

Safety Assessment of Roundup Ready[®] Cotton Event 1445

Executive Summary

Using modern biotechnology, Monsanto Company has developed Roundup Ready[®] cotton plants that confer tolerance to glyphosate, the active ingredient in Roundup agricultural herbicides, by the production of the CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein. Three genes were introduced into the cotton genome to produce this product: the *cp4 epsps* gene derived from the common soil bacterium *Agrobacterium* sp. strain CP4 which encodes the naturally glyphosate-tolerant EPSPS protein, the target site for glyphosate action; the *neomycin phosphotransferase II (nptII)* gene which provides a plant selectable marker; and the 3''(9)-*O*-aminoglycoside adenylyltransferase (*aad*) gene, a bacterial selectable marker. The *cp4 epsps* and *nptII* genes are expressed in the plants; the *aad* gene-product is not produced in the plants as this gene is under the control of a bacterial promoter.

Results from field experiments conducted in 1992, 1993 and 1994 at over 65 locations throughout the United States and commercial production of Roundup Ready cotton for four years on six million acres in United States alone, have demonstrated that cotton containing the Roundup Ready gene is tolerant to topical applications of glyphosate up to the four-leaf stage of growth. Glyphosate, the active ingredient in Roundup agricultural herbicides, has been shown to be highly effective in controlling the majority of annual and perennial grasses and broad-leafed weeds. Growers planting Roundup Ready cotton are able to reduce the number of herbicides used to control the economically destructive weeds that grow in their fields (Culpepper and York, 1998).

The following summary provides information on the methods used to develop Roundup Ready cotton, the proteins produced, as well as an overview of the food, feed and environmental safety studies that have been conducted on Roundup Ready cotton. This summary includes: molecular characterization of the DNA inserted into Roundup Ready cotton, an assessment of the safety of the produced proteins, compositional analyses of the food and feed components to evaluate substantial equivalence to conventional cotton varieties, and an assessment of the environmental safety of this product.

These studies demonstrate the food, feed and environmental safety of Roundup Ready cotton by establishing the safety of the CP4 EPSPS and NPTII proteins to humans, animals and other non-target organisms; establishing the nutritional equivalence and wholesomeness of Roundup Ready cottonseed compared to cottonseed from conventional varieties; and confirming the lack of a negative impact of Roundup Ready cotton on the environment.

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Introduction

Monsanto Company has developed Roundup Ready cotton plants that are tolerant to glyphosate. The primary mode of action of glyphosate is the competitive inhibition of the enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This enzyme is essential for the production of aromatic amino acids in plants via the shikimate pathway. When conventional plants are treated with glyphosate, the plants cannot produce the aromatic amino acids needed to survive. Roundup Ready cotton plants, developed using modern biotechnology, produce the CP4 5-enolpyruvylshikimate-3-phosphate synthase protein (CP4 EPSPS) derived from *Agrobacterium* sp. strain CP4. The CP4 EPSPS enzyme is naturally less sensitive to inhibition by glyphosate and thus imparts tolerance to Roundup agricultural herbicides.

Roundup agricultural herbicides are used as foliar-applied, non-selective herbicides, which are effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate has no pre-emergence or residual soil activity (Franz *et al.*, 1997). Furthermore, glyphosate is not prone to leaching, degrades in soil over time, and will not cause unreasonable effects to mammals, birds or fish under normal use conditions (U.S.EPA, 1993; WHO, 1994; Giesy *et al.*, 2000; Williams *et al.*, 2000).

Roundup Ready cotton offers growers an additional tool for improved weed control. Control of weeds in the cotton crop is essential, as weeds compete with the crop for sunlight, water and nutrients. Failure to control weeds within the crop results in decreased yields and reduced crop quality. In addition, weeds reduce the efficiency of the mechanical harvest of the crop. Weeds can also reduce the quality of the lint because vegetation stains the lint, reducing its potential uses and value.

Roundup Ready cotton has been produced commercially in the U.S. since 1997 and provides a number of environmental and economic benefits:

- Improved flexibility in weed control compared to herbicide programs used in conventional cotton, as specific pre-emergent herbicides that are used for prevention are replaced by a broad-spectrum post-emergent herbicide that can be used on an 'as needed' basis (Welch *et al.*, 1997; Culpepper and York, 1998).
- Less labor required due to the elimination of hand weeding and high cost, early post-directed sprays which require special equipment (McCloskey *et al.*, 1998).
- High compatibility with Integrated Pest Management and soil conservation techniques (Keeling *et al.*, 1998; Patterson *et al.*, 1998; Smart and Bradford, 1999), resulting in a number of important environmental benefits including reduced soil erosion and improved water quality (Baker and Laflen, 1979; Hebblethwaite, 1995; CTIC, 1998), improved soil structure with higher organic matter (Kay, 1995; CTIC, 2000), improved wildlife habitat (Phatak, 1998) and improved carbon sequestration (Reicosky, 1995; Reicosky and Lindstrom, 1995) and reduced CO₂ emissions (Kern and Johnson, 1993; CTIC, 2000).

