Safety Assessment of Bollgard® Cotton Event 531

Executive Summary

Bollgard cotton, developed by Monsanto and field tested since 1992, produces an insect control protein (Cry1Ac) derived from the naturally occurring soil bacterium, *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*). Production of the Cry1Ac protein in the cotton plant provides effective season-long protection against key Lepidopteran insect pests, including tobacco budworm, pink bollworm and cotton bollworm (Wilson *et al.*, 1994; Betz *et al.*, 2000). Microbial formulations of *Bacillus thuringiensis* that contain the Cry1Ac insecticidal protein have been registered in numerous countries worldwide, and have been safely used for control of Lepidopteran insect pests for more than 40 years (Luthy *et al.*, 1982, Baum *et al.*, 1999). The Cry1Ac protein produced in Bollgard cotton is nearly identical in structure and activity to the Cry1Ac protein found in nature and in commercial *B.t.k.* microbial formulations. *Bacillus thuringiensis* and *B.t.k.* microbial formulations have been shown to be specific to the target insect pests and do not have deleterious effects to non-target organisms such as beneficial insects, birds, fish, and mammals, including humans (U.S. EPA, 1988).

The primary benefits of Bollgard cotton are reduced insecticide use, improved control of target insect pests, improved yield, reduced production costs, improved profitability, reduced farming risk, and improved opportunity to grow cotton, resulting in improved economics for the cotton growers (Edge *et al*., 2001; Carpenter and Gianessi, 2001; Betz *et al.*, 2000; Economic Research Service/USDA, 2000; Falck-Zepeda *et al.*, 1998; Falck-Zepeda *et al.*, 2000; Fernandez-Cornejo and McBride, 2000; Gianessi and Carpenter, 1999; Klotz-Ingram *et al.*, 1999; Traxler and Falck-Zepeda, 1999; Xia *et al.*, 1999). Planting of Bollgard cotton since 1996 in the US has resulted in a reduction in insecticide use of 2.7 million pounds of insecticidal active ingredients and a reduction in 15 million insecticide applications (Carpenter and Gianessi, 2001). US cotton growers planting Bollgard cotton showed a 260 million pound increase in cotton production per year which resulted in an estimated $99 million increase in net income in 1999 (Carpenter and Gianessi, 2001). There also are a number of secondary benefits associated with the reduction in insecticide use, which include enhanced populations of beneficial insect and wildlife populations, reduced potential runoff of insecticides, and improved safety for farm workers by reducing potential exposure.

The genetically improved Bollgard cotton product was produced using *Agrobacterium tumefaciens*-mediated transfer of the *cry1Ac* gene into the genome of a conventional cotton variety, Coker 312, using a binary plasmid vector. The *nptII* gene, which encodes a selectable marker enzyme, neomycin phosphotransferase II (NPTII), was also present on the plasmid to facilitate selection of insect-protected plants. The NPTII protein served no other purpose and has no pesticidal properties. The plasmid also contained the antibiotic resistance *aad* gene, which encodes the bacterial selectable marker enzyme 3"(9)-O-

® Bollgard is a registered trademark of Monsanto Technology LLC

aminoglycoside adenyltransferase (AAD). This gene confers resistance to the antibiotics spectinomycin and streptomycin, and facilitated the selection of bacteria containing the plasmid in the initial steps of transforming the cotton tissue. The *aad* gene is under the control of a bacterial promoter and the encoded protein is not detected in Bollgard cotton plant tissue.

In assessing the nutritional and compositional equivalence of Bollgard cotton to conventional cotton varieties, more than 2,500 separate analyses were performed on 67 components of the cottonseed and oil. These analyses included protein, fat, moisture, calories, minerals, amino acid, cyclopropenoid fatty acid and gossypol levels. The results of these analyses clearly demonstrate that, other than the production of the Cry1Ac and NPTII proteins, Bollgard cotton is compositionally equivalent to and is as safe as conventional cotton varieties currently available (Berberich *et al.*, 1996).

The following summary provides information on the methods used to develop Bollgard cotton event 531 and a summary of the food, feed and environmental safety studies that support the safety of Bollgard cotton. In addition to the molecular characterization, the following safety studies were conducted: safety of the produced proteins, food/feed composition, and environmental safety. On the basis of this evaluation, Bollgard cotton and its processed fractions were found to be substantially equivalent to conventionally bred cotton, taking into consideration the natural variation seen among cotton varieties, with the exception of the expression of the Cry1Ac and NPTII proteins. The Cry1Ac and NPTII proteins were shown to be safe for human and animal consumption and to the environment.

Introduction

Cotton is the leading plant fiber crop produced in the world and the most important in the United States. Lepidopteran insects are the main pest problem on most of the cotton acres produced in the United States and many parts of the world. The incorporation of the *B.t.* protein into cotton reduces, and in some cases eliminates, the need to spray chemical insecticides to control the major caterpillar pests. Bollgard cotton has value not only as a replacement for insecticide applications for specific pests, but also as a pest management tool that can provide benefits above and beyond the reduction in insecticide costs (Edge *et al*., 2001; Wier *et al.*, 1998). These additional benefits include reduced risk to grower health, improved environment for beneficial insects and wildlife, and a more stable economic outlook and productivity for the cotton industry.

Bollgard cotton was produced using *Agrobacterium tumefaciens*-mediated transfer of the *cry1Ac* gene, which encodes for insecticidal activity against Lepidopteran insect pests, into the genome of Coker 312 cotton. The Coker 312 cultivar was used because of its favorable response to the tissue culture system used in the process to produce transgenic plants. Although the Coker 312 is no longer widely grown, it is still a commercially acceptable cultivar. The Bollgard trait has since been transferred to other commercial cotton varieties using traditional breeding techniques.

The *A. tumefaciens* transformation system is well understood and has been utilized for many years in the genetic modification of many dicotyledonous plants. The plasmid vector was modified so that the transformation system cannot transmit crown gall disease. This transformation system stably inserts genes from the plasmid vector into the chromosome of the plant cell. Molecular characterization showed two T-DNA (transferred DNA) inserts were integrated into the cotton genome to produce Bollgard event 531. The *cry1Ac* gene segregated in a manner consistent with the presence of a single active copy of the coding sequence and was stably transferred to numerous other commercial cotton varieties by traditional breeding techniques.

The plasmid vector within the *A. tumefaciens* used to produce Bollgard cotton event 531 contains the fully sequenced *cry1Ac, nptII* and *aad* genes (Figure 1). The *cry1Ac* gene was derived from the common soil microbe *Bacillus thuringiensis* variety *kurstaki (B.t.k.*) and encodes an insecticidal protein, Cry1Ac. This *cry1Ac* gene cassette contains an e-35S promoter, and a 7S 3' transcriptional termination sequence. The *nptII* gene encodes a selectable marker enzyme, neomycin phosphotransferase II (NPTII), which was used to identify cotton cells containing the Cry1Ac protein. The *nptII* gene is driven by a cauliflower mosaic virus 35S promoter and is followed by a nopaline synthase (*nos*) 3' region that directs polyadenylation of the mRNA. The NPTII protein served no other purpose and has no pesticidal properties. The *aad* gene encodes the bacterial selectable marker enzyme 3"(9)-O-aminoglycoside adenylyltransferase (AAD), which allowed for the selection of the *Agrobacteria* on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter, and therefore the encoded protein is not expressed in plants derived from Bollgard cotton event 531.

The gene donor organisms include cauliflower mosaic virus (e-35S promoter), *A. tumefaciens* (nopaline synthase terminator), *E. coli* (*nptII* and *aad*) and soybean (*Glycine max*; 7S 3' termination signal). Cauliflower mosaic virus and *A. tumefaciens* are phytopathogens. *E. coli* is a gram-negative, non-pathogenic bacterium used for DNA cloning and vector construction. The characteristics of the cauliflower mosaic virus and *A. tumefaciens* donor organisms do not warrant any analytical or toxicological tests, as only the specific sequenced genes encoding enzymes were transferred to the host organism.

Molecular Characterization of Bollgard Cotton

Molecular characterization of Bollgard cotton event 531 demonstrated there are two T-DNA inserts. The primary functional insert contains single copies of the full-length *cry1Ac* gene, the *nptII* gene and the *aad* antibiotic resistance gene. This T-DNA insert also contains an 892 bp portion of the 3' end of the *cry1Ac* gene fused to the 7S 3' transcriptional termination sequence. This segment of DNA is at the 5' end of the insert, is contiguous and in the reverse orientation with the full-length *cry1Ac* gene cassette and does not contain a promoter. A transcript was detected by RT-PCR, which corresponds to this 3' segment of the *cry1Ac* gene and the adjacent genomic DNA. In the unlikely

event that this RNA were translated, the resulting peptide would be highly homologous to the corresponding portion of the C-terminus of the Cry1Ac protein. The safety of this theoretical protein is addressed by the safety studies described in the following sections. The protein, if produced, would have been a constituent in all safety studies conducted with either the Cry1Ac protein or with Bollgard cotton plants or cottonseed.

