Bayer CropScience

Petition for Determination of Nonregulated Status for Glyphosate-Tolerant cotton:

GlyTol™ cotton Event GHB614

OECD Unique Identifier BCS-GHØØ2-5

The undersigned submits this petition under 7 CFR 340.6 to request that the Administrator, Animal and Plant Health Inspection Service, makes a determination that the article should not be regulated under 7 CFR 340.

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CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

COMPANY NAMES

On June 3, 2002, Bayer CropScience was formed by the acquisition of Aventis CropScience by Bayer AG. From this date, Bayer CropScience is the agricultural business unit of Bayer that is engaged in the research, development, and marketing of crop protection, seed technology, turf and ornamentals, professional pest and vector control, and home and garden products.

Some of the activities described in this report were undertaken before the acquisition. Consequently, the name Aventis CropScience may appear throughout this report. However, all inquiries regarding this report and the data contained herein should be addressed to: Bayer CropScience, P. O. Box 12014, 2 T. W. Alexander Drive, Research Triangle Park, North Carolina, 27709.

SUMMARY

Bayer CropScience LP (BCS) is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for GlyTol™ cotton event GHB614. BCS requests a determination from APHIS that GlyTol cotton event GHB614, and any progeny derived from crosses of GlyTol cotton with traditional cotton varieties, and any progeny derived from crosses of event GlyTol cotton with transgenic cotton varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. GlyTol cotton event GHB614 is considered a regulated article because it contains sequences from the plant pest, *Agrobacterium tumefaciens* (right and left border).

In the early 1970s, it was demonstrated that inhibitors of the aromatic amino acid biosynthetic pathway can have an herbicidal activity, which opened the path for the development of the glyphosate herbicide. Glyphosate is the active ingredient of a non-selective, broad-spectrum, systemic, post-emergence herbicide that has been used extensively throughout the world over the past three decades. It has a very low mammalian toxicity and low soil persistence. It is used to inhibit weeds in conservation tillage systems just prior to planting. It is also applied as a non-selective herbicide with direct spraying in orchards. Given the importance of this compound, considerable effort has been made in attempts to engineer glyphosate tolerance in various crops.

GlyTol cotton event GHB614 has been developed by BCS as an alternative herbicide tolerant cotton product. The transformation event contains the stably integrated gene *2mepsps*, which encodes the 2mEPSPS protein. The gene was introduced by *Agrobacterium*-mediated gene transfer. Southern blot analyses show GlyTol cotton event GHB614 contains one complete copy of the *2mepsps* gene.

The *2mepsps* gene was generated by introducing mutations into the wild-type *epsps* (*wt epsps*) gene from maize, leading to a double mutant EPSPS protein with two amino acid substitutions (2mEPSPS). This modification confers the protein a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, the plants bearing this gene are tolerant to glyphosate herbicides.

GlyTol cotton event GHB614 has been field tested by BCS beginning in 2002 in adapted growing regions of the United States and winter nursery. These tests have occurred at 40 sites under field release authorizations granted by USDA APHIS (USDA authorizations: 02-072-04n, 02-296-01n, 03-064-14n, 03-255-03n, 04-064-10n, 04-247-01n, 05-060-03n, 05-091-07n, 05-217-05n, 05-257-04n.) Data collected from these field trials and laboratory analyses presented herein demonstrate that GlyTol cotton: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than non-modified cotton; 3) is unlikely to increase the weediness potential of any other cultivated plant or native wild species; 4) does not cause damage to processed agricultural commodities; and 5) is unlikely to harm other organisms that are beneficial to agriculture.

Therefore, BCS requests a determination from USDA APHIS that GlyTol cotton event GHB614, and any progeny derived from crosses of GlyTol cotton with traditional cotton varieties, and any progeny derived from crosses of GlyTol cotton with transgenic cotton varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340.

CERTIFICATION

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

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ACRONYMS AND SCIENTIFIC TERMS

2mEPSPS	double mutant 5-EnolPyruvylShikimate-3-Phosphate Synthase
a.i.	active ingredient
AMS	Agricultural Marketing Services
ANOVA	ANalysis Of VAriance
APHIS	Animal and Plant Health Inspection Service
BCS	Bayer CropScience
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CI	confidence interval
cm	centimeter
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EPSPS	5-EnolPyruvylShikimate-3-Phosphate Synthase
ESA	Endngered Species Act
FDA	Food and Drug Administration
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
FW	fresh weight
g	gram
germ	germination
GlyTol	glyphosate tolerant
HPLC	High Pressure Liquid Chromatography
IC_{50}	Inhibitor concentration for 50% inhibition
ID	identification
Kb	Kilobases
kDa	kiloDalton
kg	kilogram
K_i	Inhibitior binding constant
K_m	Substrate binding constant
L	liter
LB	Left Border
lbs	pounds (1 pound = 0,454 kg)
LC/MS	Liquid chromatography/mass spectroscopy
LOD	Limit of Detection
LOQ	Limit of Quantification
MBAS	Molecular and Biochemical Analytical Services
mg	milligram
ml	milliter
mm	millimeter
mM	millimolar
µM	micromolar
MS	Mass Spectrometry
MSMA	monosodium acid methanearsoate
MW	molecular weight
MWM	molecular weight marker
µg	microgram
NA	Not Applicable
ng	nanogram
nm	nanometer
NS	not significant
ND	Not Detectable: Below the limit of detection
nm	nanometers
NT	Non-transgenic
ORF	Open Reading Frame
PEP	Phospoenolpyruvate
PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulator
PPA	Plant Protection Act of 2000
ppb	parts per billion
RAC	Raw Agricultural Product
RB	Right Border
S3P	shikimate-3-phosphate
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
T_1, T_2, etc	generations after T_0 (transformation)
T-DNA	transfer DNA from *Agrobacterium*
t-RNA	transfer Ribonucleic acid
TEP	Total Extractable Protein
™	Trademark
US/USA	United States of America
USDA	United States Department of Agriculture
WT	Wild type
wtEPSPS	Wild type 5-EnolPyruvylShikimate-3-Phosphate Synthase

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I. RATIONALE

A. Basis for the Request for Determination of Non-regulated status

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

B. Rationale for the development of glytol cotton

Cotton is the United States and the worlds leading fiber crop. In the US for the 2006 production year, cotton was grown on 15.3 million acres, the major producing states being Texas (6.4 million acres), Georgia (1.4 million acres), Mississippi (1.2 million acres), Arkansas (1.2 million acres), North Carolina (0.9 million acres) and Tennessee (0.7 million acres). The world total planted area in 2005-2006 was 85 million acres, for a production of 114.1 million bales (24.8 million tons). (USDA-FAS, 2006).

Cotton is grown in the United States using mechanized practices for planting and harvesting. Cultural practices, including irrigation and crop rotation, and herbicides are employed to control weeds. Weed management is critical to maximum cotton yield and herbicides are used on most cotton acreage grown in the United States. The grower is interested in applying a broad weed spectrum herbicide for weed control, that does not injure the crop, is cost effective, and has positive environmental attributes. Several classes of herbicides have effective broad spectrum weed control, however they may injure or kill the cotton crop when used at the application rates suggested for weed control.

Commercialization of GlyTol cotton event GHB614, following the receipt of the required regulatory approvals, including this Determination of Nonregulated Status, will offer an efficacious and environmentally friendly option to growers for weed control in cotton. The glyphosate herbicide (N-phosphonomethyl-glycine) is registered for nonselective weed control on both non-food use and food use plants. Glyphosate is a systemic, non-selective herbicide that provides effective post-emergence control of many broadleaf and grassy weeds. Its relatively slow mode of action allows movement of the herbicide throughout the plant before symptoms occur. It is highly biodegradable, has no residual activity, and has very low toxicity for humans and wild fauna (Malik *et al.* 1989). Resistance to the herbicide has now been achieved, through the insertion of a resistance gene, in over 20 commercially important plant species including cotton. Genetically engineered GlyTol cotton will provide a selective use for glyphosate, an established weed management tool to cotton producers and potentially a superior quality crop that may lead to higher yields.

Establishment of a good, uniform stand of cotton is the cornerstone for building a successful crop. Plant stand at the beginning of the season influences earliness, insect and plant growth management, and final yield potential. Cotton is especially sensitive to weed competition because it grows relatively slowly in the early stages, and does not reach full ground cover until eight or more weeks after germination. The effect of weeds on the cotton crop can be caused by competition for light, water and nutrients, and will depend on the type of weed and

the density of weed growth as well as on the soil type, rainfall and the level of fertility. Once full ground cover has been achieved cotton can compete satisfactorily with most common species of weeds, but some tall and climbing weeds can still present a problem, not only because of their effect on the yield of cotton, but also because they interfere with picking and other field operations and harbor insects. Weed foliage can impede the full impact of a spray intended to give full coverage to the leaves of the cotton plants. Late weed growth can interfere with mechanical harvesting or cause staining of the lint. Grasses and other species which shed their seeds on the open bolls add to the labor of clearing trash from the seed cotton. Weed seeds produced at this time can add to the weed problems in the subsequent crop. (Munro, 1987)

C. Adoption of glyphosate tolerant cotton

Glyphosate tolerant cotton was developed by Monsanto Co. in 1996 as Roundup Ready® cotton. This product introduced farmers to the concept of applying a broad spectrum herbicide over the top of their cotton crop. Convenience and simplicity of the application were likely drivers of the technology, but as studies were conducted and the technology understood, agricultural practices began to change to take advantage of the system. Agricultural systems such as no-till farming, which were previously considered to be infeasible, became common practice in agricultural systems utilizing herbicide tolerant products.

In 2006 over 78% of the total cotton acreage in the United States was herbicide tolerant. This acreage was concentrated in the southeast, mid-south, and southwestern cotton growing regions with over 9 million acres currently utilizing the technology. The rapid adoption of this technology in just ten years demonstrates the benefits of the technology to commercial cotton growers.

D. Benefits of glyphosate tolerant cotton

Efficacious Weed Control

Glyphosate has been shown to be an efficacious weed control system when utilized with glyphosate tolerant cotton. Broad spectrum weed control is usually achieved with a single application for pre-plant burndown, and then one in-season application. GlyTol cotton event GHB614 has a broad application window which may be used to apply glyphosate, giving growers increased flexibility in their herbicide programs. This flexibility is key in timing herbicide sprays at the ideal time in weed development, rather than a specific time during the development of the cotton plant.

Simplicity and Convenience

Glyphosate tolerant cotton provides an easy-to-use system that allows a highly efficient weed control in the crop. High efficiency allows for the cultivation of additional acreage and expansion of production operations with the existing level of infrastructure. Additionally, some equipment costs and labor may be eliminated in situations where cultivation equipment is no longer necessary, such as no-till practices.

Economic Benefit to Growers

Use of glyphosate tolerant cotton has been shown to increase grower returns in the form of higher yields and reduced overhead production costs. In 2004 the overall impact of herbicide-resistant cotton on US agriculture has been a reduction in crop production costs of $264 million and pesticide use of 14.0 million pounds. This represents 19% higher net returns in 2004 compared with 2003. Similarly, herbicide use continued to decrease by 46% in 2004 compared to 2003, mainly due to expanded acreage of biotechnology-derived herbicide-resistant cotton in 2004 (Sankula *et al.* 2005).

Environmental Benefits
The main environmental benefit of glyphosate tolerant cotton is the use of reduced and no-till production systems. These cultivation practices contribute to reductions in soil erosion from water and wind. Reduced tillage also contributes to reduced fossil fuel use, less air pollution from dust, improved soil moisture retention, and reduced soil compaction. The replacement of pre-emergence herbicides with glyphosate can also reduce herbicide concentrations in vulnerable watersheds.

E. Submissions to other regulatory agencies

Food and Drug Administration
GlyTol cotton event GHB614 is within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (FDA, 1992). In compliance with this policy, BCS will submit to FDA a food and feed safety and nutritional assessment summary for GlyTol cotton.

Environmental Protection Agency
The United States Environmental Protection Agency has authority over the use of pesticidal substances under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA) as amended (7 USA §136 *et. Seq*.). A submission for the use of a glyphosate formulation to be used on GlyTol Cotton will be presented to the Agency.

Foreign Governments
BCS intends to submit dossiers to the proper regulatory authorities of foreign governments request for import of US Cottonseed and have regulatory processes in place. These may include submissions to the relevant Regulatory Authorities in Canada, Mexico, EU, Japan, among others. Glytol cotton has been, or is currently, in field trials in cotton growing regions around the world.

II. THE COTTON FAMILY

Cotton, *Gossypium hirsutum*, has been cultivated for millennia in many parts of the world. About 90 percent of the production of cotton is *G. hirsutum*. Cotton is primarily used worldwide for its lint. Lint is produced on the seed coat, and is spun into fine strong threads. Only the United States and a few other countries have developed major commercial uses for the seed. Raw unprocessed cottonseed may be fed to ruminants in the form of cottonseed meal and hulls or the seed can be processed for oil, the primary component consumed by humans. Linters, the short fibers that remain on the hulls after the removal of the lint have both edible and non-edible use.

Cotton belongs to the genus *Gossypium*, which is in the Malvaceae or Mallow family. Other members of this family include okra, hollyhock, rose of sharon, and even such plants as teaweed, spurred anoda, and velvetleaf that are weed pests in cotton. Only the genus *Gossypium*, and a few isolated species of the other genera, is characterized by the seed hairs or trichomes, which are outgrowths of the epidermis of the seed coat. There are 50 diverse species in the genus *Gossypium*, but only four of them produce commercial-type lint (Fryxell, 1992).

The tribe Gossypieae has two specific characters: the form of the embryo (which is more complex than in the balance of the Malvaceae) and the presence of distinctive punctae in various parts of the plant but especially in the cotyledons. These punctae are now known as "gossypol glands" and are distinctive in morphology and chemical contents. They are believed to be unique to the tribe (Fryxell, 1979).

A. Cotton as a crop

Cotton, *Gossypium* spp. has been grown for its fiber for several thousand years. Its cultivation and manufacture into cloth developed independently in both the Eastern and Western Hemispheres. One of the oldest records of cotton textiles, dating back about 5,000 years, was found in the Indus River Valley in what is now Pakistan. Excavations in Peru and Mexico have uncovered cotton cloth identified as being 4,500 to 7,000 years old. Cotton fabrics have also been found in the remains of some of the ancient civilizations of Egypt and in the ruins of Indian pueblos of the Southwestern United States, dating back hundreds of years before Christ. Other products, such as cottonseed oil, cake, and cotton linters are by-products of fiber production.

Cottonseed, a raw agricultural product which was once largely wasted, is now converted into food for people, feed for livestock, fertilizer and mulch for plants, fiber for furniture padding and cellulose for a wide range of products from explosives to computer chip boards. Cotton is indeed nature's food and fiber plant. Although lint is the most valuable product from a field of cotton, it is very important to keep in mind that this versatile plant is also an important vegetable oil source. From this point of view, cotton is a food crop.

Cotton, *Gossypium hirsutum* L., is mainly produced in China, USA, India, Pakistan and Uzbekistan, with these five countries contributing to nearly 75% of world production (Table 1).

Table 1. Cotton: Production in specified countries and the world

Country	Million 480-Pound Bales			
	2002/03	***2003/04***	***2004/05***	***2005/06***
China	22.6	22.3	29.0	26.2
USA.	17.2	18.3	23.3	23.9
India	10.6	14,0	19.0	19.2
Pakistan	7.8	7.8	11.1	9.9
Uzbekistan	4.6	4.1	5.2	5.6
Turkey	4.2	4.1	4.2	3.6
Brazil	3.9	6.0	5.9	4.7
World	88.3	95.3	120.3	114.1

Source: USDA-Foreign Agriculture Service.

In the US for the 2006 production year, cotton was grown on 15.3 million acres, the major producing states being Texas (6.4 million acres), Georgia (1.4 million acres), Mississippi (1.2 million acres), Arkansas (1.2 million acres), North Carolina (0.9 million acres) and Tennessee (0.7 million acres). The world total planted area in 2005-2006 was 85 million acres, for a production of 114.1 million bales (24.8 million tons). (USDA-FAS, 2006)

The total production of cotton as an oilseed was 42.6 million tons in 2005/06 out of a world total of 389 million tons. Cottonseed oil, with a production estimated at 4.6 million tons in 2005/06, accounts only for 4% of total world oil production. With 1.3 million tons for that same year, China is by far the most important producer (USDA-FAS, 2006)

B. The taxonomy of cotton

Scientific name:	*Gossypium hirsutum* L.
Family:	Malvaceae
Genus:	*Gossypium*
Species:	*hirsutum* (2n=52, Upland cotton), *barbadense* (2n=52, Pima cotton), *arboreum* (2n=26), *herbaceum* (2n=26)
Cultivar/breeding line:	numerous varieties and breeding lines
Common name:	Cotton

The predominant type of cotton grown in the United States is *Gossypium hirsutum*, known as American Upland. The Upland type, which usually has a staple length of 1 to 1 1/4 inches, accounts for about 97 percent of the annual US cotton crop. Upland cotton is grown throughout the US Cotton Belt as well as in most major cotton-producing countries. The balance of US-grown cotton is *Gossypium barbadense*, commonly referred to as American Pima or extra-long staple (ELS). ELS cotton, which has a staple length of 1 1/2 inches or longer, is produced predominantly in California, Arizona, New Mexico, and southwest Texas, where it is particularly well adapted to the arid environmental conditions. The markets for ELS cotton are mainly high-value products such as sewing thread and expensive apparel.

C. The genetics of cotton

The genus *Gossypium* consists of 50 species, of which 4 to 5 are generally cultivated (Fryxell, 1992). The cultivated species are *G. hirsutum, G. barbadense, G. arboreum* L.*, G. herbaceum* and *G. lanceolatum* Todaro.

At least seven genomes, designated A, B, C, D, E, F, and G, are found in the genus (Endrizzi, 1984). Diploid species (2n=26) are found on all continents, and a few are of some agricultural

importance. The A genome is restricted in diploids to two species (*G. arboreum* and *G. herbaceum*) of the Old World. The D genome is restricted in diploids to some species of the New World, such as *G. thurberi*.

By far, the most important agricultural cottons are *G. hirsutum* and *G. barbadense*. These are both allotetraploids (2n=4x=52) of New World origin, and presumably of ancient cross between Old World A genomes and New World D genomes. How and when the original crosses occurred has been subject to much speculation. Euploids of these plants have 52 somatic chromosomes, and are frequently designated as AADD (they behave as disomic polyploids). Four additional New World allotetraploids occur in the genus, including *G. tomentosum*, a native of Hawaii. Due to the difference in ploidy level, *G. hirsutum* cannot cross with wild diploid cottons. *G. hirsutum* is readily cross-compatible only with other tetraploid members of the tribe Gossypium, which includes *G. tomentosum* in Hawaii, *G. darwinii* in the Galapagos, *G. mustelinum* in northeastern Brazil, *G. hirsutum* and *G. lanceolatum* in tropical/subtropical America, and *G. barbadense* in South America, as well as cultivated forms of *G. hirsutum* and *G. barbadense* (Fryxell, 1979). *Gossypium tomentosum* has been crossed with *G. hirsutum* in breeding programs; however, no commercial cotton is produced in Hawaii (Jenkins, 1993).

The New World allotetraploids are peculiar in the genus, because the species, at least in their wild forms, grow near the ocean, as invaders in the constantly disturbed habitats of strand and associated environs. It is from these "weedy" or invader species that the cultivated cottons developed (Fryxell, 1979).

D. Pollination of cotton

Gossypium hirsutum is generally considered to be a self-pollinating crop (Niles and Feaster, 1984). The morphology of cotton pollen, is heavy and somewhat sticky, does not lend itself to wind pollination. Cotton can, however, be pollinated by insects. Bees – wild bees, honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.), are the primary insect pollinators. Berger *et al*. (1988) have found that pollination by *Bombus* was more efficient than by *Apis mellifera*, which is consistent with the amount of pollen found in the hexapod. Bees collect mainly the nectar from the plants, and rarely the pollen. In addition, physical isolation with plants attractive to the bees significantly reduces the potential for pollen movement, as cotton flowers have a nectar high in glucose and low in sucrose, which probably makes it slightly repellent for bees (Moffett *et al*., 1976).

McGregor (1976) traced the movement of pollen from a cotton field surrounded by a large number of honeybee colonies. Movement of the pollen was traced by means of fluorescent particles. McGregor found that at 150 to 200 feet away from the source plant, only 1.6 percent showed the presence of the fluorescent particles. By comparison, the isolation distances for Foundation, Registered and Certified seeds in 7 CFR Part 201 are 1320, 1320 and 660 feet, respectively.

E. Weediness potential of cotton

In the United States, cotton (*G. hirsutum*) is not a weed pest and has no sexually compatible weedy relatives except perhaps *G. tomentosum* in Hawaii, which will be discussed in the next section. A number of references confirm the lack of weediness of cotton: Crockett, 1977, Holm *et al.*, 1977, Muenscher, 1980. Some feral cotton populations do exist in the US, but they are rare and found in areas hundreds of miles from commercial cotton production areas.

Cotton is a domesticated crop that requires human intervention to survive in non-cotton production area. Since cotton is an exotic species in the US and has not become a weed pest

over many centuries, there is no expectation that a new cotton variety with a single gene introduction would enhance that risk by becoming weedy in non-cotton production areas.

Within cotton production areas, the addition of the GlyTol trait (2mEPSPS protein) into domesticated cotton will not cause it to become weedy. Traditional cotton breeding has provided new cotton varieties with resistance to disease, insects and herbicides, tolerance to various environmental conditions (heat, cold, drought, etc.) and enhanced phenotypic traits, such as faster germination and rapid seedling growth. Despite the many enhanced cotton varieties, none have shown any evidence of weediness. Crops modified by molecular techniques, which are highly specific, should present no different risks than those introduced by traditional, less controlled methods. Of specific concern may be the addition of herbicide tolerance to produce GlyTol cotton, but experience with many other herbicide-tolerant crops demonstrates no change in weediness potential. For example, rapeseed, cotton, corn, soybean, tobacco, tomato and other crops have been transformed or modified to resist herbicides such as glyphosate, glufosinate, bromoxynil, and sulfonylurea without any evidence of weediness. The primary concern is with the control of volunteer plants. Yet these plants can easily be controlled by pre- or post-emergence herbicides. For example, GlyTol cotton volunteers could easily be controlled by using any number of targeted and broad-specturm herbicides used to control broadleaf weeds in agricultural systems. Of specific concern are glyphosate-tolerant corn and soybeans which are already on a majority of acreage in the United States. Potential volunteer cotton plants with the GlyTol trait can be controlled with products such as flumioxazin, metribuzin, and bentazon in soybeans, and atrazine, 2,4-D, and mesotrione in corn. Volunteer cotton with the GlyTol trait which emerges within conventional or glyphosate susceptible cotton varieties can be controlled with products such as flumioxazin during pre-plant burndown, pendimethlin, and paraquat.

F. Potential for outcrossing/gene escape in cotton

The potential for outcrossing can be defined as the ability of gene escape to wild cotton relatives. While gene flow could occur vegetatively, by seed or pollen, only pollen flow has any potential risk for cotton. Vegetative propagation is uncommon for cotton and seed dispersal (wind, birds, and animals) is rarely successful due to the properties of the boll structure. Cotton pollen is not transferred by wind due to its large, heavy and sticky nature (Niles and Feaster, 1984). Natural cross-pollination results from pollen being carried by insects, bees being the most important cotton pollinators (McGregor, 1976).

In Upland cotton, outcrossing studies suggest that pollen carryover decreases very rapidly as the distance to the closest marker pollen row increases, and that very little pollen is transferred beyond 12 meters. Vaissière (1990) prepared a report containing a literature review on cotton pollination and a summary of his study, "Pollen Dispersal and Carryover in Upland Cotton," conducted in Texas in 1983. The Texas study was conducted using a male sterile line surrounded by male fertile plants. Sixty honeybee colonies were supplied. Results showed that the pollen carryover in upland cotton decreased in proportion to the inverse of the distance to the closest pollinator row, and there was no significant pollen carryover past 12 meters.

Meredith and Bridge (1973) detected no outcrossing between adjacent plants in a study conducted in Stoneville, MS; the approximate limit of detection for the sample size and methods was approximately 0.046%.

Outcrossing data using bromoxynil-tolerant cotton is reported for seven locations in Figure 1 (Kareiva *et al*., 1994). Seed samples were collected in the border rows of Calgene's winter nursery sites in Catamarca, Argentina and Pongola, Republic of South Africa, as well as in

Stoneville, MS, USA. Sampling distance was one to 20 meters away from the bromoxynil-tolerant cotton. The frequency of outcrossing is determined by the crop and the pollinator. It is interesting to note that although the rate is higher for Argentina and South Africa (most likely due to the behavioral differences between European and African honeybees) the pattern of decline with distance is the same.

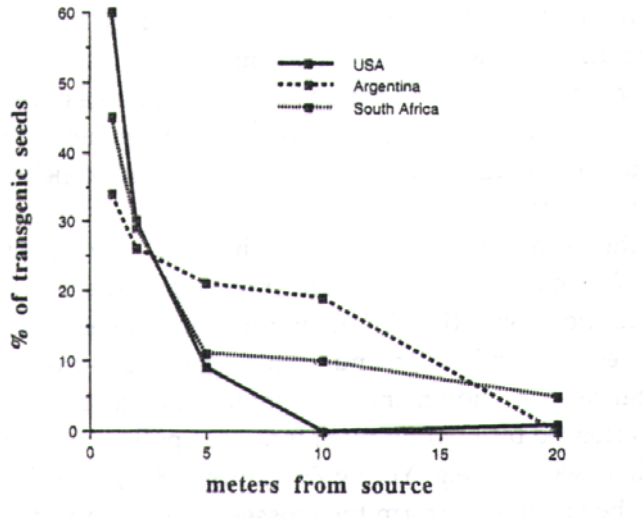

Figure 1. Outcrossing Studies with Bromoxynil-Tolerant Cotton:
The decline in transgenic cotton seeds as a function of distance away from a source for USA (five different states lumped together), Argentina and South Africa (Figure 1 in Kareiva et al., 1994). The percentage is out of the total transgenic seeds recovered at five distances (1, 2, 5 10 and 20 m away), with that total being 78 in USA, 179 in Argentina, and 728 in South Africa. The total number of seeds scored in order to obtain these transgenic dispersal events was 15024 in USA, 7632 in Argentina and 28097 in South Africa. By standardizing to a percentage the graphs are more easily compared, even though different numbers of seeds were collected at each field trial.

Recently, Van Deynze *et al*. (2005) measured pollen-mediated gene flow (PGF) in four directions over two years from commercial seed fields of bromoxynil-tolerant (BXN) and Roundup Ready (RR) cotton in the California cotton growing region, at various distances from non-transgenic cotton fields (Figure 2). The results obtained confirm -and refine- those of Kareiva *et al*. (1994), as larger distances were studied. In spite of variations due to the respective cardinal positions of the fields, the same decline with distance is observed.

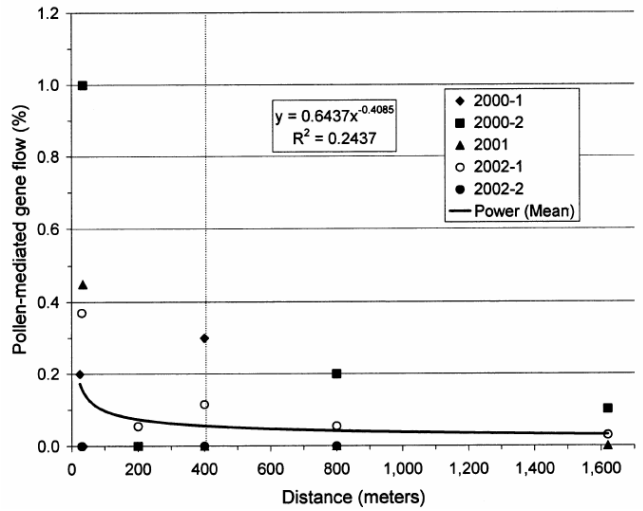

Figure 2. Pollen-mediated gene flow (PGF) in California.
Collected from neighboring fields separated by open space in five different locations in three years (Figure 2 in Van Deynze et al., 2005). PGF was calculated on the basis of samples (2000 seeds each) collected at the closest edge of solid-seeded commercial fields (25-34m), 200, 400, 800, and 1625 m from herbicide-resistant (BXN or Roundup Ready) cotton. Solid line is the best fit regression curve. Broken vertical line represents the current isolation distance for foundation seed of 400m.

In the US, there are four cotton species, two that are cultivated commercially – *G. hirsutum* L. and *G. barbadense* L. and two wild relatives – *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seemann (Fryxell, 1979). Of these four species, only three *Gossypium* species could be recipients for *G. hirsutum* - *G. hirsutum* itself, *G. barbadense* and *G. tomentosum*. *G. hirsutum* grows feral only in the southern tip of Florida and in Hawaii, which is hundreds of miles from any commercial cotton fields. *G. barbadense* is only found in very small commercial plots and is not found in wild environments in the US. Thus outcrossing to wild *G. hirsutum* or commercial plots of *G. barbadense* is unlikely.

Outcrossing of the tetraploid *G. hirsutum* to the wild diploid *G. thurberi*, which occurs in Arizona, is extremely unlikely. Crosses between these species in breeding programs have been done, but the vigor of the hybrid seed is much reduced and the plants are usually infertile. In addition, native populations of *G. thurberi* reside in the higher altitudes and are thus isolated from commercial cotton production (Fryxell, 1979). Therefore, outcrossing of commercial GlyTol cotton to *G. thurberi* is not a concern.

G. tomentosum is only found in the Hawaiian archipelago, occurring in dry coastal areas far removed from agricultural areas. The flowers of *G. tomentosum* are only receptive at night, rather than in the day as for *G. hirsutum* and moths, rather than bees generally pollinate them. Finally, outcrossing is unlikely since there are no commercial cotton production areas on the islands and there would be no selective advantage since glyphosate is not used in natural non-agricultural areas.

G. Characteristics of the recipient plant

GlyTol cotton event GHB614 has its origin in the variety Coker 312. The variety Coker 312 (PVP 7200100) is an US Protected Variety of SEEDCO Corporation, Texas. Coker 312 was developed from a cross of Coker 100 X D&PL-15 and selected through successive

generations of line selection. This variety is well suited for both dry land and irrigated production south of Lubbock, Texas (Metzer and Supak, 1990).

H. Cotton as a test system in this petition

During the development of GlyTol cotton event GHB614, the event was carried in its Coker 312 genetic background for purposes of equivalence testing. At the same time, the GlyTol trait was introgressed into commercial and/or advanced breeding varieties to evaluate performance and equivalence with the corresponding counterpart. Each trial/test in the development of this product used an appropriate control.

III. THE TRANSFORMATION SYSTEM

A. Description of the transformation system

GlyTol cotton was transformed by *Agrobacterium*-mediated gene transfer of the T-DNA from pTEM2. *Agrobacterium*-mediated gene transfer of pTEM2 results in transfer to the plant genome of the DNA fragment between the T-DNA border repeats. The left and right border repeats of *A. tumefaciens*, as described in Table 2, are also inserted into GlyTol cotton event GHB614. Even though some of the genes used in the transformation process were derived from *A. tumefaciens*, a known plant pathogen, the genes that cause crown gall disease were removed, and therefore not incorporated into the recipient plant (Deblaere *et al*., 1985).

The *2mepsps* gene is a common genetic element used in several transformations of agricultural crops as a selectable marker or as a means to confer tolerance to the herbicide glyphosate.

B. Parent line

Coker 312 is an older commercial variety of upland cotton (*Gossypium hirsutum*) which is no longer commercially cultivated. Coker 312 is well suited for transformation because of its capacity for regeneration from single cell culture.

C. Construction of the plasmid used for transformation

The vector pTEM2 is derived from pGSC1700 (Cornelissen and Vandewiele, 1989). The vector backbone contains the following genetic elements:

- the plasmid core comprising the origin of replication from the plasmid pBR322 (Bolivar *et al*., 1977) for replication in *Escherichia coli* (ORI ColE1) and a restriction fragment comprising the origin of replication from the *Pseudomonas* plasmid pVS1 (Itoh *et al*., 1984) for replication in *Agrobacterium tumefaciens* (ORI pVS1);
- a selectable marker gene (*aadA*) conferring resistance to streptomycin and spectinomycin for propagation and selection of the plasmid in *Escherichia coli* and *Agrobacterium tumefaciens* (Leemans *et al*., 1982).);
- a DNA region consisting of a fragment of the neomycin phosphotransferase coding sequence of the *nptI* gene from transposon Tn903 (Oka *et al.,*1981).

These elements are outside the T-DNA borders and are not expected to be transferred into the cotton genome. Their absence is confirmed by data presented in Section IV, Chapter E.

D. Open Reading Frames and associated regulatory regions in pTEM2

The chimeric *2mepsps* gene construct contains the promoter region of the histone H4 gene from *Arabidopsis thaliana* (Chaboute *et al.*, 1987), followed by the first intron of gene II of the histone H3.III variant of *Arabidopsis thaliana* (Chaubet *et al*., 1992), and the optimized transit peptide as described by Lebrun *et al.* (1996). The *2mepsps* coding sequence (Lebrun *et al*., 1997) is followed by the 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chaboute *et al*., 1987). This chimeric gene of pTEM2 that can be transferred to plants is denoted as "Ph4a748At-intron1 h3At-TPotpC::*2mepsps*::3'histonAt".

A map of the plasmid pTEM2 is shown in Figure 3, and a description of the DNA elements in the T-DNA is in Table 2.

Ph4a748At promoter and h3At intron

The Ph4a748At promoter sequence is derived from the histone H4 gene of *Arabidopsis thaliana* (Chaboute *et al*., 1987) and controls expression of the *2mepsps* gene. The Ph4a748At promoter, combined with the first intron of gene II of the histone H3.III variant of *Arabidopsis thaliana* (Chaubet *et al*., 1992) directs high level constitutive expression, especially in the rapidly growing plant tissues.

TPotp C

The optimized transit peptide, which contains sequences from the RuBisCO small subunit genes of corn and sunflower, targets the mature protein to the plastids, where the wild-type protein is located (Lebrun *et al*., 1996).

2mepsps gene

The wild type *epsps* gene isolated from maize was mutated using site-directed mutagenesis. Two point mutations resulted in the double mutant (*2mepsps*) gene (Lebrun *et al*., 1997). A methionine codon is added to the N-terminal of the 2mEPSPS protein sequence in order to restore the cleavage site of the optimized plastid transit peptide. The *2mepsps* gene encodes a 47 kDa protein consisting of 445 amino acids.

EPSPS (EC 2.5.1.19) is a key enzyme in the shikimate pathway. In conventionally-bred plants, EPSPS is selectively inhibited by glyphosate, leading to the death of the plants by shutting off the synthesis of aromatic amino acids and secondary metabolites (Steinrücken and Amrhein, 1980). The 2mEPSPS protein is insensitive to glyphosate inhibition, but has retained its functions in the shikimate pathway.

3' histonAt terminator

The 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chaboute *et al*., 1987) is a polyadenylation signal.

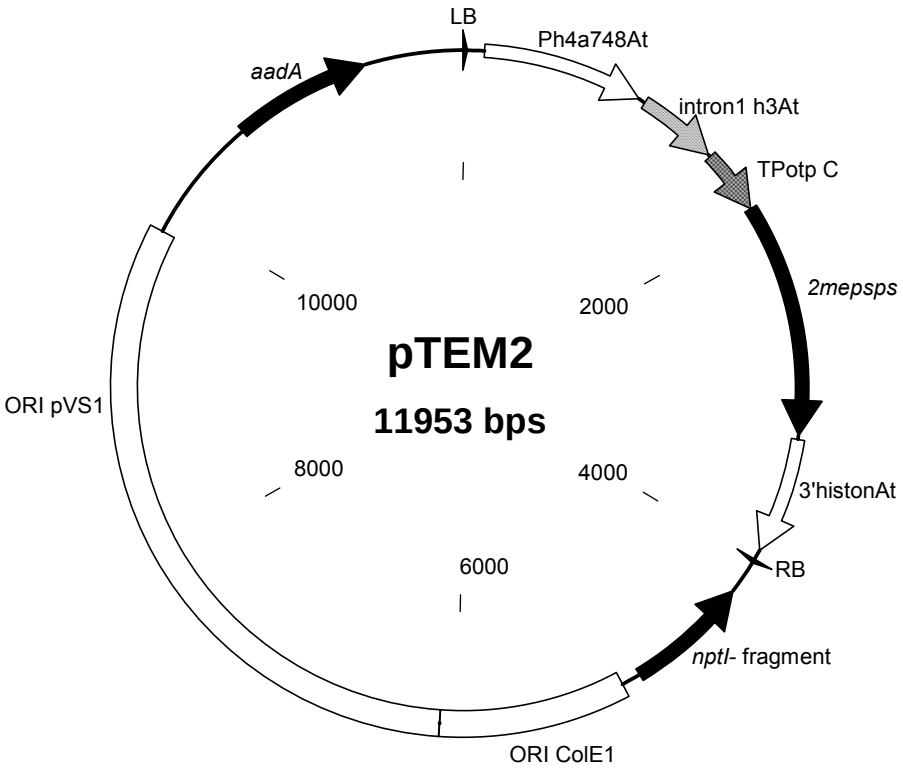

Figure 3. Vector map of plasmid pTEM2

Table 2. Genetic elements of vector pTEM2 to be inserted into the plant genome

Genetic element	Position in vector	Genetic element and function
LB	0001 - 0025	Left border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
Ph4a748At	0025 - 1036	Sequence including the promoter region of the histone H4 gene from *Arabidopsis thaliana* (Chaboute *et al*., 1987).
intron1 h3At	1037 - 1553	Sequence including the first intron of gene II of the histone H3.III variant from *Arabidopsis thaliana* (Chaubet *et al*., 1992).
TPotp C	1554 - 1926	Optimized transit peptide as described by Lebrun *et al.* (1996).
2mepsps	1927 - 3264	Coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene from *Zea mays* (corn) (Lebrun *et al*., 2003).
3'histonAt	3265 - 4007	Sequence including the 3' untranslated region of the histone H4 gene from *Arabidopsis thaliana* (Chaboute *et al*., 1987).
RB	4008 - 4032	Right border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988).