In conclusion, weeds are a severe constraint in the production of cotton worldwide. Cotton cannot compete effectively in its early growth stages and must be protected from the invasion of aggressive weeds. Present management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. The introduction of Roundup Ready cotton has reduced the number and quantity of herbicide applications resulting in improved flexibility with effective control of the weeds. The use of Roundup Ready cotton also offers environmental benefits associated with the use of conservation-tillage and integrated weed management practices.

Molecular Characterization of Roundup Ready Cotton

Roundup Ready cotton event 1445 was developed by transforming cultivar Coker 312, which was used because of its positive response to the tissue culture system. Although Coker 312 is no longer widely grown, it is still a commercially acceptable cultivar.

Roundup Ready cotton was produced by introducing the *cp4 epsps*, *nptII* and *aad* coding sequences into Coker 312 cotton-derived tissue using *Agrobacterium tumefaciens* binary single border transformation vectors (Bevan, 1984; Wang, *et al.*, 1984). The plasmid vector PV-GHGT07 (Figure 1), contains well characterized DNA segments required for selection and replication of the plasmid in bacteria, as well as a right border for initiating transfer of plasmid DNA into plant genomic DNA.

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 used in this transformation system was modified so that it could not transmit crown gall disease. This transformation system stably inserts the genes into the chromosome of the plant cell. Molecular characterization demonstrates that one T-DNA (transferred DNA) insert was integrated into the cotton genome to produce Roundup Ready cotton. This insert contains the CoMVb promoter region, *cp4 epsps*, *aad*, *nptII*, and a portion of *ori-V* coding regions. The plasmid PV-GHGT07 also contains a second gene involved in conferring glyphosate tolerance. The *gox* gene encodes the glyphosate-metabolizing enzyme glyphosate oxidoreductase (GOX), cloned from *Achromobacter sp.* Strain LBAA. However, the *gox* gene is not present in Roundup Ready cotton event 1445 and no GOX protein is produced (Nida *et al.*, 1996a).

Agrobacterium sp. strain CP4, the source of the *cp4 epsps* gene, and transposon Tn5 from *E. coli*, the source of the *nptII* gene, are both ubiquitous in nature and are not considered to be pathogenic (U.S. FDA, 1994; Harrison, *et al.*, 1996). The *nptII* gene encodes a selectable marker enzyme, neomycin phosphotransferase II (NPTII), which was used to identify transformed cotton cells containing the CP4 EPSPS protein. The *nptII* coding sequence is driven by a Cauliflower Mosaic Virus 35S promoter and is followed by a nopaline synthase (*nos*) 3' region which directs polyadenylation of the messenger RNA (mRNA). The *aad* gene encodes the bacterial selectable marker enzyme 3''(9)-O-aminoglycoside adenyltransferase

(AAD), which allowed for the selection of bacteria containing the plasmid on media containing spectinomycin or streptomycin. The bacteria were used to transform the cotton tissue. The *aad* gene is under the control of a bacterial promoter and therefore the encoded protein is not expressed in Roundup Ready cotton.

The inserted genes are inherited in the expected Mendelian pattern and have been stably maintained for greater than 10 generations of event 1445. Furthermore, the consistent commercial performance of Roundup Ready cotton over the past four years and six million acres in the US alone supports the stability of the inserted DNA and functioning of the CP4 EPSPS protein.

CP4 EPSPS and NPTII Protein Levels in Roundup Ready Cotton Plants

Enzyme linked immunosorbent assays (ELISA) (Harlow and Lane, 1988) and western blot (Matsudaira, 1987) methods were developed and optimized to estimate CP4 EPSPS and NPTII protein levels in cotton leaf and seed matrices. Data generated from 1993 samples are presented in Tables 1 and 2. CP4 EPSPS and NPTII proteins were detected in event 1445 and were not detected, as expected, in the Coker 312 parental line. The mean level of CP4 EPSPS protein in leaf tissue harvested from event 1445 in 1993 was 0.052 µg/mg tissue on a fresh weight basis. The mean level of CP4 EPSPS protein in seed from cotton event 1445 was 0.082 µg/mg tissue. The mean level for NPTII protein in leaf tissue from event 1445 was 0.045 µg/mg tissue on a fresh weight basis, and the mean level of NPTII protein in seed from cotton event 1445 was 0.0067 µg/mg tissue. These protein levels are considered to be extremely low compared to total protein levels.

The AAD protein was not detected in either leaf or seed samples at a limit of detection of 0.136 and 0.160 ng/ml leaf and seed extract, 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 CP4 EPSPS and NPTII Proteins in Roundup Ready Cotton

Safety assessments of the CP4 EPSPS and NPTII proteins expressed in Roundup Ready cotton event 1445 include protein characterization demonstrating the lack of similarity to known allergens and toxins and the long history of safe consumption of similar proteins, digestibility *in vitro*, and the lack of acute oral toxicity in mice.

CP4 EPSPS and NPTII Protein Characterization and History of Safe Consumption

The CP4 EPSPS protein expressed in Roundup Ready cotton is functionally similar to a diverse family of EPSPS proteins present in food and feed derived from plant and microbial sources (Harrison, *et al.*, 1996). The EPSPS proteins are required for the production of aromatic amino acids. The structural relationship between CP4 EPSPS and other EPSPS proteins found in food is demonstrated by comparison of the amino acid sequences with

conserved identity of the active site residues, and the expected conserved three-dimensional structure based on similarity of the amino acid sequence. The NPTII protein in Roundup Ready cotton is coded by the *nptII* sequence, derived from the Tn5 transposon which is found in a number of naturally occurring bacteria (Fuchs *et al.*, 1993a; U.S. FDA, 1994).