The second T-DNA insert contains 242 bp of a portion of the 7S 3' polyadenylation sequence from the terminus of the *cry1Ac* gene. No RNA transcript was detected by RT-PCR that corresponds to or would have been transcribed from the 242 bp 7S 3' T-DNA insert and hence no peptide is produced, as expected.

The selfed data from the crosses to other commercial cotton varieties demonstrates the stability of the transfer of the functional insert from generation to generation. The *cry1Ac* gene in Bollgard cotton has been demonstrated to be stably integrated into the chromosome based on molecular analyses, data on phenotypic expression and inheritance patterns. The results of these studies are summarized as follows:

- Southern blot analyses of numerous generations of Bollgard cotton performed over the past eight years have provided an identical Southern blot pattern, indicating stability of the functional *cry1Ac* gene insert;
- analyses (ELISA) of seed obtained from multi-site trials over eight years showed similar levels of the Cry1Ac and NPTII proteins;
- production of the Cry1Ac protein has been confirmed by immuno-detection and/or efficacy data under different environmental conditions and in numerous Bollgard cotton varieties;
- Mendelian inheritance of the Bollgard trait is observed after self-pollination or backcrossing with other cotton varieties;
- the insecticidal efficacy has been maintained during the development of this product and since its marketing in 1996 and total production on over 17 million acres planted in the U.S.; and
- the seed quality of Bollgard cotton has been maintained after transfer of the *cry1Ac* gene into different genetic backgrounds.

Based on this information, there is no evidence or likelihood of genetic or efficacy instability. These data confirm that the Bollgard trait is stably integrated in the cotton genome.

Cry1Ac and NPTII Protein Levels in Bollgard Cotton Plants

Cry1Ac and NPTII proteins are produced at low levels in the various tissues of the Bollgard cotton plant. Data generated from 1992 samples are presented in Tables 1 and 2. Cry1Ac and NPTII proteins were detected in event 531 and were not detected, as expected, in the Coker 312 parental line. The mean levels of the Cry1Ac protein in 1992

were 1.56 and 0.86 µg/gram fresh weight in leaf and raw cottonseed, respectively. The mean levels of the NPTII protein in 1992 were 3.15 and 2.45 µg/gram fresh weight, respectively for leaf and raw cottonseed. In eight years of field testing of samples from numerous sites, the mean level of the Cry1Ac protein in raw cottonseed ranged from approximately 1 to 9 µg/gram fresh weight. In raw cottonseed, the mean level of the NPTII protein ranged from 2.0 to15 µg/gram fresh weight over the same sites and years of field testing. Cry1Ac and NPTII proteins are present at low levels in whole plants collected just prior to defoliation. In field tests from the 1992 season mature Bollgard plants contained an estimated 0.08 µg Cry1Ac protein/g and 3.3 µg NPTII protein/g fresh weight of a mature, whole plant (approximately 10 µg Cry1Ac protein per plant). Two additional years of data from 1998 and 1999 field season showed Cry1Ac protein levels to range from <0.07 (limit of detection of the assay) to 0.19 µg/gram fresh weight. The Cry1Ac protein levels remained sufficiently high for effective control of the targeted insect pests throughout the season.

The Cry1Ac protein was not detected in nectar collected from Bollgard cotton using an assay with a limit of detection of 1.6 ng/g fresh weight of the nectar. The Cry1Ac protein is present in pollen at levels just above the limit of detection of the assay used to evaluate the Cry1Ac protein concentrations: 11.5 ng/g fresh weight of the pollen.

After processing, the levels of Cry1Ac protein were reduced to non-detectable levels in the major cottonseed processed products: refined oil, linter brown stock and cottonseed meal. The Cry1Ac protein was not detected by ELISA or insect bioassay in the processed cottonseed meal. The total protein content of refined cottonseed oil was found to be below the limit of detection of the assay (1.3 ppm). Linters from Bollgard cottonseed were analyzed for the presence of the Cry1Ac protein using western blot analysis and bioactivity. The Cry1Ac protein was detected at 0.1 µg/g weight of raw linters. After processing the linters to linter brownstock or to more highly purified cotton linters, the Cry1Ac protein was not detected at a limit of detection of 0.08 µg/g weight.

The AAD protein was not detected in the leaf or seed tissue from Bollgard cotton at the limit of detection of 0.008 and 0.005 µg/gram fresh weight for leaf and seed, respectively. This result was expected since the *aad* gene is driven by a bacterial promoter and was not expected to be expressed in the cotton plant.

Safety Assessment of the Cry1Ac and NPTII Proteins in Bollgard Cotton

Safety assessments of the Cry1Ac and NPTII proteins expressed in Bollgard cotton event 531 include demonstrating the lack of similarity to known allergens and toxins and the long history of safe consumption of comparable proteins in microbial formulations, rapid digestion in simulated gastric and intestinal fluids, mode-of-action/specificity of the Cry1Ac protein, and the lack of acute oral toxicity in mice.

Mode of Action and Specificity of the Cry1Ac Protein

The Cry1Ac protein is produced as an insoluble crystal in the *B. thuringiensis* microbe. The crystal protein is composed of the pro-toxin form of the protein. Insecticidal activity of the Cry1Ac protein requires that the protein be ingested. In the insect gut, the protein is solubilized due to the high pH of the insect gut and is proteolytically cleaved to the active core of the protein, which is resistant to further degradation by the insect gut proteases. The core protein binds to specific receptors on the mid-gut of Lepidopteran insects, inserts into the membrane and forms ion-specific pores (English and Slatin, 1992). These events disrupt the digestive processes and cause the death of the insect. The digestive tract tissues of non-target insects, mammals, birds and fish do not contain receptors that bind the Cry1Ac protein. Therefore the Cry1Ac protein cannot disrupt digestion and is, therefore, non-toxic to species other than Lepidopteran insects (Betz *et al*., 2000; Hofmann *et al*., 1988).

Characterization and History of Safe Consumption of the Cry1Ac and NPTII Proteins

There is a history of safe use of Cry1Ac protein in microbial *Bt*-based products (U.S. EPA, 1988; IPCS, 2000). EPA and WHO have recognized the potential for dietary exposure to Cry proteins from use of microbial sprays on food crops: "The use patterns for *B. 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" (U.S. EPA, 1998) and "*B.t.* has not been reported to cause adverse effects on human health when present in drinking-water or food." (IPCS, 2000).

The amino acid sequence of the Cry1Ac protein expressed in Bollgard cotton has been predicted based on nucleotide sequence of the coding sequence. The Cry1Ac protein produced in Bollgard cotton is >99.4% identical to the protein produced by the *B. thuringiensis* subsp. *kurstaki* (*B.t.k*) bacterial strain. Strains of *B. thuringiensis* have been used safely as commercial microbial pesticides for over 40 years. The naturally occurring Cry proteins produced in *B.t.k* have been shown to have no deleterious effects to fish, avian species, mammals and other non-target organisms (U.S. EPA, 1988; Betz *et al.*, 2000). The safety of the Cry proteins to non-target species is attributed to the highly specific mode of action, and rapid digestibility.

The NPTII protein expressed in Bollgard cotton is chemically and functionally similar to the naturally occurring NPTII protein (Fuchs *et al.*, 1993).

Digestion of Cry1Ac and NPTII Proteins in Simulated Gastric and Intestinal Fluids

In addition to the lack of receptors for the Cry1Ac protein, the absence of toxic effects in humans and other mammals is further supported by the rapid degradation of the protein in an *in vitro* gastric digestion study. The rate of degradation of the Cry1Ac protein was

evaluated separately in simulated gastric (pepsin, pH 1.2) and intestinal (pancreatin, pH 7.5) fluids: the simulated gastric and intestinal fluids were constituted based on recommended levels in U.S. Pharmacopeia, 1995. The degradation of the Cry1Ac protein was assessed by western blot analysis and insect bioactivity. The study showed that the Cry1Ac protein degrades in approximately 30 seconds upon exposure to gastric fluid (Betz *et al*., 2000). The acid conditions of the stomach denature the native conformation of the Cry1Ac protein, facilitating its rapid degradation. In intestinal fluid, the Cry1Ac protein was converted to the protease-stable form and remained intact and bioactive for at least 21 hours. This result was expected since protease-resistance core proteins of *B.t*. insecticidal proteins are known to be resistant to further trypsin digestion. In vivo, the Cry1Ac protein would be exposed to gastric conditions prior to entering the intestinal lumen. The low pH and pepsin in the stomach would be expected to either fully digest the protein or render it susceptible to intestinal digestion.

The NPTII protein was shown to degrade rapidly under simulated mammalian digestive conditions. The degradation of the NPTII protein in digestion fluids was assessed over time by western blot analysis. The enzymatic activity of the NPTII protein was shown to be destroyed after a 2-minute incubation in simulated gastric fluid and a 15-minute incubation in simulated intestinal fluid (Fuchs *et al.*, 1993).