E. Deduced amino acid sequence

The wild-type *epsps* gene was mutated using site-directed mutagenesis, giving rise to the 2mEPSPS (Lebrun *et al*., 1997). The amino acid methionine was added at the N-terminal of the protein sequence in order to restore the cleavage site of the transit peptide (De Beuckeleer, 2003).

```
  1 MAGAEEIVLQ PIKEISGTVK LPGSKSLSNR ILLLAALSEG TTVVDNLLNS EDVHYMLGAL
 61 RTLGLSVEAD KAAKRAVVVG CGGKFPVEDA KEEVQLFLGN AGIAMRSLTA AVTAAGGNAT
121 YVLDGVPRMR ERPIGDLVVG LKQLGADVDC FLGTDCPPVR VNGIGGLPGG KVKLSGSISS
181 QYLSALLMAA PLALGDVEIE IIDKLISIPY VEMTLRLMER FGVKAEHSDS WDRFYIKGGQ
241 KYKSPKNAYV EGDASSASYF LAGAAITGGT VTVEGCGTTS LQGDVKFAEV LEMMGAKVTW
301 TETSVTVTGP PREPFGRKHL KAIDVNMNKM PDVAMTLAVV ALFADGPTAI RDVASWRVKE
361 TERMVAIRTE LTKLGASVEE GPDYCIITPP EKLNVTAIDT YDDHRMAMAF SLAACAEVPV
421 TIRDPGCTRK TFPDYFDVLS TFVKN
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Figure 4. Deduced amino acid sequence for the 2mEPSPS protein

IV. CHARACTERIZATION OF GLYTOL COTTON EVENT GHB614

A. Description, history and mendelian Inheritance

During the development of GlyTol cotton event GHB614, the locus was bred into selected cotton varieties. Herbicide tolerance screenings were done in the greenhouse using glyphosate at the 1X rate. Plants were scored as tolerant (alive, no damage) or susceptible (damaged severely and dead or dying) 5-7 days post-glyphosate application. Polymerase chain reaction (PCR) testing was used to verify the transformation event.

Primary transformation event GHB614 was derived from the transformation of cotton cells as described in Section III. T_1 seed harvested from self-pollinated T_0 plants surviving a glyphosate herbicide greenhouse screen were planted in the greenhouse for seed increase and evaluation. T_1 plants were selected for survival following glyphosate herbicide application, and at each generation, plants were sprayed with glyphosate to eliminate those not expressing the *2mepsps* gene. Homozygous T_3 plants were identified by planting 25 seed and spraying with glyphosate to identify segregating seed lots. Homozygosity PCR based analysis was also performed as a secondary means of identifying homozygous plants. Selfed T_3 homozygous seed (no segregation for tolerance) was used to produce homozygous T_4 seed and was the source of the lines that were used in early event agronomic and stability studies. (Figure 5)

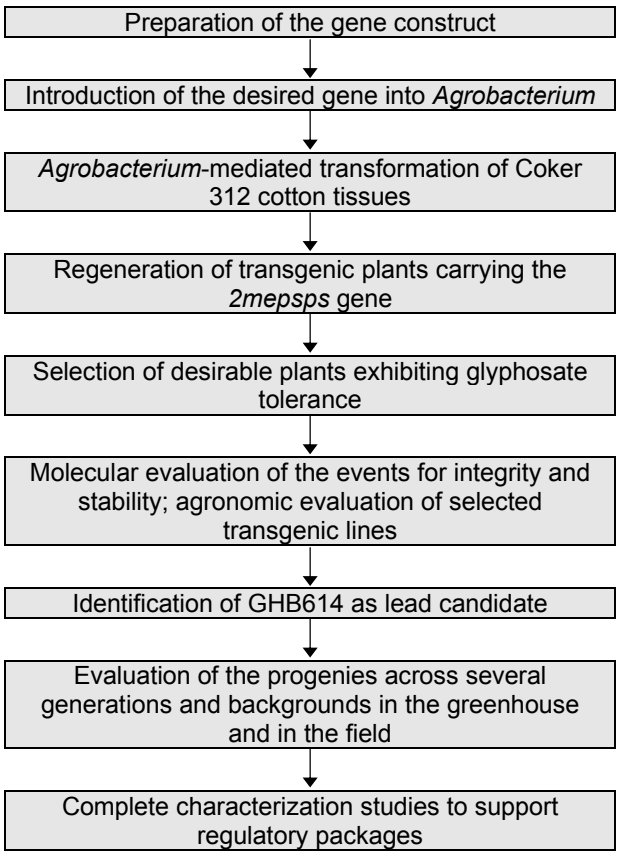

Figure 5. Flow chart for the development of GlyTol cotton event GHB614

For breeding and further confirmation of inheritance, the T_0 plant was crossed with conventional cotton, and the resulting F_1 plants were evaluated in the greenhouse. The BC_1F_1 material was planted in the greenhouse and back crossed with the same recurrent parent (Figure 6). The BC_2F_1 material was evaluated in the greenhouse for segregation of resistance. Mendelian inheritance for a single gene locus predicts one resistant plant for every one susceptible plant within BC_2F_1 progenies. Furthermore, BC_2F_2 progeny would be expected to show 3 resistant plants for every one susceptible plant. (Table 3)

In summary, all data and analyses indicate that the cotton event GHB614 behaves genetically as a single allele at one locus.

Table 3. Segregation analysis of GlyTol cotton event GHB614

Parents and zygosity for the *2mepsps* locus	Generation	Ratio	Observed		Expected		χ^2 calculated[a]
		R:S	R	S	R	S	
Hemizygous BC_2F_1 plant (conventional line A), self-pollinated (*2mepsps*/-)x(*2mepsps*/-)	BC_2F_2	3:1	28 [b]	8	27	9	0.15
Hemizygous BC_2F_2 plant crossed with conventional line B (*2mepsps*/-)x(-/-)	"F_1" population [c]	1:1	7	9	8	8	0.25
Self-pollinated hemizygous "F_1" plants (*2mepsps*/-)x(*2mepsps*/-)	"F_2" populations (pooled)	3:1	113	43	117	39	0.60
Hemizygous "F_1" plant crossed with conventional line B (*2mepsps*/-)x(-/-)	BC_1F_1 population	1:1	9	12	10.5	10.5	0.43
Hemizygous BC_1F_1 plant crossed with conventional line B (*2mepsps*/-)x(-/-)	BC_2F_1	1:1	11	6	8.5	8.5	1.47

[a] Assumes a one locus model. There was no significant difference (p=0.05) for the χ square goodness-of-fit test for the hypothesis of one locus. To reject the null hypothesis, the χ square value must be greater than 3.84, with one degree of freedom.
[b] Tested by homozygosity PCR (19 heterozygous plants and 9 homozygous plants)
[c] All "F_1" population material was generated using a hemizygous transgene donor source (BC_2F_1).
S=susceptible; R=resistant.

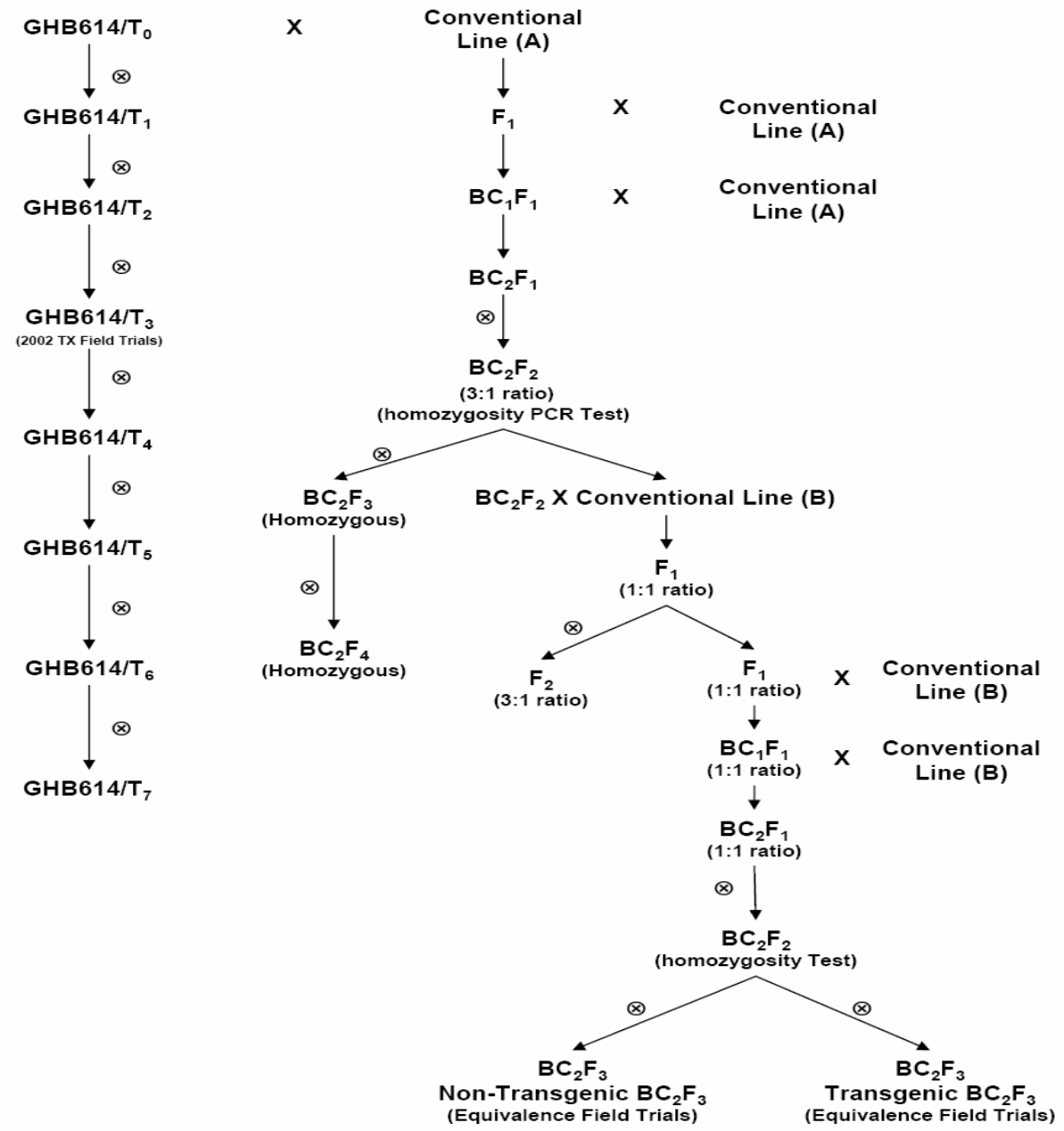

Figure 6. Breeding tree for the development of GlyTol cotton

Notes for Figure 6.

- At each generation, plants were sprayed with glyphosate to eliminate those not expressing the *2mepsps* gene
- ⊗ = self-cross
- Homozygous T_3 plants were identified by planting 25 seed, spraying with glyphosate to identify segregating seed lots. Homozygosity PCR based Invader analysis was also performed as a secondary means of identifying homozygous plants.
- Selfed T_3 homozygous seed (no segregation for resistance) was used to produce homozygous T_4 seed and was the source of the lines that were used in early event agronomic and stability studies.
- Generation BC_2F_4 (homozygous) was used for detailed insert characterization and protein expression levels.
- Generations T_3, T_4, T_5, T_6 and BC_2F_2 were used for molecular stability analyses.
- Generation T_5 was used for seed composition analysis.
- Generations T_5 and BC_2F_3 were used for replicated agronomic field tests.
- Generation T_7 was used for analyses on absence/presence of vector backbone sequences.

B. Verification of the insert and number of copies of the inserted sequences

Genomic DNA isolated from GlyTol cotton event GHB614 and control Coker 312 plants was subjected to Southern blot analysis using the different components of the transgene cassette (Ph4a748At promoter, intron1 h3At+TPotp C, *2mepsps* gene and histonAt terminator) as well as the complete T-DNA fragment, as probes. The expected and observed hybridization fragments, as well as the hybridization strategy, are shown in Appendix 3. Results of this analysis (see Figure 3.1, Appendix 3) show the presence of one 5' integration fragment and one 3' integration fragment.

These data demonstrate that the transferred DNA in event GHB614 corresponds to the DNA configuration in the pTEM2 plasmid and that a single intact copy of the gene cassette is integrated in the GlyTol cotton event GHB614.

C. Stability of the inserted DNA sequence

In order to demonstrate the stability of GlyTol cotton event GHB614, genomic DNA was prepared from several individual plants of multiple generations and different genetic backgrounds. The isolated DNA was digested with the restriction enzyme *Eco*RV, which has one recognition site in the transforming DNA. Probing *Eco*RV digested genomic GlyTol cotton DNA with the "Ph4a748At + intron1 h3At + TPotp C" probe showed the expected integration fragments in all tested samples. These integration fragments represent the junctions between the transgenic sequences and the plant DNA sequences.

The obtained results demonstrate the stability of the GlyTol cotton event GHB614 at the genomic level over different generations and different genetic backgrounds. Segregation data further confirm the stability of the insert, and show that it segregates as one dominant Mendelian locus (see Section IV, chapter A)

D. Presence of marker genes and origin of replication in the vector

The *2mepsps* gene was used as the selectable marker, therefore the same gene of interest acts as a marker. No other marker genes were present.

GlyTol cotton event GHB614 contains no vector backbone sequences as evidenced by using overlapping probes covering the complete pTEM2 vector backbone sequences (including *aadA*, ORI pSV1 and ORI ColE1). See Figure 3.13, Appendix 3.

No bacterial origin of replication is transferred with the *Agrobacterium* mediated transformation system. The inserted DNA within GlyTol cotton event GHB614 does not add a bacterial origin of replication to the wild type *Gossypium hirsutum* genome as a result of the transformation.

E. Absence of remaining parts of the vector

For the molecular verification of absence of pTEM2 vector backbone sequences in GlyTol cotton, genomic DNA was isolated from event GHB614 and control Coker 312 plants. Southern blot analysis was then performed using five overlapping probes, covering the entire vector backbone sequence. The sizes of some hybridizing fragments can be predicted by the location of restriction enzyme cleavage sites internal to the inserted DNA. Afterwards, the membranes were stripped of the vector backbone probes, and re-hybridized with a T-DNA probe, in order to demonstrate that ample GHB614 cotton genomic DNA was loaded on the gels. The positive control samples showed the expected hybridization fragment of 9131 bp. No hybridization fragments are visible in the wild-type (Coker 312) control lane.

The Southern blot analysis using overlapping probes covering the complete pTEM2 vector backbone sequences demonstrates the absence of vector backbone sequences in GlyTol cotton event GHB614.

F. The flanking regions of the inserted sequence(s)

Right and left border integration fragment
Southern blot analysis demonstrated that the transgenic glyphosate tolerant cotton event GHB614 contains one copy of the T-DNA of plasmid pTEM2. The sequence of the transgenic locus and the sequence of the pre insertion locus were determined. 5' flanking sequences and 3-prime flanking sequences were reported.

In the Southern blot analysis, a membrane containing equimolar amounts of genomic DNA prepared from leaf material was sequentially hybridized with the 3' flanking sequences of GlyTol cotton, the 5' flanking sequences, and the *2mepsps* probe. Hybridization fragments could be observed in the different *Gossypium hirsutum* genomic DNA samples after hybridization with 5' and 3' flanking sequences. This demonstrates that the flanking sequences of GlyTol cotton event GHB614 are of *Gossypium hirsutum* origin. Afterwards the identity of GlyTol cotton event GHB614 was confirmed by hybridization with the *2mepsps* probe.

PCR analysis was performed using primer-pairs targeting the 5' and 3' flanking sequences of event GHB614. Primers targeting chloroplast tRNA gene sequences were included in the reaction to serve as an internal control. In a first PCR reaction, a primer-pair targeting the flanking sequences was used to demonstrate the nature of the flanking sequence. In a second PCR reaction, the specificity of the 5' and 3' integration fragments was demonstrated. The obtained PCR results demonstrate that the 5' and 3' flanking sequences of cotton event GHB614 are of cotton plant origin.

Southern blot and PCR analyses demonstrate unequivocally that the characterized flanking sequences are of cotton plant origin.

BLASTn similarity search, and open reading frame search
Cotton plants transformed using *Agrobacterium*-mediated transformation inserting the T-DNA from vector pTEM2 into the cotton genome generated the GlyTol cotton event GHB614. Due to the insertion of the *2mepsps* gene cassette in cotton, a 5-prime and 3-prime junction, where cotton genomic DNA and inserted T-DNA are fused, was created. The junction regions were analyzed to confirm that no important cotton genes were interrupted and that no chimeric proteins would get expressed due to this insertion.

Open reading frame (ORF) and gene search tools were applied to predict the presence of potential newly created coding sequences in the 5-prime flanking genomic/insert DNA junction region and in the 3-prime flanking insert/genomic DNA junction region. Two ORFs were found, that span the 5-prime junction and none at the 3-prime junction.

The results of *in silico* analysis of the putative ORF1 and ORF2, identified in GlyTol cotton event GHB614, revealed no similarities with known toxins or allergens based on the following matching criteria:

For identification of significant similarity to an allergen:

1. a 100% identity over a linear contiguous 8 amino acid segment or,
2. a 35% identity with a known allergen and low E-value (<0.1).

For identification of significant similarity to a toxin

1. a 35% identity with a known toxin and low E-value (<0.1).

V. THE 2mEPSPS PROTEIN

A. History and background

In the early 1970s, it was demonstrated that inhibitors of the aromatic amino acid biosynthetic pathway can have an herbicidal activity (Jaworski, 1972; Baillie *et al.*, 1972). In particular, the work published by Jaworski's group opened the path for the development of the glyphosate herbicide.

In plants, as much as 20% of all fixed carbon flows through the shikimate pathway leading to the formation of the aromatic amino acids tyrosine (tyr), phenyalanine (phe) and tryptophan (trp), as well as tetrahydrofolate, ubiquinone, and vitamins K and E (Haslam, 1993; Franz *et al.*, 1997). The aromatic amino acids, in turn, serve as precursors for an array of secondary metabolites including lignin, flavonoids and alkaloids (Herrmann, 1995). The shikimate pathway occurs exclusively in plants and microorganisms including fungi. In contrast, mammals, fish, birds, reptiles, and insects must derive their aromatic compounds from their diet. For this reason, there has been interest over the last three decades in the shikimate pathway enzymes as potential targets for non-toxic herbicides and anti-microbial compounds.

Glyphosate is the active ingredient of a non-selective, broad-spectrum, systemic, post-emergence herbicide that has been used extensively throughout the world over the past three decades. It has a very low mammalian toxicity and low soil persistence. It is used to inhibit weeds in conservation tillage systems just prior to planting. It is also applied as a non-selective herbicide with direct spraying in orchards. Given the importance of this compound, considerable effort has been made in attempts to engineer glyphosate tolerance in various crops.

Study of the shikimate pathway led to the discovery of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by Amrhein *et al.*, (1980). The mode of action of glyphosate [N-(phosphonomethyl)glycine], a simple amino acid analog, was determined to be the selective inhibition of EPSP synthase (EPSPS; EC 2.5.1.19), the sixth and penultimate enzyme of the shikimate pathway (Steinrücken and Amrhein, 1980). The reaction catalyzed by EPSPS is the reversible transfer of the phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P), leading to the formation of 5-enolpyruvyl-3-shikimate phosphate (EPSP). Substrate binding to the enzyme is sequential, with S3P binding first, followed by PEP (Boocock and Coggins, 1983). The reaction catalyzed by EPSPS proceeds via C-O bond cleavage of PEP (Walsh *et al.*, 1996).

B. Characterization of the 2mEPSPS protein

a. 2mEPSPS biochemistry and mode of action

The family of EPSPS proteins is wide-spread in nature, specifically in plant, fungi and microbial sources. In higher plants, EPSPS is synthesized from a nuclear gene in the form of a cytoplasmic precursor, then imported into the plastids where it accumulates in its mature form (Kishore and Shah, 1988; Forlani *et al.*, 1994; Lebrun *et al.*, 1997). Transit peptides are typically cleaved from the mature protein following delivery to the plastids (Della-Cioppa *et al.*, 1986).

Since the 1980s, several attempts have been made to identify and characterize glyphosate-insensitive EPSPS enzyme variants from various organisms with the ultimate aim to engineer glyphosate tolerance in crop plants (Kishore and Shah, 1988). Lebrun *et al.* (1997) selected a double mutant gene from maize, which when fused to a chimeric optimized transit peptide,

generates optimal glyphosate tolerance in various crops, with no pleiotropic effects: the *2mepsps* gene encoding the 2mEPSPS protein. The *2mepsps* gene has been introduced as the source of glyphosate tolerance in the maize transgenic event GA21 which has been approved by different agencies worldwide for environment, food, and feed (OECD unique identifier MON-ØØØ21-9) (AGBIOS, 2006). Recently, glyphosate tolerance was also achieved in rice by mutagenesis of the rice *epsps* gene (Zhou *et al.*, 2006).

SDS-PAGE

The 2mEPSPS and wtEPSPS proteins were analyzed by SDS-PAGE. Analysis of the protein samples revealed an intense protein band for both the 2mEPSPS and the wtEPSPS with the same apparent molecular mass of 47 kDa. This corresponds to the deduced molecular weight based on the amino acid sequence described in Figure 4.

Western blotting

The western blot analysis reveals a single cross-reacting polypeptide for both the wtEPSPS and the 2mEPSPS proteins. The protein bands in both cases migrated with an apparent molecular mass of 47 kDa which confirms the immunoreactivity characteristics of the two proteins.

b. 2mEPSPS protein safety

The *2mepsps* gene was generated by introducing mutations into the wild-type *epsps* (wt *epsps*) gene from maize, leading to a double mutant EPSPS protein with two amino acid substitutions (2mEPSPS). These modifications confer to the protein a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, the plants bearing this gene become tolerant to glyphosate herbicides.

In order to assess any potential adverse effects to humans or animals resulting from environmental release of the crops containing the 2mEPSPS protein, Bayer CropScience (BCS) has conducted a detailed safety evaluation based on Codex Alimentarius Commission (Codex; Alinorm 03/34A). As a basis, BCS performed a series of safety studies with the 2mEPSPS protein, including homology searches of the amino acid sequence with comparison to all known allergens and toxins from large public databases, an *in vitro* digestibility assay of the protein, and an acute toxicity test in the mouse. Moreover, publicly available review documents issued by regulatory authorities, indicating that similar EPSPS protein family members are safe, have been used for supporting this safety assessment. The results of studies conducted by BCS are consistent with the published information, confirming that the crops containing this protein can be safely used as food or feed.

Assessment of the *Zea mays* source organism, the *2mepsps* gene, and the 2mEPSPS protein indicates that they are not pathogenic, allergenic, or toxic for mammals. Specifically:

History of safe use

- The source organism (*Zea mays*) is a safe crop plant widely used for food and feed with little pathogenic, toxic, or allergenic effects for humans and animals.
- The 2*mepsps* gene is composed of the same essential nucleic acids found in any food or feed DNA, which is commonly consumed as part of human or animal diets. Decades of research have indicated that dietary DNA poses no direct toxicity on human health.
- The EPSPS proteins are ubiquitous in nature, widely expressed in food and feed crops (*e.g*. soybean, tomato, maize). No health-related adverse effects have been associated with these proteins. Since the 2mEPSPS protein is derived from maize and has only two amino acid modifications, the safety profile of the novel protein is expected to remain unchanged relative to its wild-type counterpart.

- The 2mEPSPS protein is highly homologous to, and shares similar molecular weight and functionalities with other shikimate synthase proteins which have been demonstrated to be non-toxic and non-allergenic over the years through consumption. Its identity with the wtEPSPS enzyme is greater than 99.5%.
- The EPSPS proteins have a very well known and specific biochemical role in plants. The biochemical properties of the 2mEPSPS enzyme have been well characterized in comparison with the wtEPSPS protein. Except for the insensitivity to glyphosate, the change in the two amino acids results in comparable biochemical properties. The metabolic effects of the 2mEPSPS activity in plants are comparable to those of endogenous EPSPS proteins except for the insensitivity to glyphosate.
- The 2mEPSPS protein is present in glyphosate tolerant maize event GA21 (MON-ØØØ21-9), which is approved for cultivation and for food/feed use in many regions.

Lack of allergenic potential
- The 2mEPSPS protein has no amino acid sequence similarity to other known allergens, as demonstrated by overall amino acid and epitope homology searching.
- As expected, the 2mEPSPS protein has high structural similarity only to the non-allergenic *Zea mays* wtEPSPS protein and other non-allergenic EPSPS enzymes.
- The 2mEPSPS shares the same potential N-glycosylation sites as the endogenous *Zea mays* EPSPS enzyme, and both proteins are targeted to the same plastid cellular compartment. Therefore, it is unlikely that post-translational glycosylation occurs on the 2mEPSPS protein, which would lead to allergenic characteristics different from the wild-type enzyme.
- The 2mEPSPS protein is rapidly and completely degraded in human simulated gastric and intestinal fluids. This minimizes the likelihood that this protein could survive in the human digestive tract and be absorbed.

Lack of toxic potential
- The 2mEPSPS protein has no amino acid sequence similarity to other known toxins, as demonstrated by overall amino acid and epitope homology searches.
- As expected, the 2mEPSPS protein only has high structural similarity to the non-toxic *Zea mays* wtEPSPS protein and other non-toxic EPSPS enzymes.
- The 2mEPSPS protein is rapidly and completely degraded in human simulated gastric and intestinal fluids. This minimizes the likelihood that this protein could survive in the human digestive tract and be absorbed.
- There were no mortalities, clinical signs, or treatment-related effects in OF1 mice after an acute oral administration by gavage of 2mEPSPS protein at 2,000 mg protein/kg body weight.

In conclusion, it is considered that the *Zea mays* source organism is non-pathogenic and the *2mepsps* gene as well as the 2mEPSPS protein are not toxic for mammals and do not possess any of the characteristics associated with food allergens. Therefore, no adverse effects on animal and human health are to be expected by consumption of the *2mepsps* gene and the 2mEPSPS protein.

VI. EXPRESSION OF THE INSERTED SEQUENCE

Several studies were performed to quantify the 2mEPSPS protein in tissues of GlyTol cotton event GHB614. The levels of 2mEPSPS protein in event GHB614 were determined by a validated enzyme-linked immunosorbent assay (ELISA).

A. Expression of the 2mEPSPS protein

a. 2mEPSPS protein in fuzzy seed of GlyTol cotton

Nine trials were conducted in 2005; the plants were grown under conditions typical of production practices (see Appendix 2). There were six transgenic plots and three non-transgenic plots at each test site. Three of the transgenic plots were sprayed three times with glyphosate acid equivalent at 0.75 lb ai/A, and three transgenic plots were untreated. Samples of ginned cottonseed (fuzzy seed) were taken and shipped frozen to Bayer CropScience for ELISA determination of the content of 2mEPSPS protein in the raw agricultural commodity.

Results from the quantification of 2mEPSPS protein are shown in Table 4. 2mEPSPS protein was found in all fractions of transgenic fuzzy seed. More than 99.5% of the 2mEPSPS protein was found in the kernel fraction and thus also in the fuzzy seed fraction (Kernel + Lint Coat). The Lint Coat fraction contained less than 0.5% of the 2mEPSPS protein. 2mEPSPS protein content varied among different trial sites and treatments with glyphosate. The values ranged from 16.2 µg/g to 30.5 µg/g fresh weight for GHB614 cotton sprayed with glyphosate herbicide (0.75 lb g a.i. acid equivalent/acre) and from 15.8 µg/g to 25.5 µg/g FW in cotton event GHB614 receiving conventional herbicide treatment. 2mEPSPS protein was approximately 0.0093% and 0.0100%, respectively, of crude protein for fuzzy seed of GHB614 not treated or treated with glyphosate.

Analysis by ANOVA indicated significant differences between 2mEPSPS protein values with respect to site and treatment, *i.e.*, non-transgenic, non-sprayed transgenic and sprayed transgenic samples. No significant differences were found for extract and assay.

Table 4. 2mEPSPS protein levels in fuzzy seed of GlyTol cotton event GHB614 as detected by ELISA

Sample	Trial No.	[a, b] Average 2mEPSPS Content in µg/g sample, ± SD		[c] Average 2mEPSPS Content as % of crude protein, ± SD	
		Not treated with Glyphosate	Treated with Glyphosate	Not treated with Glyphosate	Treated with Glyphosate
Kernel	02-01	28.7 ± 6.5	33.6 ± 3.2	NA	NA
	03-02	40.1 ± 5.9	43.5 ± 4.5	NA	NA
	04-03	33.1 ± 3.8	39.1 ± 7.5	NA	NA
	04-04	32.0 ± 2.8	33.3 ± 5.4	NA	NA
	04-05	32.2 ± 2.2	39.9 ± 6.5	NA	NA
	04-06	29.3 ± 1.5	28.6 ± 2.6	NA	NA
	04-07	40.0 ± 3.3	39.5 ± 4.0	NA	NA
	06-08	43.9 ± 2.6	46.9 ± 4.0	NA	NA
	08-09	47.1 ± 5.6	55.8 ± 8.1	NA	NA
Average	NA	36.3 ± 7.2	40.2 ± 9.0	NA	NA
Lint Coat	02-01	0.10 ± 0.03	0.23 ± 0.24	NA	NA
	03-02	0.08 ± 0.04	0.17 ± 0.14	NA	NA
	04-03	0.10 ± 0.04	0.09 ± 0.03	NA	NA
	04-04	0.05 ± 0.02	0.05 ± 0.01	NA	NA
	04-05	0.02 ± 0.03	0.16 ± 0.18	NA	NA
	04-06	0.16 ± 0.09	0.29 ± 0.17	NA	NA
	04-07	0.06 ± 0.03	0.07 ± 0.07	NA	NA
	06-08	0.13 ± 0.06	0.14 ± 0.03	NA	NA
	08-09	0.05 ± 0.02	0.07 ± 0.03	NA	NA
Average	NA	0.08 ± 0.06	0.14 ± 0.15	NA	NA
Fuzzy Seed	02-01	15.9	18.4	0.0079	0.0090
	03-02	18.6	20.0	0.0120	0.0120
	04-03	17.5	21.5	0.0084	0.0108
	04-04	17.3	19.3	0.0085	0.0096
	04-05	19.7	20.3	0.0088	0.0087
	04-06	15.8	16.2	0.0066	0.0068
	04-07	21.9	21.8	0.0090	0.0092
	06-08	20.9	23.0	0.0107	0.0113
	08-09	25.5	30.5	0.0114	0.0129
Range in Values	NA	15.8 – 25.5	16.2 – 30.5	0.0066 – 0.0120	0.0068 – 0.0129
Average ± SD	NA	19.2 ± 3.1	21.2 ± 4.0	0.0093 ± 0.0018	0.0100 ± 0.0019

Data from Currier DQ06Q002 (2006).

[a] Results are expressed as micrograms of protein per gram of tissue on a fresh weight basis.

[b] Standard Deviation was not calculated for fuzzy seed data because the value is the weighted numerical sum of the average kernel and average lint coat values. A standard deviation was calculated for the average 2mEPSPS value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the eight sites. Standard deviations for the individual sites were based on 12 measurements (2 sample extracts assayed in duplicate from 3 replicate plots). Standard deviations for the averages of kernel and lint coat are based on 108 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at 9 sites). The data for the fuzzy seed were calculated from the amount of 2mEPSPS protein present in kernel and lint coat fractions taking into account their respective weights. [c] Average 2mEPSPS as % of crude protein is not applicable (NA) because protein determinations were not made on these samples.

b. 2mEPSPS protein content in plant parts and during the life cycle

GlyTol cotton event GHB614 (generation BC_2F_4) and Coker 312 plants were grown in a greenhouse. GlyTol cotton plants were sprayed with glyphosate herbicide at the 1-2 leaf (V1-V2) stage, and samples were collected at 16, 33, 51 and 68 days after planting (Table 5).

The 2mEPSPS protein content in young leaf tissue decreased over time from 11.16 ± 3.73 to 0.45 ± 0.22 µg/g fresh weight (FW) and was at its lowest in growth stage 4. The 2mEPSPS protein content in transgenic stem tissue remained relatively constant between growth stage 2 and growth stage 4. The 2mEPSPS protein content in transgenic root tissue increased between growth stage 2 and growth stage 4. Overall the 2mEPSPS protein levels in plant material of GHB614 cotton was highest in stage 2 leaves (7.94 ± 2.87 µg/g FW) and lowest in pollen (0.16 ± 0.00 µg/g FW).

The 2mEPSPS protein content as a percentage of TEP in young leaf tissue decreased over time and was at its lowest in growth stage 4, (range 0.028% - 0.385%). The 2mEPSPS protein content %TEP in transgenic stem tissue decreased between growth stage 2 and growth stage 4 (0.039% - 0.062%), and the 2mEPSPS protein content %TEP in transgenic root tissue increased (0.074% - 0.176%), as described in Figure 7 and Table 6.

The regulatory elements present in the construct (Table 2), have been shown to be active in meristem of green tissues (Chaboute *et al*., 1987; Chaubet *et al*., 1992; Lebrun *et al*., 2003). The cotton leaf receives the major exposure of glyphosate herbicide, which then accumulates in the meristematic parts. From the cited research, we expected GlyTol cotton event GHB614 to show high levels of 2mEPSPS protein in leaves and apices, and lesser amounts in the other organs. Indeed, the following order of 2mEPSPS expression was demonstrated:

Leaf, apex >> roots, squares >> stems, seeds >> pollen

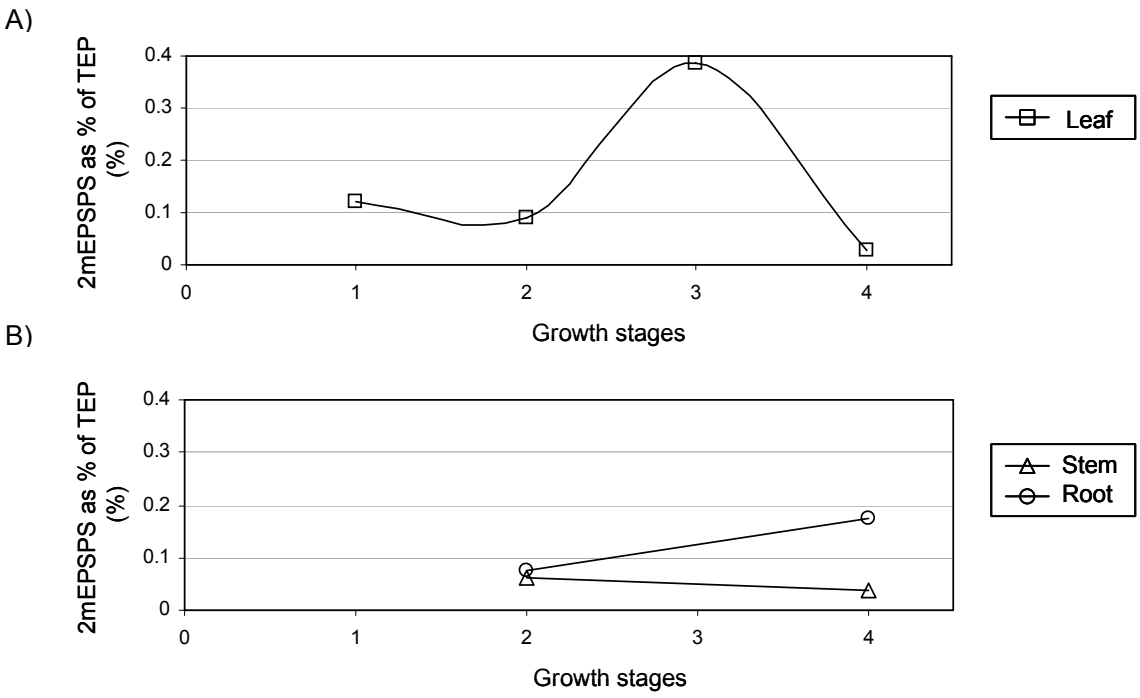

Figure 7. Average 2mEPSPS protein contents as percent of TEP in leaf, stem and root tissues at different development stages

Table 5. Growth stages for sample harvest and number of plants sampled

Stage	Growth Stage	Tissue	Days after planting	Plants sampled
1	V2-V3	young leaf	16	15
2	V4-V6	young leaf, stem, root	33	10
3	Pre-flowering	young leaf	51	10
4	Flowering	young leaf, stem, root	68	10
		apex, square, pollen		pool *

* At flowering stage, square, apex and pollen tissue from several plants were pooled, as less tissue material was available.

Table 6. 2mEPSPS protein levels in plant tissues of GlyTol cotton

Matrix	2mEPSPS Protein Contents (µg/g fresh weight) ± SD [% TEP]			
	Growth stage 1	Growth stage 2	Growth stage 3	Growth stage 4
Leaf	11.16 ± 3.73 [0.121]	7.94 ± 2.87 [0.090]	6.52 ± 7.20 [0.385]	0.45 ± 0.22 [0.028]
Stem	ND	1.94 ± 0.61 [0.062]	ND	1.58 ± 0.96 [0.039]
Root	ND	0.99 ± 1.00 [0.074]	ND	4.04 ± 1.71 [0.176]
Squares	NA	NA	NA	5.35 ± 0.25 [0.175]
Apex	ND	ND	ND	5.47 ± 0.22 [0.338]
Pollen	NA	NA	NA	0.16 ± 0.00 [0.001]

* ND = Not Determined; NA = Not Applicable

B. Expression of other parts of the insert

There is no expression of other genes (coding sequences) of the insert since the inserted sequence consists only of the 2mepsps cassette. The absence of any additional DNA from the vector used for the transformation has been documented in Section IV.E.

C. Verification of the biochemical and functional equivalence of the expressed protein

a. Equivalence of the 2mEPSPS protein produced in GlyTol cotton and in *E. coli*

Studies on potential toxicology and allergenicity for food, feed and the environment are conducted with 2mEPSPS protein expressed in *E. coli*. The results of these experiments are used to show safety of the same protein produced in GlyTol cotton event GHB614. In order to utilize the safety data of the protein produced in a microorganism for the safety assessment of the same protein produced in a genetically modified plant, it is important to confirm that the protein produced in a microorganism is representative of the protein produced in the modified plant. The 2mEPSPS protein isolated from *E. coli* was compared to the 2mEPSPS protein isolated from GlyTol cotton event GHB614, using the 6 following criteria and associated methods listed in Table 7.

Table 7. Criteria and methodologies for demonstrating equivalence between the same protein produced in a bacterium and a plant

Equivalence criteria	*Methodology*
Confirm identity of 2mEPSPS protein	Edman degradation
Comparable molecular weight	Protein mobility in SDS-PAGE
Comparable immuno-reactivity	Western blot analysis
Comparable peptide masses	HPLC/Electrospray Mass Spectrometry (LC/MS) of peptides
Glycosylation profile	Staining SDS-PAGE for glycoproteins
Comparable biological activity	Enzymatic activity

Identity

The theoretical N-terminal sequence of the 2mEPSPS protein deduced from the DNA sequence of the gene in *E. coli* and GlyTol cotton event GHB614 is: methionine, alanine, glycine, alanine, glutamic acid, glutamic acid, and isoleucine. The 2mEPSPS protein was isolated from GHB614 cotton leaves and the N-terminal sequence was determined by Edman degradation. The following primary sequence was obtained from the N-terminus: alanine, glycine, alanine, glutamic acid, and glutamic acid. This sequence is an exact match to the sequence deduced from the DNA sequence of the *2mepsps* gene for residues 2-7. These data confirm the identity of the proteins isolated from GlyTol cotton event GHB614 and show that the 2mEPSPS protein from GlyTol cotton is missing the N-terminal methionine. Post-translational modifications, such as removal of a methionine are often found in proteins from both prokaryotic and eukaryotic organisms (Bradshaw *et al*., 1998).

Molecular weight

The 2mEPSPS protein from *E. coli* and the 2mEPSPS protein purified from GlyTol cotton, using an antibody affinity column, were analyzed by SDS-PAGE. The protein from the plant and the corresponding protein from *E. coli* were denatured and analyzed by electrophoresis on a denaturing polyacrylamide gel where mobility is related to molecular weight. Standards on the gel were a series of other proteins of known molecular weight. The gel was then stained with Coomassie brilliant blue to visualize the protein bands. Appendix 3, Figure 3.19 shows the Coomassie stained gel. The electrophoretic mobilities of the 2mEPSPS protein produced in *E. coli* and in GlyTol cotton event GHB614, were indistinguishable. Both had measured electrophoretic mobilities of 26 mm. The electrophoretic mobility of each standard protein was plotted versus its respective molecular weight and an approximate molecular weight of 42 kDa was calculated from this relationship (Appendix 3, Figure 3.20). This value is close to the theoretical molecular weight of 47 kDa calculated from the amino acid sequence deduced from the DNA sequence. In addition, the SDS-PAGE gel shows that the protein had a high degree of purity.

Immuno reactivity