Digestion of CP4 EPSPS and NPTII in Simulated Gastric and Intestinal Fluids

In vitro, simulated mammalian gastric and intestinal digestive mixtures were used to assess the susceptibility of CP4 EPSPS and NPTII proteins to proteolytic digestion. Rapid degradation of the proteins correlates with limited exposure to the gastrointestinal tract and little likelihood that these proteins would be food allergens. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopeia (1995).

CP4 EPSPS protein was shown to be rapidly degraded by the components of the *in vitro* digestive system (Harrison, *et al.*, 1996). Western blot analysis demonstrated a half-life for CP4 EPSPS protein of less than 15 seconds in the simulated gastric system and less than 10 minutes in the simulated intestinal system. If the CP4 EPSPS protein did survive the gastric system, it would be rapidly degraded in the intestine. Rapidly digested proteins represent a minimal risk of conferring novel toxicity or allergy comparable to other safe dietary proteins (Astwood *et al.*, 1996; Astwood and Fuchs, 2000).

The NPTII protein was shown to degrade rapidly under simulated mammalian digestive conditions (Fuchs, *et al.*, 1993). The enzymatic activity of the NPTII protein was shown to be destroyed after a two minute incubation in simulated gastric fluids and a 15 minute incubation in simulated intestinal fluid.

Assessment of Acute Oral Toxicity of CP4 EPSPS and NPTII Proteins in Mice

Few proteins are toxic when ingested. Those that are toxic, typically act acutely. As a precaution acute and toxicity studies were performed by oral gavage dosing mice with CP4 EPSPS and NPTII. These tests were performed to directly assess any potential toxicity associated with the proteins (Harrison *et al.*, 1996; Fuchs *et al.*, 1993b). Acute administration was considered appropriate to assess the safety of CP4 EPSPS protein, since proteins that are toxic act via acute mechanisms (Sjoblad *et al.*, 1992). There were no treatment-related adverse effects in mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg. Similarly the NPTII protein caused no deleterious effects when administered by oral gavage at dosages up to 5000 mg/kg of body weight. Results from this study demonstrated that the CP4 EPSPS and NPTII proteins are not acutely toxic to mammals. This result was expected since CP4 EPSPS and NPTII proteins are readily digested in gastric and intestinal fluids *in vitro* and both proteins are from families of proteins with a history of safe consumption.

Assessment of Sequence Similarity of CP4 EPSPS and NPTII Proteins to Known Protein Toxins

Another aspect used for the assessment of potential toxic effects of proteins introduced into plants is to compare the amino acid sequence of the protein to 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 which encodes 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 CP4 EPSPS and NPTII proteins do not show meaningful amino acid sequence similarity when compared to known protein toxins present in the PIR, EMBL, SwissProt and GenBank protein databases.

Assessment of Exposure of Humans to CP4 EPSPS and NPTII from Roundup Ready Cotton

Cottonseed oil and processed cotton linters are the major cotton products used for human food (National Cottonseed Products Association, 1989). Analysis of refined cottonseed oil and processed cotton linters derived from both the parental Coker 312 control line and Roundup Ready cotton event 1445 confirmed that there is no detectable protein in either cottonseed oil or processed cotton linters (Sims *et al.*, 1996). Therefore, significant human consumption of the CP4 EPSPS and NPTII proteins present in Roundup Ready 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 Roundup Ready cottonseed oil poses no significant allergenic concerns.

Assessment of Potential Allergenicity of CP4 EPSPS and NPTII Proteins

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 (Metcalf, *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 CP4 EPSPS and NPTII proteins digestibility indicates that these proteins are readily digested.

Another significant factor contributing to the allergenicity of certain food proteins is their high concentration 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 CP4 EPSPS and NPTII proteins are present at low levels in Roundup Ready cotton plants. The CP4 EPSPS and NPTII proteins are present approximately 0.031 and 0.0025%, respectively of the total protein in seed.

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 assess 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 CP4 EPSPS and NPTII proteins were compared to these sequences. Neither the CP4 EPSPS protein nor the NPTII protein shares 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 CP4 EPSPS 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.

Characteristics of known allergenic proteins

<i>Characteristic</i>	<i>Allergens</i>	CP4 EPSPS	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 Roundup Ready Cotton

The design of a food and feed safety assessment program for a genetically engineered crop requires detailed understanding of the uses of the crop and crop products in animal and human nutrition. Cotton is the leading plant fiber crop produced in the world and is grown primarily for its fiber. Cottonseed is processed to produce animal feed ingredients. Cottonseed meal is primarily used as cattle feed, with smaller proportions of meal fractions used in feed for poultry, sheep, catfish and swine. Cottonseed serves as an excellent source of fiber and protein, particularly due to its high lysine content. Oil is the main food ingredient derived from cottonseed and is used for frying oil and in salad dressings.