Lack of Acute Oral Toxicity of Cry1Ac and NPTII Proteins in Mice

Few proteins are toxic when ingested. When proteins are toxic they are known to act by acute mechanisms and at low dose levels (Sjoblad *et al.*, 1992). Results of a mammalian acute oral toxicity study support the specificity and safety of the Cry1Ac protein. There was no evidence of toxicity even at extremely high dose levels (4200 mg/kg body weight), when the Cry1Ac protein was administered orally to mice (Betz *et al.*, 2000). Therefore, the Cry1Ac protein is not considered toxic, except to target insect pests. Also, the Cry1Ac protein produced in the cotton plant is not expected to present a risk of dermal or inhalation toxicity. First, the expression level of the Cry1Ac protein in cotton is low, and the protein is found internally within the cell walls of the plant tissues, with little or no potential for dermal or inhalation exposure. Second, proteins that are non-toxic by the oral route are not expected to be toxic by the dermal or pulmonary route. Similarly, the NPTII protein caused no deleterious effects in mice when administered by gavage at dosages up to 5000 mg/kg body weight (Fuchs *et al.*, 1993).

Lack of Sequence Similarity of Cry1Ac and NPTII Proteins to Known Protein Toxins

One method for the assessment of potential toxic effects of proteins introduced into plants is to compare the amino acid sequence of the protein to that of known toxic proteins. Homologous proteins derived from a common ancestor have similar amino acid sequences, are structurally similar and share common function. Therefore, it is undesirable to introduce a DNA that encodes for a protein that is homologous to any toxin. Homology is determined by comparing the degree of amino acid similarity between proteins using published criteria (Doolittle *et al.*, 1990). The Cry1Ac protein

does not show meaningful amino acid sequence similarity when compared to known protein toxins present in the PIR, EMBL, SwissProt and GenBank protein databases, with the exception of other Cry proteins. The NPTII protein does not show meaningful amino acid sequence similarity when compared to known protein toxins present in these protein databases.

Assessment of Exposure of Humans to Cry1Ac and NPTII Proteins from Bollgard Cotton

Cottonseed oil and processed cotton linters are the only cotton products used for human food (National Cottonseed Products Association, 1989). Analysis of refined cottonseed oil derived from both the parental Coker 312 control line and Bollgard cotton event 531 confirmed that there is no detectable protein in cottonseed oil at a limit of detection for the assay of 1.3 ppm total protein. This is consistent with other reports that conclude the absence of protein in cottonseed oil (Cottonseed Oil, 1993). Analysis of processed linters also confirmed there was no detectable protein (Sims *et al.,* 1996). Therefore, significant human consumption of the Cry1Ac and NPTII proteins present in Bollgard cotton varieties is extremely unlikely. Furthermore, direct food challenge of individuals allergic to proteins contained in the meal derived from oilseed crops (*e.g*., soybean, peanut and sunflower) with the oil from these respective crops has established that refined oil does not elicit an allergenic response (Bush *et al.,* 1985; Halsey *et al.,* 1986; Taylor *et al.,* 1981). This is consistent with the lack of detectable protein in the oil (Tattrie and Yaguchi, 1973). This information provides a strong basis to conclude that Bollgard cottonseed oil poses no significant allergenic concerns, based solely on lack of significant exposure.

Lack of Sequence Similarity of Cry1Ac and NPTII Proteins to Known Allergens

Although there are no single predictive bioassays available to assess the allergenic potential of proteins in humans (U.S. FDA, 1992), the physicochemical and human exposure profile of the protein provides a basis for assessing potential allergenicity by comparing it to known protein allergens. Thus, important considerations contributing to the allergenicity of proteins ingested orally includes exposure and an assessment of the factors that contribute to exposure, such as stability to digestion, prevalence in the food, and consumption pattern (amount) of the specific food (Metcalfe, *et al.,* 1996; Kimber *et al.,* 1999).

A key parameter contributing to the systemic allergenicity of certain food proteins is 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 food 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 Cry1Ac and NPTII proteins digestibility showed that these proteins are readily digested.

Another significant factor contributing to the allergenicity of certain food proteins is their high concentrations in foods (Taylor *et al.,* 1987; Taylor, 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). In contrast, the Cry1Ac and NPTII proteins are present at low levels in Bollgard cotton plants and are not detectable in the components of cotton that are used for food.

It is also important to establish that the protein does not represent a previously described allergen and does not share potentially cross-reactive amino acid sequence segments or structure with a known allergen. An efficient way to determine whether the added protein is an allergen or is likely to contain cross-reactive structures is to compare the amino acid sequence with that of all known allergens. A database of protein sequences associated with allergy and coeliac disease has been assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt). The amino acid sequences of the Cry1Ac and NPTII proteins were compared to these sequences and neither showed any meaningful amino acid sequence similarity with the known allergens (Astwood *et al.,* 1996).

In addition, the NPTII protein has been approved by the United States Food and Drug Administration as a processing aid food additive for tomato, cotton and canola (Food and Drug Administration, 1994), and exempted from the requirement of a tolerance as an inert ingredient by the United States Environmental Protection Agency (U.S. EPA, 1994). These approvals included an assessment of potential allergenic effects for the NPTII protein, and both agencies concluded there were no significant concerns.

In summary, these data and analyses support the conclusion that Cry1Ac and NPTII proteins are not detectable in cotton products used for human food, do not pose a significant allergenic risk, are not derived from allergenic sources, do not possess immunologically-relevant sequence similarity with known allergens, and do not possess the characteristics of known protein allergens as summarized below. This conclusion is supported by the lack of any reports of sensitization to the commercial microbial formulations and the lack of allergic concerns with the Cry proteins (McClintock *et al.,* 1995).

Characteristics of known allergenic proteins

Characteristic	***Allergens***	**Cry1Ac**	**NPTII**
Stable to digestion	yes	no	no
Stable to processing	yes	no	no
Similarity to known allergens	yes	no	no
Prevalent protein in food	yes	no	no

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

Compositional Analysis and Nutritional Assessment of Bollgard Cotton

Compositional analyses were conducted to assess the levels of key nutrients and anti-nutrients in cotton to assess whether there were any changes in these components compared to conventional cotton varieties, including the parental control from which Bollgard was generated. These data, which demonstrate that Bollgard cotton is compositionally equivalent to conventional cotton, are summarized in Tables 3 to 8. The components important for food and feed uses were assessed. These analyses included:

- Proximate analysis: protein, fat, ash, water, carbohydrate, calories (Table 3).
- Fatty acid profile: total lipid content, percentage of individual fatty acids (Table 4).
- Amino acid composition: percentage of individual amino acids (Table 5).
- Levels of three anti-nutrients: gossypol, and cyclopropenoid fatty acids and aflatoxin (Tables 6 and 7).

Results of these analyses (Tables 3 to 6) demonstrate that seed from Bollgard cotton is compositionally equivalent to, and as nutritious as, seed from the parental cotton variety and other commercial cotton varieties (Berberich *et al.*, 1996).

In addition to seed, the refined oil from Bollgard cotton was also shown to be equivalent to those products produced from the control cotton cultivar (Table 7). The refined oil was evaluated for fatty acid profile (including cyclopropenoid fatty acids), free and total gossypol content, and tocopherol levels. The fatty acid profile was typical of commercial cottonseed oil. Free gossypol was reduced to undetectable levels after processing and the tocopherol levels were comparable to levels found in commercial cottonseed oil.

Toasted cottonseed meal was evaluated for free gossypol and total gossypol content. After toasting, free gossypol was reduced to levels acceptable for feed use in both the Bollgard and conventional cotton varieties. The total gossypol content was within the acceptable range to allow the meal to be used as a protein supplement for feed (Table 8). Linters, the shorter fibers associated with the seed after ginning, are composed primarily of cellulose and are highly processed for both chemical and non-chemical uses. Yields of linters from Bollgard cotton were comparable to those of the control and to ranges reported for other cotton varieties (Table 9). Therefore, insertion of the DNA including the Cry1Ac coding sequence in the cotton genome did not alter the processing characteristics of the cottonseed.

In summary, a detailed compositional analysis has been performed on the Bollgard cotton event 531 (Berberich *et al*., 1996). Bollgard event 531 does not differ in composition from the parent or commercial cultivars regarding nutrients such as total protein, fat, fiber, ash, and amino acids, demonstrating that there is no significant quantitative or qualitative difference between Bollgard cotton event 531 and the cotton cultivar from which it was derived, or values for other reported varieties, with regard to these

components. Therefore the nutritional value of Bollgard cottonseed is substantially equivalent to other cotton varieties currently available.

In addition to the compositional studies, the nutritional wholesomeness of seed from Bollgard cotton was demonstrated by feeding rats and dairy cows diets which contained raw cottonseed from both the Bollgard cotton and control cotton cultivars. At completion of the rat study, there were no significant differences in weight gain or feed intake between rats consuming Bollgard cotton and the control cotton diet (Table 10). Results of the dairy cow study showed that the cottonseed from Bollgard cotton performed comparable to the control cottonseed. There were no significant differences in milk yield, milk composition and body condition score of the cows (Castillo *et al*., 2001). Furthermore, Bollgard cotton has been planted on a total of more than 17 million acres commercially since 1996 with no reports of differences in animal feed performance.