The same electrophoretic procedure was followed as for the SDS-PAGE analysis except there was much less protein loaded and the gel was not stained with Coomassie brilliant blue. The results of the western blot are shown in (Appendix 3 Figure 3.19, Panel B). These results show that the electrophoretic mobilities and immunoreactivities of the 2mEPSPS proteins produced in *E. coli* and GlyTol cotton event GHB614 are indistinguishable.

Peptide mass

The peptides from a tryptic digest of the 2mEPSPS protein from *E. coli* were separated by HPLC and subsequently analyzed by electrospray mass spectrometry. The sequence of the 2mEPSPS protein introduced in cotton is provided in Figure 4. The expected peptides from

the 2mEPSPS protein from *E. coli* were identified by Selected Ion Monitoring (SIM) with 93% of the 445 amino acids in 2mEPSPS (Appendix 3, Table 3.5).

The most abundant ion for each peptide from the *E. coli* 2mEPSPS protein was chosen for selected ion monitoring of the peptides produced by tryptic digestion of the 2mEPSPS protein isolated from GlyTol cotton. The N-terminal peptide was not detected in the full scan analysis of the *E. coli* 2mEPSPS protein. This was expected since the Edman degradation data obtained for the 2mEPSPS protein from *E. coli* indicated that about 80% of the N-terminal peptide is missing the methionine.

The peptides from the 2mEPSPS protein from *E. coli* were identified in the 2mEPSPS protein from GlyTol cotton event GHB614 with coverage of 91.5% of the protein. The data show that the calculated masses for the detected peptides from both proteins are identical.

Glycosylation profile
The 2mEPSPS proteins from GlyTol cotton event GHB614 and from *E. coli* were subjected to analysis by SDS-PAGE. The gel was then stained using the GlycoProfile™ III kit (Appendix 3, Figure 3.21, Panel A). Only the glycosylated standard proteins gave a strong signal with the glycoprotein stain. The non-glycosylated protein standards and the 2mEPSPS proteins from *E. coli* and GlyTol cotton event GHB614 were only very weakly visible with the glycoprotein stain, as expected. Glycosylated and non-glycosylated proteins were readily visible after staining with Coomassie brilliant blue (Appendix 3, Figure 3.21, Panel B). Absence of glycosylation for the 2mEPSPS protein from GlyTol cotton was confirmed in this analysis.

Biological activity
2mEPSPS proteins isolated from *E. coli* and from leaves of GlyTol cotton event GHB614 were shown to generate free phosphate molecules in the enzymatic assay. Based upon this evidence, we concluded that the purified proteins from *E. coli* and from GlyTol cotton are biologically active. This confirms that the proteins from these two sources were present in the correct conformation.

Conclusion
The six analytical tests described in Table 7 offer a multi-directional approach to demonstrate equivalence of the 2mEPSPS protein produced in *E. coli* and GlyTol cotton event GHB614. The results show that the 2mEPSPS protein produced in *E. coli* is representative of the 2mEPSPS protein produced in event GHB614 and that the safety data obtained for the 2mEPSPS protein produced in *E. coli* can be used to support the safety of the 2mEPSPS protein produced in GlyTol cotton event GHB614.

b. Similarity of the 2mEPSPS protein with other plant EPSPS

EPSPS is the sixth enzyme of the shikimate pathway, the metabolic pathway for the biosynthesis of aromatic compounds found in microorganisms and in plants. As such, it has been shown that EPSPS enzymes are ubiquitous in nature and are present in foods derived from plant and microbial sources. It is apparent that these proteins have a long history of safe use as endogenous components of food and feed.

The 2mEPSPS protein shows a high amino acid sequence identity to the wild-type maize EPSPS enzyme (>99.5%) as well as to other EPSPS proteins found in crops that have a long history of safe human consumption (*e.g*. rice, grape, lettuce, tomato and oilseed rape, Table 8) or in fungal and microbial food sources such as baker's yeast (Rouquié, 2006). These proteins have a long history of safe use as endogenous components of food and feed.

Essentially, there is no evidence suggesting that these proteins may be related to any type of allergenicity or toxicity to humans or other animals. Thus, exposure to the known EPSPS proteins can be deemed as innocuous as exposure to other naturally occurring proteins without inducing adverse effects.

Table 8. Comparison of the deduced amino acid sequence of 2mEPSPS to that of other EPSPS proteins

	Maize	*Rice*	*Grape*	*Lettuce*	*Tomato*	*Rape*
2mEPSPS % sequence identity	>99.5	86	79	77	75	75

VII. DETECTION TECHNIQUES FOR THE MODIFIED ORGANISM

The trait could be detected either on molecular genetic level or on protein biochemical level.

The molecular genetic detection can be performed with a PCR based method to confirm the presence of the introduced material in *Gossypium hirsutum* plant material.

The polymerase chain reaction (PCR) is a technique used to amplify a small quantity of target DNA in order to make it detectable. Most of the PCR reactions can be carried out as multiplex reactions, which means they involve more than one PCR reaction, therefore more than one target. One targets a DNA sequence endogenous to the plant; another pair targets a DNA sequence specific to the inserted transgene. The endogenous reaction acts as a control in order to determine whether plant DNA is present and that reaction conditions are sufficient to allow amplification. The transgene reaction will only amplify a product from the inserted DNA, making it possible to distinguish between non-transgenic and transgenic samples.

There are specific protocols for each transgene within each type of plant. An individual protocol usually requires optimization to account for differences between labs, matrices, or reagents. This optimization is especially important when performing multiplex reactions. Some loci are more efficiently amplified than others due to base composition, length of product, and secondary structure. In multiplex reactions, the more efficiently amplified loci compete better for the available reaction components, and will negatively influence the yield of product from the less efficient loci, making them less visible or undetectable. It is important to obtain reaction conditions that amplify equimolar quantities of both the enodogenous and transgenic sequences in a known transgenic DNA sample.

The detection tools for the protein level are based on immunoassays. These assays are a Sandwich Enzyme Linked Immunosorbent Assay (ELISA) based on the specific interaction between antibody and antigen. The wells of the solid phase are coated with affinity-purified polyclonal antibodies (capture antibodies) specifically recognizing the protein of interest from the inserted gene. The protein from the introduced gene present in the samples is bound to the capture antibody. The immobilized protein can be detected by sequential incubation with monoclonal or polyclonal antibodies (detection antibody or second antibody) recognizing the protein to be tested, and a horseradish peroxidase-conjugated polyclonal antibody (antibody conjugate) against the second antibody. A peroxidase substrate, tetramethylbenzidine, is added and converted by the peroxidase to a blue product in proportion to the amount of tested protein present in the sample. Upon the addition of the stop solution, the blue product turns yellow. The optical density of the yellow product at 450 nm reflects proportionally the amount of protein present in the sample.

Another protein detection method is the lateral flow strips. This method allows qualitative detection of the introduced protein, and can be performed under field and/or lab conditions.

The method uses a double antibody sandwich format to detect the introduced protein. Antibodies specific for the protein are present in two places in the strip. One antibody is fixed to the strip in the area where the band is expected, and its purpose is to capture the protein (if the protein is present) while the extract flows up the strip. The other antibody is found in the pad that is located near the bottom of the strip, and its purpose is to report the presence of the protein by binding to it. This detection antibody is conjugated to gold particles. When the lateral flow strip is placed in an extract from plant tissue that contains the protein of interest, the extract flows through the pad where the reporting antibody binds to the protein, if present. The extract, reporting antibody and any inserted protein flow through the strip until they come

in contact with the capture antibody. A sandwich is formed between the capture antibody, the protein of interest and with some, but not all the reporting antibody that is coupled to the gold. A second band of antibodies to the reporting antibodies capture any remaining antibody to develop the control band. The bands display as a reddish color when the gold-conjugated antibodies are captured in the specific zones on the membrane. The presence of only one band (control band) on the membrane indicates a negative sample and the presence of two bands indicates a positive sample.

Reference material (specific PCR primers, genomic DNA, seeds) of GlyTol cotton can be provided upon request, and upon agreement with BCS.

VIII. AGRONOMIC AND PHENOTYPIC EVALUATION

A. Agronomic performance and evaluation

This section of the document will describe the agronomic evaluation of the GlyTol cotton event GHB614 as compared to the non-transgenic Coker 312 control. Agronomic evaluation was also conducted between an advanced cotton strain in which GHB614 has been introgressed by standard backcrossed breeding practices and its isogenic counterpart. Equivalence evaluation was done to distinguish agronomic factors which were introgressed into the genome of the cotton plant by the transformation and those which were the result of tissue culture from the Coker 312 background. This data was compiled to demonstrate that the GlyTol cotton event GHB614 does not pose an increased plant pest risk over conventional non-transformed cotton.

Agronomic evaluations of the GlyTol cotton event GHB614 were conducted using a series of field tests between 2004 and 2005. Evaluations were made on key agronomic parameters to assess the growth habit and phenotype of the transformed lines, their reactions to biotic and abiotic stressors in their respective environments, and lint quantification and quality measurements. These parameters were designed to evaluate the GlyTol cotton event GHB614 in cotton plants to ensure commercial herbicide tolerance and agronomic performance.

GlyTol cotton event GHB614 was derived by transformation of the upland cotton variety Coker 312 to express the 2mEPSPS protein found in corn which will convey tolerance to the herbicide glyphosate. Upland cotton is grown in the southern United States. GlyTol cotton was selected based on demonstrated resistance to the herbicide glyphosate and agronomic performance. BCS intends to commercialize this trait under the trade name GlyTol. Glyphosate is widely used in herbicide-tolerant cotton and other agricultural production systems. As of 2006, 78% of the US commercial cotton acreage was planted with glyphosate-tolerant varieties (USDA-NASS, 2006).

GlyTol cotton was evaluated by comparison to the non-transgenic counterpart Coker 312 in different growing regions of the southern United States. Agronomic performance field studies were managed in a manner representative of normal agricultural practices, including conventional herbicide applications, both pre- and post- planting. In addition, the GlyTol cotton was evaluated for herbicide tolerance using glyphosate. Thus, comparisons of agronomic properties and performance of the transformed event were made under both conventional herbicide and glyphosate herbicide regimens.

GlyTol cotton was evaluated at the T_5 generation with its non-transgenic counterpart to assess seed characteristics that may contribute to weediness potential of a plant such as increased seed dormancy.

The findings of these tests show:

- No significant differences were consistently observed when the line event GHB614 is compared to Coker 312 grown under conventional herbicide regimes;
- Crop displays tolerance to glyphosate; no plant damage or adverse effects on cotton growth parameters were observed following glyphosate herbicide applications;

- The overall performance of GlyTol cotton event GHB614 was equal to or better than that of its non-transgenic counterpart;

- There was no significant difference in germination rate of either treatment between the GlyTol cotton event GHB614 and the non-transgenic counterpart and no indication of seed dormancy.

- Evaluation of the agronomic performance of GlyTol event GHB614 has identified neither safety nor environmental concerns.

Data was reviewed using analysis of variation between groups (ANOVA) at a 95% confidence interval (C.I.) across all regions, and at a 99% C.I. regionally. Where no significant differences were found, the conclusion was drawn that the transformed event did not meaningfully effect the agronomic parameter evaluated. In instances where significant differences were found, the numerical advantage was evaluated between the data point to determine if the difference was agronomically meaningful. Equivalence comparisons between the advance strain and the isogenic counterpart was also conducted to determine if the effect was due to the event or the somaclonal variation created during the tissue culture process.

B. History of field activities

GlyTol cotton event GHB614 was regenerated *via* tissue culture after transformation of the individual plant cells. In 2002, T_3 homozygus seed harvested from T_2 plants (grown in the greenhouse) were imported for planting in the first field evaluation in Texas. T_3 plants were evaluated for herbicide tolerant line selection. The T_3 line was planted again in over winter nursery in Puerto Rico in 2002 for seed increase and efficacy evaluation. T_4 seed from over winter nursery was then planted in three locations in 2003 for additional agronomic evaluation and line selection. The T_4 seed was sent to over winter nursery to produce T_5 seed which was used to generate equivalence and efficacy data on the selected line in both 2004 and 2005. In every planting, events were separated by internal border rows to prevent cross-pollination. Fields were routinely treated with insecticide to prevent possible pollen transfer by insects. Table 9 presents a summary of the field trials and associated authorization permits. Also, Appendix 2 shows a breeding diagram.

C. Agronomic performance of GlyTol cotton event GHB614

Field studies were designed to compare agronomic performance of the transformed GlyTol cotton event GHB614, with the non-transformed Coker 312 counterpart. Agronomic performance was measured with cotton plant mapping methods and observation of defined growth parameters. Samples of seed and lint were harvested to evaluate the fiber quality characteristics. The agronomic parameters used to evaluate the transgenic and non-transgenic lines are defined in Table 10.

D. Agronomic evaluation

Data from agronomic trials was taken from 17 locations in 5 states over the 2004 and 2005 growing seasons. Studies were conducted in geographic regions of the southern United States representative of the regions in which nearly 94% of the total upland cotton production occurs (Table 11 and Table 12). Trials were conducted in the southeastern, mid-southern, and southwestern regions of the United States to capture the various environmental stresses that upland cotton varieties undergo during the course of a normal production year. These regions are representative of the areas in which the majority of the herbicide tolerant cotton is

produced in the United States, and where it is anticipated that GlyTol cotton would be sold based on use of glyphosate tolerant cotton in 2006 (Table 13)

A comparison of 18 agronomic characteristics across eight locations in 2004 and nine locations in 2005 compared plant growth and plant mapping data taken by field agronomists to evaluate the growth and development of the plant. Plant mapping data was taken to evaluate the potential reproductive success of the cotton plant. The number of bolls, first position bolls, plant height, and total number of nodes are all key parameters for cotton production as it impacts the value, maturity, and development of the cotton fiber. Plant height and height to node ratio was calculated from these parameters as it is used as an indication of insufficient herbicide tolerance. These parameters are also used to determine the application timing of plant growth regulators (PGR) used to manage the height of the cotton plant which improves mechanical harvesting efficiency. Morphology ratings were taken to monitor for irregular plant development in key portions of the plant such as the leaves, flowers, and bolls. Fertility of the plant was measured in rating the number of embryos (seed) in the boll, their weight and the size of the fruit developed to house them. Fertility ratings were also taken to evaluate the fertility of the flower by the amount of pollen present and dehiscence.

Evaluation of plant mapping data and crop development data showed no significant differences in the GlyTol cotton event GHB614 and the non-transgenic Coker 312 counterpart. Findings across locations show that GlyTol cotton is similar for maturity and yield to the non-transformed counterpart in both Coker 312 and commercial cotton varieties.

No differences were noted in the morphology of the plants when compared across testing locations. All plants with the GlyTol cotton event GHB614 and their non-transgenic Coker 312 counterparts appeared to develop normally, with no abnormalities noted in field observations.

Evaluation of reproductive success yielded two results which were determined to be statistically significant. Field evaluations showed increase in seed index rating and the number of seeds per boll in the 1 x application treatment. Other treatments showed no significant differences in these parameters. Data differences were not consistent across regions or years and are attributed to environmental differences. Increased ratings in seed per boll and seed index were only seen in 2004, and not repeated in 2005. When data is reviewed by region, it was seen that these differences manifest themselves primarily in one of the three regions evaluated, and only in one treatment regimen and were not seen in equivalence evaluations. Because of the lack of consistency between years, regions, and treatment regimens, it is concluded that this difference was due to environmental conditions at the particular location, and not a representation of a difference between the GlyTol cotton and the non-transgenic Coker 312 counterpart.

E. Biotic and abiotic stress characteristics

Visual observation by the field agronomists conducting the trials in 2004 and 2005 did not find significant differences between the transformed line and the non-transgenic parent line in disease impact of the plant. Significant differences were seen in plant lodging, and chlorosis. Insect pests were not screened for these tests, as all insect populations were controlled using appropriate insecticide applications to eliminate this variable, and prevent insect pollination between plots. Data for these evaluations is found in Table 15.

Plant diseases are an issue in cotton as fungi or viral diseases can defoliate the cotton plant, cause hardlock, boll rot, or cause other conditions which can reduce yield and cotton fiber quality (UGA Extension Bulletin). Certain diseases are regional, so evaluations were visually made on all diseases present on the plant on a 1-9 scale (1 = no disease, 9 = heavy

infestation). Field trials showed no significant differences between the GlyTol cotton and the non-transformed Coker 312 for disease rating. This data shows that the transformation event does not increase susceptibility to diseases and fungi.

Lodging is an indication of the plant's ability to support its own weight and withstand weather related stress such as high wind which can be an issue in different regions of the United States. Lodging is an issue in cotton plants as plants which cannot support their fruit loads will open towards the ground which can reduce the lint quality produced. As the plant desiccates, the plant can straighten itself high enough to be harvested, but if permanent damage was done to the stem, mechanical harvesting may not be possible and the crop may be lost. Lodging was evaluated towards the end of the season to evaluate the plant's stem strength on a 1 to 9 scale (1 = erect, 9 = flattened or severely depressed). Significant differences in lodging were found in the 2005 season between the GlyTol cotton event GHB614 and non-transformed Coker 312 in the unsprayed treatment. These differences were not consistent between years, regions or treatments (Tables 15 and 18). While the difference in these evaluations was significant the degree of lodging occurring in the plots was agronomically insignificant. This is because not only was the degree of lodging minimal (a rating of approximately 2.5 out of a possible 9) the plants became erect again upon desiccation after boll crack. For these reasons, the difference in lodging was not seen as an impact of the transformation on the cotton plant.

Chlorosis was used to evaluate any adverse effects seen as a result of glyphosate application to the cotton. Chlorosis manifests itself as a yellowing or bleaching of the leaves in a pattern consistent with the spray pattern of the application and were easily identifiable. Chlorosis ratings were visually made on a scale of 1-5 (1 = no effect, 5 = severe damage) to evaluate the impact of the herbicide application to the GlyTol cotton after each application of glyphosate. A significant increase in chlorosis in the non-transgenic plots was seen in all chlorosis ratings taken in 2005 in which the non-transgenic displayed a significantly worse reaction to the herbicide glyphosate. This is to be expected as the non-transgenic cotton is susceptible to the effects of glyphosate. However, since no herbicide was to be directly applied to the control plots, this chlorosis was determined to be a result of spray drift.

F. Fiber characteristics and quality

Laboratory tests were conducted to analyze commercially important fiber qualities of harvested lint from test plots compared to the non-transformed plots. Significant differences were found in the 2004 season for fiber strength, and percent lint. Materials were generated from agronomic performance field trials in 2004 and 2005 (Table 19 and 20). Ginned cotton fiber samples from 25 bolls were taken from each plot, and sent to be analyzed at various cotton fiber analytical laboratories. Samples were analyzed for traditional cotton fiber quality parameters using high volume instrumentation (HVI).

Fiber was analyzed for fiber strength, elongation, % lint, micronaire, fiber uniformity and fiber length. These parameters are the standard classing parameters used by the United States Department of Agriculture (AMS, 2006)

Fiber strength was found to be higher in the sprayed transgenic than the non-transgenic plots in 2005, which is considered to be a positive attribute to cotton fiber. No differences in fiber strength were found between the conventionally treated plots. A reduction in % lint per plot was seen in all treatments in the southeastern and mid-southern regions in both 2004 and 2005.

During equivalence evaluation (see section VIII.G) of the transformed trait the differences in fiber characteristics no longer appeared. Backcrossing removes the majority of the Coker 312 background in which the transformation took place. Therefore, it was determined that the differences in fiber characteristics was associated with the Coker 312 background, and not the transgenic trait. Differences are most likely due to somaclonal variation resulting from the regeneration of the T_0 plants from the transformed Coker 312 parent during tissue culture.

G. Equivalence between Coker-derived and commercial varieties

Somaclonal variation can occur when plant cells are stressed in tissue culture by the surrounding environment, which can result in differences between a regenerated Coker 312 plant and the original plant from which the tissue was taken. Somaclonal variation can lead to variances in the plant that are not linked to the transgene event and are removed by backcrossing the original transformed generation with other germplasm. Coker 312 is not commercially competitive with more modern cotton germplasms. Therefore the transformed Coker 312 variety is introgressed into commercial cultivars *via* backcrossing to give a commercially viable germplasm the desired transgenic trait and to remove the Coker 312 background (Wilkins *et. al.*, 2000). Commercial germplasm was backcrossed with the T_0 generation of the Coker 312 transformant and then progressed to the BC_2F_3 generation for evaluation along with the Coker 312 variety (see Figure 6).

Data from testing of the transformed commercial variety was provided for the Mid-South and Southwestern growing regions for fiber quality and agronomic performance. Plots were set up with both the Coker 312 variety and the commercial variety with and without the GlyTol cotton event GHB614. Plots were evaluated based on 17 agronomic parameters evaluating the plant development, yield, and fiber quality of the plots. No glyphosate was applied to these trials.

Plots were evaluated across regions for significant differences between plots. Of the 68 data point comparisons, six significant differences were noted between treatments (Table 21). Five of these six differences manifested themselves in the Coker 312 background in contrast with the transformed GHB614 Coker 312 variety. The lone significant difference found in the commercial variety was in the uniformity of the cotton plants. This variation is not unexpected as the tested backcross (BC_2F_3) was only advanced two generations. Normal cotton breeding typically backcrosses a variety over five generations in order to obtain a commercially acceptable uniform cotton variety. Therefore this variance in strain uniformity is not unusual. Regardless of the number of generations, the data displayed showed that the variation was numerically minimal on a scale of 9, indicating the small difference in uniformity was agronomically insignificant.

The differences in the Coker 312 background did not appear between the commercial variety EXP9740 and its' transgenic counterpart. This shows that many of the differences displayed in the efficacy studies in 2004 and 2005 are the result of the Coker 312 background, and not the transformation event. Given the lack of significant differences across regions, and the low number of differences even within region it is determined that the overall performance of GlyTol cotton event GHB614 in the commercial variety was equal to or better than that of its non-transgenic counterpart (Table 4.1 and Table 4.2).

Table 9. Summary of field activities under USDA permits for GlyTol cotton

Notification Number	Planting Dates	Number of Locations	Type of Trial	Locations
02-072-04n	June 2002	1	Equivalence / Breeding	TX
02-296-01n	December 2002	1	Efficacy, Seed increase	PR
03-064-14n	May 2003	3	Efficacy	SC, MS, TX
03-255-03n	November 2003	1	Efficacy, seed increase	PR
04-064-10n	May 2004	9	Efficacy / Breeding	SC, NC, GA, TX, MS
04-247-01n	November 2004	1	Efficacy, seed increase	PR
05-060-03n	May-June 2005	11	Efficacy, Breeding, residue, equivalence	NC, GA, AZ, TX, AR, MS, SC
05-091-07n	May-June 2005	11	Residue, efficacy, RAC samples,	AR, FL, GA, MS, TX
05-217-05n	November 2005	1	Efficacy, Seed Increase	PR
05-257-04n	November 2005	1	Efficacy, Seed Increase	PR

*Copies of the termination reports for these field trials are provided in Appendix I

Table 10. Description of agronomic parameters for GlyTol cotton

Agronomic Characteristic	Description
Days to bloom	The number of days from planting to first bloom
Days to first open boll	The number of days from planting to first open boll
Disease ratings	A scale rating of susceptibility to disease pressure
Fertility rating	A scale rating of pollen production and viability
Fiber elongation %	Measure of the % change in length based on original fiber length
Fiber Length	Average length of the longer one-half of cotton fibers
Fiber length uniformity %	Ratio between the mean length and upper half mean length of the fibers expressed as a percentage
Fiber Micronaire	A measure of fiber fineness and maturity as indicated by specific surface area
Fiber strength	The force in grams required to break a bundle of fibers one tex unit in size (1 tex = weight in grams of 1,000 meters of fiber)
Height to node ratio	Plant height divided by total number of nodes. A measure of stress tolerance within plots.
Lint Percent	Lint weight divided by seed cotton weight, expressed as a percentage.
Number first position bolls	Total number of bolls set on first positions of fruiting branches
Number of seeds per boll	The number of ovules that are fertilized and develop into mature seed is an indication of pollination efficiency, most usually affected by heat.
Number of seeds per plant	An expression of yield component combining numbers of seed per boll and average boll retention.
Percent open bolls	Differences in percent open bolls at a given time are an indication of differences in crop maturity.
Plant height	Average plant height from cotyledonary node to terminal, expressed in inches
Plant morphology rating (leaf, flower, plant)	A scale rating of leaf, flower and boll type to evaluate physical difference in plant structure.
Seed index	Average weight in grams of 100 seed, an indication of seed size and maturity.
Total number of nodes	Number of reproductive nodes present on the main stem of the plant
Yield: Lbs. lint per acre	Productivity expressed as pounds of lint produced per acre
Number of Total Bolls	Total number of bolls on an individual cotton plant
Chlorosis	The yellowing or whitening of normally green plant tissue used as an indicator of herbicide effects on the plant
Lodging	The laying down or flattening of a plant
Boll Size	The average size of individual bolls in grams (average weight of a 25 boll sample)
Strain Uniformity	Used to evaluate the uniformity of the event on a 1 to 9 scale. (1 = uniform, 9 = highly variable)

Table 11. US cotton production in states with test sites 2001-2006

Year	Planted All Purposes (Acres)	Harvested (Acres)	Yield (lbs./A)	Production (1000 bales)	% planted (US)	% harvested (US)
2006	10,221.00	7,933.00	720.00	11,195.00	66.89%	61.90%
2005	9,485.80	9,109.00	805.40	14,617.50	66.59%	65.99%
2004	9,216.00	8,689.50	833.60	13,671.00	67.47%	66.55%
2003	9,060.00	7,738.00	712.60	9,967.00	67.21%	64.47%
2002	9,468.50	8,148.30	528.00	9,532.30	67.84%	65.62%
2001	10,397.00	8,607.50	688.00	11,008.40	65.94%	62.25%
Averages	9,641.38	8,370.88	714.60	11,665.20	66.99%	64.46%

Source: USDA National Agricultural Statistics Services

Table 12. US cotton production in representative regions 2005-2006

Year	Planted All Purposes (Acres)	Harvested (Acres)	Yield (lbs./A)	Production (1000 bales)	% planted (US)	% harvested (US)
2006	14,504.00	12,044.00	750.02	18,606.00	94.92%	93.98%
2005	13,351.30	12,912.50	832.20	21,645.20	93.72%	93.55%
Averages	13,927.65	12,478.25	791.11	20,125.60	94.32%	93.76%

Source: USDA National Agricultural Statistics Services

Table 13. Glyphosate tolerant cotton usage by region in 2006

Region	Total acres	% of total	Glyphosate tolerant acreage
Southeast (AL, GA, NC, SC, VA, FL)	3,355,000.00	87%	2,922,680.50
Mid-South (MS, LA, MO, AR, TN)	4,225,000.00	90%	3,807,878.00
Southwest (TX, OK, KS)	6,861,000.00	34%	2,315,321.60
West (CA, NM, AZ)	840,000.00	52%	439,961.20

Source: USDA Agricultural Marketing Services

Table 14. Growth habit and phenotype data across regions 2004 & 2005

Growth Habit and Phenotype									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
days to bloom	56.429	56.464	56.286	56.714	55.81	56	1.656	4.77	NS
days to 1st open boll	107.25	105.31	105.56	105.81	106.63	104.31	4.14	4.25	NS
% open bolls	46.938	50.5	52.688	53.063	53.5	51.813	1.078	23.17	NS
plant height	42.086	39.546	41.581	41.542	42.005	41.362	3.14	12.31	NS
Total # of plant nodes	16.933	16.029	16.419	16.262	16.938	16.5	1.465	14.08	NS
Height to Node Ratio	2.455	2.494	2.54	2.538	2.548	2.533	0.3716	24.87	NS
# of 1st position bolls	5.624	5.576	5.4	5.695	5.981	6.2	1.013	30.48	NS
# of total bolls	11.143	10.529	10.867	11.043	12.367	12.1	2.289	35.46	NS
Strain Uniformity	1	1	1	1	1	1	N.V.	N.V.	NS
Leaf Morphology	1	1	1	1	1	1	N.V.	N.V.	NS
Flower Morphology	1	1	1	1	1	1	N.V.	N.V.	NS
Boll morphology	1	1	1	1	1	1	N.V.	N.V.	NS
Plant Morphology	1	1	1	1	1	1	N.V.	N.V.	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

2005 Growth Habit and Phenotype									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
days to bloom	59.667	60.292	59.5	61.042	58.58	60.333	1.08	3.08	NS
days to 1st open boll	107	106.94	107.47	107.72	107.5	108.06	7.639	15.34	NS
% open bolls	43.19	46.389	41.389	42.778	41.389	40.417	3.4746	21.69	NS
plant height	28.336	29.125	28.636	28.622	29.099	28.766	1.3995	10.35	NS
Total # of plant nodes	16.681	16.622	16.811	16.626	16.97	16.907	0.4856	7.11	NS
Height to Node Ratio	1.957	1.967	1.936	1.919	1.953	1.917	0.0924	8.86	NS
# of 1st position bolls	4.804	5.015	4.889	4.815	4.789	4.704	0.5397	23.24	NS
# of total bolls	8.2	8.526	8.626	8.044	8.433	7.867	1.03	25.65	NS
Strain Uniformity	3.806	3.722	3.639	3.833	3.472	3.694	0.3845	24.95	NS
Leaf Morphology	1	1	1	1	1	1	N.V.	N.V.	NS
Flower Morphology	1	1	1	1	1	1	N.V.	N.V.	NS
Boll morphology	1	1	1	1	1	1	N.V.	N.V.	NS