Compositional analyses were performed on the cottonseed from Roundup Ready cotton event 1445, the Coker 312 parental variety and commercial cotton varieties. These analyses included:

- Proximate analysis: protein, fat, ash, water, carbohydrate, calories (Tables 3 and 4)
- Amino acid composition: levels of individual amino acids (Table 5)
- Fatty acid profile: total lipid content and percentage of individual fatty acids in raw seed (Table 6) and refined cottonseed oil (Table 7)
- Anti-nutrients: levels of gossypol in refined oil (Table 7) and seed, raw and toasted cottonseed meal (Table 8) and cyclopropenoid fatty acids in seed (Table 6) and refined cottonseed oil (Table 7)

The results of these analyses, summarized in Tables 3 to 8, demonstrate that seed from Roundup Ready cotton is compositionally equivalent to, and as nutritious as, seed from the parental cotton variety and other commercial cotton varieties (Nida *et al.*, 1996b). Isolated statistical significant values were not considered to be biologically relevant.

The major cottonseed products, refined oil and meal from Roundup Ready cotton were also shown to be equivalent to those products produced from the control cotton line. The refined oil was evaluated for fatty acid profile, free gossypol content, and tocopherol levels. The fatty acid profile of the refined oil was typical of commercial cottonseed oil (Table 7). Free gossypol was reduced to undetectable levels and tocopherol was similar to levels previously reported in the literature. The full fat flour and toasted meal were analyzed for total gossypol levels. During the processing, the gossypol that partitions into the oil is essentially completely eliminated during the subsequent refining of oil. As expected there was no detectable free gossypol in toasted meal (Table 8). Therefore, insertion of the *cp4 epsps* and *nptII* genes in the cotton genome did not alter the processing characteristics of the cottonseed.

Levels of natural toxicants contained in cotton, such as gossypol and cyclopropenoid fatty acids (sterculic, malvalic, and dihydrosterculic) were comparable for Roundup Ready event 1445 and the parental cotton line. Gossypol levels in 1445 Coker 312 background were observed to be higher than the parental Coker 312 tested in 1993, but analysis of other

commercial lines in subsequent years has confirmed the gossypol levels have not been altered (Nida et al., 1996b).

In summary, the results of numerous analytical measurements on composition and nutritional content demonstrate that Roundup Ready cottonseed is substantially equivalent to the parental variety and conventional cotton varieties. Processing is unlikely to alter the compositional components of cotton and, therefore, products derived from cottonseed will also be substantially equivalent to and as safe as current cotton-derived products.

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 which 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, transformation is the only relevant mechanism 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 Roundup Ready cotton was incorporated into the genomic DNA of the plant and represents a small fraction of cotton genome. The probability that a bacterium 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 bacteria 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.

Roundup Ready cotton contains two 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 at higher levels in the gut, it could not compromise the therapeutic efficacy of these antibiotics. The AAD enzyme needs specific cofactors at appropriate concentrations to function, which are not present in the gut. Databases of protein sequences were screened with the AAD amino acid sequence and 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-GHGT07 that was used for transformation, was not transferred into the cotton plant genome. Therefore, the antibiotic resistance genes in Roundup Ready cotton can not be mobilized by excision of the marker gene 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 gastrointestinal 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. Worldwide, four species of cotton are of agronomic importance: 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. All grow on beach strands or on small islands.

Cotton is normally considered a self-pollinating crop but can be cross-pollinated by certain insects. However, outcrossing of the *cp4 epsps* gene from Roundup Ready cotton to other *Gossypium* species or to other Malvaceous genera 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 *cp4 epsps* gene were 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 Roundup Ready gene to 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; Karieva and Morris, 1992).

Assessment of Agronomic Performance

Roundup Ready cotton event 1445 has been grown and observed at multiple locations for weediness, plant growth characteristics, susceptibility to insects and disease infection. Based on results of the field-monitoring program, there were no significant differences between

Roundup Ready cotton event 1445 and the parental Coker 312 variety. Roundup Ready cotton does not pose any different plant pest risk to other plants and the environment than non-transformed cotton varieties. Roundup Ready cotton meets all morphological, yield and quality characteristics of cotton varieties produced in the United States.

Cotton is not considered to have weedy characteristics as an annual plant grown in the United States. It does not possess any of the attributes commonly associated with weeds such as seed dormancy, long soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersal or long distance dispersal of seeds. Multiple genes control these characteristics of weeds.

Wild populations of cotton are rare, widely dispersed and confined to beach strands or to small islands (Lee, 1984). Cotton appears to be somewhat opportunistic towards disturbed land and is not especially effective in invading established ecosystems.

There is little probability that Roundup Ready cotton event 1445 or any *Gossypium* species crossing with Roundup Ready cotton could become a weed. All wild and feral relatives of cotton are tropical, woody, perennial shrubs, other than a few herbaceous perennials in northwest Australia. With the exception of *G. thurberi* and *G. sturtianum* in Australia, these cannot naturally exist even in the milder temperate regions. 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. In fact, they tend to drop fruit in response to stress. It is unlikely that expression of the CP4 EPSPS protein would impact survival either way. The only species that approaches the designation of pest is the arborescent *G. aridum*, found in parts of central western Mexico where it grows in fence rows.

