSSION

4.1 Insert number.
Test and control DNA samples were digested with *Sca*I. DP50 control DNA spiked with PV-GHBK11 was also digested with *Sca*I. Since *Sca*I does not cleave within the plasmid, a second enzyme, *Xba*I, was added to linearize the plasmid. The plasmid was linearized to facilitate its migration through the gel to serve as an accurate size estimator. The blot was probed with radiolabeled PV-GHBK11 (Appendix 1, Figure 1), the source plasmid for the linear DNA segment used in the transformation. The results are shown in Appendix 1, Figure 3. The DP50 long run (lane 1) did not produce any detectable background bands. Plasmid PV-GHBK11 mixed with DP50 short run (lanes 4 and 5)

produced the expected size band at approximately 8.7 Kb, the size of the whole plasmid, with no additional bands. The DP50B long and short runs (lanes 2 and 6) produced two bands at approximately 22 Kb and 15 Kb (very faint). Since these bands are present in both event 15985 and the DP50B control they are considered background bands associated with the *cry1Ac* event. The 15985 long and short runs (lanes 3 and 7) each produced one band not present in either the DP50 or the DP50B lanes at approximately 9.3 Kb. This result supports the conclusion that 15985 contains one segment of integrated DNA located on a 9.3 Kb *Sca*I restriction fragment.

4.2 Copy number.

Genomic DNA isolated from 15985, DP50B, DP50 (non-transgenic control) and DP50 mixed with plasmid PV-GHBK11 DNA was digested with *Sph*I. The blot was probed with PV-GHBK11 (Appendix 1, Figure 1), the source plasmid for the linear DNA segment used in transformation. The results are shown in Appendix 1, Figure 4. The DP50 long run (lane 1) did not produce any detectable background bands. Plasmid PV-GHBK11 mixed with DP50 in the short run (lanes 4 and 5) produced the expected size bands at 3.9 and 4.8 Kb; an additional faint band at 8.7 Kb in lane 5 is presumably due to undigested plasmid DNA. The DP50B long and short runs (lanes 2 and 6) produced three bands at approximately 6.4, 8.3, and 8.6 Kb. Since these bands are present in both event 15985 and the DP50B control, they are considered background bands associated with the *cry1Ac* event. The 15985 long and short runs (lanes 3 and 7) each produced two bands not present in the DP50 or the DP50B lanes at approximately 2.3 Kb and 3.5 Kb. Because the enzyme *Sph*I cuts only once within the transformation cassette, this result suggests that 15985 contains one copy of integrated DNA which produces these two restriction fragments.

4.3 *cry2Ab2* coding region intactness.

DNA from the test, controls, and control mixed with plasmid PV-GHBK11 DNA was digested with *Nco*I to release the *cry2Ab2* coding region and assess its intactness. The blot was probed with the full-length *cry2Ab2* coding region (Appendix 1, Figure 5). As expected, the DP50 non-transgenic control long run (lane 1) and the DP50B control long and short runs (lanes 2 and 6) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 in the short run (lanes 4 and 5) produced the expected ~1.9 Kb band which corresponds to the entire *cry2Ab2* coding region (Appendix 1, Figure 1). Both the 15985 long and short runs (lanes 3 and 7) also produced a 1.9 Kb band which corresponds to the expected size of an intact *cry2Ab2* coding region. This result establishes that event 15985 contains the intact *cry2Ab2* coding region, with no additional detectable fragments.

4.4 *cry2Ab2* cassette intactness.

DNA from the test, controls, and control mixed with plasmid PV-GHBK11 DNA was digested with *Bam*HI which releases the entire *cry2Ab2* cassette (*i.e.*, *cry2Ab2* coding region, the enhanced CaMV 35S promoter, and the NOS 3' polyadenylation sequence).

4.4.1 *cry2Ab2* coding region probe.

The blot was probed with the full length *cry2Ab2* coding region (Appendix 1, Figure 6). The DP50 non-transgenic control long run (lane 1) and the DP50B control long and short runs (lanes 2 and 6) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 in the short run (lanes 4 and 5) produced the expected 3.2 Kb band which corresponds to the entire *cry2Ab2* cassette (Appendix 1, Figure 1). Both the 15985 long and short runs (lanes 3 and 7) produced a band at approximately 4.0 Kb. This result indicates that the 3' end of the transformation cassette lost the *Bam*HI restriction site (Appendix 1, Figures 1 and 2) during integration into the cotton genome. The 3' sequence of the insert → plant junction, previously determined by genome walking, was verified by PCR analysis (Appendix 1, Figure 14). Sixty-six base pairs of the 3' end of the transformation cassette were shown to have been deleted, including the *Bam*HI site. The deleted nucleotides do not include any of the NOS 3' polyadenylation sequence associated with the *cry2Ab2* cassette, but only linker DNA. These results establish that the *cry2Ab2* cassette is intact. No partial *cry2Ab2* cassettes were detected.

4.4.2 Enhanced CaMV 35S promoter probe.

The blot used in section 4.4.1 was stripped and re-probed with the full length enhanced CaMV 35S promoter. The results are shown in Appendix 1, Figure 7. The DP50 long run (lane 1) did not produce any detectable background bands. Plasmid PV-GHBK11 mixed with DP50 in the short run (lanes 4 and 5) produced the expected size bands at 5.5 and 3.2 Kb with no additional bands detectable. The DP50B long and short runs (lanes 2 and 6) produced five bands at approximately 4.4, 5.3, 7.5, 9.4, and 22 Kb. Since these bands are present in both event 15985 and the DP50B control, they are considered background bands associated with the *cry1Ac* event. The 15985 long and short runs (lanes 3 and 7) both produced one band at approximately 4.0 Kb which is not present in either the DP50 or the DP50B lanes. This corresponds to the fragment predicted for the *cry2Ab2* cassette given the result obtained with the *cry2Ab2* coding region probe. A second band in the 15985 lanes resulting from hybridization to the enhanced CaMV 35S promoter associated with the *uidA* cassette is predicted but not apparent in the test lanes. The results of the NOS 3' polyadenylation sequence probe, discussed below, demonstrate that the enhanced CaMV 35S promoter sequence associated with the *uidA* cassette is present, but the 4.4 Kb band co-migrates with a 4.4 Kb background band and is not apparent. No extraneous promoters were detected.

4.4.3 NOS 3' polyadenylation sequence probe.

The blot used in section 4.4.2 was stripped and re-probed with the full length NOS 3' polyadenylation sequence. The results are shown in Appendix 1, Figure 8. The DP50 long run (lane 1) did not produce any detectable background bands. Plasmid PV-GHBK11 mixed with DP50 short run (lanes 4 and 5) produced the expected size bands at 5.5 and 3.2 Kb with no additional bands detectable. The DP50B long and short runs (lanes 2 and 6) produced one band at approximately 1.2 Kb. Since this band is present in both event 15985 and the DP50B control, it is considered background associated with the *cry1Ac* event. The 15985 long and short runs (lanes

3 and 7) each produced two bands which are not present in the DP50 or the DP50B lanes at approximately 4.0 and 4.4 Kb. The 4.0 Kb band corresponds to the fragment predicted for the *cry2Ab2* cassette, given the result from 4.4.1 above. The 4.4 Kb band was not apparent on the blot probed with the enhanced CaMV 35S promoter because it co-migrates with the 4.4 Kb background band seen on that blot. This segment is associated with the *uidA* cassette.

These results establish that the *cry2Ab2* cassette is intact and that there is a 66 bp deletion between the *Bam*HI site and the 3' end of the transformation cassette, which does not include any of the NOS 3' polyadenylation sequence at the 3' end of the *cry2Ab2* cassette. No partial *cry2Ab2* cassettes were detected.

4.5 *uidA* coding region intactness.

Genomic DNA isolated from 15985, DP50B, DP50 (non-transgenic control) and DP50 mixed with plasmid PV-GHBK11 DNA was digested with *Eco*RI and *Bgl*II to release the entire *uidA* coding region. The blot was probed with the full-length *uidA* coding region (Appendix 1, Figure 9). The DP50 non-transgenic control long run (lane 1) and the DP50B control long and short runs (lanes 2 and 6) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 short run (lanes 4 and 5) produced the expected ~1.9 Kb band, which corresponds to the entire *uidA* coding region (Appendix 1, Figure 1). Both the long and short runs of event 15985 DNA (lanes 3 and 7) also produced a 1.9 Kb band which corresponds to the expected size of an intact *uidA* coding region. This result establishes that event 15985 contains the intact *uidA* coding region, with no additional fragments detected.

4.6 *uidA* cassette intactness.

DNA from the test and control substances was digested with *Bam*HI and *Sph*I to release the entire *uidA* cassette (i.e. *uidA* coding region, the enhanced CaMV 35S promoter, and the NOS 3' polyadenylation sequence). The plasmid PV-GHBK11 was digested with *Pst*I and spiked into the DP50 short run samples after digestion (except for the NOS 3' polyadenylation sequence probe blot in which the plasmid was digested with *Bam*HI and *Sph*I). This was done to show the size of an intact full-length *uidA* cassette.