Plant Morphology	1	1	1	1	1	1	N.V.	N.V.	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 15. Biotic and abiotic stress data across regions 2004 & 2005

2004 Biotic and Abiotic Stress Data									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
Chlorosis 1	1.5	1.5	1.563	1.438	1.5	1.5	0.4866	33.26	NS
Chlorosis 2	1.313	1.5	1.25	1.313	1.313	1.5	0.4958	36.95	NS
Chlorosis 3	1.438	1.5	1.625	1.438	1.688	1.438	0.3592	25.38	NS
Lodging	1	1	1	1	1	1	N.V.	N.V.	NS
Disease	1	1	1	1	1	1	N.V.	N.V.	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

2005 Biotic and Abiotic Stress Data									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
Chlorosis 1	1.556	1.25	1.361	1.25	1.417	1.389	0.2063	34.58	a
Chlorosis 2	1.389	1.167	1.361	1.111	1.333	1.194	0.1808	33.11	abc
Chlorosis 3	1.611	1.306	1.667	1.25	1.694	1.417	0.2202	34.56	abc
Lodging	1.944	2.52	2.139	2.528	2.306	2.25	0.3841	38.54	a
Disease	1.583	1.583	1.528	1.611	1.583	1.5	0.1905	28	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 16. Reproductive quantification data across regions 2004 & 2005

2004 Reproduction Data									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
Fertility	1.044	1.031	1.019	1.025	1.1	1.038	0.3921	38.22	NS
Boll Size	5.136	5.011	5.229	4.907	5.007	4.696	0.3497	13.42	NS
Seed/Boll	26.571	24.905	26.714	23.381	25.333	23.524	1.919	12.64	b
Seed index	11.814	12.514	11.719	12.729	11.676	12.219	0.8019	11.2	b
# seeds per plant	295.240	261.330	297.950	262.520	321.000	290.710	61.04	36.5	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

2005 Reproduction Data									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
Seed index	11.87	11.75	10.35	11.913	11.192	10.488	1.088	16.63	NS
Fertility	1	1	1	1	1	1	N.V.	N.V.	NS
Boll Size	4.629	4.508	4.783	4.5	4.575	4.379	0.3713	14.04	NS
Seed/Boll	23.825	23.313	22.992	23.821	23.754	22.804	2.78	21.01	NS
# seeds per plant	125.38	126.63	113.33	121.79	131.29	111.54	31.72	37.88	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

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Table 17. Yield and fiber data across regions 2004 & 2005

2004 Yield and Fiber Data									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
yield	1049	957	1023	939	949	909	113.94	23.58	NS
fiber Length	1.215	1.205	1.218	1.21	1.218	1.211	0.0165	5.24	NS
fiber strength	30.5	31.64	30.229	31.732	30.554	32.046	1.23	7.4	bc
fiber uniformity %	85.05	85.52	84.87	85.73	85.05	85.91	2.28	5.12	NS
micronaire	4.432	4.546	4.471	4.586	4.525	4.579	0.3509	15.46	NS
fiber elongation %	7.171	7.443	7.079	7.471	7.136	7.486	0.4268	11.23	NS
% lint	39.679	38.251	39.927	37.461	39.966	37.8	0.9397	4.52	abc

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

2005 Yield and Fiber Data									
Agronomic Parameter	Not Sprayed (a)		1 x rate (b)		3 x rate (c)		Significance		
	C312	GHB614	C312	GHB614	C312	GHB614	LSD	CV	SIG
yield	848.35	793.13	869.48	853.44	837.84	823.41	95.53	26.03	NS
fiber Length	1.181	1.179	1.172	1.187	1.179	1.181	0.0476	8.85	NS
fiber strength	30.814	32.078	30.994	32.197	31.219	32.275	1.45	9.86	NS
fiber uniformity %	84.02	84.43	83.8	84.88	83.89	84.55	3.3067	8.55	NS
micronaire	4.622	4.531	4.575	4.617	4.464	4.647	0.2527	12.3	NS
fiber elongation %	7.272	7.478	7.247	7.533	7.206	7.522	0.2616	7.76	NS
% lint	38.483	36.447	39.111	36.635	38.63	36.373	2.66	13.27	NS

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

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Table 18. Equivalence data across regions

2005 Equivalence Data between Coker 312 and EXP9740 Commercial Variety							
Characteristic	C312 (a)	C312 GHB614	EXP9740 (b)	EXP9740 GHB614	CV	LSD	SIG
Yield	38.37	36.56*	39	38.73	1.5	0.6	a
% Lint	791.64	669.6*	678.15	625.55	13.5	94.53	a
Length	1.2	1.18*	1.05	1.07	1.9	0.223	a
Uniformity	84.01	84.46	82.71	82.76	0.86	0.7384	NS
Strength	30.36	30.44	31.05	31.15	3.21	1.0222	NS
Elongation	7	7.03	6.8	6.95	2.44	0.1752	NS
Micronaire	4.21	4.39*	4.58	4.44	3.74	0.1664	a
Strain Uniformity	5	4.25	4.38	5.58*	16.33	0.7677	b
Disease	1.5	1.5	1.25	1.38	26.06	0.3881	NS
Lodging	2	3.13*	4.38	4	32.13	1.0937	a
Leaf Morphology	1	1	1	1	N.V.	N.V.	NS
Flower Morphology	1	1	1	1	N.V.	N.V.	NS
Plant Morphology	1	1	1	1	N.V.	N.V.	NS
Plant Height	36.88	35.54	34.01	34.16	4.3	1.54	NS
Nodes	17.06	16.58	17.11	17.34	3.45	0.5937	NS
Height to Node	2.13	2.09	1.98	2	3.49	0.0739	NS
% Open Bolls	32.88	33.38	41.38	42.13	16.11	6.13	NS

a and b are treatment regimens and indicate in which treatments significant differences were found

H. Seed dormancy evaluation

Seed dormancy is an important characteristic in determining an increased risk of weediness from a cotton plant. One of many characteristics that make weeds successful competitors in agricultural environments is their ability to lay dormant for prolonged periods of time, and emerge when suitable conditions present themselves. Plants that do not lie dormant for prolonged periods of time are susceptible to seed decay and will not germinate if appropriate conditions are not achieved in a relatively short time (Halloin, 1975 and Woodstock *et. al*. 1985). Germination is evaluated as a measure of seed dormancy.

Seed collected immediately can display innate dormancy, an inherent condition of the maturity of the embryo that develops while the seed remains attached to the plant and immediately after detachment. Innate dormancy prevents seed from germination even through environmental conditions are optimal (Taylor & Lankford, 1970). The duration of innate dormancy can vary by variety and time (Christidis 1955). Secondary dormancy is a condition that develops to prevent germination after the seed has been detached from the plant and is exposed to the environment. Secondary dormancy can be tested or induced by exposures to low temperatures (Christidis, 1955). Germination in low temperatures is an indication of seed quality (Handbook, 1985)

Seed dormancy was evaluated in the GlyTol cotton event GHB614 to ensure that seed dormancy was not affected by the transformation of the Coker 312 germplasm or the production of the 2mEPSPS protein. Seeds were collected from the 2005 efficacy trials of transformation event GHB614 in six locations to test for effects to the seed dormancy. Seeds were split into two lots, with one lot to be germinated immediately upon harvest and the other to be germinated six months after harvest. Seeds were analyzed for germination in a warm germination environment at 86^{o} F to simulate favorable growing conditions to review the ability of the seed to perform in ideal environments. Seeds were also analyzed at 64^{o} F in a colder environment to evaluate the germination of cotton in less ideal conditions. Data was analyzed across locations at both time periods. Experimental materials and methods are detailed in Appendix 2.

No significant differences were seen in germination between the transgenic and non-transgenic seed produced from the 2005 plot sites. Normal variation was detected between those which were planted immediately after harvest, and those which were stored for six months. This indicates that transformation event GHB614 does not increase seed dormancy and therefore does not contribute to the weediness of the transformed cotton plant through increased seed dormancy.

Table 19. Mean % germination immediately after harvest over six locations

Event	Treatment	28°C Germination %	18°C Germination %	Significance
Coker 312	-	36	29	-
GlyTol event GHB614 (a)	0	42	25	NS
GlyTol event GHB614 (b)	1x	40	28	NS
GlyTol event GHB614 (c)	3x	51	38	NS
LSD (0.01)	-	31	30	-
CV	-	43	58	-

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 20. Mean % germination six months after harvest over six locations

Event	Treatment	28°C Germination %	18°C Germination %	Significance
Coker 312	-	88	83	-
GlyTol event GHB614 (a)	0	87	79	NS
GlyTol event GHB614 (b)	1x	86	80	NS
GlyTol event GHB614 (c)	3x	85	81	NS
LSD (0.01)	-	5.66	7.97	-
CV	-	3.85	5.81	-

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

I. Composition analysis

A study was conducted to obtain composition analysis data on RAC (cottonseed) samples of GlyTol cotton event GHB614 and its non-transgenic counterpart.

Cotton plants containing the GlyTol cotton event GHB614 and cotton plants representing the non-transgenic (non-transformed) counterpart were grown in the field by BCS in 2005. The transgenic seed used for planting in the field trials was cotton event GHB614. The non-transgenic counterpart seed used was Coker 312.

Nine field trials were established by BCS in typical cotton-producing areas of the southern United States of America. The plants in this study were grown under conditions typical of production practices. There were six transgenic plots and three non-transgenic plots at each test site. Three of the transgenic plots were sprayed three times with glyphosate herbicide at a target application rate of 0.75 lbs of active ingredient (glyphosate acid)/acre, and three transgenic plots were not sprayed with glyphosate. A sample of ginned cottonseed, also known as fuzzy seed, was obtained from each field plot for use in composition analysis. The 81 samples were shipped in a frozen state to Bayer CropScience/MBAS Lab, Research Triangle Park, NC. The fuzzy seeds were sub-sampled and shipped in a frozen state to the analytical facility, Eurofins Scientific, 3507 Delaware Ave., Des Moines, IA, where they were stored frozen until removed for preparation and analysis.

Composition data were obtained for 81 samples of ginned cottonseed (9 samples from each of 9 field trials). There were 27 samples from each of three groups: non-transgenic non-tolerant Coker 312 cotton, transgenic event GHB614 cotton that was not sprayed with glyphosate herbicide, and transgenic event GHB614 cotton that was sprayed three times with glyphosate herbicide.

Except for data obtained for the two oil matrices and all fatty acid data generated, the composition data were converted to percent dry matter (or other units of measure based on dry matter content, as appropriate) to compensate for variations in sample moisture (determined in the proximate analysis). Individual fatty acids are reported as relative percent of total fatty acids on a fresh weight basis. No correction of the relative fatty acid data was made for sample moisture or for crude fat content (to obtain “absolute” fatty acid quantities).

Proximates include: total protein, total fat, moisture, fiber, carbohydrate and ash. The means of the proximates are expressed on a dry matter basis, except for % moisture, of the sprayed transgenic cottonseed and the non-transgenic counterpart. The key minerals include: calcium, phosphorus, potassium, iron, magnesium and zinc, and Vitamin E (alpha-tocopherol). The values reported are corrected for moisture. In addition, the three known antinutrients found in cotton, phytic acid, cyclopropenoid fatty acids and gossypol (total and free) were also analyzed in the cottonseed samples.

Values obtained for the sample sets for cottonseed were generally within +/- 5% of the Coker 312 controls, and all measured levels fell between the published natural range for cotton. The analyses for the antinutrients are provided in Table 23. No differences were found between the GlyTol cotton event GHB614 (sprayed or unsprayed) and the non-transgenic control.

In this section, a summary of the Statistical Analysis of Compositional Data of Fuzzy Seed from First Year Field Trials of GlyTol cotton event GHB614, USA 2006 is presented.

An analysis of variance (ANOVA) was performed for all analytes at a significance level of 0.01 (α = 1%). Independent variables evaluated were the site and treatment. The null hypothesis states that there are no differences between the value of analyte (dependent variable) due to the independent variables. A small probability (p-value) means that an observed difference is unlikely to occur by chance, so the null hypothesis should be rejected. A low p-value (< 0.01) suggests that there is a significant difference caused by the effect analyzed. StatView® 5 (SAS Institute, Cary, NC) was used for ANOVA.

T-test comparisons at a significance level of 0.01 (α = 1%) were also performed using the analyte values from the following sets of data:

1. non-transgenic samples (Treatment A) and unsprayed transgenic samples (Treatment B)
2. non-transgenic samples (Treatment A) and sprayed transgenic samples (Treatment C)

All statistical analyses were done on data with full precision. Results may be rounded to two or three significant numbers.

Significant differences were observed for the interaction of site and treatment for crude fat, isoleucine, phytic acid and valine. There were no significant differences for iron for either site or treatment. Cystine and methionine are the only two variables that gave a significant difference for the treatment variable. In both of these variables the interaction p-value is close to α = 0.01, the interaction between site and treatment may have caused these variables to have a significant difference in treatment alone. For all other analytes, there were significant differences for site but not for treatment. T-tests comparing the analyte values for non-transgenic samples and unsprayed transgenic samples showed no significant difference for any of the analytes tested. Likewise, t-tests comparing the analyte values for non-transgenic samples and sprayed transgenic samples showed no significant difference for any of the analytes tested.

Table 21. Mean values for proximate and fiber compounds in cottonseed of GlyTol cotton event GHB614

Proximate and fiber compounds	Unit	Non-transgenic			Transgenic-Unsprayed			Transgenic-Sprayed			Reference
		MEAN	±	STD	MEAN	±	STD	MEAN	±	STD	ranges [a]
Moisture	%fw	9.63	±	3.42	9.42	±	2.11	8.92	±	1.39	4.0 – 15.9
Protein, Combustion	%dm	23.40	±	2.51	23.16	±	2.70	23.42	±	2.55	20.7 – 34.2
Crude Fat	%dm	17.71	±	1.46	17.15	±	1.49	17.09	±	1.37	11.8 – 36.3
Ash	%dm	4.25	±	0.31	4.26	±	0.36	4.24	±	0.34	3.3 – 5.0
Carbohydrates (calc.)	%dm	54.58	±	2.66	55.43	±	3.01	55.25	±	2.41	36.4 – 67.8
Acid Detergent Fiber	%dm	40.81	±	3.43	41.00	±	2.77	40.66	±	2.31	29.0 – 49.6
Neutral Detergent Fiber	%dm	50.06	±	3.10	50.20	±	3.53	49.66	±	2.68	39.2 – 63.4

[a] References for proximates
- Berberich *et al*., 1996.
- Bertrand *et al.* 2005.
- Calhoun *et al*., 1995.
- Lundquist, 1995.
- Nida et al*., 1996.*
- OECD, 2004.
- USCA, 1982

Table 22. Mean values for minerals and Vitamin E in cottonseed of GlyTol cotton

Minerals and Vitamin E	Unit	Non-transgenic			Transgenic-Unsprayed			Transgenic-Sprayed			Reference
		MEAN	±	STD	MEAN	±	STD	MEAN	±	STD	ranges [a]
Calcium	%dm	0.14	±	0.03	0.14	±	0.03	0.13	±	0.04	0.11 – 0.33
Iron	%dm	0.0058	±	0.0020	0.0058	±	0.0011	0.0064	±	0.0039	0.0038 – 0.016
Magnesium	%dm	0.38	±	0.03	0.38	±	0.02	0.38	±	0.02	0.31 – 0.49
Phosphorous	%dm	0.62	±	0.08	0.63	±	0.06	0.63	±	0.06	0.45 – 0.86
Potassium	% dm	1.18	±	0.08	1.18	±	0.08	1.18	±	0.08	0.99 – 1.42
Zinc	% dm	28.3	±	5.2	29.2	±	4.7	29.2	±	5.4	24.9 – 63.0
Vitamin E (alpha-tocopherol)	ppm dm	106	±	17	105	±	13	103	±	13	82 – 225

[a] References for minerals and Vitamin E
- Calhoun *et al.,* 1995.
- ILSI, 2006 (values for Coker 312).
- Lundquist, 1995.
- OECD, 2004.

Table 23. Mean values for anti-nutrients in cottonseed of GlyTol cotton

Anti-nutrients	Unit	Non-transgenic			Transgenic-Unsprayed			Transgenic-Sprayed			Reference ranges [a]
		MEAN	±	STD	MEAN	±	STD	MEAN	±	STD	
Phytic Acid	%dm	1.70	±	0.18	1.69	±	0.21	1.67	±	0.18	0.854 – 2.70
free Gossypol	%dm	0.50	±	0.07	0.48	±	0.08	0.50	±	0.08	0.47 – 1.40
total Gossypol	%dm	0.66	±	0.09	0.67	±	0.08	0.67	±	0.09	0.51 – 1.99
Malvalic acid [b]	% rel.	0.204	±	0.124	0.145	±	0.070	0.156	±	0.074	0.17 – 1.50
Sterculic acid [b]	% rel.	0.163	±	0.066	0.119	±	0.037	0.125	±	0.037	0.13 – 0.70
Dihydrosterculic acid [b]	% rel.	0.152	±	0.022	0.092	±	0.012	0.090	±	0.000	0.11 – 0.50

[a] References for minerals and Vitamin E
- Berberich *et al*. 1996 (values for Coker 312).
- Calhoun *et al*., 1995.
- ILSI, 2006 (values for Coker 312).
- Nida *et al*., 1996 (values for Coker 312).
- OECD, 2004.
- Phelps *et al*., 1965.
- Wozenski and Woodburn, 1975.

[b] For cyclopropenoid fatty acid analyses that returned a value of "< 0.10", calculations were done using a substituted value of 0.09.

Table 24. Mean values for major fatty acids in cottonseed of GlyTol cotton

Major fatty acids [a]	Unit	Non-transgenic			Transgenic-Unsprayed			Transgenic-Sprayed			Reference ranges [b]
		MEAN	±	STD	MEAN	±	STD	MEAN	±	STD	
Saturated											
C14:0 Myristic	% rel.	0.76	±	0.09	0.75	±	0.09	0.75	±	0.10	0.53 – 1.17
C16:0 Palmitic	% rel.	24.28	±	0.93	24.21	±	1.00	24.30	±	1.00	21.1 – 29.9
C18:0 Stearic	% rel.	2.35	±	0.10	2.24	±	0.12	2.24	±	0.13	2.15 – 3.40
C20:0 Arachidic	% rel.	0.30	±	0.02	0.29	±	0.02	0.29	±	0.03	0.21 – 0.48
C22:0 Behenic	% rel.	0.15	±	0.01	0.14	±	0.01	0.14	±	0.01	0.10 – 0.27
Mono-unsaturated											
C16:1 Palmitoleic	% rel.	0.62	±	0.05	0.64	±	0.05	0.65	±	0.05	0.46 – 0.88
C18:1 Oleic	% rel.	15.10	±	0.85	14.33	±	0.84	14.38	±	0.91	13.4 – 22.0
Poly-unsaturated											
C18:2 Linoleic	% rel.	54.94	±	1.82	56.14	±	1.87	55.99	±	2.04	36.3 – 64.0
C18:3 Linolenic	% rel.	0.61	±	0.04	0.45	±	0.05	0.46	±	0.04	0.08 – 0.31

[a] Fatty acid analyses that returned a value of "< 0.10" were omitted from the above table.

[b] References for major fatty acids
- OECD, 2004. (%dm values converted to relative percentages based on total lipid content of 31 .46 - 38.05% dm).
- Berberich *et al*. 1996 (values for Coker 312).
- Bertrand *et al*. 2005.
- ILSI, 2006. (values for Coker 312).
- Nida *et al.,* 1996 (values for Coker 312).

Table 25. Mean values for amino acids in cottonseed of GlyTol cotton

Amino acids	Unit	Non-transgenic			Transgenic-Unsprayed			Transgenic-Sprayed			Reference ranges [a]
		MEAN	±	STD	MEAN	±	STD	MEAN	±	STD	
Alanine	%dm	0.96	±	0.09	0.96	±	0.12	0.96	±	0.11	0.83 – 1.51
Arginine	%dm	2.60	±	0.34	2.68	±	0.48	2.62	±	0.46	2.23 – 4.40
Aspartic Acid	%dm	2.27	±	0.25	2.31	±	0.30	2.30	±	0.29	1.89 – 3.55
Cystine	%dm	0.36	±	0.04	0.35	±	0.04	0.37	±	0.06	0.25 – 0.86
Glutamic Acid	%dm	4.78	±	0.56	4.86	±	0.77	4.85	±	0.68	3.80 – 8.16
Glycine	%dm	0.96	±	0.10	0.98	±	0.13	0.98	±	0.12	0.87 – 1.58
Histidine	%dm	0.64	±	0.07	0.65	±	0.10	0.64	±	0.09	0.60 – 1.03
Isoleucine	%dm	0.69	±	0.09	0.70	±	0.12	0.70	±	0.10	0.69 – 1.17
Leucine	%dm	1.34	±	0.14	1.35	±	0.20	1.35	±	0.17	1.27 – 2.23
Lysine	%dm	1.03	±	0.10	1.04	±	0.13	1.04	±	0.11	0.97 – 1.65
Methionine	%dm	0.38	±	0.04	0.37	±	0.04	0.39	±	0.05	0.30 – 0.54
Phenylalanine	%dm	1.24	±	0.14	1.26	±	0.21	1.26	±	0.18	1.13 – 139
Proline	%dm	0.86	±	0.10	0.88	±	0.10	0.88	±	0.07	0.71 – 1.39
Serine	%dm	1.02	±	0.10	1.05	±	0.13	1.04	±	0.13	0.90 – 1.63
Threonine	%dm	0.76	±	0.07	0.78	±	0.10	0.78	±	0.09	0.64 – 1.21
Tryptophan	%dm	0.31	±	0.04	0.32	±	0.03	0.32	±	0.03	0.23 – 0.49
Tyrosine	%dm	0.59	±	0.06	0.61	±	0.09	0.61	±	0.07	0.48 – 1.17
Valine	%dm	0.97	±	0.12	0.99	±	0.17	1.00	±	0.15	0.99 – 1.67

[a] References for amino acids

- Bertrand. *et al.* 2005 (calculated from g/100g protein into %dm based on average protein content in cottonseed of 25%dm; f=0.23).
- Lawhon. *et al.*, 1977 (calculated from g/100g protein into %dm based on average protein content in cottonseed of 25%dm; f=0.23).
- OECD, 2004.

Conclusion for composition analysis

Other than the intended glyphosate tolerance, there are no additional or unintended changes in the composition of GlyTol cotton event GHB614 compared to its non transgenic counterpart, Coker 312. Fatty acids, proximates, amino acids, minerals, vitamin E and antinutrient data are all comparable between GlyTol cotton event GHB614 and its non transgenic counterpart, Coker 312.

J. Conclusions for agronomic evaluation of GlyTol cotton

A through review of GlyTol cotton event GHB614 was conducted over the 2004 and 2005 crop seasons. During these field studies, 30 different agronomic parameters were identified and evaluated to assess the impact of the transformation event GHB614 on the cotton plant. Development and maturity, environmental susceptibility to biotic and abiotic stressors, and the yield potential and quality of the cotton fiber were all evaluated to determine if the transformed Coker 312 and commercial varieties differed from the non-transformed cotton varieties of the same type.

In addition to agronomic performance composition of the seed was evaluated for any potential plant pest risks to current cotton production in the United States. Composition data provided information on gossypol, antinutrient levels, and other toxicant contents.

The overall conclusion is that there are no agronomically meaningful differences between the transformed GlyTol cotton event GHB614 and non-transformed cotton varieties evaluated. The resulting conclusion is that there are no new agronomic plant pest risks from the introduction of GlyTol cotton.

IX. ENVIRONMENTAL SAFETY/IMPACT OF NON CONTAINED USE OF GlyTol cotton

A. Potential for gene transfer/out crossing

a. Bio-geography

As discussed in Section II of this petition, only two wild *Gossypium* species are present in the US: *G. thurberi* Todaro found in mountain regions of Arizona at altitudes of 2500 to 5000 feet and *G. tomentosum* which is found in Hawaii. Only *G. tomentosum* is capable of crossing with domesticated cotton that will produce fertile offspring. There is no expected selective advantage conferred by the transfer of the Glytol trait if that cross would occur.

b. Vertical gene flow

Cotton pollination
Gossypium hirsutum is considered to be a self-pollinating crop. Cotton pollen is heavy and sticky thus cross pollination by wind is unlikely. Cotton can, however, be pollinated by insects. Honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.) are the primary insect pollinators.

As previously discussed in Section II, McGregor (1976) traced the movement of pollen from a cotton field surrounded by a large number of honeybee colonies. Movement of the pollen was traced by means of fluorescent particles. McGregor found that at 150 to 200 feet away from the source plant, only 1.6 percent showed the presence of the fluorescent particles. By comparison, the isolation distances for Foundation, Registered and Certified seeds in 7 CFR Part 201 are 1320, 1320 and 660 feet, respectively. The trend for cross pollination to decrease as the distance from the source increased has been established by several research groups over the years. (Kareiva *et al*. 1994, Sundstrom 2001, Van Deynze *et al.* 2005).

Outcrossing potential to wild/weedy relatives
The potential for outcrossing can be defined as the ability of gene escape to wild cotton relatives. Previously the USDA stated in the environmental assessment document of RoundUp Ready® cotton that 'the potential for gene introgression from genetically engineered cotton lines into wild or cultivated sexually compatible plants is very low" (USDA 1995). As discussed in section II only two wild *Gossypium* species are present in the US, *G. thurberi* Todaro and *G. tomentosum* which is found in Hawaii. Only *G. tomentosum* is capable of crossing with domesticated cotton that will produce fertile offspring. There is no expected selective advantage conferred by the transfer of the GlyTol trait if that cross would occur. Outcrossing to *G. tomentosum* is unlikely as there is no cotton production in Hawaii other than winter nursery breeding activities where isolation practices are employed, and therefore the potential for gene flow to these wild relatives is low. There are other wild relatives known to exist in Southern Florida and Puerto Rico that are capable of crossing with cultivated cotton. However, these wild relatives are found hundreds of miles from where cotton production occurs.

Outcrossing potential to feral or cultivated cotton
No feral cotton populations (domesticated plants capable of surviving outside of cultivation) of *G. barbadense* have been found in the US Cotton production fields (production of planting seed) are required to be isolated from other cotton fields to prevent cross

pollination. Therefore if any cross pollination were to occur to either *G. barbadense* or *G hirsutum* it would be from a lint production field where seed is crushed and not propagated.

c. Potential of horizontal gene flow from GlyTol cotton

Bayer CropScience is not aware of any reports of incidents of naturally occurring transgene movement from transgenic crops to sexually incompatible species.

B. Weediness potential of GlyTol cotton

In the United States, cotton (*G. hirsutum*) is not a weed pest and has no sexually compatible weedy relatives except perhaps *G. tomentosum* in Hawaii where there is no commercial cotton production. A number of references confirm the lack of weediness of cotton: Crockett, 1977, Holm et al., 1977, Muenscher, 1980. The USDA has previously determined that "cotton is not considered to be a serious, principal or common weed pest in the US"(USDA, 1995). Previous findings by the USDA of similar herbicide-tolerant cotton during environmental assessment expected no change in weediness potential. For example, two glyphosate resistant cotton events (1445 and 88913) are commercially sold today. In the environmental assessment document for a similar herbicide resistant technology RoundUp Ready Cotton the USDA stated that "the potential of gene introgression from genetically engineered cotton lines into wild or cultivated sexually compatible plants is very low" (USDA 1995, USDA 2004). The largest concern is that of volunteer plants that could become weedy in subsequent years. Volunteers are also limited by the geography in which they may exist as cotton does not survive as a perennial where freezing temperatures are reached during the winter. Volunteers can easily be controlled by crop rotation, tillage and/or pre- or post-emergence herbicides. For example, glyphosate-tolerant cotton volunteers could easily be controlled by using the herbicide glufosinate.

There is limited probability that Glytol cotton event GHB614 or any *Gossypium* species containing GlyTol cotton event GHB614 would become a weed problem. In the comparative studies presented in this petition there were no consistent significant differences in germination, dormancy, phenotypic or plant morphological characteristics between the transgenic GlyTol cotton event GHB614 and the conventional near isogenic line Coker 312 that would impact plant pest or noxious weed potential. Based on these data there was no evidence to suggest that GlyTol cotton has a higher likelihood to become a weed than conventional cotton. There were no instances in which volunteer monitoring after harvest revealed any differences in survival or persistence relative to other cotton varieties.

C. Effects on non-target organisms

The 2mEPSPS protein has a history of safe use which is described in Section V of this petition.

Compositional analysis on the plants containing 2mEPSPS protein indicates no significant changes in the overall gossypol content of the plants or antinutrient levels between GlyTol cotton event GHB614 and the non-transgenic counterpart (Section VIII.I). This indicates that the transformed cotton is no more toxic than its non-transgenic counterpart.

Composition findings are reinforced by visual field observations made by cooperators conducting field evaluations of the GlyTol cotton plants. Cooperators visually monitored all plots for differences in beneficial insect populations and types for each trial, as well as birds, pollinators, and other wildlife species. No reports of differences in populations for any of these

non-target organisms were made by cooperators making these observations. Field observations are summarized in termination reports located in Appendix I.

a. Habitat

The shift in agronomic practices as a result of herbicide tolerance technology could potentially impact the habitat for non-target organisms. Herbicide tolerant cotton has made practices such as no-till planting more viable, resulting in an "ecosystem" in the cultivated field that is less disturbed due to the lack of cultivation and reduced need to enter the field to maintain the crop. Increased cotton canopy in the field during the growing season results in increased habitat for birds, insects, and other animals to thrive (Fawcett, 2002). In addition to the increased use of practices such as no-till agriculture, reductions in soil erosion, chemical use, fuel consumption, and other reduced inputs all have a direct positive impact on the well being of species found in agricultural settings. Since weed populations are currently controlled by both cultivation and chemical applications, use of herbicide tolerant crops would at the very least, add no additional burdens on non-target organisms.

b. Containment of Protein

Because the 2mEPSPS protein is contained within the plant, potential for exposure to the protein is limited to direct feeding on the GlyTol cotton. Soil exposure is not likely to be a concern as protein production ends at senescence, and significant degradation is likely to have occurred by the time the residual cotton material is incorporated into the soil at the end of the season. Acute oral toxicity testing of the protein is described in Rouquie (2006), and the 2mEPSPS protein is not considered to be toxic to mammals, birds, or insects. Furthermore, exposures to cotton pollen containing the 2mEPSPS protein is not a concern for non-target organisms due to the low expression of the protein in the cotton pollen and due to the fact that no effects were observed in an acute toxicity study at very high levels of the protein (2,000 mg protein/kg body weight).

c. Degradation of the 2mEPSPS protein in soils

EPSPS proteins are naturally occurring and widespread. 2mEPSPS is expected to degrade in soil in the same way as other EPSPS proteins.

d. Aquatic Environments

The 2mEPSPS protein is contained in cotton tissue, is not likely to persist in soils, and exposure to aquatic organisms is therefore highly unlikely. In addition, the protein is not considered toxic to non target organisms. Therefore, it is not expected that the 2mEPSPS protein will be a source of concern to aquatic environments.

e. Metabolism of 2mEPSPS protein in animals

The 2mEPSPS protein is rapidly degraded by digestive enzymes in the simulated gastric and intestinal fluids.

f. Effects of temperature and pH on the 2mEPSPS protein

The 2mEPSPS protein is completely inactivated after 10 minutes at 60ºC.

g. Toxicity of GlyTol cotton to wildlife in environmental releases

Birds and Mammals
Because no significant changes in the overall gossypol content and antinutrient levels of the plant were detected, it is not anticipated that the GlyTol cotton event GHB614 has a higher degree of risk than non-transformed cotton. Agronomic practices adopted through the use of glyphosate tolerant cotton have resulted in reduced movement of machinery and personnel through the cotton field. This reduced disturbance of the ecosystem within and around the cotton field would be beneficial to all bird or mammal species.

Pollinators
No significant differences between the transgenic and non-transgenic cotton in the flower morphology or time to bloom were found. Additionally the 2mEPSPS protein is expressed at a very low level in cotton pollen. Because no other changes in the bloom pattern or toxicity of the cotton plant were found, it is not anticipated that pollinating species, primarily insects, would be impacted by GlyTol cotton.

Foliar Beneficial Insects
No significant differences in the development or morphology between the transgenic and non-transgenic cotton lines were found which would indicate any adverse impact on foliar beneficial insects. The reduction in disturbance of the ecosystem in the cotton field may actually increase the habitat and food supply for beneficial insects.

D. Endngered Species Considerations

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

It is not anticipated that the use of GlyTol cotton will impact any current listed species of concern. Of the total 747 plants listed as endngered, fewer than half (355) reside in states which commercially produce cotton (US FWS, 2006). Species of concern that may inhabit areas close to commercial cotton operations would not be impacted by the use of GlyTol cotton. Commercial agriculture routinely disturbs the ground in which crops are currently planted. As a result, perennial vegetative species would not grow in these areas. Additionally, because horizontal gene flow to sexually incompatible species is not an issue, there is negligible potential for exposure to the transgene contained in GlyTol cotton through sexual reproduction. Finally, the herbicide glyphosate has been shown to have no residual activity, so there are limited opportunities for an endngered specie to be exposed to the herbicide.

For these reasons, it is not believed that the use of GlyTol cotton in commercial cotton production will adversely impact endngered species of concern.

E. Effects on Current Agricultural Practices in Cotton

a. Introduction

Weeds are a significant challenge for US cotton producers which must be managed in order to successfully produce an economically viable cotton crop (Bryson, 1999). It is estimated that without weed control, crop yields in cotton would be reduced by 77% (Gianessi et. al. 2002). Weeds compete with cultivated cotton for nutrients and water in the soil, and if large enough can compete for sunlight required for photosynthesis in the plant. Because cotton prefers warmer climates, early weed control is especially important to establishing a solid stand shortly after planting in the spring when temperatures are not consistently high. It is only when soil temperatures are consistently at 75^{o}F that cotton becomes competitive with weed species (Chandler, 1984). Weeds also can host a variety of insect pests, and can interfere with the harvesting process, and can impact fiber quality by staining cotton lint during harvest. Weeds also contribute to the amount of gin trash collected during ginning, and can negatively impact equipment.