Assessment of Effect to Non-Target Organisms

Roundup Ready cotton event 1445 encodes the enzyme EPSP synthase (EPSPS). EPSPS is an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants (including cotton), and microorganisms (Levin and Sprinson, 1964), and is thus ordinarily present in food and feed derived from plant sources. Genes for numerous EPSPS proteins have been cloned, and active site domains are conserved among the known EPSPS proteins (Padgett *et al.*, 1988; 1991). Bacterial EPSPS proteins have been well characterized with respect to the three-dimensional X-ray crystal structure (Stallings *et al.*, 1991) and the detailed kinetic and chemical reaction mechanisms (Anderson *et al.*, 1990). EPSPS enzymes from a number of bacteria exhibit tolerance to glyphosate (Schulz *et al.*, 1985). CP4 EPSPS protein thus represents one of many different EPSPS proteins found in nature. EPSPS protein is considered to be ubiquitous in nature since it is present in all plants and microorganisms. Therefore, all organisms that presently feed on plants and/or microbes have historically been exposed to EPSPS proteins.

Cotton is a unique field crop in that mammals and other species that consume vegetation avoid feeding on the plant due to both the gossypol content and the morphology of the plant.

The seed is within the boll and covered with lint. The seed will not be normally found in a lint-free condition in the field. Therefore, avian species are not expected to feed on the large lint-covered seed. In addition, since the seed is not expected to enter aquatic habitats, fish should not be exposed.

The wholesomeness and safety of cottonseed from Roundup Ready cotton event 1445 was assessed by feeding cottonseed meal to bobwhite quail. No mortality occurred in birds fed up to 100,000 ppm (10% w/w) raw cottonseed meal in the diet. This feeding level approximates consumption of 400 seeds/kg body weight per bird. The no observed effect level (NOEL) was considered to be greater than 100,000 ppm. Based on the parameters measured, the wholesomeness of meal from Roundup Ready cottonseed was comparable to that of the parental line when fed in the diet to quail.

Impact on Biodiversity

Since the naturally-occurring EPSPS proteins are considered innocuous in nature and non-toxic to fish, avian species, insects, mammals and other species, and exposure to these species is not likely due to their feeding preferences, no adverse effects to wildlife are expected from the commercialization of these plants.

Assessment of Genetic Stability

The *cp4 epsps* gene responsible for conferring glyphosate tolerance in cotton event 1445 has been demonstrated to be stably integrated into the chromosome. This conclusion is based on molecular analyses, data on phenotypic expression, and inheritance patterns. The results of these studies are summarized as follows:

- molecular analyses of plants from the R3 to R5 generations establish that the introduced genes are maintained in the same chromosomal location;
- analyses of seed obtained from multi-site trials using R4 and R5 generations showed no marked change in expression of CP4 EPSPS protein;
- the level of tolerance to glyphosate herbicides has been maintained for at least four generations and during the production of over 6 million acres of commercial cotton produced in the US over the last three years;
- expression of CP4 EPSPS protein has been confirmed under different environmental conditions and in many cotton lines with different genetic backgrounds;
- Mendelian inheritance of the glyphosate tolerance trait is observed after self-pollination or backcrossing with other cotton varieties;
- seed quality (germination, vigor) of Roundup Ready cotton is maintained after transfer of the *cp4 epsps* gene into cotton from different genetic backgrounds.

In summary, it is concluded that the inserted genes in Roundup Ready cotton event 1445 are stably integrated and the line is phenotypically and genetically stable over several generations, and in various environments. The genetic stability has been confirmed with the

successful commercialization of Roundup Ready cotton varieties over the past four years. Based on this information, the likelihood of instability is considered to be negligible.

Assessment of Resistance to Glyphosate

Today, some 109 herbicide-resistant weed biotypes have been identified; over half of them are resistant to the triazine family of herbicides (Holt and Le Baron, 1990; LeBaron, 1991; Shaner, 1995). Resistance has usually developed because of the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action, long residual activity of the herbicide with the capacity to control weeds year-long, and frequent applications of the same herbicide without rotation to the other herbicides or cultural control practices. Using these criteria, and based on current use data, glyphosate is considered to be a herbicide with a low risk for weed resistance (Benbrook, 1991). Nonetheless, questions have been raised as to whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds resistant to that particular herbicide. This concern is based on the assumptions that the use of the herbicide will increase significantly, and possibly that it will be used repeatedly in the same location. However, other increases in glyphosate use over the previous years have been more significant than the projected increase associated with the introduction of Roundup Ready crops. Although it cannot be stated that evolution of resistance to glyphosate will not occur, the development of weed resistance to glyphosate is expected to be a very rare event because:

1. weeds and crops are inherently not tolerant to glyphosate, and the long history of extensive use of glyphosate has resulted in few instances of resistant weeds (Bradshaw *et al.*, 1997);
2. glyphosate has many unique properties, such as its mode of action, chemical structure, limited metabolism in plants, and lack of residual activity in soil, which make the development of resistance unlikely;
3. selection for glyphosate resistance using whole plant and cell/tissue culture techniques was unsuccessful, and would, therefore, be expected to occur rarely in nature under normal field conditions.