4.6.1 *uidA* coding region probe.

The blot was probed with the full length *uidA* coding region (Appendix 1, Figure 10). As expected, the DP50 non-transgenic control long run (lane 1) and the DP50B control long and short runs (lanes 2 and 6) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 short run (lanes 4 and 5) produced the expected 2.8 Kb band, which corresponds to the entire *uidA* cassette (Appendix 1, Figure 1). Both the 15985 long and short runs (lanes 3 and 7) produced an approximately 2.5 Kb band. This result indicates that a portion of the *uidA* cassette was not present. The 5' insert → plant junction, previously determined by genome walking, was verified by PCR analysis (Appendix 1, Figure 14). It had been demonstrated previously that 284 bp of the 5' portion of the transformation cassette were deleted. These results establish that the *uidA* cassette is missing approximately 260 bp of the 5' promoter sequence and 24 bp of

polylinker DNA derived from the multiple cloning site of the plasmid. Odell et al. (1985) showed that such a deletion should not affect accurate transcription initiation. No additional partial *uidA* cassettes were detected with the *uidA* coding region probe.

4.6.2 Enhanced CaMV 35S promoter.
The blot used in section 4.6.1 was stripped and re-probed with the full length enhanced CaMV 35S promoter. The results are shown in Appendix 1, Figure 11. The DP50 long run (lane 1) did not produce any background bands. Plasmid PV-GHBK11 mixed with DP50 short run (lanes 4 and 5) produced the expected size bands at 1.5 and 2.8 Kb with no additional bands detected. The DP50B long and short runs (lanes 2 and 6) produced five bands at approximately 4.3, 4.6, 5.0, 6.6, and 8.5 Kb. Since these bands are present in both event 15985 and the DP50B control they are considered background bands associated with the *cry1Ac* event. The 15985 long and short runs (lanes 3 and 7) each produced two bands at approximately 2.5 and 1.0 Kb not present in the DP50 or the DP50B lanes. The 2.5 Kb band corresponds to the fragment predicted for the *uidA* cassette. The 1.0 Kb band results from the enhanced CaMV 35S promoter associated with the *cry2Ab2* cassette (Appendix 1, Figure 1). No extraneous promoters were detected.

4.6.3 NOS 3' polyadenylation sequence probe.
The blot was probed with the full length NOS 3' polyadenylation sequence. The results are shown in Appendix 1, Figure 12. The DP50 long run (lane 1) did not produce any detectable background bands. Plasmid PV-GHBK11 mixed with DP50 short run (lanes 4 and 5) produced the expected size bands at 3.8 and 2.2 Kb with no additional bands detected. The DP50B long and short run (lanes 2 and 6) produced one band at approximately 1.2 Kb. Since this band is present in both event 15985 and the DP50B control it is considered background associated with the *cry1Ac* event. The 15985 long and short runs (lanes 3 and 7) each produced two bands not present in the DP50 or the DP50B lanes at approximately 2.5 and 2.3 Kb. The 2.5 Kb band corresponds to the fragment predicted for the *uidA* cassette. The 2.3 Kb band results from NOS 3' polyadenylation sequence associated with the *cry2Ab2* cassette (Appendix 1, Figure1).

These results confirm that the *uidA* cassette is missing approximately 260 bp of the 5' end of the enhanced CaMV 35S promoter but is otherwise intact.

4.7 Analysis for backbone fragments.
Genomic DNA isolated from event 15985, DP50B, DP50 (non-transgenic control) and DP50 mixed with plasmid PV-GHBK11 DNA was digested with *Kpn*I. The blot was probed with the entire backbone sequence (Appendix 1, Figure 13). The DP50 long run (lane 1) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 DNA (lanes 4 and 5) produced one band at the expected size of 2.6 Kb for the entire backbone (Appendix 1, Figure 1). The DP50B long and short run (lanes 2 and 6) produced a single band at approximately 22 Kb. Since this band is present in both event

15985 and the DP50B control it is considered background associated with the *cry1Ac* event. The 15985 long and short runs (lanes 3 and 7) contained the 22 Kb background band with no additional hybridization. This result establishes that event 15985 does not contain any detectable plasmid backbone sequence.

4.8 Genomic flanking sequence.

PCR was performed on genomic DNA to confirm the insert → plant junction sequences at the 5' and 3' ends of the 15985 insert. The results of these PCRs are shown in Appendix I, Figure 14. As expected, the non-transgenic samples did not yield a PCR product when either the 5' or 3' primer set was used (lanes 3 and 7). The DP50B sample (*cry1Ac* control event) did not yield products with either primer pair (lanes 4 and 8), as expected. An alternate Cry2Ab2 event, 15813, also did not yield products when either primer set was used (lanes 2 and 6). The 15985 genomic DNA yielded the correct size products of 230 bp at the 5' end using primers A and B (lane 1) and 869 bp at the 3' end using primers C and D (lane 5). This PCR analysis confirmed the 5' and 3' border sequences of 15985.

5. CONCLUSIONS

The insect protected cotton event 15985 was produced by particle acceleration technology using a *Kpn*I DNA segment containing the *uidA* and *cry2Ab2* cassettes. The 15985 event contains one new DNA insert. This insert is located on a 9.3 Kb *Sca*I fragment. This insert contains one complete copy of the *cry2Ab2* cassette linked to one copy of the *uidA* cassette, which is missing approximately 260 bp at the 5'end of the enhanced CaMV 35S promoter. PCR was used to verify the 5' and 3' junction sequences of the insert with the plant genome, as well as the intactness of the 5' and 3' ends of the insert. Event 15985 does not contain any detectable plasmid backbone sequence resulting from the *cry2Ab2* transformation. Based on the enzymes used in this study, the restriction pattern of the *cry1Ac* insert is not changed by the insertion of the *cry2Ab2* and *uidA* DNA.