Horizontal Gene Transfer and the Assessment of Marker Genes

Horizontal gene transfer is defined as the transfer of DNA from one species to another. With respect to crop plants that are developed through biotechnology, a number of assessments have been performed to evaluate the possibility that antibiotic resistance marker genes used to facilitate the selection of the transformed plants might be transferred to bacteria either in the field or in animals that have consumed the crop. The reason for the assessment is that some species of bacteria found in soil, in the rumen or in the intestine can receive DNA from other organisms through three mechanisms of transfer (Morrison, 1996; Davison, 1999). However, only one mechanism, transformation, is relevant to the possible transfer of DNA from plants to bacteria and subsequent expression of the encoded protein product. The other two mechanisms, conjugation (exchange of plasmid DNA between compatible bacteria) and transduction (viral transfer of DNA into bacteria) are specific to restricted forms of transfer and are not relevant to the potential transfer of DNA from plants (Thomson, 2000). In general, bacterial species differ markedly in their ability to accept DNA from the environment, and the frequency of transformation even under ideal circumstances is very low. The DNA that was transferred into cotton to produce Bollgard cotton was incorporated into the genomic DNA of the plant and represents a small fraction of cotton genome. The probability that a bacteria would take up the marker genes from the transformation is the same as from any other randomly chosen piece of DNA from the plant.

Horizontal Gene Transfer in the Field

The factors affecting possible "horizontal" gene transfer between genetically modified plants expressing antibiotic resistance marker genes and microorganisms in the environment has been extensively studied (Prins and Zadoks, 1994; Schlüter *et al.*, 1995; Nielsen *et al.*, 1998; Smalla *et. al*., 2000). To date, there is no experimental evidence that any antibiotic resistance marker gene from a plant has transformed a bacterium either in laboratory conditions, or in the field (Broer *et al.*, 1996; Schlüter *et al*., 1995; Nielsen *et al.*, 1997). Most bacteria in natural environments are not competent to accept DNA. Even under

laboratory conditions, studies specifically designed to detect the transfer of functional marker genes from plants into bacteria have failed to demonstrate such an occurrence.

Horizontal Gene Transfer from Food and Feed Products

In addition to the field environment, several studies have addressed the potential for the horizontal transfer of antibiotic selectable marker genes from transgenic plants to microflora in the gut of humans, ruminants or other animals. The probability of this event occurring is virtually zero (Prins and Zadoks, 1994; Schlüter *et al.*, 1995; Nielsen *et al.*, 1998, Beever and Kempe, 2000).

If a marker gene were to be transferred, an important question would be whether there is any added risk regarding the abundance of antibiotic resistant bacteria. Recently, Smalla *et al.*, (2000), published a thorough review of the potential hazard associated with horizontal gene transfer of an antibiotic resistant marker from a plant to a microorganism and concluded that "it is unlikely that antibiotic resistance genes used as markers in transgenic crops will contribute significantly to the spread of antibiotic resistance in bacterial populations". As such, the risk associated with an antibiotic resistant marker in a modified crop is considered minimal.

Bollgard cotton contains two antibiotic marker genes, *aad* and *nptII*. The *aad* gene was isolated from transposon Tn7 that is commonly found in gram-negative bacteria (Shaw *et al.*, 1993). If a gut bacterium were to acquire the *aad* gene, it would have no selective advantage in the absence of spectinomycin or streptomycin. The AAD protein is ubiquitous in nature and therefore consumed as part of our natural diet. Even if the AAD protein were present in the gut, it could not compromise the therapeutic efficacy of these antibiotics, as the AAD enzyme needs specific cofactors at appropriate concentrations to function, which are not found in the gut. Databases of protein sequences were screened with the AAD amino acid sequence; no similarities to known toxins or allergens were revealed (Kärenlampi, 1996). The *nptII* gene was isolated from transposon Tn5, which is found in a number of gram-negative bacteria, including strains that naturally colonize the human gut (Kärenlampi, 1996). Additionally, if the *nptII* gene was transferred to a microbe, it would not be expressed unless it was integrated into a region containing a bacterial promoter as the *nptII* gene is regulated by a plant promoter.

The origin of replication for plasmid maintenance at high copy number in *E. coli*, *ori322*, contained on the plasmid PV-GHBK04 that was used for transformation, was not transferred into the cotton plant genome. Therefore the antibiotic resistance genes in Bollgard cotton can not be mobilized by excision of the marker gene with other inserts to create a functional plasmid. The DNA would have to be integrated into the recipient's genome or plasmid in order to replicate and be passed on through reproduction.

The question of the transfer of antibiotic resistance marker genes has recently been discussed in detail by scientific experts in the European Union in relation to an application to market an insect-protected maize under Directive 90/220 and at a seminar

organized by the biomolecular engineering commission and the genetic engineering commission. The European Commission requested the opinion of three Scientific Committees, which focused in particular on the risks of transfer of the *bla* gene that confers ampicillin resistance to bacteria in the gastro-intestinal tract of humans and animals. The scientific committees concluded that '(a) the possibility of transfer of a functional *bla*-gene construct' is virtually zero, and (b) that if the virtually impossible event occurred, it would have no clinical significance' (http:/europa.eu.int/comm/food/fs/sc/oldcomm6/out01_en.html). A similar conclusion was reached by Salyers, 1998.

Environmental Assessment

Cotton

Cotton is of the genus *Gossypium*, of the tribe Gossypieae, and of the family *Malvaceae*. Four species of cotton are of agronomic importance worldwide: the two diploid Asiatic species, *G. arboreum* and *G. herbaceum*, and the two allotetraploid New World species, *G. barbadense* and *G. hirsutum*. Although the diploid species remain important in restricted areas of India, Asia, and Africa, the two New World species account for approximately 98% of world cotton fiber production. Wild species of *Gossypium* typically occur in arid parts of the tropics and sub-tropics. Wild populations *of G. hirsutum* are relatively rare and tend to be widely dispersed.

Outcrossing Potential

Cotton is predominantly a self-pollinating crop, but can be cross-pollinated by certain insects. However, outcrossing of the *cry1Ac* gene from Bollgard cotton to other *Gossypium* species or to others of the malvacea family is extremely unlikely for the following reasons (Percival *et al.*, 1999):

- Cultivated cotton is an allotetraploid and is incompatible with cultivated or wild diploid cotton species; therefore, it cannot cross and produce fertile offspring.
- Although outcrossing to wild or feral allotetraploid *Gossypium* species can occur, commercial cotton production generally does not occur in the same geographical locations as the wild relatives. For example, outcrossing to *G. tomentosum* in Hawaii is possible, but no commercial cotton is grown in Hawaii.
- There are no identified non-cotton plants that are sexually compatible with cultivated cotton.

If the *cry1Ac* gene were to be transferred to a wild population of a tetraploid cotton species, and if this was considered undesirable, the size of the plants, their perennial growth habit, their restricted habitat and their low natural fecundity would make them easy to control. Crossing of the insect protection trait into other cultivated cotton genotypes is possible should the plants be in close proximity; however, studies have shown that this occurs at a very low frequency and is not considered to be a concern as it is unlikely to cause any adverse impact to the environment (Green and Jones, 1953; Mehetre, 1992).

Agronomic Performance

Field test data concerning yields and visual observations of agronomic properties including susceptibility to diseases and insects indicate that Bollgard 531 cotton is not different in agronomic performance compared to non-modified varieties. Bollgard cotton does not pose any different plant pest risk to other plants and the environment than conventional cotton varieties as demonstrated by the following information:

Weediness Potential

Bollgard cotton does not have any different weediness characteristics than other conventional cotton varieties. Cotton is not considered to have weediness characteristics, such as seed dormancy, soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle or high seed output and dispersal. Bollgard cotton does not exhibit different agronomic or morphological traits compared to controls, which would confer a competitive advantage over other species in the ecosystem in which it is grown. Also, there is little probability that any Gossypium species crossing with Bollgard cotton could become more weedy. All wild and feral relatives of cotton are tropical, woody, perennial shrubs other than a few herbaceous shrubs (Percival *et al.*, 1999). In most instances, the distribution of these species is determined by soil and climatic conditions. As perennials, the plants are not particularly programmed to produce seed each year. Based on these mechanistic arguments and field experience there is no indication that insertion of the *cry1Ac* gene into the cotton genome would have any effect on the weediness traits of the cotton plant.

Lack of Effect to Non-target Organisms

There is extensive information about microbial preparations of *Bacillus thuringiensis* subsp. *kurstaki (B.t.k)* containing Cry proteins, including the Cry1Ac protein, that demonstrate that these proteins are non-toxic to non-target organisms (U.S. EPA, 1988; Betz *et al.*, 2000). The literature has established that the Cry proteins are extremely selective for the lepidopteran insects, bind specifically to receptors on the mid-gut of lepidopteran insects and have no deleterious effect on beneficial/non-target insects.