Environmental Assessment Conclusions

In summary, assessments indicate that the risks present with Roundup Ready cotton are equivalent to or not greater than those already present with traditional cotton varieties. Agronomic evaluations consisting of plant vigor, growth habit characteristics and general disease susceptibility have shown Roundup Ready cotton to be equivalent to the parental Coker 312 cotton variety. Finally, data generated to support the registration of Roundup agricultural herbicides and almost 30 years of use experience with glyphosate demonstrate that these herbicides will not cause unreasonable adverse effects to humans, mammals or other non-target organisms under normal use conditions. In addition, the data demonstrate that the use of these herbicides in cotton is not expected to cause unreasonable adverse effects to the environment.

Summary

The introduction of Roundup Ready cotton has reduced the number and cost of herbicide applications, and offers considerable environmental benefits due to its fit with conservation tillage systems. The introduced CP4 EPSPS protein is similar to other EPSPS proteins that are ubiquitous in nature. Detailed food, feed and environmental safety assessments confirm the safety of this product. The analyses included: 1) detailed molecular characterization of the introduced DNA; 2) safety assessments of the expressed CP4 EPSPS and NPTII proteins; 3) compositional analysis of cottonseed, oil and meal; and 4) environmental impact assessment of the cotton plants. These studies demonstrate that the CP4 EPSPS protein is safe to non-target organisms, including humans, animals and beneficial insects. Additionally, Roundup Ready cotton plants and cottonseed were shown to be as safe and nutritious 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.

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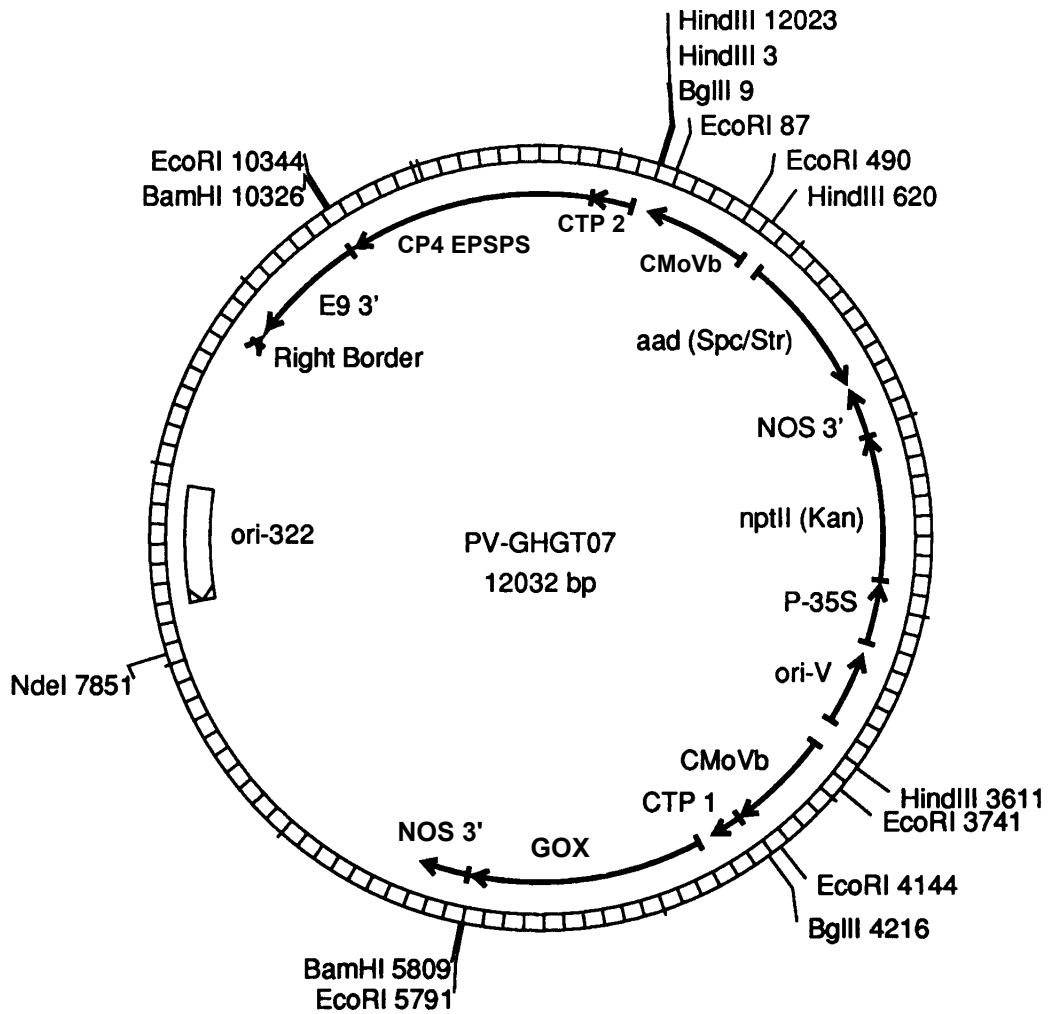


Figure 1. Plasmid Map of PV-GHGT07 used to produce Roundup Ready cotton event 1445.