6. REFERENCES

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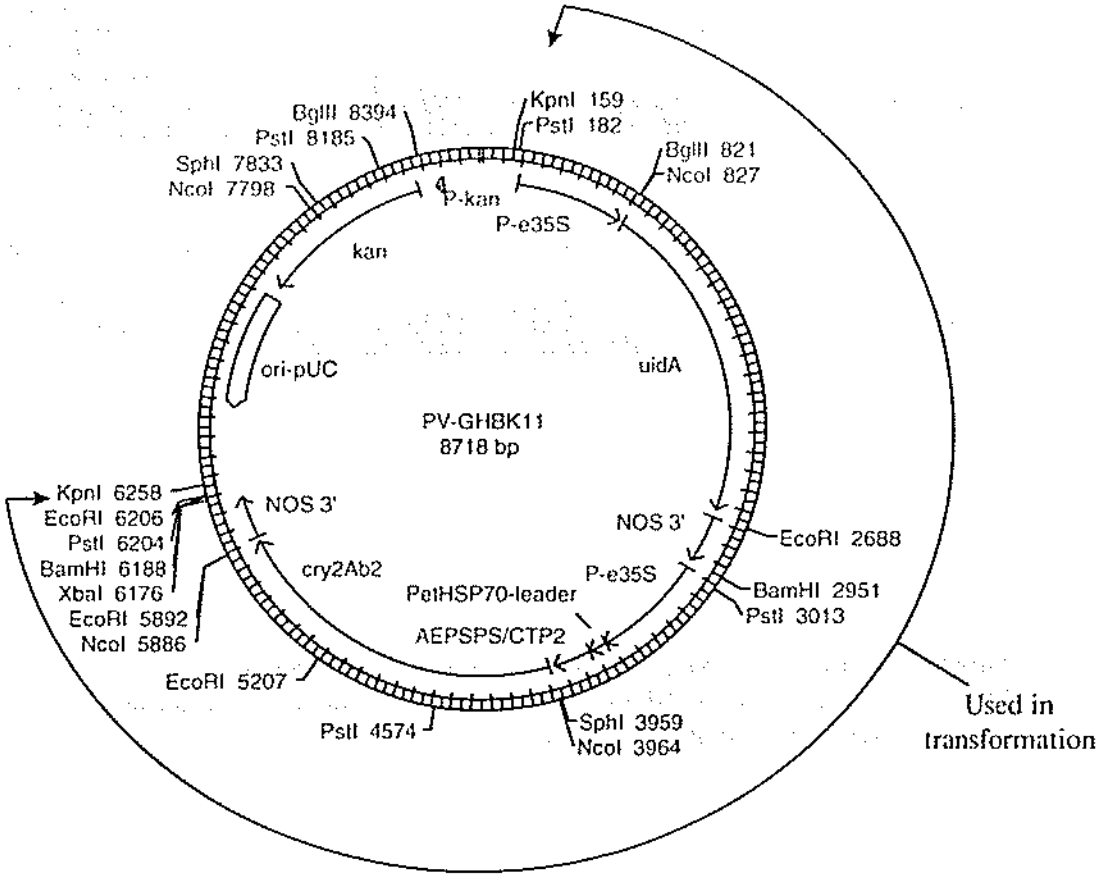
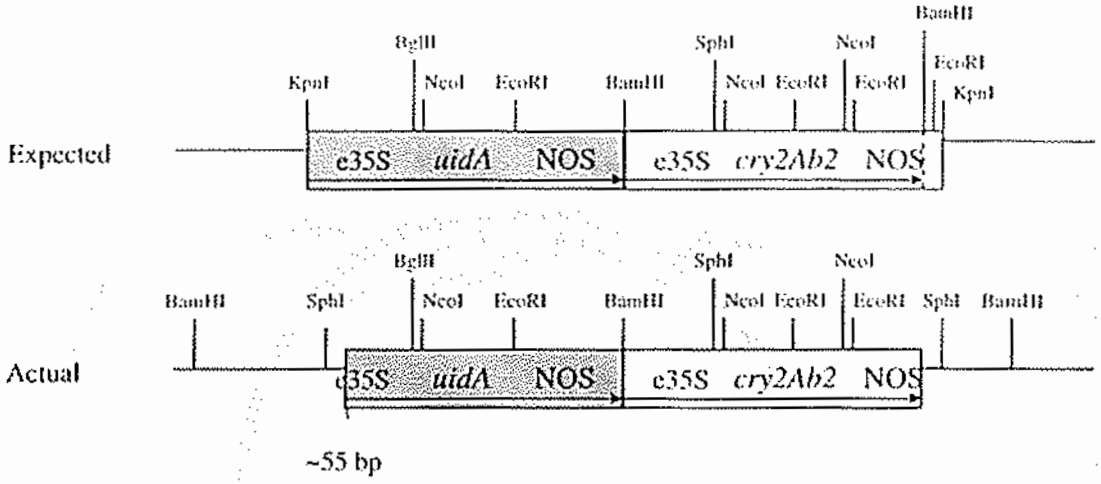
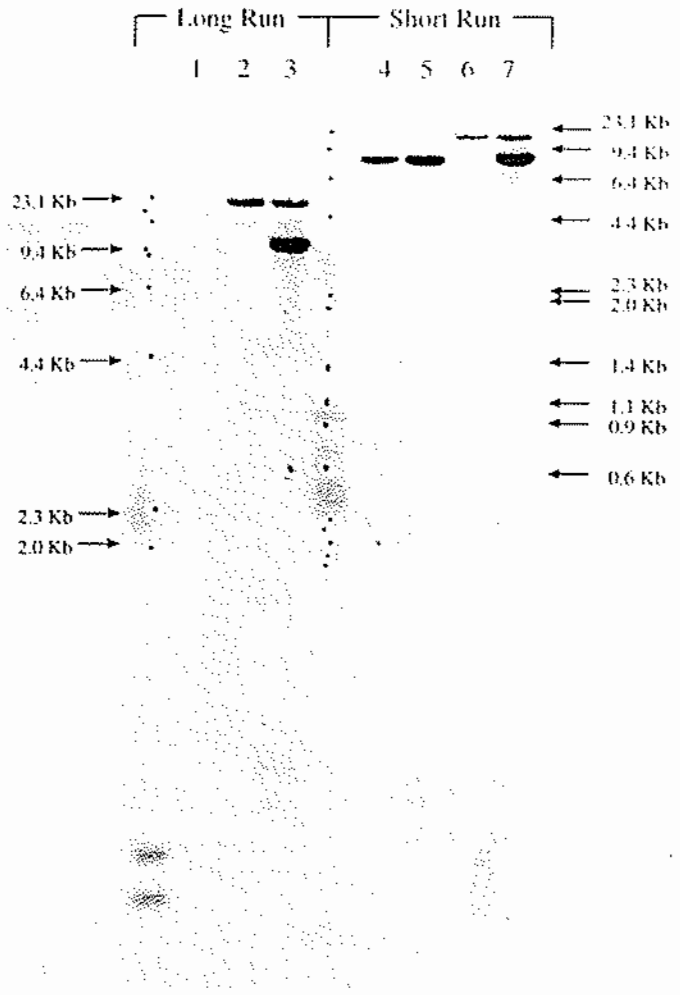


Appendix 1, Figure 1. Plasmid Map PV-GHBK11
The *Kpn*I segment of PV-GHBK11 plasmid was used to generate insect protected cotton event 15985.



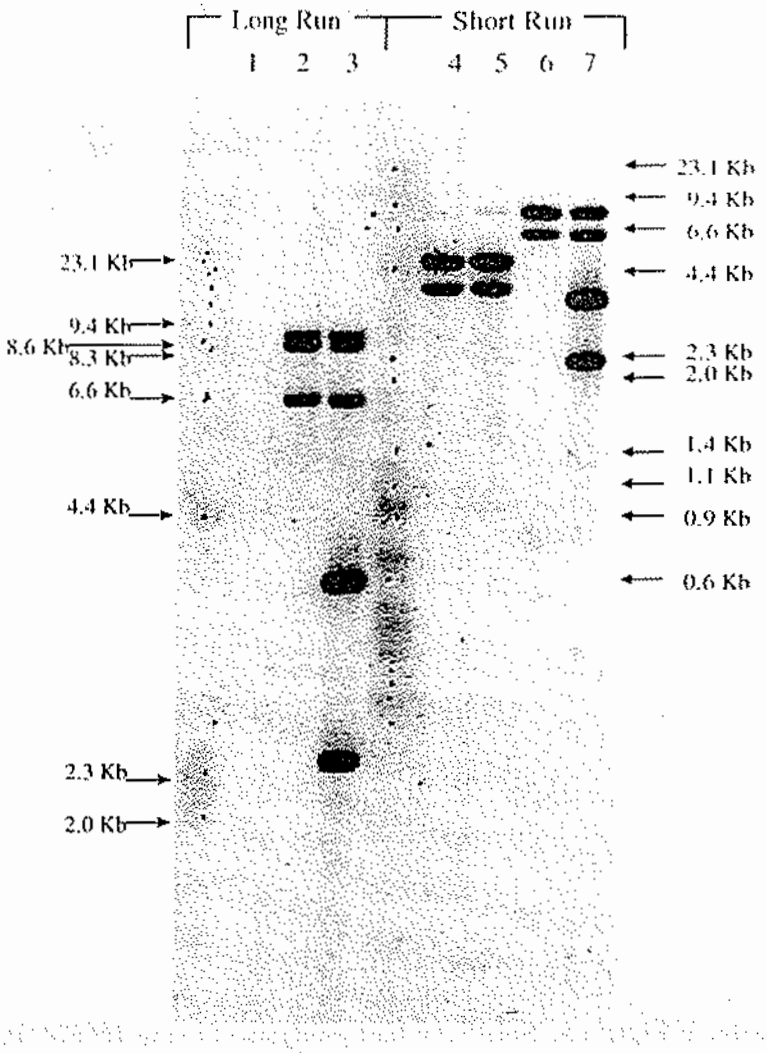
Appendix 1, Figure 2. Map of DNA segment PV-GHBK11L
The DNA segment, PV-GHBK11L, was used to generate insect-protected cotton event 15985 by particle acceleration technology.



Appendix 1, Figure 3. Southern blot analysis of event 15985: insert number analysis. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Sca*I. The DP50 short run samples were also digested with *Xba*I. The blot was probed with ^{32}P-labeled PV-GHBK11. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

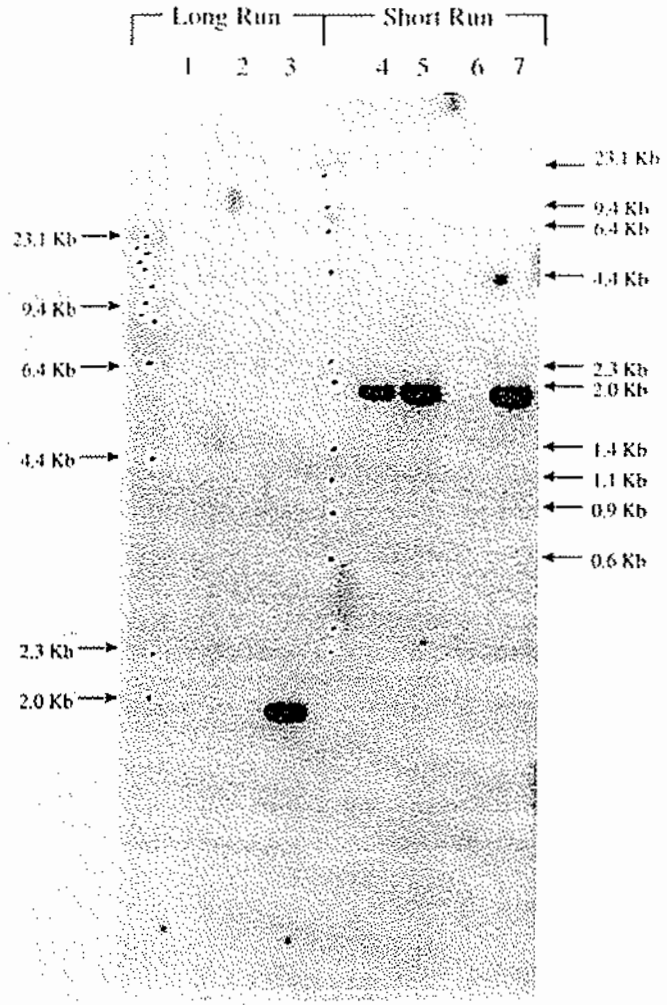
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 4. Southern blot analysis of event 15985: copy number analysis
Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Sph*I. The blot was probed with ^{32}P-labeled PV-GHBK11. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