Cotton has been grown across 13-15 million acres over the past 5 years (Table 26). Greater than 95% of these acres receive a herbicide application for weed control in 2005 (USDA, NASS 2006). Many acres are often treated multiple times using herbicide tolerant cotton during pre-plant burndown and at least one application post-emergence. Standard treatments of the past included several herbicides and several application timings along with cultivation. A typical conventional herbicide program includes the use of a pre-emergence or pre-plant incorporated herbicide followed by a post-emergence herbicide application. Trifluralin, pendimethylin and acephate are the most common herbicides used other than glyphosate (USDA, NASS 2006). These 3 herbicides are utilized on approximately 30% of cotton acreage. Glyphosate is the most widely used cotton herbicide and is utilized on over 70% of the total cotton acreage. Pyrithiobac sodium and MSMA are used post-emergence on 7-9% of the US cotton acreage (USDA, NASS 2006).

Table 26. US cotton production nationwide 2001-2006

Year	Planted All Purposes(1)	Harvested (1)	Yield (2)	Production (3)	Price per Unit (4)	Value of production (5)
2006	15,281.00	12,816.00	774.00	20,659.00		
2005	14,245.40	13,802.60	83.00	23,890.20	0.49	5,574,119.00
2004	13,658.60	13,057.00	855.00	23,250.70	0.44	4,853,730.00
2003	13,479.60	12,003.40	730.00	13,255.20	0.63	5,516,761.00
2002	13,957.90	12,416.60	665.00	17,208.60	0.46	3,777,132.00
2001	15,768.50	13,827.70	705.00	20,302.80	0.32	3,121,848.00
Averages	14,398.50	12,987.22	635.33	19,761.08	0.47	4,568,718.00

1 – Thousand acres
2 – Pounds
3 – Thousand Bales
4 – Dollars / lbs
5 – Thousand Dollars

The main weed species across all cotton include redroot pigweed (*Amaranthus retroflexus*) and other amaranth species, morning glories (*Ipomoea* spp), cocklebur (*Xanthium strrumarium*), Johnsongrass (*Sorghum halepense*), crabgrass (*Digitaria* spp), barnyardgrass and watergrass (*Echinochloa* spp), sicklepod (*Cassia obtusifolia*), and Texas panicum (*Panicum texanum*). Cotton is grown across the southern United States in 4 distinct regions (southeast, mid-south, southwest and west). Weed species infestations change across these regions and weed control methods are adjusted accordingly.

Table 27. Common weed Species in US Cotton Production

Common Name	Scientific Name	Region
Morning glory	*Ipomoea* spp	SE, MS, SW, W
Prickly sida	*Sida spinosa*	SE, MS, SW
Sicklepod	*Senna obtusifolia*	SE, MS, SW
Pigweed spp	*Amaranthus* spp	SE, MS, SW
Nutsedge spp	*Cyperus* spp	SE, MS, SW, W
Velvet leaf	*Abutilon theophrasti*	SE, MS
Smartweed spp	*Polygonum* spp	SE, MS, SW
Tropic croton	*Croton glandulosus* var. *septentrionalis*	SE, MS, SW
Hemp sesbania	*Sesbania herbacea*	SE, MS, SW
Redvine	*Brunnichia ovata*	MS
Johnsongrass	*Sorghum halep*	SE, MS, SW
Common Cockleburr	*Xanthium strumarium*	SE, MS, SW
Nightshade spp	*Solanum* spp	SE, MS, SW, W
Lambsquarter spp	*Chenopodium* spp	SE, MS, W
Field blindweed	*Convolvulus arvensis*	SW, W
Grass spp	Various species	SE, MS, SW, W
Texas panicum	*Panicum texanum*	SW, W

SE = Southeast MS = Mid-south MW = Midwest W = West
Source: 2001 proceedings, SWSS vol. 54; NCSU Crop profiles 2006

Prior to the development of herbicide tolerant crops, control of these diverse species required the use of multiple herbicide families and multiple applications. Development of crops which are tolerant to broad spectrum herbicides has changed agricultural tillage, weed control, and ecological practices. The volume of herbicide sprayed has been reduced greatly using herbicide tolerant cotton varieties (Sankula *et al*., 2005). Additionally, cultivation of herbicide tolerant cotton has provided multiple benefits in the form of reduced inputs to manage cultivated crops, and reduced losses of those inputs from the field due to erosion, run-off, and waste (USDA-ERS, 2002).

b. US cotton production

Cotton is the fourth highest grossing crop in the United States behind corn, soybeans, and wheat (USDA-NASS 2006). Originally from tropic origins, cotton production in the United States occurs where a sufficient number of heat units can be obtained to properly grow cotton. While cotton varieties differ by region, cotton requires at least 120 days above 15^{o}C to grow, and at least 200 frost free days to conduct preparatory activity and harvest the cotton lint once grown (Waddle, 1984). Cotton is limited in its production geography primarily by the climate of the region. Aside from temperature requirements, cotton requires adequate moisture at appropriate times to ensure production of fruit and lint.

In 2006 cotton was produced in 17 states ranging from California to Virginia, and was planted on over 15 million acres (USDA-NASS, 2006). Upland cotton is the most commonly cultivated cotton species with approximately 97% of the total cotton acreage in the US planted in upland varieties (USDA-NASS, 2006). The remaining cotton acreage is planted in Pima Extra Long Staple (ELS) cotton. These varieties are grown in the western regions of the United States, primarily in California.

Cotton production is divided into four distinct regions of the country (Table 28). Each region has different environmental, soil, climate, and weather conditions that impact the production and evaluation of the cotton plant.

Table 28. Cotton producing regions of the United States

Region	*States in Region*	*Approximate Acreage**
Southeast	VA, NC, SC, GA, FL, AL	3,355,000
Mid-South	MS, LA, AR, TN, MO	4,225,000
Southwest	TX, OK, KS	6,924,000
West	NM, AZ, CA	840,000

Source: National Agricultural Statistics Services
*Approximate acreage for the 2006 cotton production season

The southeast and mid-south cotton growing regions display many similar qualities, and have similar environmental conditions corresponding with their respective latitudes and longitudes. Soil variation can be great with soil types ranging from extremely light sandy soils to heavy clay porous soils with some high organic soils in some parts of the east coast and Mississippi flood plain. Acreage and inputs for these regions are similar, as are weed species encountered. Insect pressure can be of similar type, although infestations of these pests vary greatly by geographic location.

The southwestern production region has the largest acreage, mostly in Texas, but also has the lowest production per acre (USDA-NASS, 2006). This is primarly due to the arid conditions and the availability of water to irrigate cotton fields in locations. As a result, cotton production can vary widely in these regions based on the availability of moisture to the cotton crop. Production is more aligned with arid agricultural practices, with many insect pests and weed species which are not present in the southeast and mid-south regions.

The western production region is similar to the southwestern region in that production occurs in arid conditions. However, water is more readily available to these production systems, as a result the western production region has the highest yields per acre of any region (USDA-NASS, 2006). This enhanced production also has a corresponding increase in production costs associated with water and land values. Many sucking insect species present in the southeast and mid-south regions are not present in this region, but are replaced with different insect pest challenges such as white fly and pink bollworm. This area also uses the lowest percentage of transgenic crops on a per acre basis due to the higher degree of control that growers have over production through water management (USDA-AMS, 2006). Because of water controls weed problems are not considered to be as much of an issue in these production regions, although they can be severe if not managed.

c. Production Considerations

Pre-season
Pre-season production considerations consist of field preparation, crop rotation, variety selection, and crop management planning. Commercial operations usually decide well in advance and purchase equipment and supplies according.

Field preparation includes the bedding or preparing of rows for planting, cultivation and early season weed management, and fertilization of the field for the anticipated nutritional needs of the crop. Field preparation also depends on which crop will be planted in a particular field as crop rotation considerations are made with regard to managing weeds, insect pests, soil nematodes, and plant diseases.

Varieties available for selection vary greatly by region with varieties bred for a particular geographic region's climate and available moisture. Other deciding factors include transgenic traits, output potential, fiber quality, and disease and pest resistance.

These factors influence the decisions the growers must make in order to plan the purchase of their chemicals, fertilizers, and seed. Often growers purchase these supplies in advance in anticipation for their needs during the growing season. Early purchasing decisions provide economic benefit to a grower's operation. The more predictable a crop's production is the easier it is to make timely and correct predictions on future needs of the crop, and keep input costs low.

Planting/early season
Production practices for planting and early season management of a crop vary greatly by region. Row spacing and plant density are impacted by the cost of inputs (*e.g.* seed and chemicals) as well as factors such as irrigation practices, insect pests native to the production region, and the variety of cotton selected. Planting timing is largely based on soil temperature and moisture, as well as availability of oxygen in the soil. Soil depth is also an important factor, as cotton is slow to germinate and is especially vulnerable during this time period to weed, disease, and insect pressures.

Mid-season
Mid-season cotton concerns revolve primarily around the control of insect pests and retaining fruit on the plant. Attention to moisture and nutritional needs is also important as deficiencies in moisture and key micronutrients can result in poor fiber development prior to boll crack and shed of fruit. Weed management is also important as many weed species of concern can still develop in an established cotton field which can cause yield drag, impact harvest efficiency, and the quality of the fiber. Many regions use PGRs to keep the height of the cotton manageable and production energy toward reproduction and away from vegetative growth.

Late season & harvest
Timely harvest is key to protecting a crop of cotton. Cotton fiber becomes vulnerable once exposed to the elements after cracking. Leaving the cotton fiber exposed to weather can result in loss of the cotton fiber or damage to the quality. Weed management again is an issue towards harvest as populations of large established weeds can effect or damage cotton harvesting equipment. At this time chemical defoliation or desiccation occurs to remove the vegetative tissue from the cotton plant to minimize these impacts. Vegetative tissue remaining on the plant will be harvested with the lint and impact the quality of the fiber by increasing the volume of gin trash, and possibly staining the cotton fiber.

d. Problem weeds

Conventional methods of control
Successful weed control utilizing conventional methods is achieved by a combination of crop rotation, cultivation, and herbicides.

Crop rotation allows for the use of complimentary chemical and agricultural practices. Certain weeds do not grow well in other crops, therefore reducing the weed seed bank of the seed so in subsequent years there is no build-up of weed populations from recurrent cotton plantings.

Herbicide is the most effective and direct form of weed control. Herbicides are used in pre-plant burndown applications where established weed populations are removed prior to planting. Herbicide formulations are also available for broadcast and directed application post-emergence to help establish the stand of the cotton to provide competitive advantage over weed species (Ferrell, 2006). Many herbicides used in herbicide tolerant cotton production systems (including glyphosate) have no residual soil activity, which contributes to their more favorable environmental profile. Herbicides used in conventional systems often have residual soil activity to increase the duration of the herbicidal effect, and to reduce the number of herbicide applications made to a field. Late into the season, hooded spray applications of herbicides, which would normally be harmful to cotton crops, may be applied between cotton rows to help reduce the population of weed species. Should herbicide application fail to control weed populations, mechanical cultivation can be used to remove weed species from between cotton rows.

Other weed management programs have been attempted over the course of cotton production which have been effective in some cases, but the methods mentioned above are by far the most commonly used methods of weed control used in conventional cotton production systems.

Volunteer management
GlyTol cotton is sensitive to many other chemicals registered for pre-plant burndown and post-emergence in cotton. All cotton varieties are sensitive to many herbicides, such as 2,4-D, used for weed management in monocotyledon crops such as corn in rotational systems. Additionally, other herbicides, such as glufosinate-ammonium (Liberty®) and flumioxazin (Valor®), can be used for burndown in no-till planting systems common in herbicide tolerant cropping systems.

In conventional cultivation systems, post-directed sprays of herbicides such as MSMA in combination with traditional cultivation would be successful in removing volunteer cotton plants.

In rotational situations with other glyphosate tolerant crops such as corn or soybeans, many herbicides, (*e.g.* 2,4-D) are used for broadleaf control in monocotyledon crops (*i.e*. corn). Soybean crops can use soil incorporated, pre-plant, and post-emergence herbicides to control a broad spectrum of broadleaf plants, such as cotton. Products such as Lexon® and Lorox® and others are available should cotton volunteers emerge. A listing of available herbicides for control of broadleaf weeds which are labeled for glyphosate tolerant corn, soy, and cotton is found in Tables 29, 30 and 31.

Glyphosate tolerant weeds

As described above, glyphosate tolerant cotton volunteers can be managed through a variety of conventional herbicide management techniques. This same conclusion can be made for weeds that may have developed tolerance to glyphosate. Utilization of crop rotation and different herbicide chemistries for pre-plant weed control are methods that can be utilized to control glyphosate tolerant weeds. Additionally, areas that have not reported glyphosate tolerant weeds can extend the usefulness of glyphosate tolerant cotton by rotating this system with other herbicide tolerant cottons such as bromoxynil tolerant or glufosinate-ammonium tolerant cotton, in addition to conventional weed management programs.

Table 29. Broadleaf Corn Herbicides

Pre-emergence Broadleaf Herbicides	
Chemical	**Trade Name**
Atrazine	AAtrex, etc.
Mesotrione	Callisto
acetochlor + atrazine	Degree Xtra, Fultime, Harness Xtra, Bullet, etc.
Simazine	Princep, Princep Calliber 90
dimethenamid + atrazine	Guardsman Max
S-metolachlor + atrizine	Bicep II Magnum
Pendimethlin	Prowl
rimsulfuron + thifensulfuron methyl	Basis
Post-Emergence Broadleaf Herbicides	
Chemical	**Trade Name**
Bentazon	Basagran
acetochlor + atrazine	Degree Xtra, Fultime, Harness Xtra, Bullet, etc.
dimethenamid + atrazine	Guardsman Max
Atrazine	Aatrex
nicosulfuron + rimsulfuron + atrizine	Basis Gold
Bromoxinyl	Buctril
Carfentrazone	Aim (EC)
dicamba, dimethylamine salt	Banvel, clarity, etc.
flumichlorac pentel ester	Resource
Mesotrione	Callisto
thifensulfuron methyl	Harmony GT
2,4-D	Various brands
glufosinate-ammonium*	Liberty
imazethapyr + imazapyr	Lightning

Table 30. Broadleaf Soybean Herbicides

Pre-plant Broadleaf Herbicides	
Chemical	**Trade Name**
Imazaquin	Scepter
Metribuzin	Sencor
Pre-emergence Broadleaf Herbicides	
Chemical	**Trade Name**
S-metolachlor	Dual Magnum
Pendimethlin	Prowl
Clomazone	Command 3 ME
Flumetsulam	Python
Flumioxazin	Valor
Imazaquin	Scepter
Linuron	Linex, etc.
Metribuzin	Sencor
Paraquat	Gramoxone Max
Post-Emergence Broadleaf Herbicides	
Chemical	**Trade Name**
Acifluorfen	Ultra Blazer
Bentazon	Storm, Basagran, etc.
cloransulam – methyl	Amplify
chlorimuron ethyl	Classic
thifensulfuron methyl	Harmony
flumichlorac penthyl ester	Resource
Lactofen	Stellar
Fomesafen	Reflex
Imazomox	Raptor
Imazaquin	Scepter
Imazethapyr	Pursuit

Table 31. Broadleaf Cotton Herbicides

Pre-plant Broadleaf Herbicides	
Chemical	**Trade Name**
paraquat	Gramoxone Max
glufosinate	Ignite
Pendimethlin	Prowl
trifluralin	Treflan
fluometuron	Cotoran
Pre-emergence Broadleaf Herbicides	
Chemical	***Trade Name***
pryrithiobac sodium	Staple
fluometuron	Cotoran
clomazone	Command 3 ME
Pendimethlin	Prowl
Post-Emergence Broadleaf Herbicides	
Chemical	**Trade Name**
pryrithiobac sodium	Staple
Fluometuron	Cotoran
Trifloxisulfuron	Envoke
Glufosinate-Ammonium*	Ignite
Diruon	Direx
Linuron	Layby Pro
Flumioxazin	Valor
S-metolachlor	Dual Magnum
Lactofen	Cobra
Promethryn	Caparol
Trifloxisulfuron	Suprend
Paraquat	Gramoxone Max

F. Summary of Environmental Safety/Impact on non-contained use of GlyTol Cotton

GlyTol cotton event GHB614 was evaluated for agronomic impacts during seed germination and dormancy studies, protein safety assessment, composition analysis, and agronomic performance evaluation. These assessments of GlyTol cotton and the 2mEPSPS protein were conducted across a wide variety of environmental and climatic conditions which are representative of the majority of upland cotton acres produced in the United States. These assessments demonstrate that GlyTol cotton event GHB614 does not pose a greater plant pest potential than conventional cotton produced in the United States.

The environmental impacts of pollen transfer to other cotton varieties is not considered to be an issue with the production of GlyTol cotton. The limited range of movement of cotton pollen described in Section II coupled with the low acute oral toxicity of the 2mEPSPS protein demonstrates that the opportunities for exposure and the impacts of this exposure are minimal. Additionally, the opportunities for outcrossing with sexually compatible cotton species is highly unlikely due to the limited number of species, and their isolation from cotton production regions in the United States. Agronomic evaluation of GlyTol cotton included seed germination evaluations to rate the potential for increased dormancy of GlyTol cottonseed. No increases in seed dormancy were found for the range of temperatures expected for cotton producing regions. Therefore the agronomic consequences of introduction of GlyTol cotton

are also expected to be minimal due to the wide range of methods of control of transgenic cotton, and cotton's inability to establish itself as a major weed species.

The resulting conclusion is that GlyTol cotton event GHB614 is not expected to have an adverse impact on non-target organisms found in and around agricultural production systems, or to the environment around these regions.

X. STATEMENT OF GROUNDS UNFAVORABLE

Data generated from agronomic tests and molecular characterization indicate that no unfavorable ground are associated with GlyTol cotton event GHB614.

Therefore Bayer CropScience requests that GlyTol cotton no longer be considered a regulated article under 7 CFR 340.

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Appendix 1. FIELD TRIALS TERMINATION REPORTS 2002-2005

*Termination Reports Formatted for USDA Petition

Termination Report 1 - 02-072-04n

USDA Field Termination Report

Notification No.: 02-072-04n

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

Permittee: Aventis CropScience (Now Bayer CropScience LP) Research Triangle Park, NC

Regulated Article: Herbicide-tolerant, glyphosate-tolerant; Cotton (*Gossypium hirsutum*)

Site Release Information: Release information for Lubbock Co., TX is as follows:

Acreage Planted	Date Planted	Date Terminated
<1.0	6/25/02	12/7/02

Purpose of Release: The purpose of the release was to test the efficacy of the glyphosate-tolerant (HT) cotton plants. The performance of transgenic cotton with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was inspected twelve (12) times during the growing season (7/2/02, 7/9/02, 7/10/02, 7/11/02, 7/14/02, 7/17/02, 8/4/02, 8/14/02, 8/15/02, 9/15/02, 10/9/02, 11/12/02) for agronomic growth characteristics and disease and insect pest infestation.

Observations were recorded from first square through open boll growth stages on both the transgenic and nontransgenic plants. Plant emergence patterns were similar for both the transgenic and nontransgenic plots, with rates ranging from 75 to 80%. The initial stand count was highly variable between the transgenic cotton lines.

One species of insect pests was noted: bollworm (7/14/02 and 8/4/02). Damage ratings ranged from slight to moderate. No differences were recorded in either the diversity or density of insect pest species found between the transgenic and nontransgenic counterpart.

Two (2) species of beneficial insects were observed: ladybug (8/15/02 and 9/15/02) and lacewing (9/15/02).

No disease susceptibility was noted on the transgenic or non-transgenic plants during any of the visits made on 7/10/02, 8/14/02, 10/9/02 or 11/12/02.

Results: No agronomic, insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic cotton plants and the nontransgenic counterpart. The only difference noted in the transgenic and nontransgenic counterpart plants was in the desired trait – tolerance to glyphosate herbicide – in the transgenic cotton plants, whereas the nontransgenic plants were susceptible to treatment with glyphosate.

Plant Disposition: Harvest occurred on 12/7/02. Following harvest, all remaining plant material was disked under.

Volunteer Monitoring: The plot area was visually inspected for volunteer cotton plants seven (7) times during the following growing season, until no volunteers had been observed for three (3) consecutive post-season monitoring visits.

Post-Season Volunteer Monitoring		
Date	**No. Plants Observed/Stage**	**Method of Destruction**
4/30/03	1 to 10 plants	Mechanically Cultivated
5/16/03	None	
5/30/03	None	
7/16/03	1 to 10 plants	Mechanically Cultivated
8/15/03	None	
9/2/03	None	
9/15/03	None	

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

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

Weather Synopsis: Weather notations indicate the site experienced normal climatic conditions during the growing season.

Containment Measures: A 40-foot wide perimeter of nontransgenic cotton plants surrounded the test plot to minimize pollen flow. All cotton that bordered the test plot was destroyed at the conclusion of the trial. The test plot and border area were monitored the following growing season for volunteer cotton plants.

Termination Report 2 - 02-296-01n

USDA Field Termination Report

Notification No.: 02-296-01n

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

Permittee: Bayer CropScience LP, Research Triangle Park, NC

Regulated Article: Herbicide-tolerant, glyphosate-tolerant; Cotton (*Gossypium hirsutum*)

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

Acreage Planted	Date Planted	Germination Data Transgenic *vs.* Non-transgenic	Date Terminated
0.71	12/5/02	**% Emergence/Seedling Vigor** >50% *vs* >50% on 11/30/02 **Initial Stand Count Percentage** >80% *vs* >80% on 12/6/02 **Final Stand Count Percentage** 40-60% *vs* >80% on 12/20/02	Harvest occurred on 4/16/03 and 4/17/03. The plot was mechanically cultivated 4/23/03.

Purpose of Release: The purpose of the release was to test the efficacy of the glyphosate-tolerant cotton plants. The performance of transgenic cotton with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was inspected twelve (12) times during the growing season (11/26/02, 11/30/02, 12/6/02, 12/17/02, 12/18/02, 12/20/02, 1/2/03, 2/15/03, 2/23/03, 3/10/03, 4/15/03, 4/16/03) for agronomic growth characteristics and disease and insect pest infestation.

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

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

Two (2) species of beneficial insects were observed: cucumber beetle (12/20/02) and honeybees (3/10/03).

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

Results: No agronomic, insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic cotton plants and the nontransgenic counterpart. The only difference noted in the transgenic and nontransgenic counterpart plants was in the desired trait – tolerance to glyphosate herbicide – in the transgenic cotton plants, whereas the nontransgenic plants were susceptible to treatment with glyphosate.

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

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

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

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

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

Weather Synopsis: Weather notations indicate the site experienced typical climatic conditions during the growing season.

Containment Measures: The Sabana Grande test site is a 59-acre farm. The site produces no commercial crops. A 40-foot-wide perimeter of nontransgenic cotton plants surrounded the test plot to minimize pollen flow. Border rows were not harvested but destroyed at trial conclusion. Border areas were monitored (as part of the test plot) for volunteer cotton plants during the period that followed.

Termination Report 3 - 03-064-14n

USDA Field Termination Report

Notification No.: 03-064-14n

Applicant No.: GLY-3A-Cotton-MR

Permittee: Bayer CropScience LP Research Triangle Park, NC

Regulated Article: Herbicide-tolerant, Glyphosate-tolerant; Cotton (*Gossypium hirsutum*)

Site Release Information: All releases authorized under this notification were planted. Shown below is the acreage amount, planting date and termination date for each site.

County/State	Acreage Planted	Date Planted	Date Terminated
Washington/MS	1.8	5/23/03	10/22/03
Dillon/SC	1.92	5/27/03	11/22/03
Lubbock/TX	2.0	5/26/03 and 6/9/03	1/10/04

Purpose of Release: The purpose of the releases was for breeding, to obtain analytical data, and to evaluate the efficacy and agronomic characteristics of the transgenic herbicide-tolerant cotton plants. The performance of the transgenic cotton with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

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

At each location, the transgenic cotton plants exhibited normal growth and development. Heavy rainfall in Dillon Co. caused some stunting to occur. Hail and wind damage necessitated a replant in Lubbock Co.

County/State	Germination Data Transgenic *vs.* Non-transgenic
Washington/MS	**% Emergence/Seedling Vigor** 90% *vs*. 90% on 5/30/03 **Initial Stand Count Percentage** 90% *vs.* 90% on 6/7/03 **Final Stand Count Percentage** 85% *vs*. 75% on 10/20/03
Dillon/SC	**% Emergence/Seedling Vigor** 98-100% *vs*. 98-100% on 6/6/03 **Initial Stand Count Percentage** 90-100% *vs.* 90-100% on 7/15/03 **Final Stand Count Percentage** 90% *vs*. 90% on 9/15/03
Lubbock/TX	**% Emergence/Seedling Vigor** 82.3% *vs*. 86.6%on 7/14/03 **Initial Stand Count Percentage** 82.3% *vs*. 86.6% on 7/14/03 **Final Stand Count Percentage** 87.3 *vs*. 86.6% on 8/15/03

Plots were visually inspected for plant diseases and insects. Insect species were categorized as pests and beneficials. Observations were noted as follows:

County or District/State or Territory	Observations/Dates		
	Fungi/Diseases	Insect Pests	Beneficial Insects
Washington/MS	None observed 5/30/03, 6/20/03, 7/17/03 or 9/30/03.	Light infestation of plant bugs was observed 6/24/03 and 8/14/03.	Lady beetles were observed on 5/30/03, 7/17/03 and 8/14/03.
Dillon/SC	Nematode root-knot and *Fusarium* boll rot were noted 7/17/03 and 9/5/03.	Very light infestations of thrips, whiteflies, aphids, bollworms, and armyworms were observed (6/12/03, 7/1/03, 7/7/03, 7/14/03, 7/17/03, 7/25/03 and 8/30/03).	Ants and ladybugs were observed 6/12/03, 7/7/03, and 7/25/03.
Lubbock/TX	None visible on 7/16/03 and 8/15/03.	Light to moderate infestations of bollworms and leafminers noted 7/28/03 and 8/15/03. Aphids were present on 10/20/03.	Adult hooded beetle, lacewings and ladybugs seen on 7/28/03 and 8/15/03.

Results: No insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic cotton plants and the nontransgenic plants. A phenotypic difference was exhibited between the transgenic and nontrangenic cotton plants in terms of herbicide tolerance.

Plant Disposition: Harvest occurred 10/20/03 at the Washington Co. site. Following harvest, remaining vegetative material was destroyed 10/21/03 and 10/22/03. Cotton stalks were mowed and disked, and lint and seed were burned. The Dillon Co. plot was harvested 11/22/03. Harvested cotton was dumped back into the field and disked under. The Lubbock Co. site was harvested 10/27/03. Remaining plant material for this trial was burned and disked under on 1/10/04.

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

Post-Season Volunteer Monitoring			
County or District/State or Territory	**Date**	**No. Plants Observed/Stage**	**Method of Destruction**
Washington/MS	4/5/04	None	
	4/13/04	None	
	4/28/04	None	
	5/6/04	None	
	5/14/04	None	
Dillon/SC	5/15/04	None	
	6/21/04	None	
	7/15/04	None	
	8/21/03	None	
	9/30/03	None	
Lubbock/TX	4/2/04	None	
	4/16/04	1 to 10 plants	Plants were removed by hand.
	4/30/04	None	
	5/13/04	1 to 10 plants	Plants were removed by hand.
	5/28/04	None	
	6/15/04	None	

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

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

Weather Synopsis: Weather conditions for Washington Co. were hot and dry. Dillon Co. experienced a wet season from planting through harvest. Initially, climatic conditions for Lubbock Co. were wet but became normal later in the season.

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

Termination Report 4 - 03-255-03n

USDA Field Termination Report

Notification No.: 03-255-03n

Applicant No.: GLY-3B-Cotton-MR

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

Regulated Article: Herbicide-tolerant, glyphosate-tolerant; Cotton (*Gossypium hirsutum*)

Site Release Information: The Sabana Grande District, PR site was planted:

Acreage Planted	Dates Planted	Date Terminated
6.96	11/24/03 and 11/29/03	5/7/04

Purpose of Release: The trial was established to generate seed from the transgenic cotton plants for research purposes. The performance of the transgenic cotton with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was visually inspected eight (8) times during the growing season (12/11/03, 12/19/03, 12/31/03, 1/7/04, 2/15/04, 3/10/04, 4/30/04, and 5/7/04) for agronomic growth characteristics and disease and insect pest infestation.

Observations were recorded from plant emergence through harvest on both the transgenic and nontransgenic plants. Both the transgenic and nontransgenic plants germinated well (>80%) and grew vigorously.

Three (3) species of insect pests were noted: grasshoppers (12/19/03), aphids (1/7/04), and boll weevils (2/15/04). Populations ranged from light to moderate. No differences were recorded in either the diversity or density of insect pest species found between the transgenic and nontransgenic counterpart.

Two (2) species of beneficial insects were observed: cucumber beetles (12/19/03) and honeybees (3/10/04).

No disease susceptibility was noted on the transgenic or nontransgenic plants during any of the visits made on 12/19/03, 1/7/04, 3/10/04, 4/30/04 or 5/7/04.

Results: No agronomic, insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic cotton plants and the nontransgenic counterpart. The only difference noted in the transgenic and nontransgenic counterpart plants was in the desired trait – tolerance to glyphosate herbicide – in the transgenic cotton plants, whereas the nontransgenic plants were susceptible to treatment with glyphosate.

Plant Disposition: Harvest occurred over a two-week period. The plot planted 11/24/03 was harvested 4/26/04 – 4/30/04. The plot planted 11/29/03 was harvested 5/3/04 – 5/7/04. Following harvest, all remaining plant material was mechanically cultivated.

Volunteer Monitoring: The plot area was visually inspected for volunteer cotton plants six (6) times during the following growing season, until no volunteers had been observed for three (3) consecutive post-season monitoring visits.

Post-Season Volunteer Monitoring		
Date	**No. Plants Observed/Stage**	**Method of Destruction**
5/31/04	>50 plants/V2	Mechanically Cultivated
6/21/04	11 to 50 plants/V2	Mechanically Cultivated
7/13/04	1 to 10 plants/V3	Mechanically Cultivated
8/17/04	None	
8/31/04	None	
9/10/04	None	

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

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

Weather Synopsis: Weather notations indicate the climatic conditions at the beginning of the season was wet, followed by a normal/typical growing season.

Containment Measures: The Sabana Grande test site is a 59-acre farm. The site produces no commercial crops. A 40-foot-wide perimeter of nontransgenic cotton plants surrounded the test area to minimize pollen flow. Border rows were not harvested but destroyed at trial conclusion. Border areas were monitored (as part of the testing area) for volunteer cotton plants during the period that followed.

Termination Report 5 - 04-064-10n