Table 1. Levels of CP4 EPSPS, NPTII, and AAD Proteins in Cotton Leaf Tissue in 1993

<i>Analyte</i>	<i>µg/mg tissue fresh weight</i>	
	<i>Coker 312^a</i>	<i>Line 1445^b</i>
CP4 EPSPS mean	ND	0.052
range	NA	0.027-0.101
std dev.	NA	0.016
NPTII mean	ND	0.045
range	NA	0.019-0.084
std dev.	NA	0.014
AAD mean	ND	ND
range	NA	NA
std dev.	NA	NA

^a Single extract of leaf samples, one protein loading per sample, three replicate samples per line for five sites.

^b Single extract of leaf samples, one protein loading per sample, four replicate samples per line for one site, three replicate samples per line for five sites. Of the five sites, the Arizona site with 3 replicate samples had two replicate samples from pooled adjacent sub-plots and the third sample was from a single sub-plot. ND=non-detectable; NA=not applicable;

Table 2. Levels of CP4 EPSPS, NPTII, and AAD Expression in Cottonseed Tissue in 1993

<i>Analyte</i>	<i>µg/mg tissue fresh weight</i>	
	<i>Coker 312^b</i>	<i>Line 1445^a</i>
CP4 EPSPS mean	ND	0.082
range	NA	0.058-0.117
std dev.	NA	0.017
NPTII mean	ND	0.0067
range	NA	0.0050-0.0104
std dev.	NA	0.0010
AAD mean	ND	ND
range	NA	NA
std dev.	NA	NA

^a Single extract of seed samples, one protein loading per sample, three replicate samples per line for five sites, four replicate samples per line for one site.

^b Single extract of seed samples, one protein loading per sample with one exception that had two protein loadings, three replicate samples per line for five sites, four replicate samples per line for one site.

ND=non-detectable; NA=not applicable;

Table 3. Summary of Proximate Analysis of Cottonseed from 1993 U.S. Field Trials

Characteristic	Non-transgenic Parent Coker 312		Roundup Ready cotton Cotton Event 1445	
	Mean	Range	Mean	Range
Protein %	27.8	24.6-28.9	29.6*	25.6-31.3
Fat %	23.4	20.5-24.8	23.8	19.5-26.1
Ash %	4.5	4.1-4.9	4.70	4.2-5.2
Carbohydrate %	44.4	41.9-46.2	41.9*	39.2-44.0
Calories/100g	498	483-505	500	477-512
Moisture%	11.6	9.1-14.1	11.1	9.0-13.0

Protein, fat, ash, carbohydrate, and calories reported on dry weight basis.

Six samples analyzed per line (one from each of six sites)

* Statistically significant from Coker 312 parental control.

Table 4. Literature Ranges of Cotton Components

Component	Range or Mean Value	Literature Reference
Protein %	18.8-22.9	Turner <i>et al.</i> , 1976.
	23.5-29.5	Cherry <i>et al.</i> , 1978a.
	12-32	Kohel <i>et al.</i> , 1985.
Fat (oil) %	23.2-25.7	Cherry <i>et al.</i> , 1978b.
	21.4-26.8	Cherry <i>et al.</i> , 1978a.
Ash %	4.1-4.9	Cherry <i>et al.</i> , 1978b.
	3.8	Belyea <i>et al.</i> , 1989.
Moisture	5.4-10.1	Cherry <i>et al.</i> , 1978a.

Table 5. Amino Acid Composition of Cottonseed from Roundup Ready Cotton Event 1445 and Coker 312; 1993 US Field Trials

	<i>Literature</i>		<i>Coker 312</i> ²	<i>RR 1445</i> ²
	<i>Max</i> ¹	<i>Min</i> ¹		
Aspartic acid	9.5	8.8	9.58	9.57
Threonine	3.2	2.8	3.47	3.44
Serine	4.4	3.9	4.75	4.70
Glutamic Acid	22.4	20.5	18.91	19.57
Proline	4.0	3.1	4.06	4.03
Glycine	4.5	3.8	4.28	4.25
Alanine	4.2	3.6	4.06	4.05
Cysteine	3.4	2.3	1.72	1.62
Valine	4.7	4.3	4.24	4.28
Methionine	1.8	1.3	1.95	1.75
Isoleucine	3.4	3.0	3.10	3.12
Leucine	6.1	5.5	5.96	5.96
Tyrosine	3.3	2.8	2.86	2.90
Phenylalanine	5.6	5.0	5.22	5.25
Lysine	4.6	4.2	4.73	4.68
Histidine	2.8	2.6	3.01	2.96
Arginine	12.3	10.9	11.62	11.59
Tryptophan	1.4	1.0	1.03	1.05

¹ Lawhon *et al.*, 1977

² Amino acids reported as g/100 g protein. Value reported is mean of six samples, one from each field site.