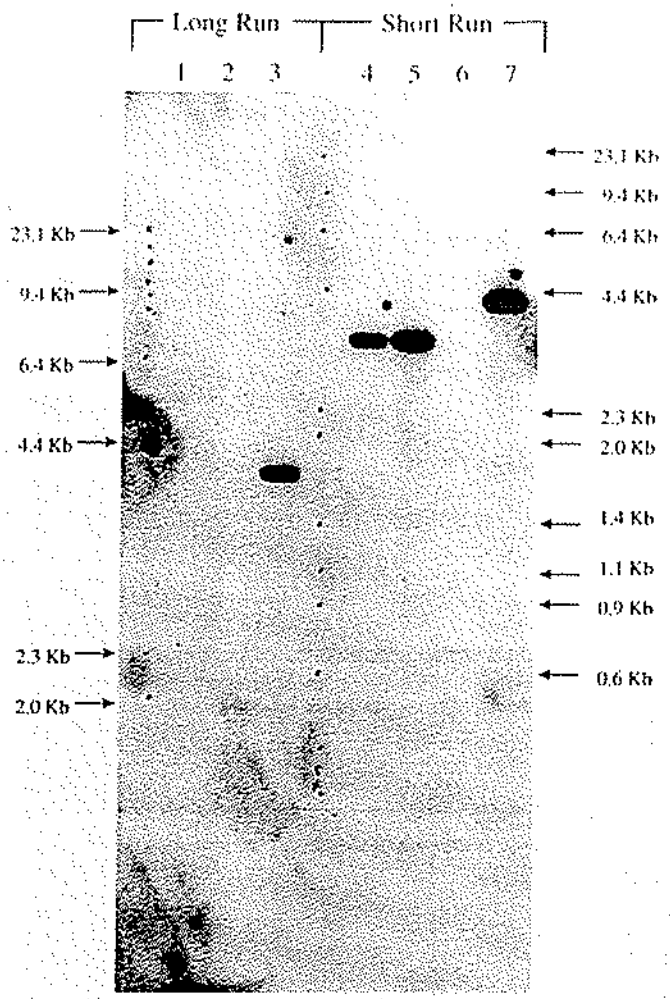
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 5. Southern blot analysis of event 15985: *cry2Ab2* coding region intactness. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Nco*I. The blot was probed with ^{32}P-labeled *cry2Ab2* coding region. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

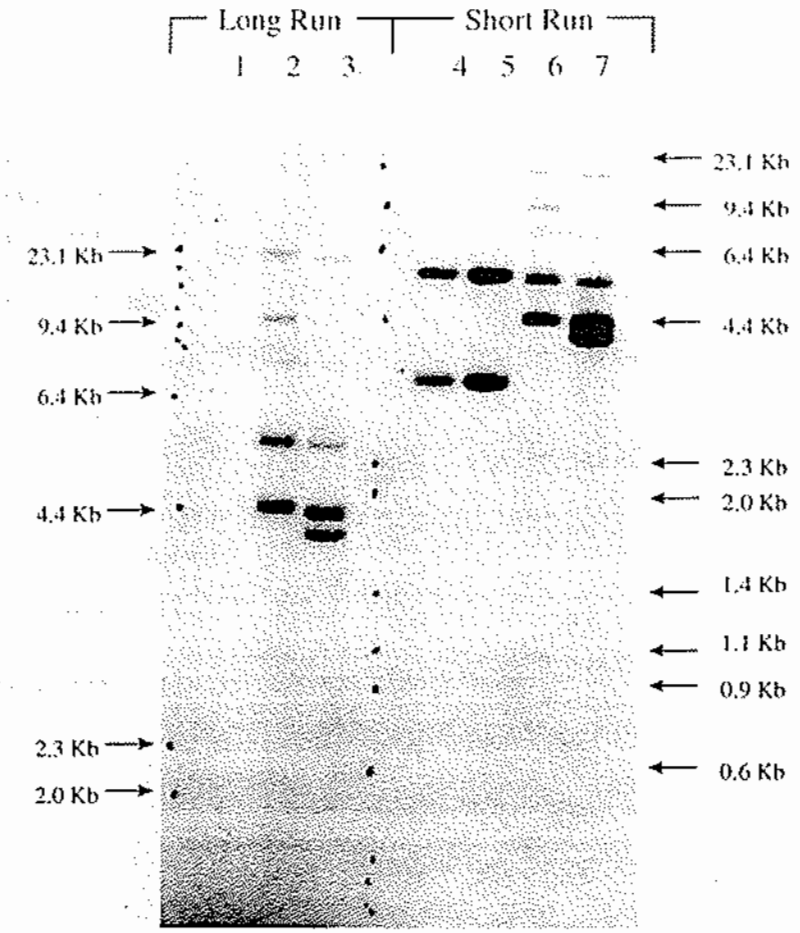
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 6. Southern blot analysis of event 15985: *cry2Ab2* cassette intactness - *cry2Ab2* probe. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI. The blot was probed with ^{32}P-labeled *cry2Ab2* coding region. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

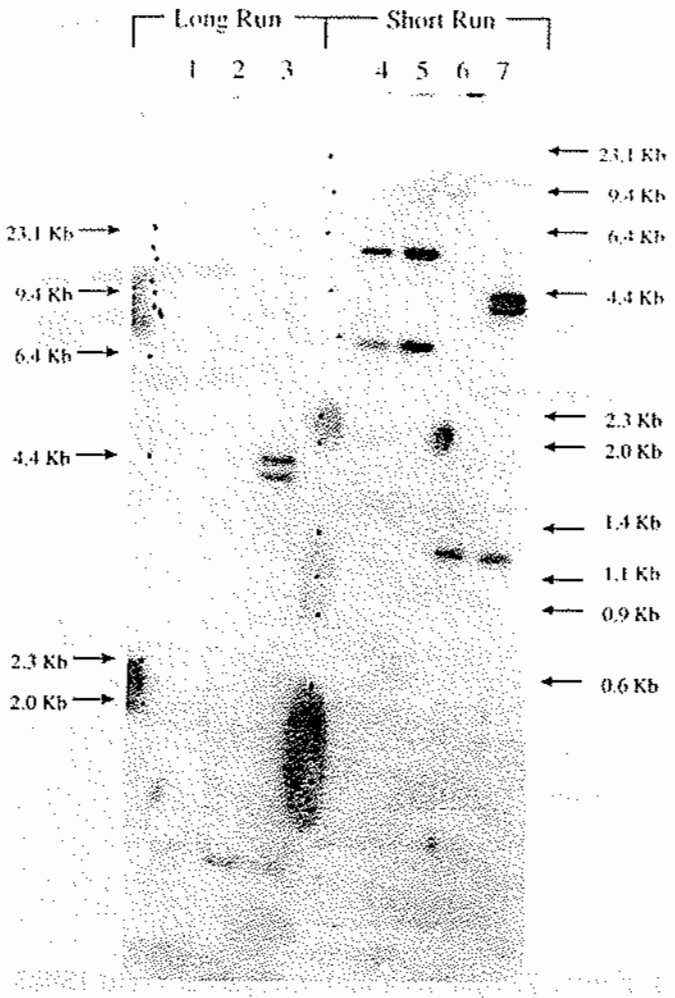


Appendix 1, Figure 7. Southern blot analysis of event 15985: *cry2Ab2* cassette intactness -enhanced CaMV 35S promoter probe

Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI. The blot was probed with ^{32}P-labeled enhanced CaMV 35S promoter probe. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