To confirm and expand on results obtained for the microbial products that contain the same Cry1Ac protein as Bollgard cotton, the potential impact of the Cry1Ac protein on non-target organisms was assessed on several representative organisms. The non-target insect species included larvae and adult honey bee (*Apis mellifera* L.), a beneficial insect pollinator; green lacewing larvae (*Chrysopa carnea*), a beneficial predaceous insect commonly found on cotton and other cultivated crops; parasitic Hymenoptera (*Nasonia vitripennis*), a beneficial parasite of the housefly; the ladybird beetle (*Hippodamia convergens*), a beneficial predacious insect which feeds on aphids and other plant bugs commonly found on stems and foliage of weeds and cultivated plants; and Collembola (*Folsomia candida* and *Xenylla grisea*) non-target soil organisms (Betz *et al.*, 2000).

There were no deleterious effects on the growth and development of the insects and test organisms (Betz *et al.*, 2000). No effects were observed when *Folsomia candida* and *Oppia nitens* (Acari: Orbatidae) were fed transgenic cotton leaf material containing the Cry1Ac protein (Yu *et al.*, 1997; Betz *et al.*, 2000).

An assessment of potential impacts to birds present in Bollgard fields was conducted. Bobwhite quail chicks were fed diet containing 10% raw cottonseed meal from Bollgard

cotton and control cotton. This feeding level of cottonseed approximates consumption of 400 seeds/kg body weight per bird. There was no difference in the feed consumption or weight gain for chicks eating diet with Bollgard cottonseed meal versus the control cottonseed meal (Betz *et al*., 2000).

The purified Cry1Ac protein was administered orally, by gavage, to male and female mice at 500, 1000 and 4200 mg/kg body weight. The growth and feed consumption of the mice was unaffected by the Cry1Ac protein (Betz *et al.*, 2000). The levels of protein exposed to the mice represented a safety factor of more than 50,000 times the amount that a cow would consume when eating raw cottonseed.

Environmental fate of Cry1Ac protein

USDA has conducted environmental assessments of Cry proteins and has issued findings of no significant impact (FONSI) for the Cry1Ac protein (USDA, 1995). Cry protein crystals have been found to degrade readily in the field due to solar radiation and temperature (Palm *et al*., 1993, 1994, 1996).

The environmental fate of purified Cry proteins has been extensively studied. The published literature has demonstrated that Cry protein adsorption to soil is rapid and complete within 30 minutes (Venkateswerlu and Stotzky, 1992). Numerous other studies of the biodegradation and binding of Cry proteins in soil have been conducted, including Tapp *et al.,* (1994), Tapp and Stotzky (1995; 1998), Crecchio and Stotzky (1998), Koskella and Stotzky (1997). These studies demonstrate that isolated Cry proteins could bind to clay particles and humic acids in artificial soil mixes.

The Cry1Ac protein levels were measured in whole mature plants obtained from field tests in 1992 and 1993 at the end of the season. Those data were used to estimate the amount of Cry1Ac protein that would enter the environment after harvest when the plants are plowed into the soil. For the two years evaluated (1992 and 1993), the load to the soil was estimated to be 1.44 and 0.6 grams Cry1Ac protein per acre, respectively. Based upon these values, an *in vitro* soil degradation study was conducted using insecticidal activity to measure degradation of the protein. This study showed that the Cry1Ac protein was rapidly degraded in the soil in both the purified form of the protein and as part of the cotton plant tissue. The half-life of the Cry1Ac protein in plant tissue was calculated to be 41 days, which is comparable to the degradation rates reported for *B.t.* microbial formulations (Betz *et al.*, 2000). The half-life for the purified protein was less than 20 days. These values are similar to the degradation rates observed by Palm et al., (1993, 1994, 1996) for transgenic plants producing Cry proteins.

Summary

The introduction of Bollgard cotton has reduced the number and cost of insecticide applications needed for control of cotton bollworm, tobacco budworm and pink bollworm, and offers a number of secondary benefits. The introduced Cry1Ac protein is

comparable to Cry proteins that have been safely used for over 40 years. Detailed food, feed, and environmental safety assessments confirm the safety of this product. These analyses included: 1) detailed molecular characterization of the introduced DNA; 2) safety assessments of the expressed Cry1Ac and NPTII proteins; 3) compositional analysis of cottonseed, oil and meal; and 4) environmental impact assessment of the Cry1Ac protein and Bollgard cotton plants. These studies demonstrate the Cry1Ac protein poses minimal risk to non-target organisms, including humans, animals and beneficial insects. Based on the available data and experience collected to date, Bollgard cotton poses comparable or fewer risks to the environment as traditional cotton treated with commercially approved insecticides. Rather, the reduction of insecticide applications as a result of using Bollgard affords significant environmental benefit. Additionally, Bollgard cotton plants, cottonseed, cottonseed oil and fiber were shown to be equivalent to and hence as safe as conventional cotton varieties.

Information and data contained within this document have been provided to regulatory authorities for review. Regulatory review continues as we update regulatory files and make submissions to additional countries globally.

References

Astwood, J.D. and R.L. Fuchs. 1996. Food allergens are stable to digestion in a simple model of the gastrointestinal tract. Journal of Allergy and Clinical Immunology 97: 241.

Astwood, J.D., J.N. Leach, and R.L. Fuchs. 1996. Stability of food allergens to digestion *in vitro.* Nature Biotechnology 14:1269-1273.

Baum, J.A. T.B. Johnson, and B.C. Carlton. 1999. *Bacillus thuringiensis* natural and recombinant bioinsecticide products. *In* Methods in Biotechnology. Pp 189-209. Vol 5. Biopesticides: Use and Delivery (F.R. Hall and J.J. Mean (eds). Humana Press, Inc., Totowa, N.J.

Beever, D.E., and C.F. Kemp. 2000. Safety issues associated with the DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures. Nutrition Abstracts and Reviews Series B: Livestock feeds and feeding. 70: 175-182.

Belyea, R.L., B.J. Steevens, R.J. Restrepo, and A.P. Clubb. 1989. Variation in composition of by-product feeds. J. Dairy Sci. 72:2339-2345.

Berardi, L.C. and L.A. Goldblatt. 1980. Gossypol. Pp. 184-237. *In*: Toxic Constituents of Foodstuffs.. 2nd Ed. Liener, I.E. (ed.), Academic Press, New York.

Berberich, S.A., J.E. Ream, T.L. Jackson, R. Wood, R. Stipanovic, P. Harvey, S. Patzer, and R.L. Fuchs. 1996. The composition of insect-protected cottonseed is equivalent to that of conventional Cottonseed. J. Agri. Food Chem. 44:365-371.

Betz, F.S., B.G. Hammond and R.L. Fuchs. 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regulatory Toxicology and Pharmacology 32:156-173.

Broer, I., W. Droge-Laser, and M. Gerke. 1996. Examination of the putative horizontal gene transfer from transgenic plants to agrobacteria. Pp 67-70. *In* E.R. Schmidt, T. Hankeln (eds.). Transgenic organisms and biosafety. Springer Verlag Berlin, Heidelberg, New York.

Bush, R.K., S.L. Taylor, J.A. Nordlee, and W.W. Busse. 1985. Soybean oil is not allergenic to soybean-sensitive individuals. J. Allergy Clin. Immunol. 76(2): 242-245.

Carpenter, J.E. and L.P. Gianessi. 2001. Agricultural biotechnology: updated benefit estimates. National Center for Food and Agricultural Policy, Washington, D.C.

Castillo, A.R., M.R. Gallardo, M. Maciel, J.M. Giordano, G.A. Conti, M.C. Gaggiotti, O. Quaino, C. Gianni, and G.F. Hartnell. 2001. Effect of feeding dairy cows with either Bollgard BollgardII, Roundup Ready or control cottonseeds on feed intake, milk yield and milk composition. J.Dairy Sci., Vol 84, suppl. 1, Abstract 1712.

Cherry, J.P., J.G. Simmons, and R.J. Kohel. 1978a. Potential for improving cottonseed quality by genetic and agronomic practices. Pp 343-364. *In* Nutritional Improvement of Food and Feed Proteins; Friedman, M. (ed.), Plenum Press: New York.

Cherry, J.P., J.G. Simmons, and R.J. Kohel. 1978b. Cottonseed composition of national variety test cultivars grown at different Texas locations. Pp 47-50. *In* Proceedings of the Beltwide Cotton Production Research Conference, Dallas, TX, (J.M. Brown, (ed.) National Cotton Council: Memphis, TN.

Cherry, J. P. 1983. Cottonseed oil. J. Am. Oil Chem. Soc. 60(2):360-367.

Cherry, J.P. and H.R. Leffler. 1984. Chapter 13: Seed. Pp 511-569. *In* Cotton; Kohel, R.J., and C.F. Lewis, (eds.) No. 24 in AGRONOMY Series; American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc. Publishers: Madison, WI.

Cottonseed Oil. 1993. L.A. Jones and C.C. King (eds.), National Cottonseed Products Associations, Inc. and the Cotton Foundation, Memphis, TN.

Crecchio, C. and .G. Stotzky. 1998. Insecticidal activity and biodegradation of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound to humic acids from soil. Soil Bio. Biochem., 30:463-470.