USDA Field Termination Report

Notification No: 04-064-10n

Applicant No: GLY-4A-Cotton-MR

Permittee: Bayer CropScience LP Research Triangle Park, NC 27709

Regulated Article: Cotton tolerant to the herbicide Glyphosate

Site Release Information: The trial was released on nine (9) sites:

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Jefferson Co. / GA	2.85	5/22/04	10/25/04	40' isolation buffer
Coahoma Co. / MS	0.52	5/23/04	11/15/04	40' isolation buffer
Washington Co. (1) / MS	0.52	5/20/04	11/10/04	40' isolation buffer
Washington Co. (2) / MS	3.90	5/22/04	11/12/04	40' isolation buffer
Halifax Co. / NC	2.7	5/24/04	11/13/04	40' isolation buffer
Dillon Co. / SC	2.95	5/21/04	11/29/04	40' isolation buffer
Dawson Co. / TX	1.78	5/20/04	12/1/04	40' isolation buffer
Lubbock Co. / TX	2.0	5/25/04	12/18/04	40' isolation buffer
Swisher Co. / TX	1.65	5/19/04	12/13-14/04	40' isolation buffer

Purpose of Release: This trial was established to evaluate the performance of the glyphosate tolerant trait in the experimental cotton varieties tested. Additionally, breeding activities were conducted at some sites for future test and breeding work.

Observations: Trial sites were observed at various times throughout the growing season, usually on a monthly basis. At least four (4) observations were made for insect and disease pressures, germination rates, beneficial insect populations, any occurrence of increased weediness characteristics, and for any phenotypical differences between transgenic and non-transgenic lines. Most sites showed very little or no insect or disease pressure, no differences in beneficial insect populations, and no evidence of increased weediness in the crop. However, the Swisher Co. and Dawson Co. TX sites detected slightly variable germination rates with the transgenic variety showing an almost 10% decrease in stand count at the end of the season. Additionally, the Dawson Co. TX site displayed high levels of Fusarium wilt virus in both the transgenic and non-transgenic varieties. The Jefferson Co. GA site displayed low levels of hard lock, most likely due to the low levels of plant bug and lepidopteron insect pressure present.

Plant Disposition: All plots were harvested, and taken to yield, with residual material being disked into soil after harvest. Most sites are still under volunteer monitoring.

Volunteer Monitoring: Volunteer monitoring is still being conducted on most sites, and is scheduled to be completed by the end of December 2005.

Weediness Characteristics: There was no indication that either the transgenic or non-transgenic lines developed weediness characteristics in the trial.

Non-target Organisms: There was no indication of population differences in beneficial insect populations or other indication of an adverse effect by the transgenic line on beneficial insects.

Weather Synopsis: Most sites from the mid-Atlantic region to Mississippi reported above average rainfall for the season, with the end of the summer and early fall cooler than normal. Most locations in

Texas reported higher than average rainfall early in the planting season, with below average rainfall mid season and cooler temperatures late in the season.

Containment Measures: Plots were spatially isolated from other cotton by a 40 foot wide buffer of commercially available non-transgenic cotton. This buffer was destroyed prior to harvest, and is included in volunteer monitoring efforts.

Termination Report 6 - 04-247-01n

USDA Field Termination Report

Notification No: 04-247-01n

Applicant No: GLY-4B-Cotton-MR

Permittee: Bayer CropScience LP Research Triangle Park, NC 27709

Regulated Article: Cotton tolerant to the herbicide Glyphosate

Site Release Information: The trial was released on only one site:

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Sabana Grande / PR	7.18 A	11/10/04 11/11/04 11/15/04 12/18/04	4/8/05 4/11-21/05 4/22-30/05 5/1-3/05 5/19/05 5/23/05	Some sites were spatially isolated by 660' from other cotton, some plot adjacent to border of farm were enclosed in a 40' buffer

Purpose of Release: This trial was established as an over-winter nursery for seed production for the 2005 trial season. Additionally, plots were evaluated for their resistance to glyphosate.

Observations: Observations were made of the various plantings of Glyphosate tolerant cotton multiple times for each planting between the end of January until harvest. There was no difference in germination and stand counts, plant disease, and insect pest population between the transgenic and non-transgenic varieties. Additionally, there was no indication of adverse effects on beneficial insect populations (mostly honey bees) or an increase in the weediness characteristics of the transgenic treatment. No significant phenotypical differences were noted during the growing season.

Plant Disposition: Plants were harvested between April and May of 2005, and seed retained for future plot work. Remaining plant material in the field was cultivated into the soil after harvest.

Volunteer Monitoring: Volunteer monitoring is currently being conducted on the trial site, scheduled to be completed on 11/15/05.

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

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

Weather Synopsis: Weather patterns were reported as being normal for this area and time of the year.

Containment Measures: Plots were spatially isolated from other cotton by a distance of 660 ft. Additionally, plots that were adjacent to the border of the test site were surrounded by a 40' buffer of non-transgenic cotton.

Termination Report 7 - 05-060-03n

USDA Field Termination Report

USDA Notification Number: 05-060-03n

Applicant Internal Number: GLY-5B-Cotton-MR

Permittee: Bayer CropScience LP Research Triangle Park, NC 27709

Regulated Article: *Gossypim hirsutum* varieties tolerant to glyphosate herbicide

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

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Pinal Co., AZ	9.38	5/28/05	12/17/05	Isolation border
Bulloch Co., GA	2.0	6/8/05	11/11/05	Isolation border
Coahoma Co., MS	2.44	5/27/05	10/6/05	Isolation border
Washington Co., MS (#1)	3.66	5/26/05	10/13/05	Isolation border
Washington Co., MS (#2)	4.65	6/7/05	10/14/05	Isolation border
Halifax Co., NC	2.0	5/27/05	11/18/05	Isolation border
Dillon Co., SC	2.62	6/3/05	12/2/05	Isolation border
Lubbock Co., TX (#1)	1.9	6/7/05	11/11/05	Isolation border
Lubbock Co., TX (#2)	1.7 and 5.2	5/21/05 and 5/23/05	11/12/05 and 11/23/05	Isolation border
Lubbock Co., TX (#3)	1.5	5/18/05	12/12/05	Isolation border

Purpose of Release: This trial was established to evaluate the performance of cotton varieties tolerant to glyphosate herbicide.

Observations:
Pinal Co., AZ: No differences in emergence, disease pressure, insect pest populations, and beneficial insect populations were noticed between the transgenic and non-transgenic varieties. Slight differences were noted in the growth of the plants at different times during the season, which appeared to normalize as the season progressed. This indicates a variety response between the two lines. Cooperator did notice that the transgenic varieties appeared to lodge less during boll opening, with a difference in lodging counts as high as 30%.

Bulloch Co., GA: No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Coahoma Co., MS: No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Washington Co., MS (#1): No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Washington Co., MS (#2): No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Halifax Co., NC: No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Dillon Co., SC: No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Lubbock Co., TX (#1): Emergence rates in the transgenic variety were reported to be higher (approximately 20%) than in the non-transgenic, which were seen through all evaluations of plant stand. No other differences in disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Lubbock Co., TX (#2): Emergence rates in the transgenic variety were reported to be higher (approximately 20%) than in the non-transgenic, which were seen through all evaluations of plant stand. No other differences in disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties. This site was planted in two locations, one of which was inappropriately harvested, as reported to USDA. All material in connection with this harvest was destroyed by incineration.

Lubbock Co., TX (#3): No differences in emergence, disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics were noticed between the transgenic and non-transgenic varieties.

Plant Disposition:
Pinal Co., AZ: Seed harvested and then dumped in plot and burned. Remaining material was incorporated into the trial site.
Bulloch Co., GA: Samples taken for analysis; all remaining harvested material was destroyed.
Coahoma Co., MS: All material harvested from this plot was destroyed.
Washington Co., MS (#1): All material harvested from this plot was destroyed.
Washington Co., MS (#2): All material harvested from this plot was destroyed.
Halifax Co., NC: Samples taken for analysis; all remaining harvested material was destroyed.
Dillon Co., SC: Samples taken for analysis; all remaining harvested material was destroyed.
Lubbock Co., TX (#1): All material harvested from this plot was destroyed.
Lubbock Co., TX (#2): All material harvested from this plot was destroyed. Material which was inadvertently removed from the field site was contained and destroyed with contaminated commercial material as reported to USDA compliance division.
Lubbock Co., TX (#3): All material harvested from this plot was destroyed.

Volunteer Monitoring: Volunteer monitoring is currently being conducted on these sites, with all volunteer monitoring scheduled to be completed on 12/17/06.

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

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects. Various beneficial insect populations were present native to the regions of the specific trials.

Weather Synopsis:
Pinal Co., AZ: Normal for this area, with the exception of an extended dry and warm fall.
Bulloch Co., GA: Dry late summer, followed by a wet fall.
Coahoma Co., MS: Mostly hot and dry through out the season; Major storms reported in September, with no plot damage.

Washington Co., MS (#1): Mostly hot and dry through out the season; Major storms reported in September, with no plot damage.
Washington Co., MS (#2): Mostly hot and dry through out the season; Major storms reported in September, with no plot damage.
Halifax Co., NC: A mostly dry summer, with a small hail storm in July (no plot damage). Fall rainfall above average.
Dillon Co., SC: Conditions were normal for this area, and time of year, with a dry period in the month of August.
Lubbock Co., TX (#1): Weather conditions were reported as normal for this area.
Lubbock Co., TX (#2): Weather conditions were reported as normal for this area.
Lubbock Co., TX (#3): Weather conditions were reported as normal for this area.

*Damage from Hurricane Rita to all plots in the affected area was reported to USDA as a summary of plot conditions following the Hurricane.

Containment Measures: All plots were contained using an isolation border that measured at least 40 feet at each border of the field perimeter.

Termination Report 8 - 05-091-07n

USDA Field Termination Report

USDA Notification Number: 05-091-07n

Applicant Internal Number: GLY-5B-Cotton-MR

Permittee: Bayer CropScience LP Research Triangle Park, NC 27709

Regulated Article: *Gossypim hirsutum* varieties tolerant to glyphosate herbicide

Site Release Information: Trials utilizing this trait were conducted at twelve (12) locations:

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Crittenden Co., AR	0.57	5/18/05	10/11/05	660 ft. isolation distance
Drew Co., AR	1.57	5/19/05	10/18/05	Isolation border
Jackson Co., AR	1.81	5/26/05	11/3/05	Isolation border
Escambia Co., FL	1.643	5/27/05	11/17-19/05	Isolation border
Tift Co., GA	1.72	6/8/05	11/17/05	Isolation border
Tate Co., MS (#1)	1.66	5/31/05	10/27/05	Various*
Tate Co., MS (#2)	1.84	6/4/05	10/20/05	Isolation border
Washington Co., MS	2.5	5/26/05	11/4/05	Isolation border
Hockley Co., TX	1.61	6/3/05	11/16/05	Isolation border
Uvalde Co., TX	7.27	5/20/05	10/6/05 and 10/8/05	Isolation border
Wharton Co., TX (#1)	1.98	6/8/05	10/27/05	Isolation border
Wharton Co., TX (#2)	1.58	6/6/05	10/27/05	Isolation border

* Tate Co., MS (#1) site was isolated using a combination of 660 ft. isolation and isolation border. This was reported to USDA as a potential compliance incident, which was resolved without further investigation.

Purpose of Release: This trial was established to evaluate the performance of cotton varieties tolerant to glyphosate herbicide, and obtain samples for analysis.

Observations:

Crittenden Co., AR: Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Drew Co., AR: Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Jackson Co., AR: Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Escambia Co., FL: Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Tift Co., GA: Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application. Cooperator reported excellent tolerance to herbicide treatment in transgenic varieties.

Tate Co., MS (#1): Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Tate Co., MS (#2): Cooperator observed no differences in plant disease pressure, insect pest populations, beneficial insect populations, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application. Cooperator reported significant increase in emergence in the transgenic lines over the non-transgenic lines (15-23% higher). Additionally, cooperator noted that transgenic AND non-transgenic lines displayed much clearer signs of drought stress than the isolation border which was of a different variety. This was most likely a varietal difference due to the fact that both transgenic and non-transgenic varieties show equal signs of stress from lack of water.

Washington Co., MS: Cooperator observed no differences in emergence, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application. A slight difference in disease pressure (a bit vs. none) was observed in one of the transgenic treatment plots. Additionally, plots suffered slight plot damage from Hurricane Rita and were blown over as the result of high winds. Bolls were not yet open, so no material left the perimeter of the field plot, and was therefore not reported as a potential compliance incident.

Hockley Co., TX: Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Uvalde Co., TX: Although differences were noted between different varieties, cooperator observed no differences in plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application. Cooperator did notice that in one variety, there was a significant difference in the emergence of the transgenic (92%) versus the non-transgenic (50%). This leveled off somewhat during the course of the season, but transgenic stand was still significantly better than the non-transgenic stand (87% vs. 65%). Cooperator noted excellent tolerance in the transgenic lines to glyphosate.

Wharton Co., TX (#1): There was not a non-transgenic variety to compare the transgenic plants to, but no observations were made which could be an indication of differences from a non-transgenic variety of the same type.

Wharton Co., TX (#2): Cooperator observed no differences in emergence, plant disease pressure, insect pest populations, beneficial insect populations, phenotypical differences, or weediness characteristics between the transgenic and non-transgenic varieties prior to herbicide application.

Plant Disposition:
Crittenden Co., AR: All material harvested from this location was destroyed.
Drew Co., AR: All material harvested from this location was destroyed.
Jackson Co., AR: All material harvested from this location was destroyed.
Escambia Co., FL: All material harvested from this location was destroyed.
Tift Co., GA: All material harvested from this location was destroyed.
Tate Co., MS (#1): All material harvested from this location was destroyed.
Tate Co., MS (#2): Samples were taken and sent for analysis in RTP, NC. AI remaining harvested from this location was destroyed.
Washington Co., MS: Samples harvested and shipped to Lubbock, TX for storage; remaining material harvested from this location was destroyed.
Hockley Co., TX: All material harvested from this location was destroyed.

Uvalde Co., TX: Approximately 150 lbs. of harvested material sent to Navasota, TX for processing and analysis; most material sent to TX A & M for Feeding study; remaining harvested material was destroyed.
Wharton Co., TX (#1): Samples harvested and shipped to Navasota, TX for processing and analysis; remaining harvested material destroyed.
Wharton Co., TX (#2): Samples harvested and shipped to Navasota, TX for processing and analysis; remaining harvested material destroyed.

Volunteer Monitoring: Volunteer monitoring is currently being conducted on this site, and is scheduled for completion on 11/2/06 in MS and 10/24/06 in TX.

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

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects. Various beneficial insect populations were present with adult and larval lady beetles observed.

Weather Synopsis:
Crittenden Co., AR: Normal temperatures for this region, with below average rainfall.
Drew Co., AR: Cooperator reported below average rainfall for this region.
Jackson Co., AR: Weather conditions were reported to be normal for this region.
Escambia Co., FL: Weather conditions were reported to be normal for this region.
Tift Co., GA: Temperatures were reported to be normal, with below average rainfall in the fall.
Tate Co., MS (#1): Average rainfall was reported to be significantly below normal.
Tate Co., MS (#2): Average rainfall was reported to be significantly below normal.
Washington Co., MS: Early season rainfall was reported as significantly below normal, resulting in delayed maturity of the trial.
Hockley Co., TX: Weather conditions were reported to be normal for this region. Cooperator reported a hail storm on 7/6/05 in which the plots sustained slight damage. This damage occurred prior to the reproductive cycle, and did not affect the trials development, and therefore was not reported to USDA as a potential compliance incident.
Uvalde Co., TX: Cooperator reported that regional temperatures were the hottest in 10 years, and rainfall was significantly below average.
Wharton Co., TX (#1): June and July were reported to be extremely hot and dry, followed by normal rainfall and above average temperatures. Cooperator reported minor wind damage from Hurricane Rita on 9/24/05, but with no loss of material from the plot.
Wharton Co., TX (#2): June and July were reported to be extremely hot and dry, followed by normal rainfall and above average temperatures. Cooperator reported minor wind damage from Hurricane Rita on 9/24/05, but with no loss of material from the plot.

**Damage from Hurricane Rita to all plots in the affected area was reported to USDA as a summary of plot conditions following the Hurricane.

Containment Measures: With the exception of Crittenden Co., AR which utilized a 660 ft. isolation distance, all plots were contained by an isolation border which was at least 40 ft. wide around the perimeter of the field plots.

Termination Report 9 - 05-217-05n

USDA Field Termination Report

USDA Notification Number: 05-217-05n

Applicant Internal Number: HT-5B-GH-MR

Applicant: Bayer CropScience LP Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum;* tolerant to glyphosate herbicide

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

County / State	Acreage Planted	Dates Planted	Date(s) Terminated	Isolation Method(s)
Sabana Grande, PR	2.74	11/11/05, 11/16/05 and 12/5/05	4/7/06, 5/1/06, 5/2/06 and 5/4/06	Isolation border

Purpose of Release: Trials were established to evaluate the performance of cotton varieties tolerant to glyphosate herbicide.

Observations: Cooperator noted no significant differences in the emergence, plant pests, beneficial insect populations, disease pressure, physical plant characteristics, or weediness characteristics between the transgenic and non-transgenic plots.

Plant Disposition: Cooperator confirmed that all seed sent by Bayer was used in trial creation. A total of four plots were planted. Two plots were planted 11/11/05. One of the two plots was destroyed before flowering on 12/19/05, and the other was harvested 5/1/06. A third plot was planted 11/16/05, and was harvested on 4/7/06 and 5/2/06. The fourth plot was planted 12/5/05 and harvested 5/4/06. All material harvested from these plots was sent to the Bayer facility in Lubbock, Texas. Residual plant material was incorporated into the trial sites.

Volunteer Monitoring: Volunteer monitoring is currently being conducted in each plot area and is scheduled for completion one year from harvest.

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

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

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

Plot Damage: No damage to plots was reported.

Containment Measures: Plots were contained utilizing a 40 ft isolation border.

Termination Report 10 - 05-257-04n

USDA Field Termination Report

USDA Notification Number: 05-257-04n

Applicant Internal Number: HT-5C-GH-MR

Applicant: Bayer CropScience LP Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum;* tolerant to glyphosate herbicide

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

County / State	Acreage Planted	Dates Planted	Date(s) Terminated	Isolation Method(s)
Sabana Grande, PR	0.68	11/22/05	4/22/06, 4/24/06 – 4/26/06	660 ft Isolation distance

Purpose of Release: This trial was established to evaluate trait performance in selected cotton varieties.

Observations: Cooperator noted no differences in the emergence, plant pests, beneficial insect populations, disease pressure, physical plant characteristics, or weediness characteristics between the transgenic and non-transgenic plots.

Plant Disposition: Cooperator confirmed that all seed sent by Bayer was used in trial creation. Harvest occurred 4/22/06, 4/24/06, 4/25/05 and 4/26/06. All harvested material was sent to the Bayer facility in Lubbock, Texas. Residual plant material was incorporated into the trial site.

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

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

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

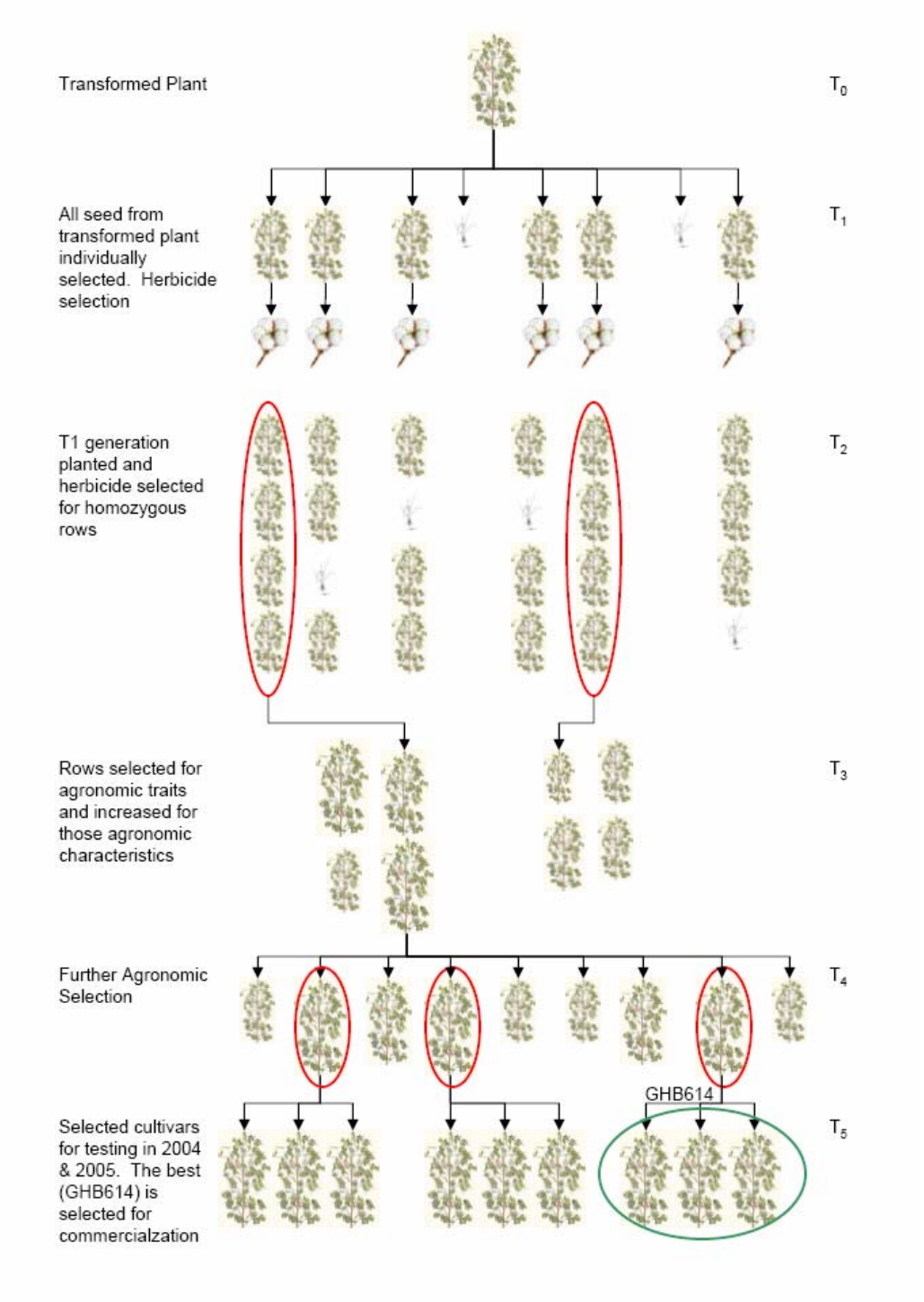
Weather Synopsis: Cooperator reported normal growing conditions for this region.

Plot Damage: No damage to plots was reported.

Containment Measures: Plot was contained utilizing a 660 ft isolation distance.

Appendix 2. MATERIALS AND METHODS- PRODUCT CHARACTERIZATION

Breeding diagram



Materials and methods for molecular characterization – DNA tests

Materials
DNA for the analyses was isolated from leaves of GlyTol cotton event GHB614 and the control (Coker 312) produced in the greenhouse. The references included the plasmid pTEM2 (Figure 3) that was used to produce GlyTol cotton event GHB614. For Southern blot analysis of cotton genomic DNA, digested DNA of plasmid pTEM2 (approximately 0.1, 1 10 genomic copies equivalent) was mixed with genomic DNA of Coker 312 (negative control) and separated by electrophoresis on agarose gels. Phage Lambda – *Pst*I digested (Fermentas Life Sciences) was used as a molecular weight marker for size estimation of the DNA fragments.

Identity of the materials
Plants of the transgenic seedlots were treated with glyphosate in order to eliminate eventual non-transgenic plants. The identity of the greenhouse produced material was confirmed by PCR analysis prior to use, to confirm the presence or absence of GHB614, as appropriate. In the case of segregating seedlots, the zygosity of the harvested plants was determined by means of zPCR. The integrity of the isolated DNA was verified in each Southern analysis by observation of the DNA samples on an ethidium bromide stained agarose gel. The identity of the materials used in generational stability analyses was confirmed by chain-of-custody documents and by PCR analysis.

DNA preparation for Southern blot and PCR analyses
Harvested plant tissues were directly transferred and frozen in liquid nitrogen, then stored in an ultrafreezer until DNA preparation. Leaf material, stored in the ultrafreezer, is stable for at least 10 years. Genomic DNA was extracted following standard procedures, and stored at 4°C. Plasmid DNA was prepared from an *E.coli* cell strain containing plasmid pTEM2. Concentration of the different DNA preparations was determined by measuring the fluorescence of the Quant-iT™ PicoGreen® dsDNA Reagent.

Approximately 10 µg of genomic DNA of each material was digested with restriction enzymes (see Chapter IV following the procedure indicated by the manufacturer. Digestions took place in a total reaction volume of 50 µl, and the digests were incubated overnight at 37°C.

Probe template DNA was prepared by means of PCR amplification using the *Taq* DNA polymerase or the Expand enzyme, following standard procedures. Probe templates were [α-^{32}P] labeled.

Southern blot analysis of genomic DNA.
Digested genomic DNA samples were loaded on 1% TAE agarose gels and separated based on size, following standard procedures (Sambrook *et al*., 1989).

An appropriate dilution of the restriction enzyme digested pTEM2 was prepared. With a single copy integration of the transgene into the *Gossypium hirsutum* genome, ten µg of genomic heterozygous DNA would correspond to ca. 26.6 pg of pTEM2 plasmid DNA[*Gossypium hirsutum* genome size: 4.5x10^{9}bp (Arumuganathan and Earle, 1991)], pTEM2 size: 11953 bp]. The amount representing approximately 0.1, 1, 10 plasmid copies per genome was added to 10 µg of digested non-transgenic DNA. This reconstitution sample served as a positive control and was used to show that the hybridizations were performed under conditions allowing

hybridization of the probe with target sequences. Phage Lambda DNA digested with *Pst*I was included as size standard.

PCR preparation of DNA probes

The DNA templates (*2mepsps* gene, Ph4a748At promoter, intron 1 h3 At +TPotp C, and 3' histone At regulatory elements, as well as complete T-DNA) used for probe preparation were synthesized by means of polymerase chain reaction (PCR) amplification, using the Expand™ High Fidelity PCR system (Boehringer Mannheim). Five hundred pg of target DNA were mixed with 10 pmoles of each primer, 200 µM of each deoxyribonucleoside triphosphate, 5 µl expand high fidelity Buffer 2 and 2.6 Units Expand High Fidelity polymerase enzyme in a 50 µl PCR reaction. The amplification of the different products was performed under the following conditions: 95°C for 4 minutes, 5 cycles at 94°C for 1 minute, 57°C for 1 minute, 72°C for 2 minutes, 25 cycles at 94°C for 15 seconds, 60°C for 45 seconds, 72°C for 2 minutes, and 1 cycle at 72°C for 10 minutes. Aliquots of each product were separated on 1% (w/v) agarose gel in 1X TAE buffer and visualized by ethidium bromide staining to verify that the amplified fragments were of the expected size.

The DNA templates were labeled using the 'Ready-to-go DNA labeling system' from Amersham Biosciences. Unincorporated nucleotides were removed by separation on a micro Bio-Spin-30 column from Bio-Rad.

Materials and methods for protein characterization tests

Studies designed to evaluate the 2mEPSPS protein for characteristics associated with food allergens and toxins were conducted using highly purified 2mEPSPS protein produced by *Escherichia coli* expressing the *2mepsps* gene. Six analytical tests show that the 2mEPSPS protein produced in *E. coli* is representative of 2mEPSPS protein produced in GlyTol cotton event GHB614.

Materials
The plant-produced 2mEPSPS protein was isolated from greenhouse-grown plants of GlyTol cotton event GHB614. The identity of the plants was confirmed by PCR. Leaf extract was purified on an antibody affinity column, and the purified protein solution was stored at -10 C or lower until further analyses were performed. The antibody affinity column used for this purification was purchased from Pierce (Rockford, IL, product number 44894), and was prepared using a covalently attached monoclonal antibody specific for 2mEPSPS.

The 2mEPSPS protein reference standard (BCS reference standard, Batch N° LEJ5837, purity 99.52%) was produced in *E. coli*, and purified following a modification of the method of Priestman *et al.* (2005). The protein solution is stored in an Ultrafreezer.

Analysis by N-terminal sequencing
The affinity purified 2m EPSPS protein was loaded onto the PVDF membrane of a sample preparation cartridge (Applied Biosystems, cat# 401950) according to manufacturer's instructions The membrane was punched out and was sent to Eurosequence bv (Groningen, The Netherlands) for analysis of the N-terminal amino acid sequence of the protein by Edman degradation.

Analysis by SDS-PAGE
SDS-PAGE was performed using a Novex Bis-Tris 12% polyacrylamide gel (InVitrogen, CA, product number NP0341BOX) and a MOPS SDS running buffer according to the manufacturer's instructions. The gel was stained with Coomassie brilliant blue in methanol acetic acid water (0.125% Coomassie brilliant blue, 50% methanol, 10% acetic acid, 40% water) for 1 hour and then destained in methanol, acetic acid, water (50% methanol, 10% acetic acid, 40% water) for 1 hour and finally in several changes of methanol, acetic acid, water (5% methanol, 7% acetic acid, 88% water) until the background was clear. BenchMark™ molecular weight markers from InVitrogen Life Technologies (product number 100747-012) were used.

Analysis by western blotting
Western blotting was performed in the same electrophoresis system as used for SDS-PAGE and the gel was blotted to PVDF membranes (New England Nuclear, MA, product number NEF1001) according to the instructions provided by InVitrogen. The proteins in the gel were transferred out of the gel perpendicular to the direction of the first electrophoresis. They were adsorbed to the membrane giving an exact replica of the positions of all the proteins in the gel. The membrane was then exposed to a monoclonal antibody to the 2mEPSPS protein and through a series of additional steps a luminescent tag was attached to the bound antibody to reveal the position of the protein of interest. The second antibody was a horse radish peroxidase (HRP) linked anti-mouse antibody. All reagents except the monoclonal anti-2mEPSPS antibodies used for western blotting were obtained from Amersham Pharmacia Biotech (NJ) as an ECL Plus luminescent detection kit (product number RPN 2108).

MagicMark™ XP molecular weight markers from InVitrogen Life Technologies (product number LC5602) were used.

Analysis by HPLC/Electrospray Mass Spectrometry

The 2mEPSPS protein from *E. coli* and the 2mEPSPS protein from the GlyTol cotton event GHB614 sample were digested for 1 hour at 37 °C in Rapid Gest (Waters Corporation) containing 4 mM DTT. The peptides of the 2mEPSPS protein from *E. coli* were separated by HPLC and a full scan mass spectrum was obtained for individual peptides using an electrospray mass spectrometer equipped with a quadrupole ion detector. Two HPLC columns were used. One was suited for separation of large and small peptides and the other was suited for separation of peptides smaller than about 3 kDa. Individual peptides from the *E. coli* 2mEPSPS protein were identified by their mass to charge ratio (calculated from the amino acid sequence for that peptide and the number of expected charges). The most abundant charge state was normally used for selected ion monitoring (SIM) of the peptides produced by GlyTol cotton. The peptides from GlyTol cotton event GHB614 were analyzed under the same HPLC and mass spectrometer conditions as the peptides from the 2mEPSPS protein from *E. coli.* The presence of the selected ion (identified in the *E. coli* 2mEPSPS protein) at the expected retention time demonstrates the presence of that peptide in the 2mEPSPS protein from GlyTol cotton. The peak height of the selected ion had to be 3X background to be identified.

Glycoprotein staining analysis

The 2mEPSPS proteins purified from *E. coli* and from GlyTol cotton were separated by SDS-PAGE as described previously. A set of glycoprotein molecular weight standards was included on the gel. This set of marker proteins forms an alternating ladder of glycosylated and non-glycosylated proteins. The presence of sugar residues on the proteins was tested using the GlycoProfile™ III fluorescent glycoprotein detection kit (part number PP0300) from Sigma-Aldrich Co., MO. After staining with the GlycoProfile™ detection kit, the gel was stained with Coomassie brilliant blue as described above for the analysis by SDS-PAGE.

Analysis of enzymatic activity

The enzymatic activity of the purified 2mEPSPS proteins was analyzed according to Forlani *et al*. (1994). EPSPS activity was measured in the forward direction using shikimate-3-phosphate and phosphoenol pyruvate as substrates. The amount of released inorganic phosphate during catalysis was determined according to the method described by Lanzetta *et al*. (1979) with minor modifications.

Materials and methods for protein levels in seeds

Seed samples analyzed in this study were produced under field conditions in 2005 alongside the materials for composition analysis, and were grown from seed lot # 05XEEPR SD INC Field A. The field-produced seed samples were assayed by PCR testing to confirm their identity.