No statistically significant differences from the Coker 312 control

Table 6. Lipid and Fatty Acid Composition of Cottonseed from Roundup Ready Cotton Event 1445 and Coker 312; 1993 US Field Trials

<i>Component</i>	<i>Coker 312^{a,b}</i>		<i>Roundup Ready Event 1445^{a,b}</i>	
	<i>Mean</i>	<i>Range^d</i>	<i>Mean</i>	<i>Range</i>
Lipid	32.65	31.16-33.93	32.24	30.21-34.48
Myristic (14:0)	0.97	0.89-1.17	0.95	0.84-1.03
Pentadecanoic (15:0)	1.00	0.46-2.27	0.56	0.27-0.80
Palmitic (16:0)	27.70	25.82-28.55	26.76	26.00-27.76
Palmitoleic (16:1)	0.64	0.56-0.77	0.65	0.61-0.69
Stearic (18:0)	2.68	2.43-3.41	2.67	2.29-3.02
Oleic (18:1)	15.28	13.94-15.80	15.49	14.39-16.76
Linoleic (18:2)	43.18	36.32-47.27	45.90	43.93-46.98
Linolenic (18:3)	0.16	0.08-0.31	0.21	0.13-0.38
Arachidic (20:0)	0.24	0.21-0.29	0.29	0.24-0.34
Behenic (22:0)	0.15	0.10-0.27	0.17	0.11-0.38
Malvalic (C-17)	0.43	0.25-0.58	0.41	0.21-0.58
Sterculic (C-18)	0.71	0.52-0.92	0.70	0.56-0.98
Dihydrosterculic (C-19)	1.12	0.34-3.39	0.58	0.27-1.07
Unidentified ^c	3.81	1.97-7.07	3.15	1.85-4.33

^a Value of lipid is % of sample weight. Value of fatty acid is % of total lipid.

^b Values presented are means and ranges of six samples, one seed sample of each line from each of six sites.

^c "Unidentified" are sum of peaks labeled as "A" and "B" in the raw data.

^d Range denotes the lowest and highest individual assay for each plot.

No statistically significant differences from the Coker 312 control

Table 7. Fatty Acid Profile and Gossypol Content of Refined Oil from Cotton Lines 1445 and Coker 312, 1993

<i>Fatty Acid</i>	<i>Lit Range</i>	<i>Refined Oil (% of total fatty acids)</i>	
		<i>Coker 312</i>	<i>Line 1445</i>
Myristic (14:0)	(0.5-2.5) ¹ (0.68-1.16) ²	0.95	0.84
Pentadecanoic (15:0)		0.40	0.43
Palmitic (16:0)	(17-29) ¹ (21.63-26.18) ²	25.54	25.14
Palmitoleic (16:1)	(0.5-1.5) ¹ (0.56-0.82) ²	0.64	0.61
Stearic (18:0)	(1.0-4.0) ¹ (2.27-2.88) ²	2.46	2.41
Oleic (18:1)	(13-44) ¹ (15.17-19.94) ²	15.03	14.53
Linoleic (18:2)	(33-58) ¹ (49.07-57.64) ²	50.10	51.27
Linolenic (18:3)	(0.1-2.1) ¹ (0.23) ³	0.14	0.16
Arachidic (20:0)	(<0.5) ¹ (0.41) ³	0.26	0.27
Behenic (22:0)	(<0.5) ¹	0.12	0.08
Sterculic	(0.08-0.56) ⁴	0.44	0.50
Malvalic	(0.22-1.44) ⁴	0.35	0.56
Dihydrosterculic (C-19)	NA	0.23	0.23
Unidentified fatty acid	NA	1.97	1.79
Total gossypol	<0.06% ²	ND	ND
Free gossypol	<0.031% ²	ND	ND
α-tocopherol ⁵	136-660 ⁶	670	588

¹ Ranges adopted by the FAO/WHO Codex Alimentarius committee on fats and oils (Cottonseed Oil, 1993).

² Cherry and Leffler, 1984. Reported for LCP flour.

³ Cherry, J.P., 1983.

⁴ Phelps, et.al., 1965.

⁵ α-tocopherol reported as mg/kg of oil

⁶ Rossel, 1991; Dicks, 1965.

Values reported for crude cottonseed oil.

ND Not Detected. NA Not Available.

Table 8. Gossypol Levels Determined in Seed, Raw Meal, and Toasted Meal from Roundup Ready Cotton Event 1445 and Coker 312; 1993 US Field Trials

	<i>% Total Gossypol</i>		<i>% Free Gossypol</i>
	<i>Mean</i>	<i>Range</i>	
Seed			
Coker 312	1.19	(0.99-1.46) ^a	NA
Event 1445	1.32*	(1.13-1.63)	NA
Full Fat Flour			
Coker 312	1.05 ^b	NA	0.70
Event 1445	1.35	NA	0.83
Toasted meal			
Coker 312	0.99	NA	ND
Event 1445	1.30	NA	ND

* Values are statistically significant compared to the Coker 312 at p=0.05 using a pooled variance t-test. Data generated in 1997 US field trials with Roundup Ready cotton varieties DP50, DP5415 and DP5690 (range 0.81-0.92) had no significant differences in gossypol levels compared to their non-transgenic controls (range 0.76-1.02).

^a Values reported for seed samples are the means and ranges of six samples per line; one sample from each of six sites.

^b Values reported from full fat flour (kernel) and toasted meal samples are one value obtained from processing fractions generated from the composite of seed from six sites.

NA = Not Applicable

ND = Not Detectable