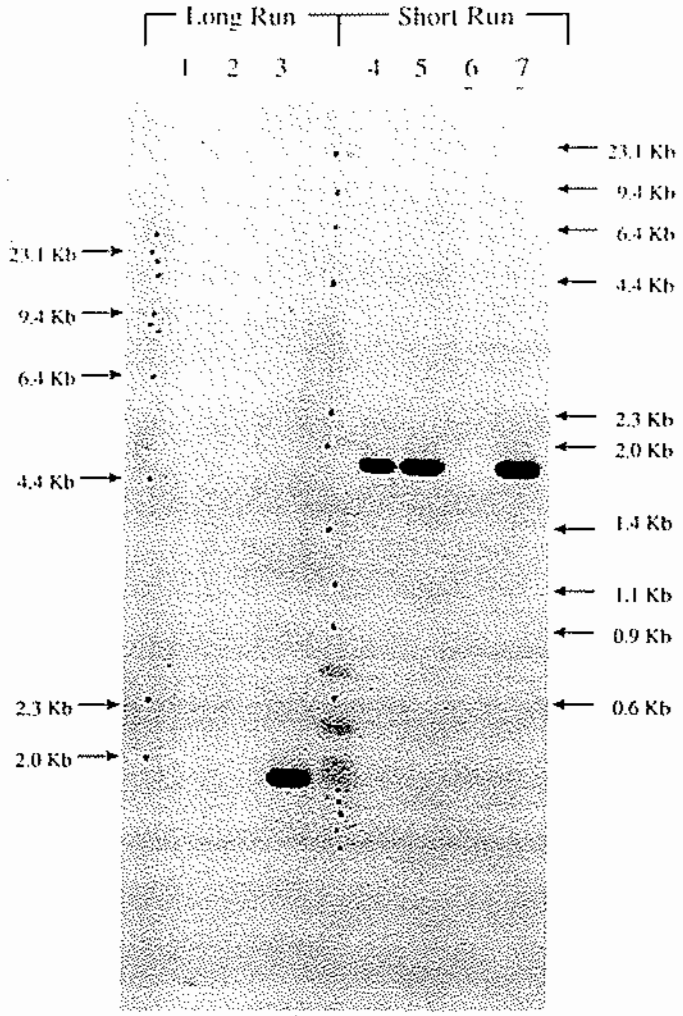
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 8. Southern blot analysis of event 15985: *cry2Ab2* cassette intactness - NOS probe. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI. The blot was probed with ^{32}P-labeled NOS 3' polyadenylation sequence. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

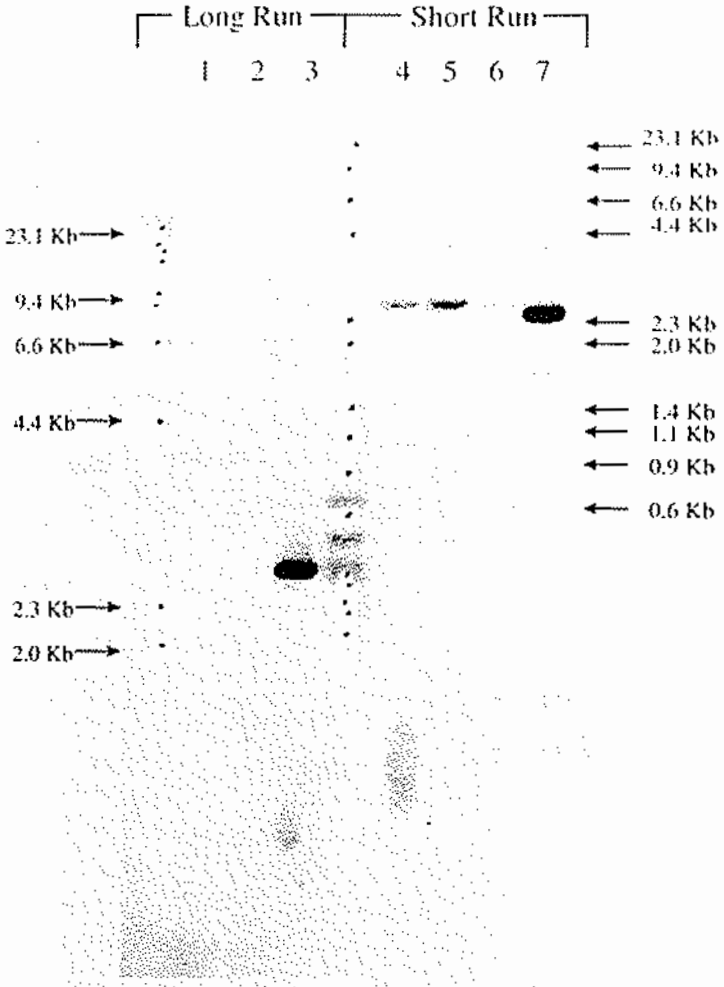
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 9. Southern blot analysis of event 15985: *uidA* coding region intactness. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Bgl*II and *Eco*RI. The blot was probed with ^{32}P-labeled *uidA* coding region. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

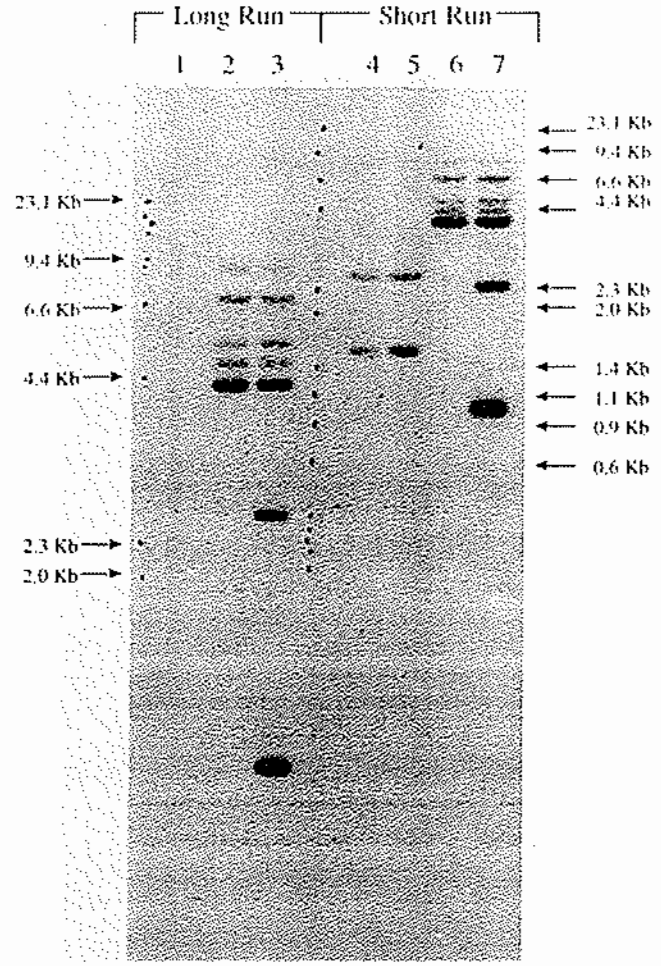
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 10. Southern blot analysis of event 15985: *uidA* cassette intactness - *uidA* probe. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI and *Sph*I. Plasmid DNA was digested with *Pst*I and spiked into the DP50 genomic samples prior to precipitation. The blot was probed with ^{32}P-labeled *uidA* coding region. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

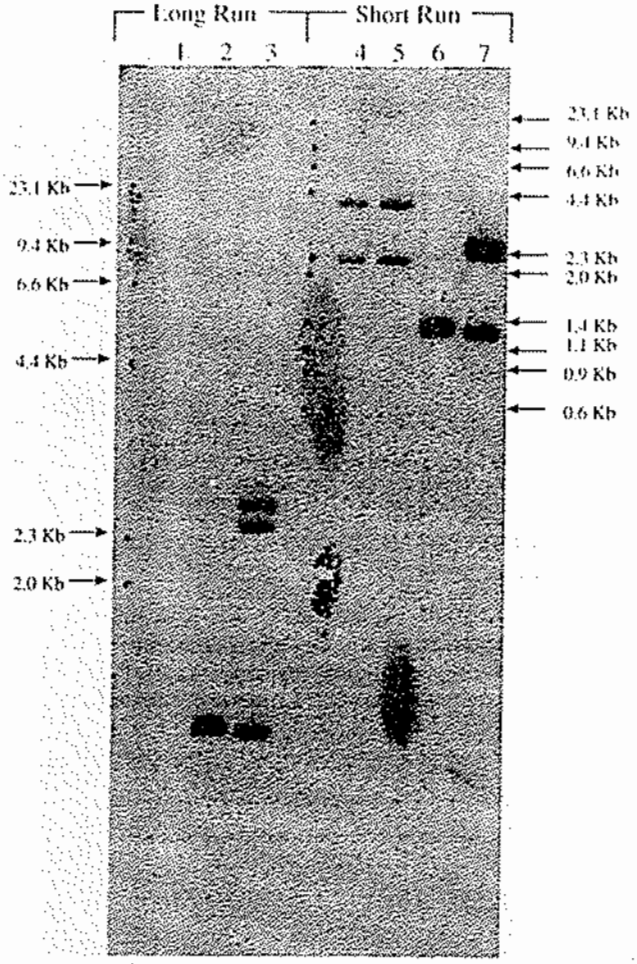


Appendix 1, Figure 11. Southern blot analysis of event 15985: *uidA* cassette intactness - enhanced CaMV 35S promoter probe

Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI and *Sph*I. Plasmid DNA was digested with *Pst*I and spiked into the genomic samples prior to precipitation. The blot was probed with ^{32}P-labeled enhanced CaMV 35S promoter probe. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