Davison, J. 1999. Genetic exchange between bacteria in the environment. Plasmid 42, 73-91.

Dicks, M.W. 1965. Vitamin E content of foods and feeds for human and animal consumption. Univ. Wyoming Agricultural Experiment Station. Bulletin 435.

Doolittle, R.F, D.F. Feng, K.L Anderson, and M.R. Alberro. 1990. A naturally occurring horizontal gene transfer from a eukaryote to a prokaryote. J. Molecular Evolution 31:383-388.

Economic Research Service/USDA. 2000. Geneticallyengineered crops: has adoption reduced pesticide use? Pp13-17. Agricultural Outlook, August 2000.

Edge, J.M., J.H. Benedict, J.P. Carroll, and H.K. Reding. 2001. Bollgard Cotton: An assessment of global economic, environmental, and social benefits. J. Cotton Science 5:1-8.

English, L. and S.L. Slatin. 1992. Mode of action of delta-endotoxin from *Bacillus thuringiensis*: a comparison with other bacterial toxins. Insect Biochem. Molec. Biol., 22(1):1-7.

FAO (Food and Agriculture Organization). 1995. Report of the FAO Technical Consultation on Food Allergies, Rome, Italy, November 13-14, 1995. FAO, Rome.

Falck-Zepeda J.B., G. Traxler, and R.G. Nelson. 1998. Rent creation and distribution from biotechnology innovations: the case of Bt cotton and herbicide-tolerant soybeans in 1997. Agribusiness 16:1-25. ISAAA Briefs No. 14 ISAAA, Ithaca, NY.

Falck-Zepeda J.B., G.Traxler, and R.G. Nelson. 2000. Surplus distribution from the introduction of a biotechnology innovation. Am J of Agric Economics 82:360-369.

Fernandez-Cornejo J, and W.D. McBride. 2000. Genetically engineered crops for pest management in U.S. agriculture: farm level effects. Economic Research Service/U.S. Department of Agriculture-Agricultural Economic Report (AER)-786.

Fuchs, R.L., J.E. Ream, B.G. Hammond, M.W. Naylor, R.M. Leimgruber and S.A. Berberich. 1993. Safety assessment of the neomycin phosphotransferase II (NPTII) protein. Bio/Technology 11:1543-1547.

Fuchs, R.L. and J.D. Astwood. 1996. Allergenicity assessment of foods derived from genetically modified plants. Food Technology 50: 83-88.

Gianessi, L.P. and J.E. Carpenter. 1999. Agricultural Biotechnology: Insect Control Benefits. National Center for Food and Agricultural Policy. http://www.bio.org/food&ag/ncfap.htm

Green, J.M. and M.D. Jones. 1953. Isolation of cotton for seed increase. Agron. J. 45:366-368.

Halsey, A.B., M.E. Martin, M.E. Ruff, F.O. Jacobs and R.L. Jacobs. 1986. Sunflower oil is not allergenic to sunflower seed-sensitive patients. J. Allergy Clin. Immunol 78(3):408-410.

Hofmann, C., P. Lüthy, R. Hutter, and V. Pliska. 1988. Binding of the delta endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). Eur. J. Biochem. 173: 85-91.

IPCS (International Programme on Chemical Safety). 2000. Environmental Health Criteria, 217: *Bacillus thuringiensis*. http://www.who.int/pcs/docs/ehc_217.html

Kärenlampi, S. 1996. Health effects of marker genes in genetically engineered food plants. TemaNord 530.

Kimber, I., N.I. Kerkvliet, S.L. Taylor, J.D. Astwood, K. Sarlo, and R.J. Dearman. 1999. Toxicology of protein allergenicity: prediction and characterization. Toxicological Sciences 48:157-162.

Klotz-Ingram, C., S. Jans, J. Fernandez-Cornego and W. McBride. 1999. Farm-level production effects related to the adoption of genetically modified cotton for pest management. AgBioForum 2(2):73-84.

Kohel, R.J., J. Glueck, and L.W. Rooney. 1985. Comparison of cotton germplasm collections for seed-protein content. Crop Sci. 25:961-963.

Koskella, J. and G. Stotzky. 1997. Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. Appl. Environ. Microbiol. 63(9):3561-3568.

Lawhon, J.T., C. M. Cater, and K.F. Mattil. 1977. Evaluation of the food use potential of sixteen varieties of cottonseed. J. Amer. Oil Chem. Soc. 54:75-80

Lüthy, P., J.L. Cordier, and H.M. Fischer. 1982. "*Bacillus thuringiensis* as a bacterial insecticide: basic considerations and applications", Pp 35-74. *In* Microbial and Viral Pesticides. E. Kurstak (ed.), Marcel Dekker, Inc., New York.

McClintock, J.T., C.R. Schaffer and R.D. Sjoblad. 1995. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. 45:95-105.

Mehetre, S.S. 1992. Natural crossing in cotton (*Gossypium Sp*.): Its significance in maintaining variety purity and production of hybrid seed using male sterile lines. J. Cotton Res. & Dev. 6(2):73-97.

Metcalfe, D.D., J.D. Astwood, R. Townsend, H.A. Sampson, S.L. Taylor, and R.L. Fuchs. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Reviews in Food Science and Nutrition 36(S):S165-S186.

Morrison, M. 1996. Do ruminal bacteria exchange genetic material? J. Dairy Sci. 79, 1476-1486.

National Cottonseed Products Association. 1989. Cottonseed and Its Products, 9th ed. Memphis TN.

Nielsen, K.M., F. Gebhard, K. Smalla, A.M. Bones, and J.D. van Elsas. 1997. Evaluation Of possible horizontal gene transfer from trangenic plants to the soil bacterium *Acinetobacter calcoaceticus* Bd413. Theoretical and Applied Genetics 95(5/6):815 - 821.

Nielsen, K.M. , A.M. Bones, K. Smalla, and J.D. van Elsas. 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria - A rare event? FEMS Microbiology Reviews 22:79 - 103.

Palm, C.J., R.J. Seidler, K.K. Donegan, and D. Harris. 1993. Transgenic plant pesticides: Fate and Persistence in soil. Plant Physiol. Suppl. 102, 166.

Palm, C.J., K.K. Donegan, D. Harris, and R.J. Seidler. 1994. Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* delta-endotoxin from transgenic plants. Mol. Ecol. 3:145-151.

Palm, C.J., D.L. Schaller, K.K. Donegan,, and R.J. Seidler. 1996. Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki* delta-endotoxin. Can. J. Microbiol. 42:1258-1262.

Percival, A. E., J.F. Wendel, and J.M. Stewart. 1999. Taxonomy and Germplasm Resources. *In* Cotton: Origin, History, Technology, and Production. Pp 33-63. Smith, W.C. (ed.). John Wiley and Sons, Inc. chapter 1.2.

Prins, T.W. and J.C. Zadoks. 1994. Horizontal gene transfer in plants, a biohazard? Outcome of a Literature review. Euphytica 76:133-138.

Rossell, J.B. 1991. Vegetable oils and fats. Pp 261-327. *In* Analysis of Oilseeds, fats, and fatty foods. Rossell, J.B., and J.L.R. Pritchard (eds.). Elsevier Applied Science Publisher, Ltd.; New York.

Salyers, A. 1998. Genetically engineered foods:safety issues associated with antibiotic resistance genes. 'http://www.healthsci.tufts.edu/apua/salyersreport.htm'

Schlüter, K., J. Fütterer, and I. Potrykus. 1995. "Horizontal" gene transfer from a transgenic potato line to a bacterial pathogen (*Erwinia chrysanthemi*) occurs - if at all- at an extremely low frequency. Bio/Technology 13:1094-1098.

Shaw, K. J., P.N. Rather, R.S. Hare, and G.H. Miller. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside modifying enzymes. Microbiol. Rev. 57:138-163.

Sims, S.R., S.A. Berberich, D.L. Nida, L.L. Segalini, J.N. Leach, C.C. Ebert, and R.L. Fuchs. 1996. Analysis of expressed proteins in fiber fractions from insect-protected and glyphosate-tolerant cotton varieties. Crop Sci. 36(5):1212-1216.

Sjoblad, R.D., J.T. McClintock and R. Engler. 1992. Toxicological considerations for protein components of biological pesticide products. Regulatory Toxocology and Pharmacology 15:3-9.

Smalla, K., S. Borin, H. Heuer, F. Gebhard, J.D. van Elsas, and K. Nielsen. 2000. Horizontal transfer of antibiotic resistance genes from transgenic plants to bacteria. *In* Proceedings of the 6th International Symposium on the Biosafety of GMOs. Pp 146-154. Fairbairn, C, G. Scoles, and A. McHughen (eds.). University Extension Press, University of Saskatchewan; Saskatoon, Saskatchewan.

Tapp, H., and G. Stotzky. 1995. Insecticidal activity of the toxins from *Bacillus thuriengiensis* subsp. *kurstaki* and *tenebrionis* adsorbed and bound on pure and soil clays. Appl. Environ. Microbiol. 61(5):1786-1790.