An *E.coli* produced 2mEPSPS protein standard (BCS batch # NB2903405P183) was used as a reference for analysis, and to fortify non-transgenic samples for validation and recovery studies. BSA (Sigma-Aldrich Chemical Company Product number P-0914) was used as a reference substance to determine total extractable protein in the Bradford assay.

Field design

Cotton plants containing the transgenic GlyTol cotton event GHB614 and cotton plants representing the non-transgenic (non-transformed) counterpart Coker 312 were field tested by Bayer CropScience in 2005 under USDA notification 05-091-07n. Trials were conducted in EPA Regions II, III, IV, VI and VIII in the following locations: Trial number 02-01-Tift County, Georgia; Trial number 03-02-Escambia County, Florida; Trial number 04-03-Jackson County, Trial number 04-04-Crittenden County, Arkansas; Trial number 04-05-Drew County, Arkansas; Trial number 04-06-Tate County, Mississippi; Trial number 04-07-Tate County, Mississippi; Trial number 06-08-Wharton County, Texas; Trial number 08-09-Hockley County, Texas, which are typical cotton growing regions of the southeastern United States. The plants in this study were grown under conditions typical of production practices. There were six transgenic plots and three non-transgenic plots at each test site. Three of the transgenic GlyTol cotton event plots were sprayed three times with glyphosate herbicide, and the other six plots were untreated. Each application of glyphosate herbicide was made at a rate of 0.75 pounds of active ingredient (glyphosate acid equivalent) per acre. One sample of ginned cottonseed (fuzzy seed) was obtained from each test plot. The samples were shipped frozen to the laboratories of Bayer CropScience. Shipping and storage of the regulated seed was carried out under applicable USDA regulations and Bayer CropScience guidelines.

Certificates of analysis (COA) were produced by the BCS QA Laboratory in Lubbock, TX and at BCS, Research Triangle Park for seed shipped to the nine field test sites for planting. The data showed that the transgenic cottonseed that was planted in the field was indeed GlyTol cotton, and that the non-transgenic seed (Coker 312) supplied to the field contained less than 1% of GlyTol cotton seed with 95% confidence. Adventitious presence of several other cotton genotypes was also checked in the GHB614 and Coker 312 seed. There were no positive results in these analyses indicating the seed lots contained very low, if any, amounts of other cotton genotypes.

Sample preparation

Fuzzy seed was difficult to grind to homogeneity with dry ice due to the residual lint adhering to the seed. During the grinding with dry ice, some of the seed broke away from the lint but the lint rapidly became entangled in the blades of the grinder making further grinding impossible. Gently grinding the fuzzy seed in a mortar and pestle in the presence of liquid nitrogen produced a relatively clean seed fraction (designated as a kernel sample) and a sample of short strands of cotton lint and seed coat material, which is identified as lint coat sample for this study. The lint coat samples produced by gentle grinding in liquid nitrogen were easily separated from the kernel fraction because they held together in a loose intertwined agglomerate that could be manually transferred to a separate tube. The lint coat fraction was manually inspected for entangled kernels, and when these were found they were

added to the kernel fraction. The weights of the kernel and lint coat fractions were recorded for each sample of fuzzy seed that was analyzed. This made it possible to reconstruct the amount of 2mEPSPS protein in the fuzzy seed as it was received from the field.

Each of the frozen kernel samples (equivalent to approximately 20 grams of fuzzy seed) was ground in a Waring Laboratory Blender prechilled with dry ice, adding dry ice as necessary to ensure the samples remained frozen during preparation. In between samples, the blender was washed with soapy water using a brush, rinsed twice with hot water and twice with deionized water, and dried with an air stream. The ground samples were stored in a freezer at approximately -20°C for overnight or longer to allow the dry ice to dissipate before extraction. The lint coat samples were not ground in dry ice. They were used directly in all the assays.

Protein extraction

The 2mEPSPS protein was extracted from raw agricultural products of cotton using a buffer described in the publication of Xin *et al*., (1988). The extraction buffer provided with the ELISA plates by the manufacturer was used. A representative sample (approximately 0.5 g) of ground sample was mixed with the extraction buffer (5 mL for lint coat and 50 mL for kernel) in a 50 mL polypropylene centrifuge tube, shaken for 15 minutes at ~ 4°C on a shaker (IKA-SCHÜTTLER MTS 4) at 250 rpm and then centrifuged at approximately 4100 x g for 5 minutes at ~ 4°C. The supernatant was transferred to a clean centrifuge tube for another cycle of centrifugation at approximately 18000 x g for 5 minutes at ~ 4°C. The clear supernatant was then used for 2mEPSPS and TEP analyses. Duplicate extracts were prepared for each sample.

Bioassay

A commercial ELISA kit is not available for 2mEPSPS protein at the time of these tests, but a validated ELISA available at Bayer CropScience was used to measure the amount of 2mEPSPS present. The amount of 2mEPSPS in the total protein extracts was measured using a quantitative ELISA developed by Strategic Diagnostics Inc. (SDI, Newark, DE, USA). Before the analysis was performed the Limit of Detection (LOD) was determined for each tissue.

Serially-diluted sample extracts were applied to ELISA plates at 100 µL/well. This was followed by a period of incubation on a shaker at 900 rpm at room temperature. The 2mEPSPS protein that was present in the samples was bound to the capture antibody. Unbound material was removed by rinsing the wells 4 times with wash solution. The plate was subsequently incubated with a second antibody, which recognizes 2mEPSPS protein, followed by a series of rinses with wash solution. The plate was then incubated in the same way with a third antibody conjugated to horseradish peroxidase and washed the same way.

A peroxidase substrate, Tetramethylbenzidine (TMB), was then added and converted by the peroxidase to a blue product in proportion to the amount of protein present in the sample. The reaction was stopped with 0.5 M H_2SO_4 and the color changed to yellow. The resulting color development was measured in a microplate reader (Molecular Devices THERMOmax) at 450nm.

Validation

The ELISA procedures were validated for kernel and lint coat samples from a non-transgenic counterpart cotton line (Coker 312) using the protein standards 2mEPSPS and BSA. A validation was performed for 2mEPSPS using the non-transgenic kernel and lint coat samples fortified at the concentrations listed in Table 2.1. The standards were added to the extraction buffer at the indicated concentrations prior to extraction in 5 replicates. Each replicate was

analyzed using duplicate wells. A summary of the validation data is shown for kernel and lint coat matrices in Table 2.1.

Limit of detection and limit of quantification

The limit of detection (LOD) is determined for each matrix using the average standard curve and the concentration derived from the background optical density (OD) of the negative control samples. The LOD is the concentration corresponding to an OD value three standard deviations above the mean background OD.

Table 2.1. Validation of sample extraction for the 2mEPSPS ELISA with fortified non-transgenic controls of kernel and lint coat

Sample ID	Validation of Cotton Seed Kernel for 2mEPSPS (Field Sample ID: 04-03, BTID: 1001B)					Validation of Lint Coat for 2mEPSPS (Field Sample ID: 04-03, BTID: 1001B)				
2mEPSPS Fortified (ng/mL)	2mEPSPS Detected (ng/mL)[a] Mean ± SD		% 2mEPSPS Recovery Mean ± SD		2mEPSPS Recovery % CV	2mEPSPS Detected (ng/mL) Mean ± SD		% 2mEPSPS Recovery Mean ± SD		2mEPSPS Recovery % CV
15	15.6 ±	0.7	104 ±	5	4.79	14.6 ±	1.2	97.6 ±	7.7	7.89
3.75	1.81 ±	0.15	99.9 ±	5.5	5.50	3.00 ±	0.36	79.9 ±	9.7	12.2
1.875	0.73 ±	0.13	96.4 ±	8.0	8.27	1.35 ±	0.23	72.3 ±	12.3	17.1
0.938	0.01 ±	0.18	77.9 ±	14.1	18.0	0.82 ±	0.18	87.7 ±	19.2	21.9

[a] The 2mEPSPS protein detected and its recovery are expressed as the average of 8 data points from duplicate extracts of 4 samples at each fortification level using non-transgenic matrix.

The limit of detection is expressed in the unit of concentration (ng/mL) and the unit of weight ratio (ng/g matrix, *i.e.* ppb) calculated based on the extraction of an amount of the matrix with a known volume of extraction buffer, *e.g.*, 1 g of matrix/10ml extraction buffer. The data are summarized in Table 2.2. An absorbance reading giving rise to a 2mEPSPS concentration above this limit of detection level is assumed to be greater than the zero dose reading.

The limit of quantification (LOQ) is given by the lowest concentration of the standard that meets the criteria for the LOQ. Validity criteria are a) analyte recoveries from fortified matrix samples are ≥ 60 % and ≤ 130 % and b) the coefficient of variance (relative standard deviation) is less than 25%. When a lower recovery is caused by the nature of a specific matrix or the effect of a process, the lowest concentration of the standard that gives a coefficient of variance equal to or less than 25% is used as the LOQ. Values below the LOD are reported as ND (Non-detectable) and values below the LOQ but above the LOD are reported as '<LOQ'. The LOQ values are determined by inspection from Table 2.1.

Protein determination

Protein determinations were made in order to confirm that protein was extracted from the samples. The Bradford assay (Bradford, 1976; Sedmak, 1977) was used to determine the concentration of total extractable protein (TEP). The assay relies on the binding of the dye Coomassie blue G250 to protein. The anionic form of the dye, which binds to protein, has a maximum absorption at 595 nm. The amount of absorption at 595 nm produced is therefore correlated to the protein concentration. Bovine Serum Albumin (BSA) was used as protein standard at 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL in the assay.

Table 2.2. Limits of detection and quantification of 2mEPSPS protein in raw agricultural commodities of cotton as detected by enzyme assay.

Protein Analyte	Matrix	Extraction Ratio (g matrix/mL extraction buffer)	LOD		LOQ	
			(ng/mL)	ng/g Sample	(ng/mL)	ng/g Sample
2mEPSPS	Kernel	1:200	0.694	139	0.938	188
2mEPSPS	Lint Coat	1:60	0.479	28.7	0.938	56.3

The total extractable protein was determined for each sample extract. Duplicate 10µL aliquots of the sample extract were placed in wells of a 96-well plate (Costar No. 3590) and 200 µL of Bradford Reagent (Sigma-Aldrich Chemical Company, Product number: B-6916) was added. After 12 ± 3 minutes of incubation on a shaker (IKA-SCHÜTTLER MTS 4) at 700 rpm at room temperature, the optical density (OD) was measured in a microplate reader (Molecular Devices THERMOmax) at 595 nm.

Protein analyte content

SoftMax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of 2mEPSPS protein. Absorbance units were adjusted for the buffer blank and then any background due to the matrix was subtracted, using values from wells containing non-transgenic extracts, assayed on the same plate. This correction was used for all samples except the transgenic seed as discussed in the following paragraph. The absorbance readings corrected for both buffer blank and non-transgenic background were converted to the protein concentration using the standard curve.

A set of wells containing samples of the corresponding non-transgenic matrix was always included on a plate for background subtraction. The appropriate background corrections for the transgenic kernel samples were obtained from background values of a non-transgenic kernel sample (BTID 1001B), which was diluted on the same plate and to the same extent as the transgenic kernel sample. Thus the dilution of the non-transgenic sample used for background subtraction was the same as the dilution of the transgenic sample that was required to place the OD reading in the center portion of the standard curve. To obtain negative samples for use in background subtraction, several small subsamples of kernel from sample BTID 1001B were ground and tested by ELISA. Only those subsamples which gave results < LOD were used as the negative controls for the transgeinc kernel samples.

The absorbance assays give results in units of ng of analyte per milliliter of extract that are then converted into ng or µg of analyte per gram of fresh sample. As different tissues have different protein and water contents, the results are also expressed in this report as percent of Crude Protein and as percent of Total Extractable Protein (TEP). Samples were analyzed for crude protein and moisture at Eurofins and for TEP at the Molecular and Biochemical Analytical Services Laboratories, BCS, NC. The calculations and conversions were done as described below.

The values of percent crude protein on a fresh weight basis were used to calculate the 2mEPSPS protein concentration as percent of Crude Protein. Percent moisture of each matrix is also determined for reference. The following is the conversion formula used:

$$\text{Average 2mEPSPS as \% Crude Protein} = \frac{\text{Average µg 2mEPSPS/ g sample}}{\text{\% Crude Protein FW/ g matrix}} \times 10^{-2}$$

The values of analyte protein as percent of TEP were calculated using the values generated by ELISA assay [Average analyte protein (µg/g sample)] and the values generated by the Bradford Assay [Average TEP (mg/g sample)]. The following is the conversion formula used:

$$\text{Average 2mEPSPS Protein as \% Total Extractable Protein} = \frac{\text{Average µg 2mEPSPS protein/g sample}}{\text{Average mg TEP / g sample}} \times 10^{-1}$$

Protein content (Bradford) µg

SoftMax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of protein from the Bradford assay (Bradford, 1976; Sedmak, 1977). The optical density was converted to the TEP concentration using the standard curve. The data point for the dilution falling nearest the center of the standard curve was used. If two points were near the center of the curve, the data for the least diluted sample was used.

Statistical analysis

Descriptive statistics (mean, standard deviation, and coefficient of variance) were calculated for each sample matrix and treatment (Devore and Peck, 1986). An analysis of variance (ANOVA) was performed on the data for 2mEPSPS protein content in kernel and lint coat fractions at a significance level of 0.01 (α = 1%). Independent variables evaluated were the site, treatment, extract and assay. The null hypothesis states that there are no differences between the values of analyte protein content (dependent variable) due to the independent variables. A small probability (p-value) means that an observed difference is unlikely to occur by chance, so the null hypothesis should be rejected. A low p-value (< 0.01) suggests that there is a significant difference caused by the effect analyzed. StatView® 5 (SAS Institute, Cary, NC) was used for ANOVA. BCS conducted all statistical analyses. All statistical analyses were done on data with full precision. Results may be rounded to two or three significant numbers.

Results

The average amounts of 2mEPSPS protein, the average amounts of TEP and the average amounts of 2mEPSPS protein as a percent of TEP for sprayed and non-sprayed fuzzy seed are provided in Tables 2.3 and 2.4. The total amount of TEP in non-transgenic samples is given for comparison in Table 2.5.

Table 2.3. Average 2mEPSPS protein concentration, Total Extractable Protein (TEP) concentration and 2mEPSPS protein concentration as a percent of TEP for non-sprayed fuzzy seed samples

BTID	Field Sample ID DQ05B001-	Average ng 2mEPSPS Protein/g Sample	Average mg TEP/g in 2mEPSPS Sample	2mEPSPS as % TEP
1000D, 1000E, 1000F	02-01	15.9	32.8	0.049
1015D, 1015E, 1015F	03-02	18.6	27.1	0.069
1001D, 1001E, 1001F	04-03	17.5	34.4	0.051
1002D, 1002E, 1002F	04-04	17.3	29.4	0.059
1003J, 1003K, 1003L	04-05	19.7	31.4	0.063
1004D, 1004E, 1004F	04-06	15.8	33.9	0.047
1005D, 1005E, 1005F	04-07	21.9	43.2	0.051
1016D, 1016E, 1016F	06-08	20.9	29.0	0.072
1017D, 1017E, 1017F	08-09	25.5	34.0	0.086
Range of values		15.8 – 25.5	27.1 – 43.2	0.047 – 0.086

Table 2.4. Average 2mEPSPS protein concentration, Total Extractable Protein (TEP) concentration and 2mEPSPS protein concentration as a percent of TEP for sprayed fuzzy seed samples

BTID	Field Sample ID DQ05B001-	Average µg 2mEPSPS Protein/g Sample	Average mg TEP/g in 2mEPSPS Sample	2mEPSPS as % TEP
1000G, 1000H, 1000I	02-01	18.4	33.7	0.055
1015G, 1015H, 1015I	03-02	20.0	27.1	0.074
1001G, 1001H, 1001I	04-03	21.5	31.8	0.067
1002G, 1002H, 1002I	04-04	19.3	31.8	0.061
1003M, 1003N, 1003O	04-05	20.3	27.5	0.074
1004G, 1004H, 1004I	04-06	16.2	33.4	0.049
1005G, 1005H, 1005I	04-07	21.8	41.9	0.052
1016G, 1016H, 1016I	06-08	23.0	29.3	0.078
1017G, 1017H, 1017I	08-09	30.5	35.4	0.086
Range of values		16.2 – 30.5	27.1 – 35.4	0.049 – 0.086

Table 2.5. Average Total Extractable Protein in Non-Transgenic Fuzzy Seed Samples Analyzed for 2mEPSPS Protein

BTID	Field Sample ID DQ05B001-	mg TEP per g Sample
1000A, 1000B, 1000C	02-01	33.0 ± 3.4
1015A, 1015B, 1015C	03-02	23.9 ± 0.6
1001A, 1001B, 1001C	04-03	29.8 ± 3.0
1002A, 1002B, 1002C	04-04	28.9 ± 0.7
1003A, 1003B, 1003C	04-05	31.3 ± 1.5
1004A, 1004B, 1004C	04-06	33.3 ± 1.5
1005A, 1005B, 1005C	04-07	36.1 ± 2.7
1016A, 1016B, 1016C	06-08	35.2 ± 2.3
1017A, 1017B, 1017C	08-09	35.9 ± 3.3
Range of values		23.9 – 36.1

Materials and methods for protein levels in plant parts and during the life cycle

In order to analyze the presence of 2mEPSPS protein in plant parts, samples were crushed, extracted and the TEP content was determined using the Bradford method (Bradford, 1976).

Materials

Samples from 6 different tissues of GlyTol cotton event GHB614 were harvested separately covering 4 different growth stages of the plant. Transgenic and non-transgenic plants were chosen randomly out of a starting population of 240 plants. In the first growth stage (V2-V3) leaf specimens from 15 plants were taken, in the other growth stages separate specimens per tissue were harvested from 10 plants (see Table 2.6). In the first growth stage (V2-V3) and the third growth stage (pre-flowering) young leaf tissue was sampled. In the second and fourth growth stage (V4-V6 and flowering) stem, root and young leaf tissues were sampled. Square, apex and pollen tissues were also sampled in the fourth growth stage. Identical specimens were taken from the non-transgenic cotton line. Specimens were stored at -70°C at the facilities of Bayer BioScience N.V. (Ghent, Belgium), until further analysis.

Genomic DNA of the transgenic and non-transgenic reference seeds used to grow the plants were analyzed for the presence or absence of the *2mepsps* coding sequence to confirm the identity of the plant material analyzed in this study, using a zygosity PCR (zPCR).

An *E. coli* produced 2mEPSPS protein standard (BCS batch # NB2903405P183) was used as a reference for analysis, and to generate a standard curve.

Table 2.6. Growth stages for harvest of tissue samples and number of plants sampled

Stage	Growth stage	Tissue	Days after planting	Plants sampled
1	V2-V3	Leaf	16	15
2	V4-V6	Leaf, stem, root	33	10
3	Pre-flowering	Leaf	51	10
4	Flowering	Leaf, stem, root	68	10
		Apex, square, pollen		Pool *

*At flowering stage, square, apex and pollen tissues were polled from several plants, as less material was available.

Sample preparation and protein extraction

For the expression analysis of 2mEPSPS protein 5 separate specimens per tissue were chosen randomly from the material harvested. Square, apex and pollen tissues were pooled from several plants. Each tissue, except for pollen, was ground for 30 seconds in a Waring blender pre-cooled with dry ice. Frozen tissue specimens, together with dry ice, were ground until all crushed material was homogeneous, adding dry ice as necessary. All the crushed powder with dry ice was collected in 50mL Falcon tubes and stored overnight at -20°C to remove all carbon dioxide by leaving a small opening for evaporation. Pollen tissue was processed without crushing.

For each transgenic specimen an appropriate amount was weighed into 2 separate 50mL Falcon tubes. Because of the limited amount of tissue, one 50mL Falcon tube was prepared with the appropriate amount of crushed square, apex or pollen tissue. For non-transgenic

specimens one 50mL falcon tube was prepared. The measured weights are shown in Table 2.7.

Table 2.7. Dilution rate of crushed tissue material used in expression analysis

Tissue	Amount of tissue mg	Volume extraction buffer mL	Dilution
Leaf	250	7.50	1:30
Stem	250	7.50	1:30
Root	250	3.75	1:15
Square	250	7.50	1:30
Apex	250	7.50	1:30
Pollen	25	0.75	1:30

The above volumes of extraction buffer were added to the crushed tissue samples in pre-cooled 50mL Falcon tubes. After 30 minutes of shaking at 250rpm at 7°C, the tubes were centrifuged at 4000g for 20 minutes at 4°C. Supernatants were collected in deepwell refill tubeholders resulting in one sample for non-transgenic tissue. Collections were made in duplicate for transgenic tissue, as well as for transgenic and non-transgenic pollen.

Limit of detection
After crushing and extraction of the non-transgenic samples, the Limit of Detection (LOD) was determined using pre-coated prototype 2mEPSPS ELISA plates developed by SDI (Newark DE, USA, lot #NB2285-61-1). This assay is a sandwich ELISA based on the specific interaction between antibody and antigen. The wells of the ELISA plate were coated with monoclonal antibodies (capture antibodies), and the captured protein was detected by polyclonal antibodies. Both antibodies were raised against the 2mEPSPS produced in bacteria. The polyclonal antibody was linked to a horseradish peroxidase conjugate. A peroxidase substrate, tetramethylbenzidine, was added and converted to a blue product in proportion to the amount of 2mEPSPS protein present in the sample. Upon the addition of an acidic solution to stop the reaction, the blue product turned yellow. The optical density of the yellow product at 450nm reflected proportionally the 2mEPSPS protein content present in the sample. Absorbances were measured using the multifunctional monochromator Safire^2 (Tecan, Grödig, Austria). A standard curve was included with 2mEPSPS protein produced in bacteria and used in a dilution series of 15 – 7.5 – 3.75 – 1.875 – 0.938 – 0.469 – 0 ng/mL.

After extraction all samples were analyzed in duplicate resulting in 20 measurements of the individual non-transgenic tissues. For pollen tissue, as the available amount was limited, only 10 measurements were performed originating from one extraction.

The standard curve was fitted to the data points, obtained from the standard dilution series, using a second order equation in the Graphpad Prism 4 software (version 4.03, GraphPad Software, Inc.).
The LOD was determined as follows:
The absorbance or optical density (OD) of the non-transgenic samples was determined and values were corrected for the buffer blank.
The average OD was calculated and subtracted from each of the individual OD values.

The average of the individual corrected OD values generated in step (2) was determined. This is the average adjusted OD value and is mathematically zero.
The standard deviation of the individual corrected OD values generated in step (2) was determined and multiplied by 3.
The standard deviation generated in step (4) was added to the average adjusted OD value determined in step (3).
The resulting OD value was used to extrapolate the (2m)EPSPS protein content from the standard curve obtained with the Graphpad Prism 4 software. This value corresponds to the LOD expressed as ng per mL.
The value obtained in step (6) was converted to µg/g by multiplying by the dilution factor.

Calculation of the LOD gives a 99% probability of detecting 2mEPSPS protein in a tissue sample when it is present in an amount equal to the LOD. So, there is a 99% probability that a concentration of 2mEPSPS at or just above the LOD is positive (Table 2.8).

Table 2.8. LOD for the 2mEPSPS ELISA in the different tissues

Matrix	LOD $x10^{-3}$ µg/g
Leaf	4.47
Stem	8.34
Root	27.33
Square	27.33
Apex	8.10
Pollen	16.08

An ELISA absorbance resulting in a 2mEPSPS concentration equal to or above the LOD level is assumed to represent a positive detection.

Determination of Total Extractable Protein content

After extraction the TEP was measured according to the Bradford protein assay using bovine serum albumin as reference protein and measuring the Optical Density (OD) at 595nm (Bradford, 1976).

Protein analyte content
After crushing and extraction of the specimens, the presence or absence of the 2mEPSPS protein in all transgenic and non-transgenic samples was determined by ELISA. The final dilutions used for the analysis of all transgenic tissues at the different growth stages in the ELISA are described in Table 2.9. Non-transgenic tissue samples were analyzed in the same dilutions to correct for the matrix background. All tissue samples were analyzed in duplicate.

Table 2.9. Final dilutions for the GHB614 cotton tissues

Matrix	Final dilutions			
	Growth stage 1	Growth stage 2	Growth stage 3	Growth stage 4
Leaf	1:900	1:900	1:1800	1:300
Stem	ND	1:210	ND	1:420
Root	ND	1:150	ND	1:150
Square	NA	NA	NA	1:210
Apex	ND	ND	ND	1:210
Pollen	NA	NA	NA	1:50

* ND: Not Determined, NA: Not Applicable

For the analysis of the 2mEPSPS protein contents in undiluted non-transgenic samples, all tissues were analyzed in duplicate.

A positive control and a standard curve were included based on 2mEPSPS protein produced in bacteria. The standard curve was made in a dilution series of 15 – 7.5 – 3.75 – 1.875 – 0.938 – 0.469 – 0 ng/ml.

Calculations and conversions

The measured absorbances of all transgenic tissue samples were corrected with the average signal of the buffer-only sample (blank). Then the obtained values were corrected for the specific tissue background. In this case the values of the diluted non-transgenic tissue samples were subtracted from the transgenic values. All non-transgenic tissue samples were corrected in an identical manner. The corrected measured absorbances of all sample tissues were converted to concentrations of 2mEPSPS per mL by fitting them to the standard curve. The standard curve was fitted to the data points, obtained from the standard dilution series, using a second order equation in the Graphpad Prism 4 software (version 4.03, GraphPad Software, Inc.). These 2mEPSPS protein concentrations were converted into µg/g fresh weight taking into account the applied dilution factor. To correct for the different protein contents in tissues, the 2mEPSPS protein concentrations were also expressed as percent of TEP.

Statistics

Descriptive statistics (mean, standard deviation, and coefficient of variance) were calculated for each tissue and growth stage using Microsoft® Excel 2002. Outliers were determined by the Grubb´s test, also known as the maximum normalized residual test, at the 95% confidence level. The Grubb´s test, applied to the population means (averages) and standard deviations, detects one outlier at a time. Outliers within the absorbance values of 10 buffer blank samples were omitted from the calculation of the average of the buffer blank. This average was then subtracted from the absorbance values of the transgenic, non-transgenic and the standard serial dilution samples. Then outliers within the absorbance values of non-transgenic samples were determined before subtracting the average of these background samples from the transgenic or non-transgenic absorbance values to correct for the specific background tissue. These calculations were performed by Bayer BioScience N.V. (Ghent, Belgium).

An analysis of variance (ANOVA) was performed on the data for 2mEPSPS protein contents in all different transgenic tissues at a significance level of 0.01 (α = 1%). These calculations

were performed using StatView®5 (SAS Institute, Cary, NC, USA) by BCS (Research Triangle Park, NC, USA). Independent variables evaluated were the growth stage, extraction process and the assay itself, the extraction process and sampling of extracts for ELISA analysis.

Materials and methods for agronomic efficacy studies

Materials

Materials for efficacy evaluation were created at field sites in 2004 and 2005 in the southeastern, mid-southern, and mid-western regions of the United States. Eight locations in 2004, and nine locations in 2005 in five states were used to produce the reference material for fiber analysis, and the plants used in agronomic performance. Material was obtained from the three treatment regimes of the transformed cotton and their corresponding non-transgenic counterpart in the Coker 312 variety.

Characterization of the Materials

Identity of the materials was preserved through chain of custody documentation. Chain of custody documentation was utilized to identify the materials shipped to their respective field sites for proper identification of the evaluated plots in the field. Harvested materials contained chain of custody documentation for samples sent from the field to analytical laboratories to preserve identity.

Performing Facility and Experimental Methods

Trials in 2004 and 2005 were utilized to characterize and evaluate agronomic performance of the selected event, and develop materials for nutritional and compositional testing. Trials were conducted in three geographic regions of the United States.

Field studies were managed in a manner representative of normal agricultural practices for inputs including, but not limited to:

- Conventional herbicide treatments, both pre- and post- planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide applications
- Growth regulator application
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants

All trials received similar agronomic treatments for the care and upkeep of the plots. Insect pressure was strictly controlled to ensure that the transgenic lines did not cross pollinate with non-transgenic lines and to eliminate this variable. Field studies utilized an experimental treatment regime which compared the transformed event GHB614 sprayed, and unsprayed compared to the non-transformed counterpart of the Coker 312 upland cotton variety.

Trials were performed using a randomized complete block design using two row plots with four replications and four treatments (Table 2.10). A total of 30 agronomic parameters were used to measure the growth and development of the plant, and provide visual observations on the effect of any biotic and abiotic stressors upon the field plots across regions. Of the agronomic parameters observed, seven determined yield quality and quantity, 18 were conducted to evaluate growth habit and agronomics, three measured impact of herbicide treatments (abiotic stress), and two measured biotic stress factors. These parameters were selected as key indicators of commercial and agronomic importance to commercial cotton growers, and the

ability of the crop to perform under a variety of stresses from the different growing regions around the county.

Table 2.10. Treatment schedule for agronomic field tests in 2004 and 2005

Label	Treatment	Description
UTC	Not sprayed	Non-transgenic unsprayed
Control	Not sprayed	Transgenic GHB614 unsprayed
1x	Glyphosate	450 g a.i. glyphosate per acre
3x	Glyphosate	1350 g a.i. glyphosate per acre

Plant mapping was conducted on 10 consecutive plants in the plots which were representative of general field conditions. Plant height, number of nodes, first fruiting position, and total boll count were taken as a measure of agronomic performance throughout the year. This data showed plotted the development and potential reproductive success of the cotton plant as an indication of the yield of the plant. Other agronomic parameters were evaluated on a visual rating of 1-9 (1 = most favorable rating and 9 = least favorable rating, specific units depending on factor evaluated).

Disease and lodging were observed in the test plots as biotic stress factors which may express differently in the transformed and non-transformed plots. Additionally, Chlorosis was evaluated as a potential abiotic stressor at three different times corresponding with the herbicide applications made to the transgenic lines. These parameters were observed through visual observation and rated on a scale of 1-5 (1 = no impact, 5 = severe impact). Insect populations were not evaluated as this variable was controlled through conventional insecticide applications as needed to both the transformed and non-transformed lines, and was therefore not an influencing factor in the tests.

Statistical Analysis

Analysis of variance (ANOVA) between groups was calculated to analyze data for significant differences. All treatments were analyzed in comparison to their non-transgenic counterpart across regions, regionally, and locally. Data was reviewed using a confidence interval of 95%.

Materials and methods for agronomic equivalence studies

Materials

Materials for efficacy evaluation were created at field sites in 2005 in the mid-southern, and southwestern regions of the United States. Three locations were chosen in each of the major cotton growing regions to evaluate equivalence of the GlyTol cotton event GHB614 in Coker 312 and commercial cotton varieties. Material was obtained from the three treatment regimes of the transformed cotton and their corresponding non-transgenic counterpart in the Coker 312 variety.

Characterization of the Materials