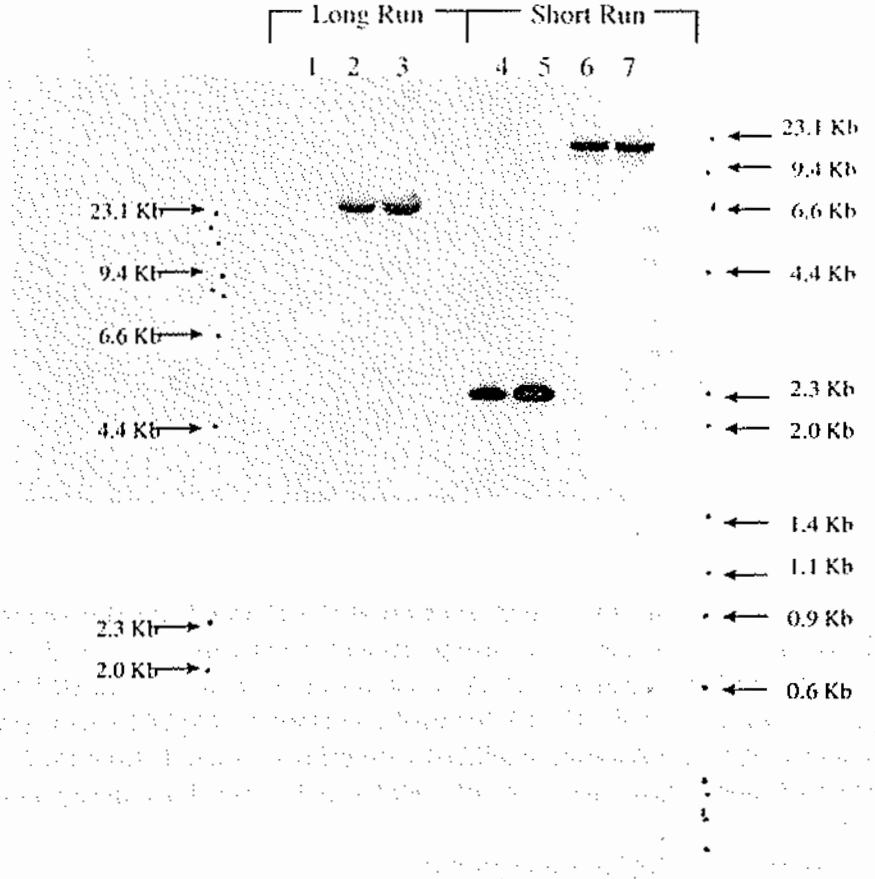
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 12. Southern blot analysis of event 15985: *uidA* cassette intactness - NOS probe. Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue (15985 and DP50B samples) and seed (DP50 sample) were digested with *Bam*HI and *Sph*I. The blot was probed with ^{32}P-labeled NOS 3' polyadenylation sequence. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers .

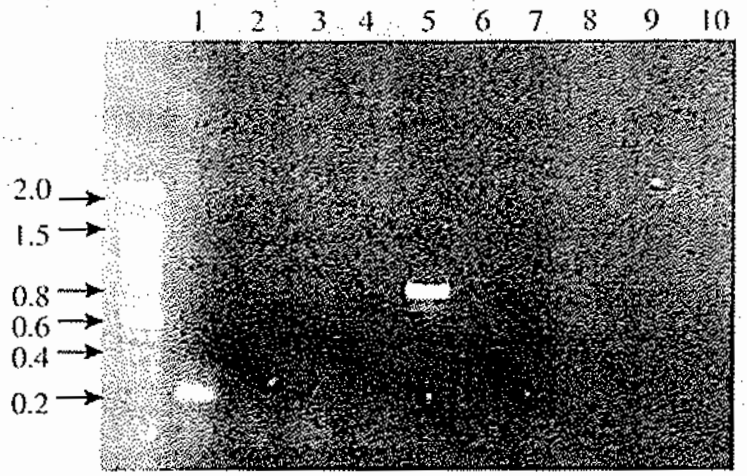


Appendix 1, Figure 13. Southern blot analysis of event 15985: analysis for backbone sequences

Ten micrograms of DP50, DP50B and 15985 genomic DNA isolated from leaf tissue were digested with *Kpn*I. The blot was probed with ^{32}P-labeled full length backbone sequence. Lane designations are as follows:

Lane 1: DP50 (Long Run)
2: DP50B (Long Run)
3: 15985 (Long Run)
4: DP50 spiked with 5.15 pg of PV-GHBK11 (Short Run)
5: DP50 spiked with 10.3 pg of PV-GHBK11 (Short Run)
6: DP50B (Short Run)
7: 15985 (Short Run)

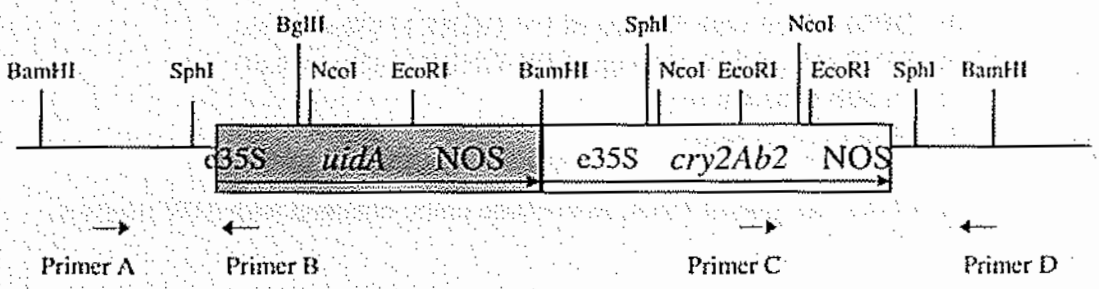
→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 1, Figure 14. PCR confirmation of the 5' and 3' border sequences of the 15985 insert. PCR was performed using primers specific to the 5' and 3' border sequences for 15985 on genomic DNA isolated from leaf tissue from DP50 (non-transgenic control), DP50B (Cry1Ac control), an alternate Cry2Ab2 transgenic event, 15813, and 15985. DNAs were amplified with primers A and B from the 5' end of 15985 and primers C and D from the 3' end of 15985 (see below). Lane designations are as follows:

Lane 1: 10 µl of 5' 15985 reaction product
2: 10 µl of 5' alternate Cry2Ab2 reaction product
3: 10 µl of 5' DP50 (non-transgenic) negative control reaction product
4: 10 µl of 5' DP50B (Cry1Ac) negative control reaction product
5: 10 µl of 3' 15985 reaction product
6: 10 µl of 3' alternate Cry2Ab2 reaction product
7: 10 µl of 3' DP50 (non-transgenic) negative control reaction product
8: 10 µl of 3' DP50B (Cry1Ac) negative control reaction product
9: 10 µl of 5' no template negative control reaction product
10: 10 µl of 3' no template negative control reaction product

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.



Appendix 2. Molecular Analysis of the Stability of Cotton Event 15985

PURPOSE

The purpose of this study was to assess by Southern blot analysis the genetic stability of the additional DNA inserted into Bollgard cotton line DP50B to produce the Cry2Ab Bollgard II cotton event 15985 across five plant breeding generations. The restriction enzyme *SphI* generates a unique Southern blot banding pattern fingerprint for event 15985 when probed with the *cry2Ab* coding region. Genomic DNA from the R1, R2, R3, R4, and 2 BC2F3 generations (Appendix 2, Figure 1) was digested, blotted, and probed with the entire *cry2Ab* coding region to assess the stability of the inserted DNA over time and breeding generations.

MATERIALS AND METHODS

The test substance for this study was the Bollgard II cotton event 15985. The following five plant breeding generations were analyzed: R1, R2, R3, R4, and 2 BC3F2 commercial track varieties (Appendix 2, Figure 1). The control substances for this study were cotton lines DP50 (non-transgenic) and Bollgard event DP50B (*cry1Ac* event 531, negative for *cry2Ab*). Plasmid PV-GHBK11, the source plasmid, served as the primary reference substance in these analyses. The plasmid, mixed with DNA from the DP50 control substance, was used as a size indicator and a positive hybridization control in Southern blot analysis. Additionally, molecular size markers from Boehringer Mannheim [Molecular Weight Markers II (23.1 Kb-0.6 Kb) and IX (1.4 Kb-0.072 Kb), catalog #236 250 and #1449 460, respectively] were used for size estimations.