Tapp, H., and G. Stotzky. 1998. Persistence of the insecticidal toxin from *Bacillus thuriengiensis* subsp. *kurstaki* in soil. Soil Biol. Biochem. 30:471-476.

Tapp, H., L. Calamai, L. and G. Stotzky. 1994. Adsorption and binding of the insecticidal proteins from *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* on clay minerals. Soil Biol Biochem. 26:663-679.

Tattrie, N.H., and M. Yaguchi. 1973. Protein -content of various processed edible oils. J. Inst. Can. Sci. Technol. Aliment. 6(4):289-290.

Taylor, S.L., W.W. Busse, M.I. Sachs, J.L. Parker, and J.W. Yunginger. 1981. Peanut oil is not allergenic to peanut-sensitive individuals. J. Allergy Clin. Immunol 68(5):372-375.

Taylor, S.L., R.F. Lemanske Jr., R.K. Bush, and W.W. Busse. 1987. Food allergens: structure and immunologic properties. Ann. Allergy 59(5):93-99.

Taylor, S.L. 1992. Chemistry and detection of food allergens. Food Technology 46: 146-152.

Thomson, J. 2000. Gene transfer: Mechanisms and Food Safety Risks. Topic 11. Biotech 00/13. Joint FAO/WHO Expert Consultation on foods derived from biotechnology. 29 May-2 June, 2000. World Health Organization, Geneva, Switzerland.

Traxler G., and J. Falck-Zepeda. 1999. The distribution of benefits from the introduction of transgenic cotton varieties. AgBioForum 2(2):94-98.

Turner, J.H., H.H. Ramey Jr. and S. Worley Jr.. 1976. Influence of environment on seed quality of four cotton cultivars. Crop Sci. 16:407-409.

USDA. 1995. Availability of Determination of Non-regulated status for genetically engineered cotton. Federal Register 60(134):36096-36097.

U.S. EPA. 1988. Guidance for the re-registration of pesticide products containing *Bacillus thuringiensis* as the active ingredient. NTIS PB 89-164198.

U.S. EPA. 1994. Neomycin phosphotransferase II; tolerance exemption. Federal Register 59(187):49351-49353.

U.S. EPA. 1998. R.E.D. Facts: *Bacillus thuringiensis*. EPA 738-F-98-001.

U.S. FDA. 1992. Statement of policy: Foods derived from new plant varieties. Federal Register 57(104):22984-23005.

U.S. FDA. 1994. "Secondary Direct Food Additives Permitted in Food for Human Consumption; Food Additives Permitted in Feed and Drinking Water of Animals; Aminoglycoside 3' Phosphotransferase II" Federal Register 59(98):26700-26711.

United States Pharmacopeia. 1995. Volume 23. United State Pharmacopeial Convention, Inc., Rockville, MD.

Venkateswerlu, G. and G. Stotzky. 1992. Binding of the protoxin and toxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* on clay minerals. Current Microbiol. 25:225-233.

Wier, A.T., J.W. Mullins, and J.M. Mills. 1998. Bollgard Cotton - Update and economic comparisons including new varieties. *Proceedings of the Beltwide Cotton Conference 2:* 1039-1040. *National Cotton Council.* Memphis, TN.

Wilson, F.D., H.M. Flint, W.R. Deaton, and R.E. Buehler. 1994. Yield, yield components, and fiber properties of insect-resistant cotton lines containing a *Bacillus thuringiensis* toxin gene. Crop. Sci. 34:38-41.

Xia, J.Y., J.J. Cui, L.H. Ma, S.L. Dong and X.F. Cui. 1999. The role of transgenic *Bt* Cotton in integrated insect pest management Acra Gossypii Sin. 11:57-64.

Yu, L., R.E. Berry and B.A. Croft. 1997. Effects of *Bacillus thuringiensis* toxins in transgenic cotton and potato on *Folsomia candida* (*Collembola: Isotomidae*) and *Oppia nitens* (*Acari: Orbatidae*). J. Econ. Entomol. 90(1):113-118.

Figure 1: Plasmid map of PV-GHBK04

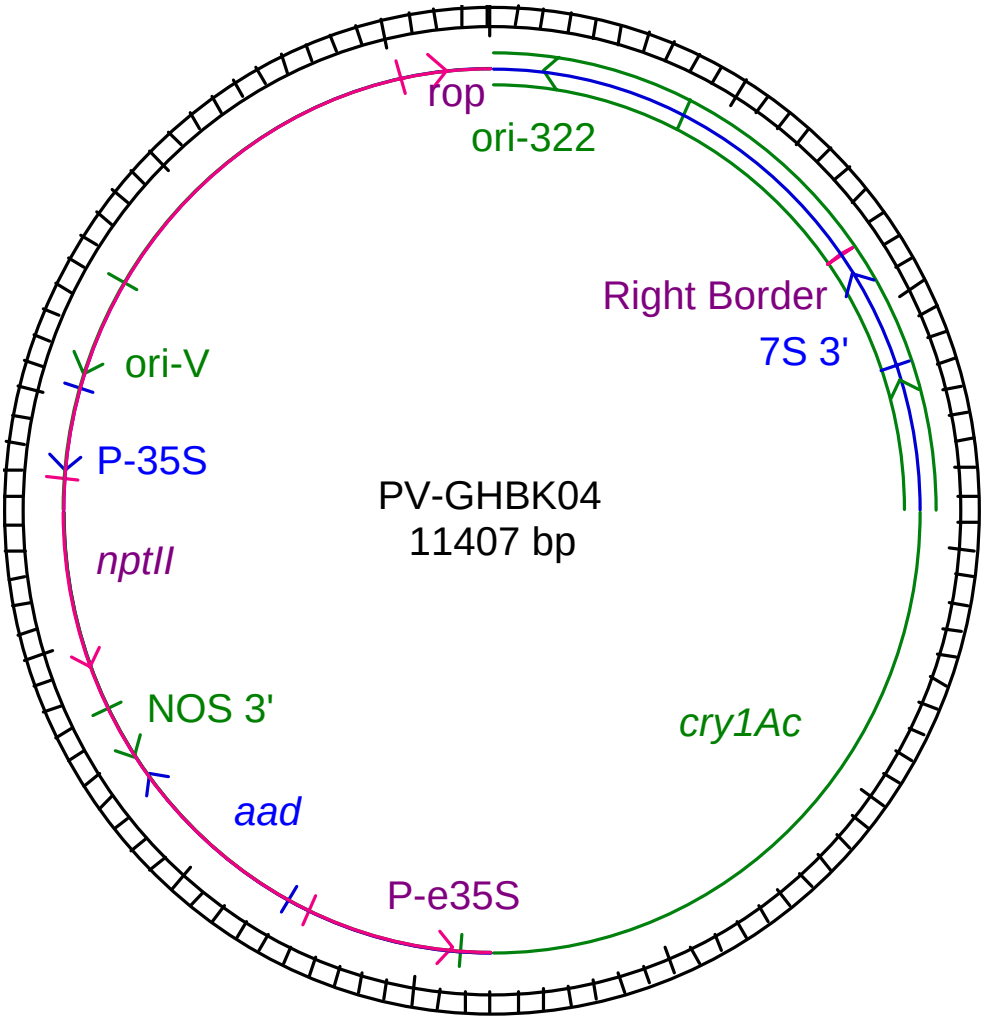

Table 1. Levels of Cry1Ac, NPTII, and AAD Proteins in Cotton Leaf Tissue in 1992

	µg/g tissue fresh weight	
Analyte	***Coker 312*** [a]	***Line 531*** [a]
Cry1Ac mean	ND	1.56
range	NA	1.18-1.94
std err.	NA	0.15
NPTII mean	ND	3.15
range	NA	2.46-3.84
std err.	NA	0.270
AAD mean	ND	ND
range	NA	NA
std dev.	NA	NA

[a] Mean expression level across all field test locations. N=36, 6 samples per each of six sites.
ND=non-detectable; NA=not applicable

Table 2. Levels of Cry1Ac, NPTII, and AAD Protein Expression in Cottonseed Tissue in 1992

	µg/g tissue fresh weight	
Analyte	***Coker 312***[a]	***Line 531***[a]
Cry1Ac mean	ND	0.86
range	NA	0.40-1.32
std err.	NA	0.18
NPTII mean	ND	2.45
range	NA	1.97-2.93
std err.	NA	0.19
AAD mean	ND	ND
range	NA	NA
std err.	NA	NA

[a] Mean expression level across all field test locations. N=36, 6 samples per each of six sites.
ND=non-detectable; NA=not applicable;

Table 3. Proximate Analysis of Cottonseed from Bollgard Cotton Event 531 and Coker 312 (1993 Field Trials)

Component	C312		Bollgard 531		Literature range/mean value	Literature reference
Characteristic†	**Mean**[1]	(Range)‡	**Mean**[2]	(Range)‡		
Protein %	27.00	(23.3-28.4)	27.56	(22.8-31.0)	18.8-22.9 23.5-29.5 12-32	Turner, *et al.*, 1976. Cherry, *et al.*, 1978a. Kohel, *et al.*, 1985.
Fat %	22.96	(19.6-25.1)	23.23	(22.2-25.8)	23.2-25.7 21.4-26.8	Cherry, *et al.*, 1978b. Cherry, *et al.*, 1978a.
Ash %	4.63	(4.3-5.0)	4.53	(3.9-4.7)	4.1-4.9 3.8	Cherry, *et al.*, 1978b. Belyea, *et al.*, 1989.
Carbohydrate %	45.40	(42.8-47.6)	44.68	(42.0-46.7)		
Calories/100g	496.32	(479-508)	498.11	(495-511)		
Moisture %	12.36	(9.6-15.9)	13.43	(11.2-14.7)	5.4-10.1	Cherry, *et al.*, 1978a.