Genomic DNA from multiple generations of insect-protected cotton event 15985 was digested with the restriction enzyme *SphI* and subjected to Southern blot hybridization analysis to assess the genetic stability of the DNA containing the *cry2Ab* transgene. DNA extracted from leaf tissue was used for all of the analyses in this report except for the nontransgenic DP50 sample which was isolated from seed according to the method of Rogers and Bendich (1985). Leaf tissue was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Approximately 1 g of the ground leaf tissue was transferred to 13 ml centrifuge tube containing 6 ml of the extraction buffer [2.5 ml DNA extraction buffer (350 mM Sorbitol, 100 mM Tris pH 7.5, 5 mM EDTA), 2.5 ml Nuclei lysis buffer (200 mM Tris pH 7.5, 50 mM EDTA, 2 M NaCl, 2% CTAB), and 1 ml Sarkosyl (5% solution)]. The samples were incubated at 65°C for 30-60 minutes with intermittent mixing. Four and a half milliliters of a mixture of chloroform:isoamyl alcohol (24:1) at room temperature were added to the samples. The suspension was mixed for 2 to 3 minutes, and the two phases separated by centrifugation for 15 minutes at ~2,300 rpm at 4°C. The aqueous (top) layer was removed using a transfer pipet and placed into a 13 ml centrifuge tube. Five milliliters of 100% isopropanol were added, and the tubes were mixed by inversion to precipitate the DNA. The precipitated DNA was pelleted by centrifuging at ~2,300 rpm for 5 minutes at 4°C. The pellet was washed with approximately 1 ml of 70% ethanol and centrifuged for an additional 5 minutes at ~2,300 rpm at 4°C . Alternatively, the DNA was spooled from the isopropanol precipitation using a pipet tip and placed directly into 1 ml of 70% ethanol. The sample

was then pelleted in a microcentrifuge at 14,000 rpm for 2-5 minutes. The DNA was dried for 4 minutes in a vacufuge and re-dissolved in TE overnight in a 4°C refrigerator.

The purified genomic DNA was quantitated using a Hoefer DyNA Quant™ 200 Fluorometer (San Francisco, CA)(SOP BR-EQ-0065-01) with Boehringer Mannheim Molecular Weight Marker IX used as a calibration standard. Approximately 10 µg of genomic DNA from the test and control lines were used for the restriction enzyme digests. Overnight digests were performed at 37°C according to SOP GEN-PRO-010-01 in a total volume of 500 µl using 100 units of restriction enzyme. One of the nontransgenic control digests was spiked with 10 pg of PV-GHBK11. After digestion, the samples were precipitated by adding 1/10 volume (50 µl) of 3M NaOAc and 2 volumes (1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a -20°C freezer for at least one hour. The digested DNA was pelleted by centrifugation, washed with 70% ethanol, vacuum dried for 4 minutes, and re-dissolved at room temperature in water.

Digested DNAs were separated on a 0.8% agarose gel in 1X TBE buffer according to SOP GEN-PRO-003-01. The gel was electrophoresed overnight at 48 V and the voltage increased to 80 V in the morning. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide for approximately 20 minutes and photographed. Plasmid PV-GHBK11 DNA was isolated from overnight *E. coli* cultures. Probe template homologous to the *cry2Ab2* coding region was prepared by PCR using PV-GHBK11 as the template. Approximately 25 ng of *cry2Ab2* probe template were labeled with ^{32}P-dCTP using the random priming method (RadPrime DNA Labeling System, Life Technologies). The radiolabeled probe was purified using a Sephadex G-50 column (Boehringer Mannheim).

Southern blot analysis (Southern, 1975) was performed according to SOP GEN-PRO-025-02. Following electrophoresis, the gel was incubated in depurination solution (0.125 N HCl) for ~ 10 minutes followed by denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes, and then neutralizing solution (0.5 M Tris-HCl pH 7, 1.5 M NaCl) for ~30 minutes. The DNA from the agarose gel was transferred to Hybond-N™ nylon membranes (Amersham) using a Turboblotter™ (Schleicher & Schuell). The DNA was allowed to transfer for 4½ hours (in 20X SSC) and covalently cross-linked to the membrane with a UV Stratalinker™ 1800 (Stratagene) set to autocrosslink. The blot was prehybridized for 30 minutes in an aqueous solution of 0.5 M sodium phosphate, 7% SDS (w/v), and 0.1 mg/ml *E. coli* tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 16 hours at approximately 65°C. The membrane was washed four times in an aqueous solution of 0.1% (w/v) SDS and 0.1X SSC for 15 minute intervals at 65°C. The exposure of the blot was generated using Kodak Biomax MS™ film in conjunction with one Kodak Biomax MS™ intensifying screen.

RESULTS AND DISCUSSION

DNA from the R1, R2, R3, R4, and 2 BC2F3 generations of Bollgard II cotton event 15985, controls, and control mixed with plasmid PV-GHBK11 DNA was digested with

*Sph*I. The blot was probed with the full-length *cry2Ab2* coding region (Appendix 2, Figure 2). As expected, the DP50 non-transgenic control (lane 8) and the DP50B control (lane 7) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 (lane 9) produced the expected ~3.8 Kb band. Each event15985 generation (lanes 1-6) produced the predicted ~2.3 Kb border segment (produced from the *Sph*I restriction site at position 3959 in plasmid PV-GHBK11 and an *Sph*I restriction site in the plant DNA). No differences in banding pattern were observed between DNA extracted from the R1, R2, R3, R4, or 2 BC2F3 generations. This demonstrates the genetic stability of the inserted DNA in samples spanning five plant breeding generations.

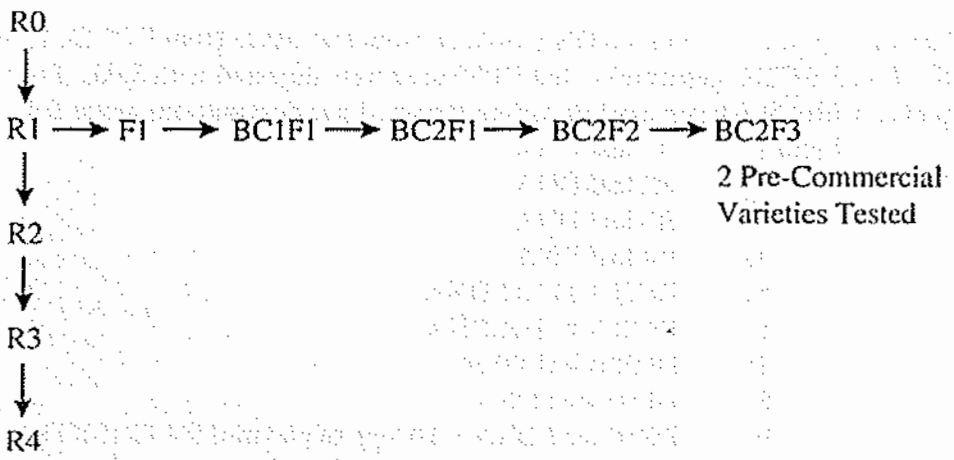
CONCLUSIONS

The genetic stability of the inserted DNA was demonstrated by Southern blot fingerprint analysis on 15985 genomic DNA spanning five plant breeding generations. No differences in banding pattern were observed among DNA extracted from the R1, R2, R3, R4, or 2 BC2F3 generations. This demonstrates that the *cry2Ab* DNA insert in Bollgard II cotton event 15985 is stable in the plant genome across five plant breeding generations.

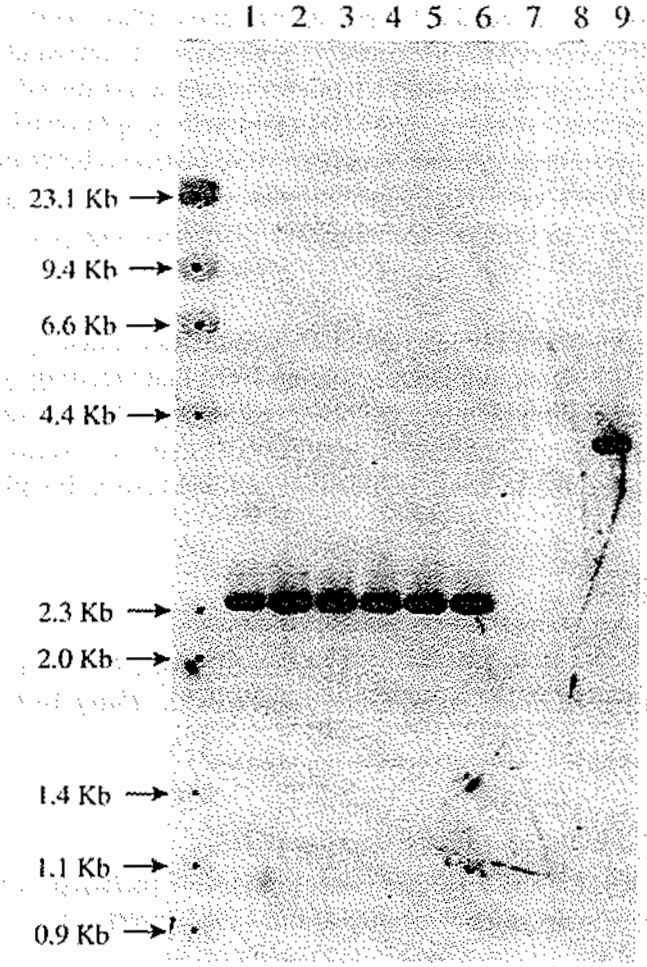
REFERENCES

Rogers, S.O. and Bendich, A.J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* 5, 69-76.