† Protein, fat, ash, carbohydrate, and calories reported as percent dry weight of sample.

‡ Range denotes the lowest and highest individual values across sites for each cultivar or event .

[1] Value reported is least squares mean of five samples, one from each field site where bulk seed from cultivar C312 was collected.

[2] Value reported is least squares mean of four samples, one from each field site where bulk seed from Bollgard cotton event 531 was collected. No statistically significant differences from the Coker 312 control.

Table 4. Lipid and Fatty Acid Composition of Cottonseed from Bollgard Event 531 and Coker 312(1993 Field Trials)

Component	*C312*[a,b]		*Bollgard 531*[a,b]	
	Mean	*Range*[c]	*Mean*	*Range*
Lipid	33.5	30.9-35.5	33.5	30.8-35.9
Myristic (14:0)	0.94	0.67-1.07	0.88	0.75-0.98
Pentadecanoic (15:0)	0.40	0.32-0.60	0.62	0.32-0.90
Palmitic (16:0)	26.5	24.8-27.8	26.3	25.1-27.2
Palmitoleic (16:1)	0.64	0.48-0.71	0.61	0.54-0.64
Margaric (17:0)	0.16	0.13-0.20	0.18	0.14-0.27
Stearic (18:0)	2.63	2.32-3.26	2.90	2.71-3.26
Oleic (18:1)	15.3	14.8-16.0	16.8	14.8-19.1
Linoleic (18:2)	47.8	46.4-49.9	45.6	41.6-49.0
Linolenic (18:3)	0.20	0.13-0.29	0.14	0.13-0.18
Arachidic (20:0)	0.29	0.26-0.31	0.28	0.22-0.33
Behenic (22:0)	0.15	0.12-0.17	0.14	0.13-0.15
Malvalic (C-17)	0.37	0.22-0.45	0.38	0.23-0.47
Sterculic (C-18)	0.59	0.48-0.70	0.62	0.54-0.69
Dihydrosterculic (C-19)	0.36	0.29-0.50	0.49	0.24-0.84

[a] Value of lipid is % of dry sample weight. Value of fatty acid is % of total lipid.

[b] Values presented are least squares mean and ranges of five samples for C312 and four samples for Bollgard event 531.

[c] Range denotes the lowest and highest individual value across sites for each variety.

No statistically significant differences from the Coker 312 control

Table 5. Amino Acid Composition of Cottonseed† from Bollgard Cotton Event 531 and Coker 312 (1993 Field Trials)

	Literature			
Amino Acid	**Max[1]**	**Min[1]**	**C312[2]**	**Bollgard 531[3]**
Aspartic acid	9.5	8.8	9.72	9.49
Threonine	3.2	2.8	3.40	3.42
Serine	4.4	3.9	4.62	4.67
Glutamic Acid	22.4	20.5	19.56	18.21*
Proline	4.0	3.1	4.22	4.03
Glycine	4.5	3.8	4.32	4.18
Alanine	4.2	3.6	4.12	4.03
Cysteine	3.4	2.3	1.60	1.68
Valine	4.7	4.3	4.50	4.09*
Methionine	1.8	1.3	1.48	1.94*
Isoleucine	3.4	3.0	3.26	3.02*
Leucine	6.1	5.5	5.98	5.93
Tyrosine	3.3	2.8	2.92	3.08*
Phenylalanine	5.6	5.0	5.32	5.28
Lysine	4.1	3.9	4.50	4.73*
Histidine	2.8	2.6	2.72	2.94*
Arginine	12.3	10.9	11.20	11.68
Tryptophan	1.4	1.0	1.04	1.00

† Amino acids reported as mg/kg dry weight of protein in the cottonseed.

[1] Lawhon, 1977 (only one relevant reference could be cited, resulting in a narrow range for comparison that is probably not representative of variation in levels of amino acids between different cotton varieties).

[2] Value reported is least squares mean of five samples, one from each field site where bulk seed from cultivar C312 was collected.

[3] Value reported is least squares mean of four samples, one from each field site where bulk seed from Bollgard event 531 was collected.

* Significantly different from the control cultivar C312, at the 5% level (paired t-test). Additional years' data have shown these differences are not consistent across years and in different genetic backgrounds indicating that differences observed in 1993 are not attributable to the genetic trait.

Table 6. Aflatoxin and Gossypol Levels Determined in Cottonseed from Bollgard Cotton Event 531 and Coker 312 (1993 Field Trials)

	% Total Gossypol†		Aflatoxin
Variety	**Mean**	**Range**	
C312	1.16[a]	(0.97-1.43)[a]	ND[b]
Bollgard 531	1.10	(0.86-1.29)	ND

† Gossypol expressed as percent dry weight of seed: literature range is 0.39 - 1.7% (Berardi and Goldblatt, 1980)

[a] Values reported for seed samples are the least squares mean (from statistical analyses); ranges represent the lowest and highest values among six samples per variety, one sample per site where bulk seed samples collected.

[b] Not detected at a limit of detection of 1 ppb

Table 7. Fatty Acid† Profile of Refined Oil from Bollgard Cotton Event 531 and Coker 312 (1993 Field Trials)

Fatty Acid	Literature Range	*Refined Oil (% of total fatty acids)* Coker 312	Bollgard 531
Myristic (14:0)	(0.5-2.5) [1] (0.68-1.16)[2]	0.98	0.77
Palmitic (16:0)	(17-29)[1] (21.63-26.18)[2]	25.42	25.08
Palmitoleic (16:1)	(0.5-1.5) [1] (0.56-0.82)[2]	0.64	0.58
Stearic (18:0)	(1.0-4.0)[1] (2.27-2.88)[2]	2.53	2.67
Oleic (18:1)	(13-44)[1] (15.17-19.94)[2]	14.92	15.89
Linoleic (18:2)	(33-58)[1] (49.07-57.64)[2]	50.27	50.88
Linolenic (18:3)	(0.1-2.1)[1], (0.23)[3]	0.16	0.17
Arachidic (20:0)	(<0.5)[1], (0.41)[3]	0.21	0.30
Behenic (22:0)	(<0.5)[1]	0.12	0.13
Malvalic (C-17	(0.22-1.44)[4]	0.36	0.46
Sterculic (C-18	(0.08-0.56)[4]	0.48	0.43
Dihydrosterculic (C-19)	NA	0.22	0.16
Total gossypol	0.01% (1ppm)[2]	0.09	ND
Free gossypol	0.01% (1 ppm)[2]	ND	ND
α-Tocopherol[5]	136 - 660 [6]	638	568

[1]Ranges adopted by the FAO/WHO Codex Alimentarius committee on fats and oils (Cottonseed oil, 1993).
[2]Cherry and Leffler, 1984.
[3]Cherry, J.P., 1983.
[4]Cottonseed Oil, 1993. Values reported for crude cottonseed oil.
[5] α-tocopherol reported as mg/kg of oil
[6] Rossel, 1991; Dicks, 1965
ND = Not Detected, NA = Not Available

Table 8. Total and Free Gossypol Levels Determined in Raw Cottonseed Meal, and Toasted Meal from Bollgard Cotton Event 531 and Coker 312*

	% Total Gossypol	% Free Gossypol
Raw Meal		
C312	1.06	0.667
Bollgard 531	1.05	0.687
Toasted Meal		
C312	1.11	0.011
Bollgard 531	0.87	0.008

* Values were obtained from analysis of one composite sample comprised of seed from all field sites where bulk cottonseed were collected in the 1993 field test.

Table 9. Yield of Linters Fractions from Processing Cottonseed

	Yield (lbs)	% Yield	% Yield across Cultivars
C312	5.3	11.8	9.9-12.4[1], 8.4[2]
Bollgard 531	7.5	14.7	

[1] Cherry and Leffler, 1984.
[2] Cottonseed and its Products, 1989.

Table 10. Summary of Rat Weight Gain in One month Rat feeding study with Bollgard Cottonseed Meal*

		Male Body Weight/g		Female Body Weight/g	
		Pre-test	**28 days**	**Pre-test**	**28 days**
C312	Mean	174.5	321.9	140.0	191.9
	Std. Dev.	10.29	18.87	6.51	10.31
	Sample size	10	10	10	10
Bollgard 531	Mean	174.8	323.8	140.9	189.3
	Std. Dev.	10.38	14.39	8.15	10.31
	Sample size	10	10	10	10

* Dietary concentration of approximately 10%