Appendix 2, Figure 1. 15985 Breeding History.
The R1, R2, R3, R4 and 2 BC2F3 generations were included in Southern blot analyses to assess the molecular genetic stability of the 15985 insert.



Appendix 2, Figure 2. Southern blot stability analysis of event 15985: R1, R2, R3, R4, and 2 BC2F3 generations.



Ten micrograms of genomic DNA isolated from leaf tissue from DP50B, 15985 R1, R2, R3, R4 , 2 BC2F3 generations and DP50 seed were digested with *Sph*I. The blot was probed with ^{32}P-labeled *cry2Ab* coding region. Lane designations are as follows:

Lane 1:	R1 leaf DNA
2:	R2 leaf DNA
3:	R3 leaf DNA
4:	R4 leaf DNA
5:	BC2F3 #1 leaf DNA
6:	BC2F3 #2 leaf DNA
7:	DP50B leaf DNA
8:	DP50 seed DNA
9:	DP50 seed DNA + 10.3 pg of plasmid PV-GHBK11

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

Appendix 3. Materials and Methods Used in Compositional Analyses

Compositional Analytical Methods

Ginned and acid delinted cottonseed from each location was shipped under ambient conditions to Covance Laboratories, Inc. for compositional analyses. Seed samples were analyzed for proximates (protein, fat, ash, carbohydrate, moisture, fiber, calories), amino acids, fatty acids, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), total gossypol, cyclopropenoid fatty acids and aflatoxins content. Carbohydrate values in seed were determined by calculation. Cottonseed oil and meal samples that were processed at Texas A&M from cottonseed samples pooled across all locations were shipped frozen on dry ice to Covance Laboratories, Inc. Fatty acid, cyclopropenoid fatty acids, vitamin E and gossypol analyses were performed on the oil samples and cottonseed meal samples were analyzed for gossypol levels. The methods used by Covance Laboratories, Inc. are summarized in Table 1.

Processing Summary

Cottonseed samples were ginned and acid delinted at Monsanto, St. Louis prior to shipment to Covance for compositional analyses of the seed. Ginned cottonseed was shipped to Texas A&M for processing into oil and meal samples. The ginned seed was saw delinted at the processing facility rather than at Monsanto, and mechanically cracked to separate the hull material from the kernel. The kernel material was then flaked and heated and fed into an expander/extruder. The material was then hexane extracted, and miscella refined. After refining, the oil and soapstock were separated by centrifugation. The refined oil was then bleached and deodorized. The soapstock was added back to the solvent-extracted meal and toasted.

Statistical Analysis of the Data

Statistical analyses of the composition data were conducted by Certus International, Inc., Chesterfield, MO. Analytes with fifty percent or more of the observations at or below the LOD of the assay were excluded from statistical analysis. A range of values was determined for the non-transgenic and reference lines but these were not included in the statistical analysis. Cottonseed samples from all plots were analyzed for all components. Fatty acid, cyclopropenoid fatty acid, vitamin E and gossypol content in cottonseed oil were measured for a single sample per line that was composited from all eight field locations. The gossypol content of cottonseed meal was also measured for one composite sample per line.

Statistical analyses of the seed composition data were conducted using a mixed model analysis of variance. Combined site analyses used the model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + Lt_{ij} + e_{ijk},$$

where U = overall mean, T_i = line effect, L_j = random location effect, $B(L)_{jk}$ = random block effect with location effect, Lt_{ij} = random location by line interaction effect and e_{ijk} = residual error. For each compositional analysis component, the test event 15985 was

compared to the parental control line DP50B. Data for seed compositional analysis components from additional commercial reference lines were not included in the statistical analysis. However, a range of values was determined for the transgenic, non-transgenic and combined reference lines. SAS® software was used to generate all summary statistics and perform all analyses. Report tables present p-values from SAS as either <0.001 or as the actual p-value truncated to three decimal places. A p-value less than 0.05 means that the difference is significant at a level of 5%.

Appendix 3, Table 1. Summary of Analytical Methods Used for Compositional Analysis of Bollgard II Cotton Event 15985.

Type of Analysis	Method Description	Limit of Detection	Protocol Name and/or Reference
Protein	Nitrogenous compounds were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. Following alkalinization, the ammonia was distilled and titrated with a standard acid. The percent nitrogen was calculated and converted to protein using the factor 6.25.	0.1%	AOAC Official Methods, 16th Edition, 1998, Methods 955.04 and 979.09, modified Bradstreet, R. B., 1965, The Kjeldahl Method for Organic Nitrogen, Academic Press: New York, New York Kalthoff, I.M. and E.B. Sandell. Quantitative Inorganic Analysis. MacMillan, N.Y. (1948), modified.
Fat	The sample was weighed into a cellulose thimble containing sand or sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was evaporated, dried and weighed.	0.1%	AOAC Official Methods, 1998, 16th Edition, Method 960.39, modified.
Carbohydrates	The total carbohydrate level was calculated by difference using the fresh weight-derived data.	1.0%	USDA, 1973, Energy Value of Foods, Agriculture Handbook No. 74, pp. 2-11.
Calories	Calories were calculated using the Atwater factors with the fresh weight-derived data.	1.0 Kcal/100g	USDA, 1975, Composition of Foods, Agriculture Handbook No. 8, pp. 159-160.
Ash	The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine the percent ash.	0.1%	AOAC Official Methods, 1998, 16th Edition, Fourth revision, Method 923.03, modified.

Type of Analysis	Method Description	Limit of Detection	Protocol Name and/or Reference
Crude fiber	Crude fiber was quantitated as the loss on ignition of dried residue remaining after digestion of the sample with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions under specific conditions.	0.1%	AOAC Official Methods, 1998, 16th Edition, Fourth revision, Method 962.09, modified.
Moisture	The sample was dried in a vacuum oven at 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture.	0.1%	AOAC Official Methods, 16th Edition, 1998, Methods 926.08 and 925.09, modified
Fatty acids, including cyclopropenoid fatty acids	The total lipid fraction was extracted using $CHCl_3$/MeOH and quantitated gravimetrically. Free FA were extracted with ethyl ether and hexane and converted to their phenacyl derivatives with 2-bromoacetophenone. Quantitation was by UV-HPLC.	0.100%	Wood, R. 1986. High Performance Liquid Chromatography Analysis of Cyclopropene Fatty Acids, Biochemical Archives, 2:63-71, modified.
Vitamin E	The sample was saponified and extracted with ethyl ether. Quantification was by HPLC on a silica column.	0.100 - 0.200 mg/100g	Cort, W.M., Vincente, T.S., Waysek, E.H., and Williams, B.D., 1983, Vitamin E Content of Feedstuffs Determined by High-Performance Liquid Chromatographic Fluorescence, Jo. Ag. Food Chem. 31:1330-1333, modified.

Type of Analysis	Method Description	Limit of Detection	Protocol Name and/or Reference
Amino acids	Three methods were used to obtain the full profile: **Tryptophan** - NaOH hydrolysis **Sulfur-containing AA** - oxidation with performic acid, then HCl hydrolysis **Other AA** - HCl hydrolysis Once hydrolyzed, quantitation was by automated AA analyzer.	0.1 mg/g	AOAC Official Methods, 16th Edition, Fourth revision, 1998, Method 982.30, modified
Minerals	The sample was dried, precharred, and ashed overnight at 500°C ± 50°C. The ashed sample was treated with HCl, taken to dryness, and put into a solution of 5% HCl. The amount of each element was determined at appropriate wavelengths by comparing inductively coupled plasma emission of the unknown sample relative to the standards.	Ca 20.0 ppm Cu 0.500 ppm Fe 2.0 ppm Mg 20.0 ppm Mn 0.30 ppm P 20.0 ppm K 100 ppm Na 100 ppm Zn 0.40 ppm	Dahlquist, R.L., and Knoll, J.W., 1978, Inductively Coupled Plasma-Atomic Emission Spectrometry: Analysis of Biological Materials and Soils for Major, Trace, and Ultra Trace Elements, Applied Spectroscopy, 32:1-29, modified. AOAC Official Methods, 16th Edition, Fourth revision, 1998, Methods 984.27 and 985.01, modified

Type of Analysis	Method Description	Limit of Detection	Protocol Name and/or Reference
Total gossypol	The sample was extracted using a complexing reagent containing acetic acid, 3-amino-1-propanol, and dimethylformamide. The solution was filtered and the total gossypol was reacted with aniline. The dianilinogossypol was quantitated spectrophotometrically against a standard curve.	0.00500%	AOCS, Fifth Edition, 1997, BA 7-58 and BA 8-78, modified
Aflatoxins	The sample was moistened with dilute HCl, and extracted with $CHCl_3$. A portion of the extract was purified on a silica gel column and eluted with CH_2Cl_2 and acetone. The eluent was concentrated and quantitated by HPLC.	1.0 ppb	Proceedings of the 3rd International Congress of Food Science and Technology, pp. 705-711, modified. Aflatoxins in Cottonseed Product, Thin Layer and Liquid Chromatographic Methods, 1988, JAOAC, 71(1):26.052-26.060, modified.