Identity of the materials was preserved through chain of custody documentation. Chain of custody documentation was utilized to identify the materials shipped to their respective field sites for proper identification of the evaluated plots in the field. Harvested materials contained chain of custody documentation for samples sent from the field to analytical laboratories to preserve identity. Additionally, two leaf samples were taken from two individual plants per plot for PCR analysis to confirm identity of plots in the tests.

Performing Facility and Experimental Methods

Equivalence trials in 2005 were utilized to characterize and evaluate equivalence of the GlyTol™ Cotton GHB614 in Coker 312 and commercial cotton varieties. Trials were conducted in three geographic regions of the United States (see Table 9).

Field studies were managed in a manner representative of normal agricultural practices for inputs including, but not limited to:

- Conventional herbicide treatments, both pre- and post- planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide applications
- Growth regulator application
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants

All trials received similar agronomic treatments for the care and upkeep of the plots. Glyphosate herbicide was not applied to any treatment, and insect pressure was strictly controlled to ensure that the transgenic lines did not have an advantage over the unconverted recurrent parent lines. Field studies utilized an experimental treatment regime which compared the non-transformed Coker 312 and commercial varieties with the transformed GlyTol cotton event GHB614 in the same varieties.

Trials were performed using a randomized complete block design using two row plots with four replications of four treatments (Table 2.11). A total of 16 agronomic parameters were used to measure the growth and development of the plant, and provide visual observations on the effect of any biotic and abiotic stressors upon the field plots across all locations. An additional 11 parameters were evaluated in the mid-southern regions. Of the 27 potential agronomic parameters observed, seven determined yield quality and quantity, 15 were conducted to evaluate growth habit and agronomics, and five were visual measures of biotic and abiotic

stress (chlorosis, disease and lodging). These parameters were used to evaluate equivalence in crop development, fiber qualities, and environmental tolerance.

Table 2.11. Treatment schedule for Equivalence Studies Conducted in 2005

Label	Treatment	Description
Coker 312	Not sprayed	Non-transgenic Coker 312 variety
Commercial	Not sprayed	Non-transgenic Commercial Cotton Variety
Coker 312/GHB614	Not sprayed	Coker 312 variety transformed with GHB614
Commercial/GHB614	Not sprayed	Commercial cotton variety backcrossed to incorporate the GHB614 trait

Plant mapping was conducted on 10 consecutive plants in the plots which were representative of general field conditions. Plant height, number of nodes, first fruiting position, and total boll count were taken as a measure of agronomic performance throughout the year. This data showed plotted the development and potential reproductive success of the cotton plant as an indication of the yield of the plant.

Disease and lodging were observed in the test plots as biotic stress factors which may express differently in the transformed and non-transformed plots. Additionally, Chlorosis was evaluated as a potential abiotic stressor at three different times corresponding with the herbicide applications made to the transgenic lines. These parameters were observed through visual observation and rated on a scale of 1-9 (1 = no impact, 9 = severe impact). Insect populations were not evaluated as this variable was controlled through conventional insecticide applications as needed to both the transformed and non-transformed lines, and was therefore not an influencing factor in the tests.

Statistical Analysis

Analysis of variance (ANOVA) between groups was calculated to analyze data for significant differences. All treatments were analyzed in comparison to their non-transgenic counterpart across regions, regionally, and locally. Data was reviewed using a confidence interval of 95%.

Materials and methods for seed germination studies

Materials

Materials were created in efficacy trials conducted at nine locations in 2005. Samples were taken from each plot replicate from locations in the southeastern, mid-southern, and southwestern United States.

Characterization of the Materials

Identity of the materials was preserved through chain of custody documentation. Chain of custody documentation was utilized to identify the materials shipped to their respective field sites for proper identification of the evaluated plots in the field. Harvested materials contained chain of custody documentation for samples sent from the field to analytical laboratories to preserve identity

Performing Facility and Experimental Methods

Seed germination assays were conducted at Bayer Commercial Seed Laboratory in Lubbock, TX. Seed germination studies were conducted at two different temperatures to determine if the transformed line altered the dormancy of the cottonseed. Different temperatures and timings were used to simulate seed just produced from a viable cotton plant and seed stored at lower temperatures to simulate commercial seed practices. Four hundred seeds each of Coker 312/GBH614 and the non-transgenic counterpart, were collected on six different locations immediately upon harvest. Seed samples were divided into two seed lots of 200 seeds each, lot A and B. The seed lots were either germinated immediately upon harvest at 28°C and 18°C or stored at room temperature for six months and then germinated (storage of seed is done to complete the desiccation of freshly harvested seed. Freshly harvested seed with too much moisture impacts the germination, so storage for six months is done to simulate commercial practice). The seed lots were then submitted to a standard warm and cool germination test and evaluated for germination. Breaking dormancy is not an absolute requirement for cotton seeds, and a cold treatment or period of storage is sometimes used to increase the number of germinating seeds. The data show that the period of storage greatly increased germination rate while reducing germination variability of both the transgenic and non transgenic counterpart.

Statistical Analysis

Analysis of variance (ANOVA) between groups was calculated to analyze data for significant differences. All treatments were analyzed in comparison to their non-transgenic counterpart across regions, regionally, and locally. Data was reviewed using a confidence interval of 95%.

Materials and methods for fiber quantification studies

Materials

Materials were generated from agronomic performance field trials in 2004 and 2005 (see table 9). Ginned cotton fiber from 25 boll samples were taken from each plot, and sent to be analyzed at various cotton fiber analytical laboratories (ITC, Star, USDA, etc.). Samples were analyzed for traditional cotton fiber quality parameters using high volume instrumentation (HVI).

Characterization of the Materials

Identity of the materials was preserved through chain of custody documentation. Chain of custody documentation was utilized to identify the materials shipped to their respective field sites for proper identification of the evaluated plots in the field. Harvested materials contained chain of custody documentation for samples sent from the field to analytical laboratories to preserve identity.

Performing Facility and Experimental Methods

Fiber was analyzed using HVI standard procedures at Star Labs in Knoxville, TN and the International Textile Center in Lubbock, TX. Fiber was analyzed for fiber strength, elongation, % lint, micronaire, fiber uniformity and fiber length. These parameters are the standard classing parameters used by the United States Department of Agriculture. Measurements for color and trash were not taken because the samples were hand harvested. Samples harvested by hand do not have the same issues as fiber harvested with cotton picking equipment, therefore making these measurements irrelevant.

Statistical Analysis

Analysis of variance (ANOVA) between groups was calculated to analyze data for significant differences. All treatments were analyzed in comparison to their non-transgenic counterpart across regions, regionally, and locally. Data was reviewed using a confidence interval of 95%.

Materials and methods for composition analysis

Field design

Cotton plants containing the GlyTol cotton event GHB614 and cotton plants representing the non-transgenic (non-transformed) counterpart Coker 312 were field tested by Bayer CropScience in 2005 under USDA notification 05-091-07n at nine replicated sites. Trials were conducted in EPA Regions II, III, IV, VI and VIII in the following locations: Trial number 02-01-Tift County, Georgia; Trial number 03-02-Escambia County, Florida; Trial number 04-03-Jackson County, Trial number 04-04-Crittenden County, Arkansas; Trial number 04-05-Drew County, Arkansas; Trial number 04-06-Tate County, Mississippi; Trial number 04-07-Tate County, Mississippi; Trial number 06-08-Wharton County, Texas; Trial number 08-09-Hockley County, Texas, which are typical cotton growing regions of the southeastern United States.

The trials were randomized at each field trial site. There were six transgenic plots and three non-transgenic plots at each test site. Each plot was separated by border areas of 20 feet (or 6 rows) of planted non-transgenic cotton, and the entire plot area was surrounded by 40 ft (or 12 rows) of planted non-transgenic cotton. Each plot was clearly and uniquely identified by a suitable means (e.g., labeled stakes or flags) and related to a permanent field marker. Three of the GlyTol cotton event GHB614 plots were sprayed three times with glyphosate herbicide, and the other six plots were untreated. Each application of glyphosate herbicide was made at a rate of 0.75 pounds of active ingredient (glyphosate acid equivalent) per acre.

Nine samples of ginned cottonseed (fuzzy seed) were collected from each trial. Replication was provided from the triplicate plots of each planted regimen, rather than from multiple samples from each plot. Harvest was done by hand or mechanical means as appropriate to the method typical of the trial site area. One sample of ginned cottonseed (fuzzy seed) was obtained from each test plot. Each sample was representative (a composite) of cotton bolls harvested from multiple areas within the plot. Ginning was carried out at the field trial locations with small “research scale” cotton gins. A total of 81 samples was shipped frozen to the laboratories of BCS. The fuzzy seeds were sub-sampled and shipped in a frozen state to the analytical facility, Eurofins Scientific, 3507 Delaware Ave., Des Moines, IA, where they were stored frozen until removed for preparation and analysis.

Shipping and storage of the regulated seed was carried out under applicable USDA regulations and BCS guidelines. Any remaining plant material, including unused seeds, was destroyed at the conclusion of each field trial by incineration or disking into the plot area. Each field trial area was monitored for volunteers in 2006, with the results reported to Bayer CropScience - BioScience Regulatory Affairs.

Characterization of the material

Certificates of analysis (COA) were produced by the BCS QA Laboratory in Lubbock, TX and at BCS, Research Triangle Park for seed shipped to the nine field test sites for planting. The data showed that the transgenic cottonseed that was planted in the field was indeed cotton GHB614, and that the non-transgenic seed (Coker 312) supplied to the field contained less than 1% of GlyTol cotton event GHB614 seed with 95% confidence. Adventitious presence of several other cotton genotypes was also checked in the GHB614 and Coker 312 seed. There were no positive results in these analyses indicating the seed lots contained very low, if any, amounts of other cotton genotypes.

Analytical procedures

Eighty-one samples of ginned cottonseed were prepared for analysis by grinding and were analyzed for the following composition analytes or parameters (Table 2.12).

Table 2.12. Analytical methods

Parameter (Analyte)	Method
Moisture	AOCS Ba 2a-38 (1989)
Crude Fat	AOAC 920.39 (1990)
Crude Protein	AOCS Ba 4e-93 (1995)
Ash	AOAC 942.05 (1990)
Acid Detergent Fiber (ADF)	ANKOM$^{200/220}$ Fiber Analyzer
Neutral Detergent Fiber (NDF)	ANKOM$^{200/220}$ Fiber Analyzer
Carbohydrates	Difference between 100 and the sum of crude protein, fat, moisture and ash
Ca	AOAC 984.27 (modified)
P	AOAC 965.17 (1995 modified)
K	AOAC 984.27 (modified)
Fe	AOAC 984.27 (modified)
Mg	AOAC 984.27 (modified)
Zn	AOAC 984.27 (modified)
Vitamin E (alpha-Tocopherol)	AOAC 969.40 (GC)
Amino Acids	AOAC 982.30 (1990) Sec. D, F
Fatty Acid Profile, % Relative	AOCSCE2-66, CE1E-91
Cyclopropenoid Fatty Acids*	Covance method procedure MP-CPFA-MA
Phytic Acid	NP Analytical Laboratories Method Mnemonic: PYEX (Revised 01-13-01)
Gossypol (total)	AOCS Ba 8-78 (1983, reapproved 1997)
Gossypol (free)	Method version of AOCS Ba 7-58 (reapproved 1997) and JAOCS vol. 59, no. 12, pp. 546-549 (Dec. 1982) (Modified)

*Cyclopropenoid Fatty Acids comprise malvalic acid, sterculic acid and dihydro-sterculic acid.

Some analyses were conducted at another Eurofins laboratory in Memphis, TN. Cyclopropenoid Fatty Acids* were analyzed at Covance Laboratories in Madison, WI. Sub-samples were shipped frozen to these laboratories from the Des Moines laboratory after the samples were prepared by grinding.

The results were reported on a sample fresh-weight basis. A dry-matter weight basis summary of the appropriate analytes that were adjusted for their reported moisture content was also reported. Fatty acids were reported as % relative and are not adjusted for moisture content. Mean and standard deviation values were calculated for the reported analytes/parameters across the nine field locations and by treatment regime. StatView® 5 (SAS Institute, Cary, NC) was used for ANOVA. BCS conducted all statistical analyses.

Appendix 3. CHARACTERIZATION OF GLYTOL COTTON

Verification of the insert

Genomic DNA was isolated from GlyTol cotton event GHB614 and control Coker 312 plants, and isolated DNA samples were subjected to Southern blot analysis using the different components of the transgene cassette (Ph4a748At promoter, intron1 h3At+TPotp C, *2mepsps* gene and histonAt terminator) as well as the complete T-DNA fragment, as probes (Table 3.1).

Several aliquots of GlyTol cotton event GHB614 genomic DNA were digested with the restriction enzymes *Eco*RV, *Bgl*II, *Pvu*II, *Ase*I, *Nco*I, *Hind*III, *Afl*III, *Bam*HI and *Kpn*I. See Figure 3.1 and Table 3.2 to locate restriction sites in pTEM2. After separation of the DNA by electrophoresis, the DNA was transferred to two nylon membranes and hybridized with four overlapping gel purified ^{32}P-labeled probes, covering the complete vector backbone A fifth ^{32}P-labeled probe was also utilized, which covers the entire T-DNA. Lanes contained approximately 10 µg of restricted DNA. The amount of restricted pTEM2 in positive control lanes is equivalent to one copy of the plasmid integrated in 10 µg of cotton DNA. The probed membranes were visualized by autoradiography. Electronic scans of the autoradiographs are presented in this document. Standard molecular biology methods were used (Sambrook *et al.*, 1989).

Table 3.1. Probes used in Southern Hybridization of GlyTol cotton event GHB614

DNA probes	Position in Vector pTEM2	Size (bp)
Ph4a748At	0001 → 1089	1089
intron1 h3At+TPotp C	1071 → 1920	850
2mepsps	1934 → 3284	1351
3'histonAt	3239 → 3991	753
complete T-DNA	0001 → 3991	3991

The expected and observed hybridization fragments are described in Table 3.2.

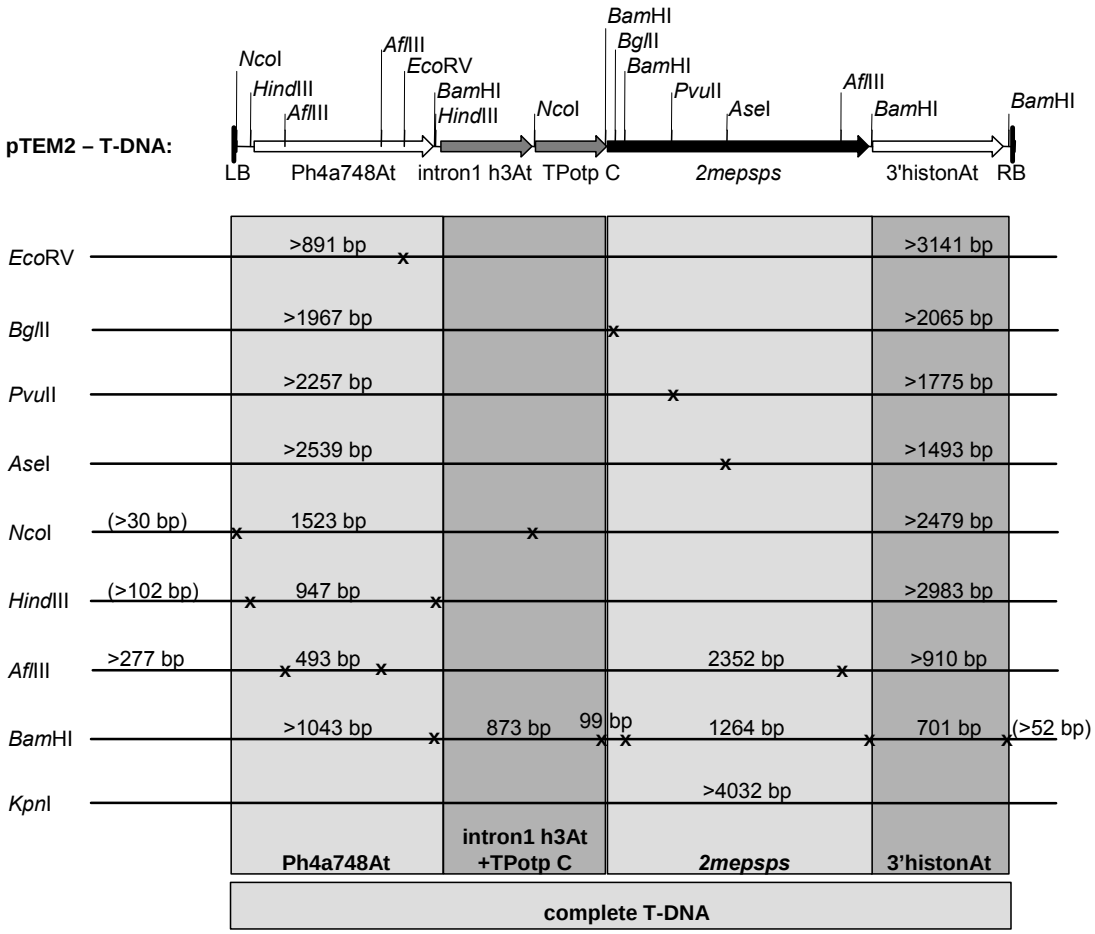


Figure 3.1. Schematic Drawing of the Hybridization Strategy

Southern blot hybridization results obtained with genomic DNA of cotton event GHB614 (digested with *Eco*RV, *Bgl*II, *Pvu*II, *Ase*I, *Nco*I, *Hind*III, *Afl*III, *Bam*HI and *Kpn*I) probed with Ph4a748At, intron1 h3At+TPotp C, *2mepsps*, 3'histonAt and the complete T-DNA show the presence of one 5' integration fragment and one 3' integration fragment. This observation is indicative of a single copy integration of the T-DNA sequences.

These data demonstrate that the transferred DNA in GlyTol cotton corresponds to the DNA configuration as designed in the pTEM2 plasmid and that a single copy of the gene cassette is integrated in GlyTol cotton event GHB614.

Table 3.2. Expected and observed hybridization fragments in Southern Analysis of GlyTol cotton event GHB614

Digest	Probes									
	Ph4a748At		intron1 h3At + TPotp C		2mepsps		3'histonAt		Complete T-DNA	
	Expected (bp)	Observed (bp)	Expected (bp)	Observed (bp)	Expected (bp)	Observed (bp)	Expected (bp)	Observed (bp)	Expected (bp)	Observed (bp)
*Eco*RV	>891, >3141	5000, 10250	>3141 bp	10250	>3141	10250	>3141	10250	>891, >3141	5000, 10250
*Bgl*II	>1967	>14000	>1967	>14000	>1967[5], >2065	--, .9200	>2065	9200	>1967, >2065	>14000, .9200
*Pvu*II	>2257	4000	>2257	4000	>2257, >1775	4000, 7200	>1775	7200	>2257, >1775	4000, 7200
*Ase*I	>2860	6600	>2860	6600	>2860, >1172	6600, 1500	>1172	1500	>2860, >1172	6600, 1500
*Nco*I	>30 bp[1], 1523	--, 1500	1523, >2479	1500, 6000	>2479	6000	>2479	6000	1523, >2479	1500, 6000
*Hind*III	>102[7], 947, >2983[2]	--, 910, --	>2983	9000	>2983	9000	>2983	9000	>102[7], 947, 2983	910, 9000
*Afl*III	>277, 493, 2352	1450, 510, 2300	2352	2300	2352, >910	2300, 970	>910	970	>277, 493, 2352, >910	1450, --, 2300, 970
*Bam*HI	>1043, 873[3]	>14 kb, --	873, 99[9]	860	99 bp[9], 1264	1210	1264[6], 701, >52[8]	--, 690, --	>1043, 873, 99[9], 1264, 701, >52[8]	>14000, 860 --, 1210 690; --
*Kpn*I	>4032	>14000	>4032	>14000	>4032	>14000	>4032	>14000	>4032p	>14000
WT - *Nco*I	none	--	none	--	none	--	none	--	none	--
WT + pTEM2 - *Nco*I	10430[4], 1523	--, 1500	10400, 1523	10400, 1500	10430	10400	10430	10400	10430, 1523	10400, 1500

Some fragments were not visible due to a small overlap with the probe used:
(1) Overlap of 30 bp (5) Overlap of 34 bp
(2) Overlap of 40 bp (6) Overlap of 41 bp
(3) Overlap of 46 bp (7) Overlap of 102 bp
(4) Overlap of 30 bp (8) Overlap of 11 bp
(9) Fragment will probably not be visible because of its small size and a limited overlap with the probe used
"--" = no hybridization

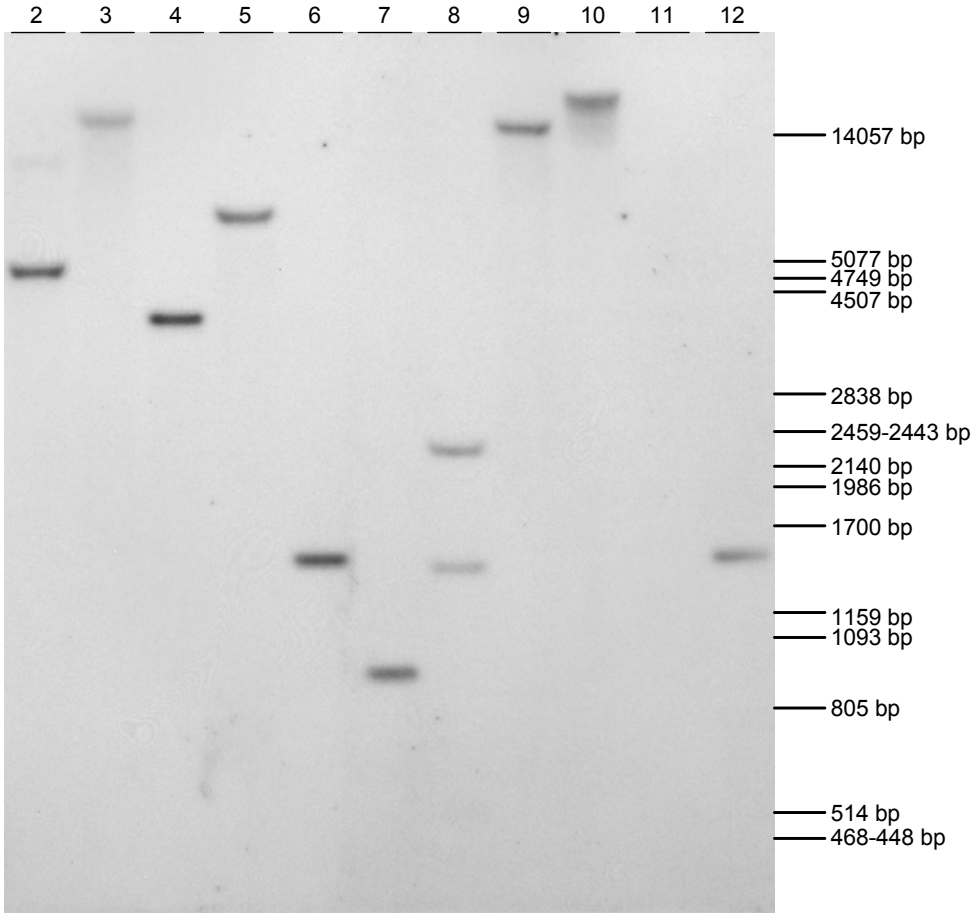


Figure 3.2. Verification of the GHB614 cotton insert – Ph4a748At probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed with the Ph4a748At probe (1089 bp fragment of pTEM2).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol Cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol Cotton event GHB614 – *Bgl*II digested
Lane 4: GlyTol Cotton event GHB614 – *Pvu*II digested
Lane 5: GlyTol Cotton event GHB614 – *Ase*I digested
Lane 6: GlyTol Cotton event GHB614 – *Nco*I digested
Lane 7: GlyTol Cotton event GHB614 – *Hind*III digested
Lane 8: GlyTol Cotton event GHB614 – *Afl*III digested
Lane 9: GlyTol Cotton event GHB614 – *Bam*HI digested
Lane 10: GlyTol Cotton event GHB614 – *Kpn*I digested
Lane 11: Negative control (variety Coker 312) – *Nco*I digested
Lane 12: Negative control (variety Coker 312) – *Nco*I digested + 1 copy pTEM2 - *Nco*I digested
Lane 13: MWM Phage Lambda – *Pst*I digested

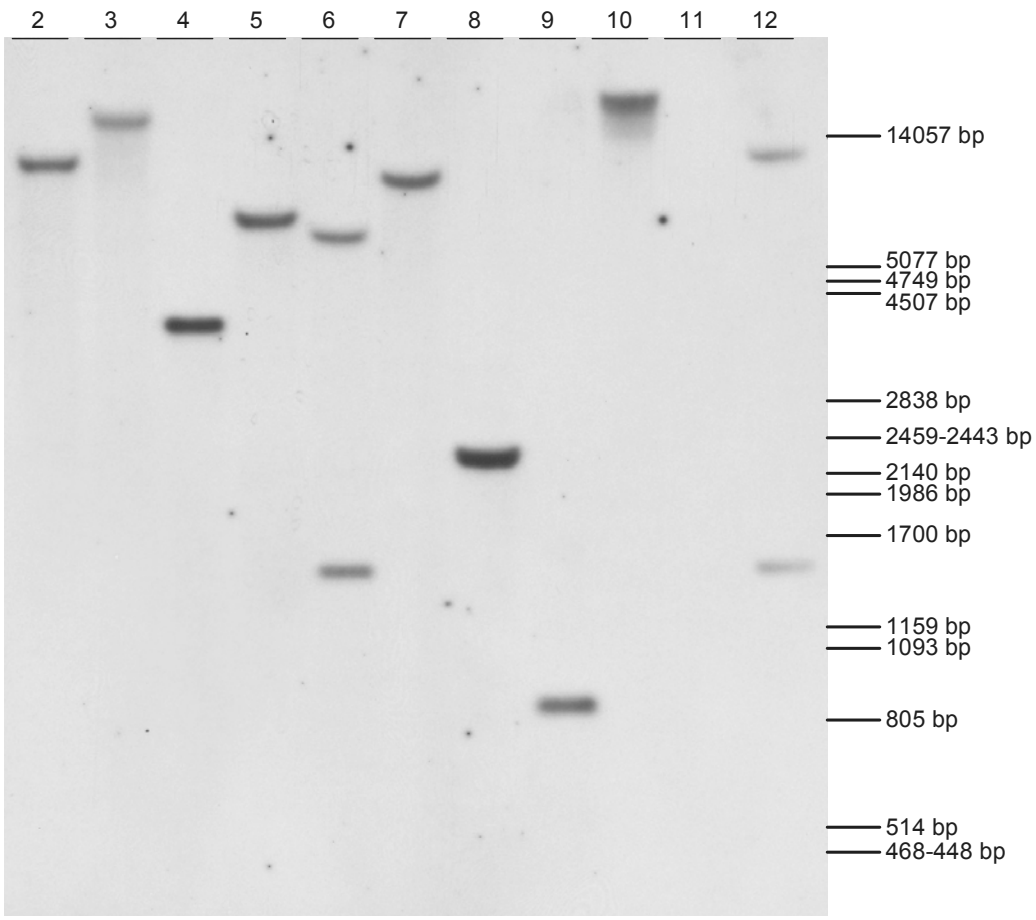


Figure 3.3. Verification of the GHB614 cotton insert - intron1 h3At+TPotp C probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed with the intron1 h3At+TPotp C probe (850 bp fragment of pTEM2).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol Cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol Cotton event GHB614 – *Bgl*II digested
Lane 4: GlyTol Cotton event GHB614 – *Pvu*II digested
Lane 5: GlyTol Cotton event GHB614 – *Ase*I digested
Lane 6: GlyTol Cotton event GHB614 – *Nco*I digested
Lane 7: GlyTol Cotton event GHB614 – *Hind*III digested
Lane 8: GlyTol Cotton event GHB614 – *Afl*III digested
Lane 9: GlyTol Cotton event GHB614 – *Bam*HI digested
Lane 10: GlyTol Cotton event GHB614 – *Kpn*I digested
Lane 11: Negative control (variety Coker 312) – *Nco*I digested
Lane 12: Negative control (variety Coker 312) – *Nco*I digested + 1 copy pTEM2 - *Nco*I digested
Lane 13: MWM Phage Lambda – *Pst*I digested

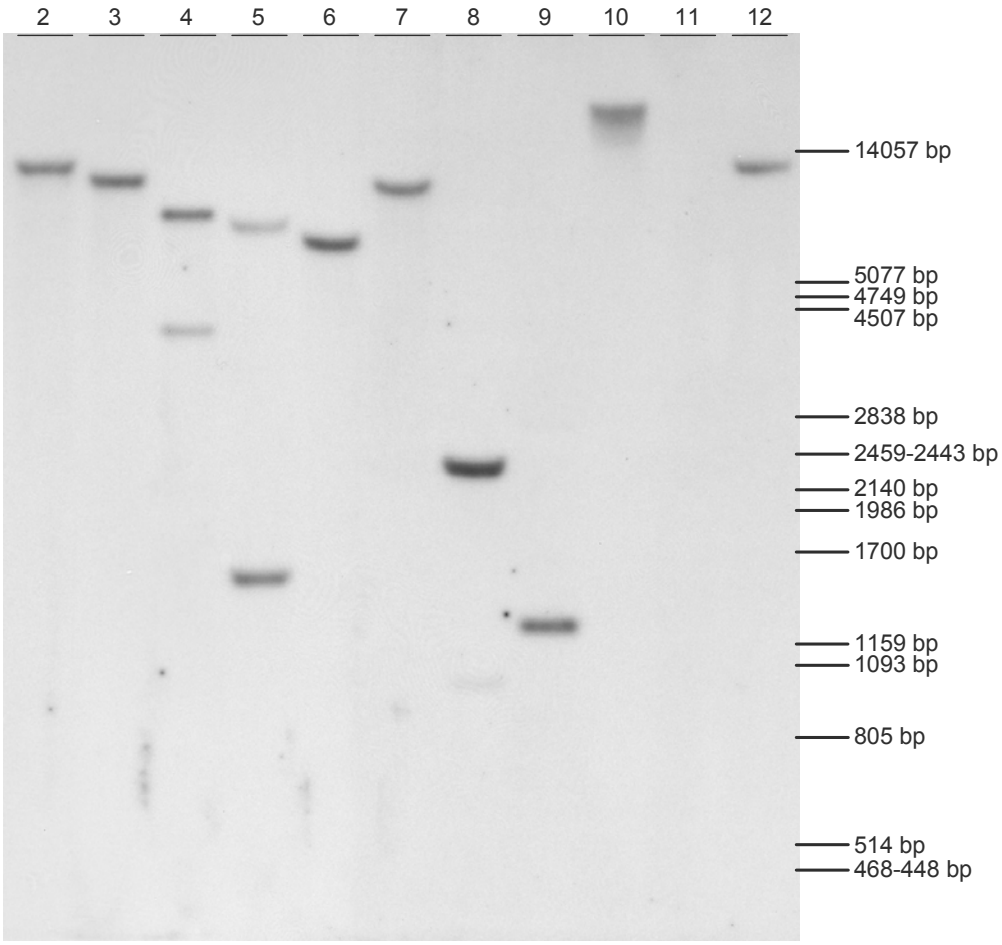


Figure 3.4. Verification of the GHB614 cotton insert – 2mepsps probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed with the *2mepsps* probe (1351 bp fragment of pTEM2).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol Cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol Cotton event GHB614 – *Bgl*II digested
Lane 4: GlyTol Cotton event GHB614 – *Pvu*II digested
Lane 5: GlyTol Cotton event GHB614 – *Ase*I digested
Lane 6: GlyTol Cotton event GHB614 – *Nco*I digested
Lane 7: GlyTol Cotton event GHB614 – *Hind*III digested
Lane 8: GlyTol Cotton event GHB614 – *Afl*III digested
Lane 9: GlyTol Cotton event GHB614 – *Bam*HI digested
Lane 10: GlyTol Cotton event GHB614 – *Kpn*I digested
Lane 11: Negative control (variety Coker 312) – *Nco*I digested
Lane 12: Negative control (variety Coker 312) – *Nco*I digested + 1 copy pTEM2 - *Nco*I digested
Lane 13: MWM Phage Lambda – *Pst*I digested

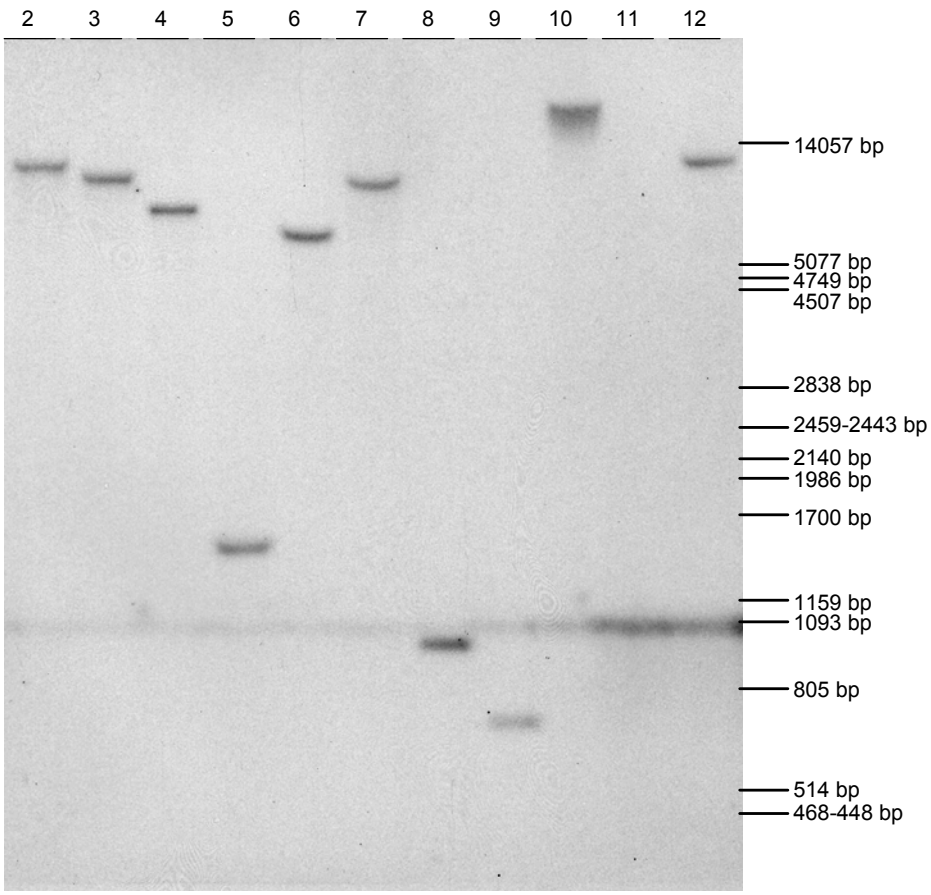


Figure 3.5. Verification of the GHB614 cotton insert - 3'histonAt probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed with the 3'histonAt probe (753 bp fragment of pTEM2)*.

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol Cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol Cotton event GHB614 – *Bgl*II digested
Lane 4: GlyTol Cotton event GHB614 – *Pvu*II digested
Lane 5: GlyTol Cotton event GHB614 – *Ase*I digested
Lane 6: GlyTol Cotton event GHB614 – *Nco*I digested
Lane 7: GlyTol Cotton event GHB614 – *Hind*III digested
Lane 8: GlyTol Cotton event GHB614 – *Afl*III digested
Lane 9: GlyTol Cotton event GHB614 – *Bam*HI digested
Lane 10: GlyTol Cotton event GHB614 – *Kpn*I digested
Lane 11: Negative control (variety Coker 312) – *Nco*I digested
Lane 12: Negative control (variety Coker 312) – *Nco*I digested + 1 copy pTEM2 - *Nco*I digested
Lane 13: MWM Phage Lambda – *Pst*I digested

* After hybridization of the membrane with the 3'histonAt probe, a horizontal black line was visible over the complete blot at the height of ca. 1100 bp. This line comes from damage to the membrane, which leads to aspecific hybridization. All hybridization fragments on this height of the membrane are clearly visible. Therefore, this aspecific hybridization line has no impact on the interpretation of the study results.

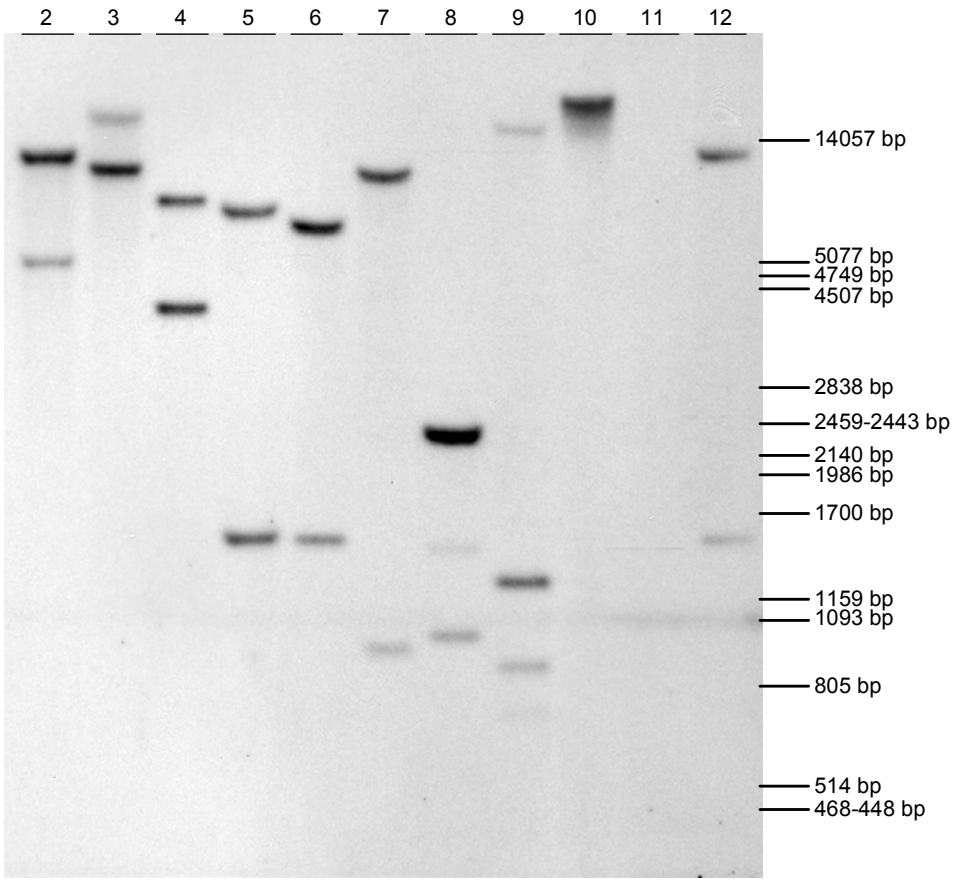


Figure 3.6. Verification of the GHB614 cotton insert – Complete T-DNA probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed with the complete T-DNA probe (3991 bp)*

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol cotton event GHB614 – *Bgl*II digested
Lane 4: GlyTol cotton event GHB614 – *Pvu*II digested
Lane 5: GlyTol cotton event GHB614 – *Ase*I digested
Lane 6: GlyTol cotton event GHB614 – *Nco*I digested
Lane 7: GlyTol cotton event GHB614 – *Hind*III digested
Lane 8: GlyTol cotton event GHB614 – *Afl*III digested
Lane 9: GlyTol cotton event GHB614 – *Bam*HI digested
Lane 10: GlyTol cotton event GHB614 – *Kpn*I digested
Lane 11: Negative control (variety Coker 312) – *Nco*I digested
Lane 12: Negative control (variety Coker 312) – *Nco*I digested + 1 copy pTEM2 - *Nco*I digested
Lane 13: MWM Phage Lambda – *Pst*I digested

* After hybridization of the membrane with the complete T-DNA probe, a horizontal black line was visible over the complete blot at the height of ca. 1100 bp. This line comes from damage to the membrane, which leads to aspecific hybridization. All hybridization fragments on this height of the membrane are clearly visible. Therefore, this aspecific hybridization line has no impact on the interpretation of the study results.

Genetic stability

Stability of the inserted DNA over several generations and backgrounds

In order to demonstrate the stability of GlyTol cotton event GHB614 over multiple generations and in different genetic backgrounds, Southern blot analysis was performed using GHB614-C312-T_3, GHB614-C312-T_4, GHB614-T_5, GHB614-C312-T_6, and GHB614-C312-BC_2F_2. Isolated DNA from leaf tissue was digested with the restriction enzyme *Eco*RV, which has one recognition site in the transforming DNA (Figure 3.7). The digested genomic DNA from GlyTol cotton event GHB614 was probed with "Ph4a748At+ intron1 h3At + TPotp C" and showed the expected 5' and 3' integration fragments, of approx. 4850 bp and 9100 bp respectively, in all tested samples thus showing the stability of the event GHB614 at the genomic level (see from Figure 3.8 to 19). Segregation data further confirm the stability of the insert, and show that it segregates as one dominant Mendelian locus.

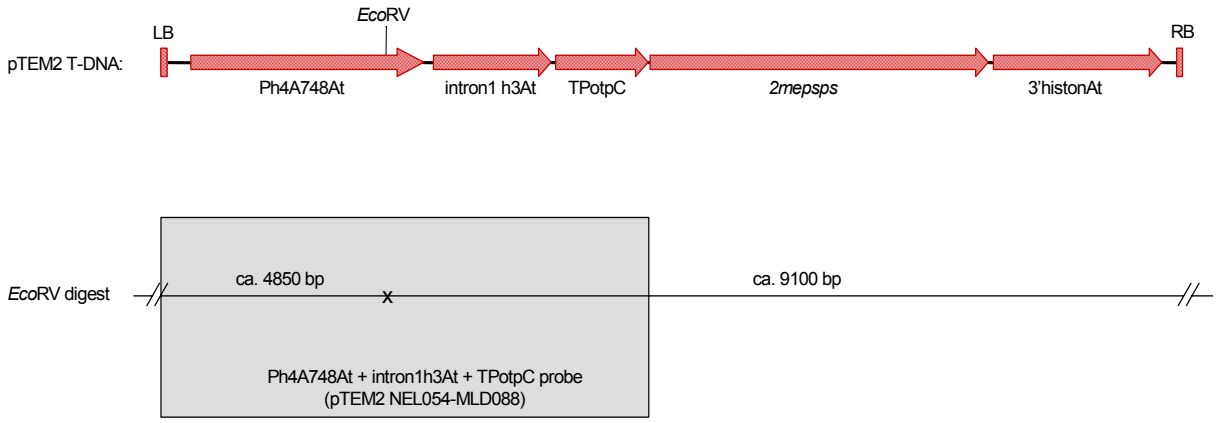


Figure 3.7. Schematic drawing of the T-DNA of pTEM2 with indication of the *Eco*RV restriction site and position of the probe used

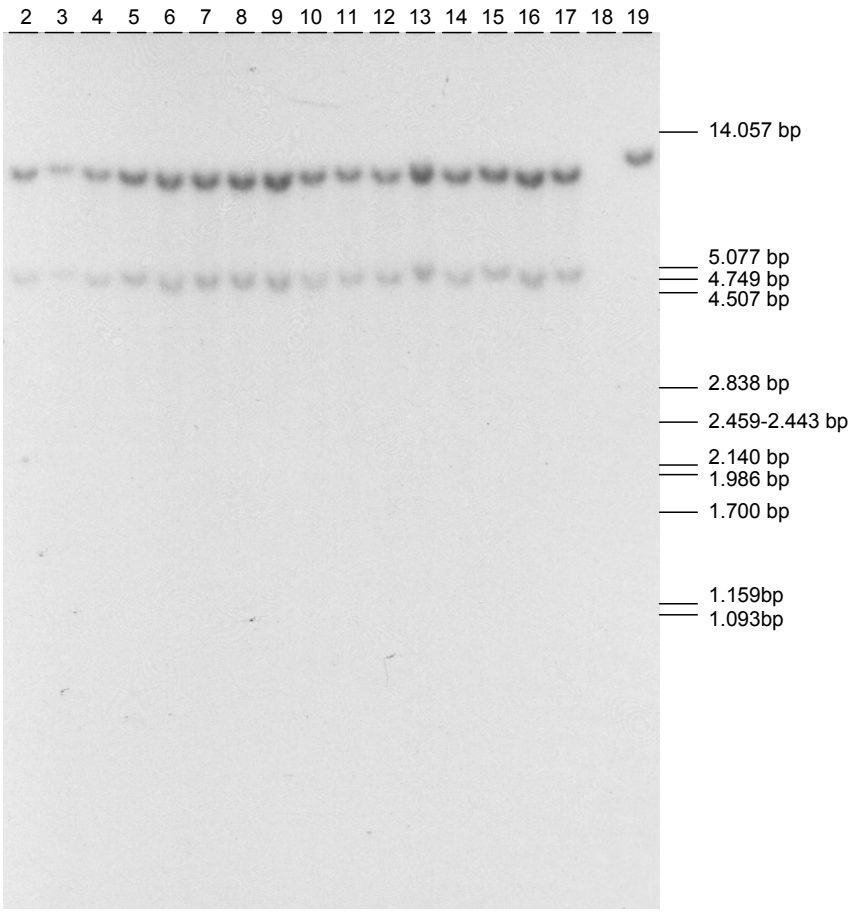


Figure 3.8. Demonstration of the stability of GlyTol cotton event GHB614 – Generation T_3

Genomic DNA was isolated from GlyTol cotton event GHB614 plants (generation T_3) and from the non-transgenic counterpart (Coker 312). Genomic DNAs (7 µg) were digested with *Eco*RV and probed with part of the T-DNA (1920 bp fragment of pTEM2).

Lane 2: GlyTol Cotton GHB614 - T_3- plant 1 - EcoRV
Lane 3: GlyTol Cotton GHB614 - T_3- plant 2 - EcoRV
Lane 4: GlyTol Cotton GHB614 - T_3- plant 3 - EcoRV
Lane 5: GlyTol Cotton GHB614 - T_3- plant 4 - EcoRV
Lane 6: GlyTol Cotton GHB614 - T_3- plant 5 - EcoRV
Lane 7: GlyTol Cotton GHB614 - T_3- plant 6 - EcoRV
Lane 8: GlyTol Cotton GHB614 - T_3- plant 7 - EcoRV
Lane 9: GlyTol Cotton GHB614 - T_3- plant 8 - EcoRV
Lane 10: GlyTol Cotton GHB614 - T_3- plant 9 - EcoRV
Lane 11: GlyTol Cotton GHB614 - T_3- plant 10 - EcoRV
Lane 12: GlyTol Cotton GHB614 - T_3- plant 11 - EcoRV
Lane 13: GlyTol Cotton GHB614 - T_3- plant 12 - EcoRV
Lane 13: GlyTol Cotton GHB614 - T_3- plant 12 - EcoRV
Lane 14: GlyTol Cotton GHB614 - T_3- plant 13 - EcoRV
Lane 15: GlyTol Cotton GHB614 - T_3- plant 14 - EcoRV
Lane 16: GlyTol Cotton GHB614 - T_3- plant 15 - EcoRV
Lane 17: GlyTol Cotton GHB614 - T_3- plant 16 - EcoRV
Lane 18: WT var. Coker 312 - EcoRV
Lane 19: WT var. Coker 312 + 1 copy pTEM2 – EcoRV

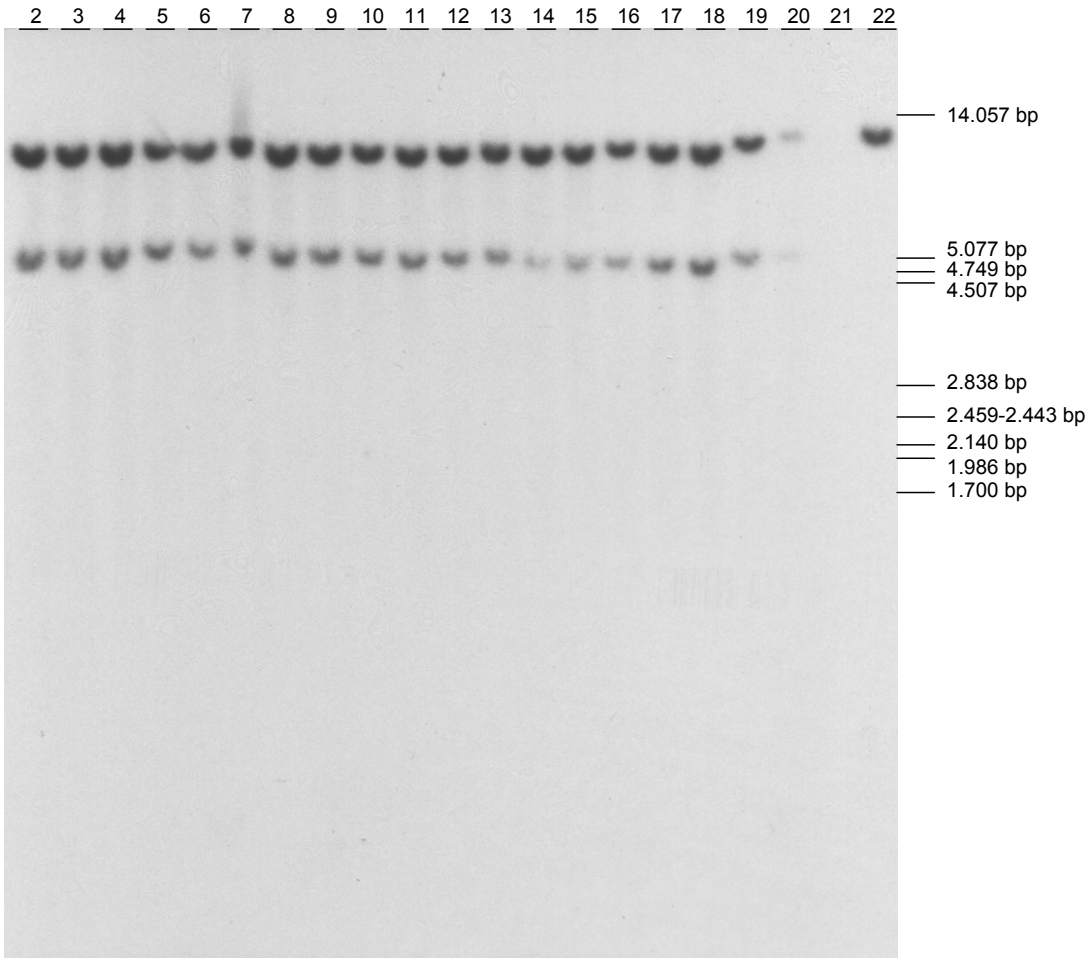


Figure 3.9. Demonstration of the stability of GlyTol cotton event GHB614 – Generation T_4

Genomic DNA was isolated from GlyTol cotton event GHB614 plants (generation T_4) and from the non-transgenic counterpart (Coker 312). Genomic DNAs (7 µg) were digested with *Eco*RV and probed with part of the T-DNA (1920 bp fragment of pTEM2).

Lane 2: GlyTol cotton GHB614 - T_4 - plant 1 - *Eco*RV
Lane 3: GlyTol cotton GHB614 - T_4 - plant 2 - *Eco*RV
Lane 4: GlyTol cotton GHB614 - T_4 - plant 3 - *Eco*RV
Lane 5: GlyTol cotton GHB614 - T_4 - plant 4 - *Eco*RV
Lane 6: GlyTol cotton GHB614 - T_4 - plant 5 - *Eco*RV
Lane 7: GlyTol cotton GHB614 - T_4 - plant 6 - *Eco*RV
Lane 8: GlyTol cotton GHB614 - T_4 - plant 7 - *Eco*RV
Lane 9: GlyTol cotton GHB614 - T_4 - plant 8 - *Eco*RV
Lane 10: GlyTol cotton GHB614 - T_4 - plant 9 - *Eco*RV
Lane 11: GlyTol cotton GHB614 - T_4 - plant 10 – *Eco*RV
Lane 12: GlyTol cotton GHB614 - T_4 - plant 11 - *Eco*RV
Lane 13: GlyTol cotton GHB614 - T_4 - plant 12 - *Eco*RV
Lane 14: GlyTol cotton GHB614 - T_4 - plant 13 - *Eco*RV
Lane 15: GlyTol cotton GHB614 - T_4 - plant 14 - *Eco*RV
Lane 16: GlyTol cotton GHB614 - T_4 - plant 15 - *Eco*RV
Lane 17: GlyTol cotton GHB614 - T_4 - plant 16 - *Eco*RV
Lane 18: GlyTol cotton GHB614 - T_4 - plant 17 - *Eco*RV
Lane 19: GlyTol cotton GHB614 - T_4 - plant 18 - *Eco*RV
Lane 20: GlyTol cotton GHB614 - T_4 - plant 19 - *Eco*RV
Lane 21: WT var. Coker 312 - *Eco*RV
Lane 22: WT var. Coker 312 + 1 copy pTEM2 - *Eco*RV

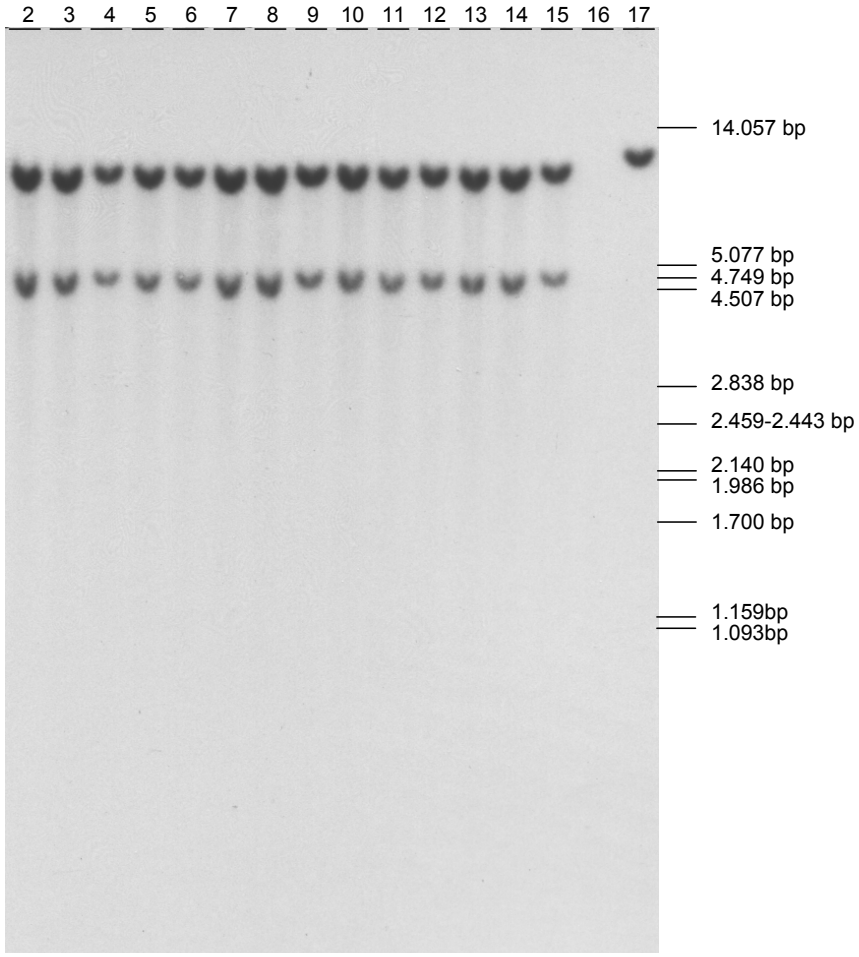


Figure 3.10. Demonstration of the stability of GlyTol cotton event GHB614 – Generation T_5

Genomic DNA was isolated from GlyTol cotton event GHB614 plants (generation T_5) and from the non-transgenic counterpart (Coker 312). Genomic DNAs (7 µg) were digested with *Eco*RV and probed with part of the T-DNA (1920 bp fragment of pTEM2).

Lane 2: GlyTol cotton GHB614 - T_5 - plant 1 - *Eco*RV
Lane 3: GlyTol cotton GHB614 - T_5 - plant 2 - *Eco*RV
Lane 4: GlyTol cotton GHB614 - T_5 - plant 3 - *Eco*RV
Lane 5: GlyTol cotton GHB614 - T_5 - plant 4 - *Eco*RV
Lane 6: GlyTol cotton GHB614 - T_5 - plant 5 - *Eco*RV
Lane 7: GlyTol cotton GHB614 - T_5 - plant 6 - *Eco*RV
Lane 8: GlyTol cotton GHB614 - T_5 - plant 7 - *Eco*RV
Lane 9: GlyTol cotton GHB614 - T_5 - plant 8 - *Eco*RV
Lane 10: GlyTol cotton GHB614 - T_5 - plant 9 - *Eco*RV
Lane 11: GlyTol cotton GHB614 - T_5 - plant 10 - *Eco*RV
Lane 12: GlyTol cotton GHB614 - T_5 - plant 11 - *Eco*RV
Lane 13: GlyTol cotton GHB614 - T_5 - plant 12 - *Eco*RV
Lane 14: GlyTol cotton GHB614 - T_5 - plant 13 - *Eco*RV
Lane 15: GlyTol cotton GHB614 - T_5- plant 14 - *Eco*RV
Lane 16: WT var. Coker 312 - *Eco*RV
Lane 17: WT var. Coker 312 + 1 copy pTEM2 - *Eco*RV

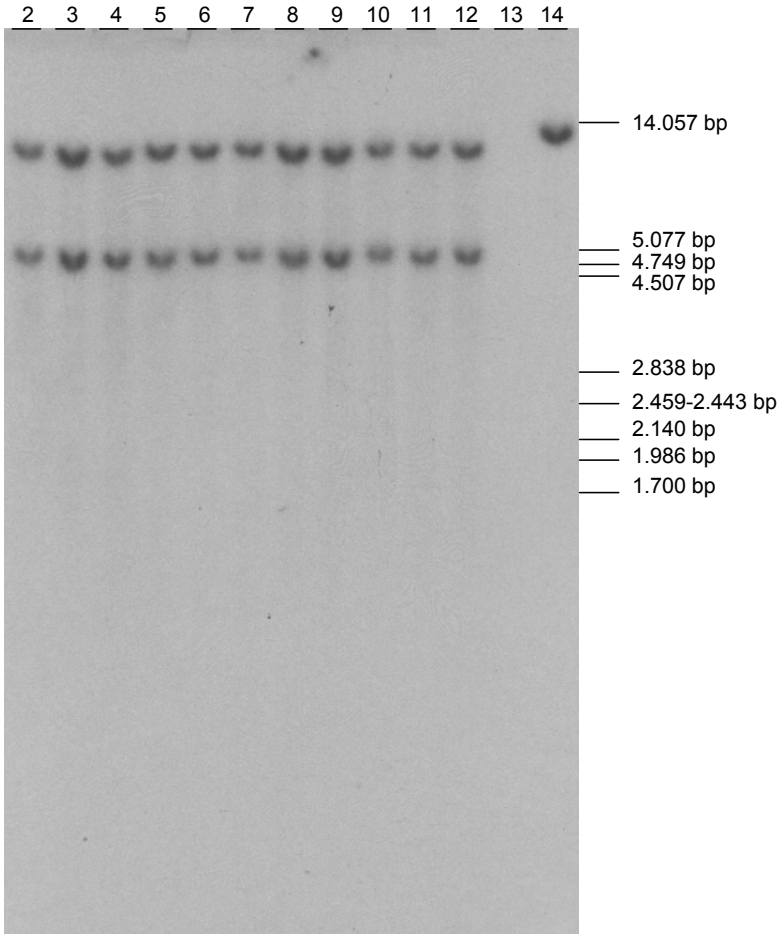


Figure 3.11. Demonstration of the stability of GlyTol cotton event GHB614 – Generation T_6

Genomic DNA was isolated from GlyTol cotton event GHB614 plants (generation T_6) and from the non-transgenic counterpart (Coker 312). Genomic DNAs (7 µg) were digested with *Eco*RV and probed with part of the T-DNA (1920 bp fragment of pTEM2).

Lane 2: GlyTol cotton GHB614 - T_6 - plant 1 - *Eco*RV
Lane 3: GlyTol cotton GHB614 - T_6 - plant 2 - *Eco*RV
Lane 4: GlyTol cotton GHB614 - T_6 - plant 3 - *Eco*RV
Lane 5: GlyTol cotton GHB614 - T_6 - plant 4 - *Eco*RV
Lane 6: GlyTol cotton GHB614 - T_6 - plant 5 - *Eco*RV
Lane 7: GlyTol cotton GHB614 - T_6 - plant 6 - *Eco*RV
Lane 8: GlyTol cotton GHB614 - T_6 - plant 7 - *Eco*RV
Lane 9: GlyTol cotton GHB614 - T_6 - plant 8 - *Eco*RV
Lane 10: GlyTol cotton GHB614 - T_6 - plant 9 - *Eco*RV
Lane 11: GlyTol cotton GHB614 - T_6 - plant 10 - *Eco*RV
Lane 12: GlyTol cotton GHB614 - T_6 - plant 11 - *Eco*RV
Lane 13: WT var. Coker 312 - *Eco*RV
Lane 14: WT var. Coker 312 + 1 copy pTEM2 - *Eco*RV

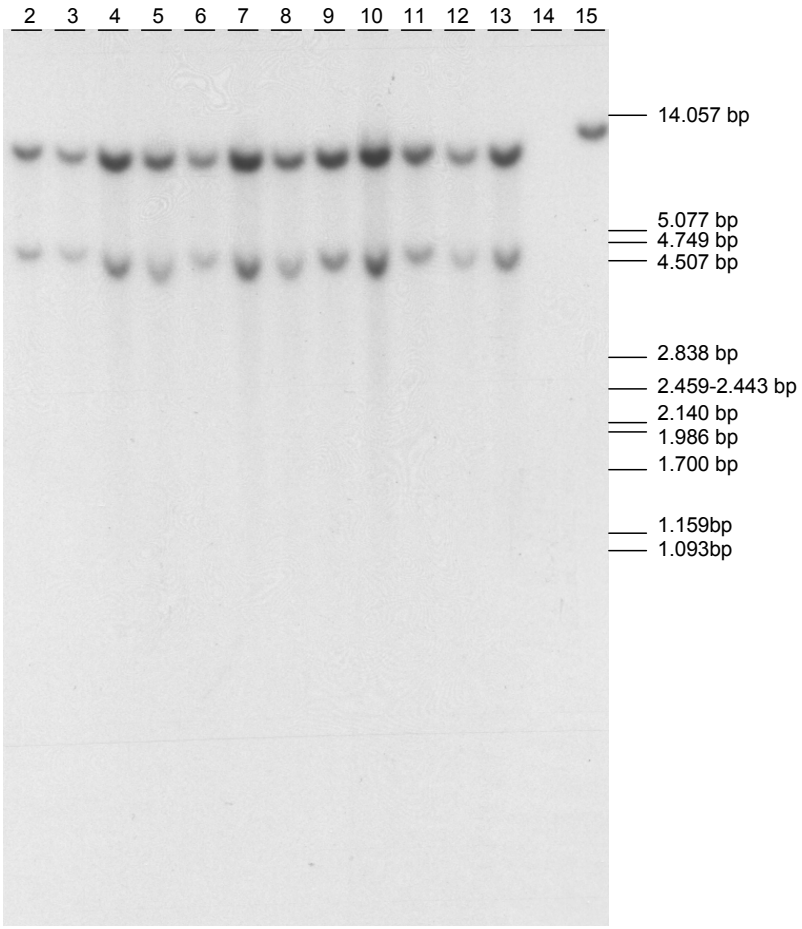


Figure 3.12. Demonstration of the stability of GlyTol cotton event GHB614 – Generation BC_2F_2

Genomic DNA was isolated from GlyTol cotton event GHB614 plants (generation BC_2F_2) and from non-transgenic cotton (Coker 312). Genomic DNAs (7 µg) were digested with *Eco*RV and probed with part of the T-DNA (1920 bp fragment of pTEM2).

Lane 2: GlyTol cotton GHB614 - BC_2F_2 - plant 1 - *Eco*RV
Lane 3: GlyTol cotton GHB614 - BC_2F_2 - plant 2 - *Eco*RV
Lane 4: GlyTol cotton GHB614 - BC_2F_2 - plant 3 - *Eco*RV
Lane 5: GlyTol cotton GHB614 - BC_2F_2 - plant 4 - *Eco*RV
Lane 6: GlyTol cotton GHB614 - BC_2F_2 - plant 5 - *Eco*RV
Lane 7: GlyTol cotton GHB614 - BC_2F_2 - plant 6 - *Eco*RV
Lane 8: GlyTol cotton GHB614 - BC_2F_2- plant 7 - *Eco*RV
Lane 9: GlyTol cotton GHB614 - BC_2F_2 - plant 8 - *Eco*RV
Lane 10: GlyTol cotton GHB614 - BC_2F_2 - plant 9 - *Eco*RV
Lane 11: GlyTol cotton GHB614 - BC_2F_2 - plant 10 - *Eco*RV
Lane 12: GlyTol cotton GHB614 - BC_2F_2 - plant 11 - *Eco*RV
Lane 13: GlyTol cotton GHB614 - BC_2F_2 - plant 12 - *Eco*RV
Lane 14: WT var. Coker 312 - *Eco*RV
Lane 15: WT var. Coker 312 + 1 copy pTEM2 - *Eco*RV

Absence of the vector backbone

For the molecular verification of the absence of pTEM2 vector backbone, genomic DNA was isolated from GlyTol cotton event GHB614 and control Coker 312 plants. Southern blot analysis was performed using five overlapping probes, covering the entire vector backbone sequence. Afterwards, the membranes were stripped of the vector backbone probes, and re-hybridized with a T-DNA probe (PT006), in order to demonstrate that ample GlyTol cotton genomic DNA was loaded on the gels. The positive control samples showed the expected hybridization fragment of 9131 bp. No hybridization fragments are visible in the wild-type (Coker 312) control lane.

- *Eco*RV cuts once in the GHB614 inserted sequences. Therefore, two integration fragments are expected after hybridization of *Eco*RV digested GHB614 genomic DNA with T-DNA sequences. The 5' integration fragment should be more than 891 bp; the 3' integration fragment larger than 3141 bp. *Eco*RV digested GHB614 genomic DNA shows two integration fragments: 4800 ± 50 bp and 9000 ± 200 bp. From previous analyses, it is known that the 4800 ± 50 bp fragment represents the 5' border fragment, while the 9000 ± 200 bp fragment is derived from the 3' border fragment.

- There is no *Not*I recognition sequence in the genomic DNA of GHB614 cotton. Therefore, only one integration fragment is expected after hybridization of *Not*I digested genomic DNA of GlyTol cotton event GHB614 with T-DNA sequences. Indeed, one hybridization fragment larger than 14 Kb was observed.

The results of these analyses are provided below for each probe and are summarized in Table 3.3.

PT001 probe
In the DNA positive controls, the expected 9131 bp *Not*I fragment was observed. No hybridization signals could be observed in the GlyTol cotton event GHB614 samples or in the Coker 312 wild type DNA (negative control) (Figure 3.14).

PT002 probe
In the *Not*I digested positive control samples, the expected fragments of 1290 bp, 1532 bp ,and 9131 bp were observed. No hybridization signals could be observed in the lanes containing GlyTol cotton event GHB614 samples or in the lane containing WT control DNA (Figure 3.15)

PT003 probe
The positive control shows the expected pTEM2 – *Not*I fragments of 1290 and 1532 bp. GlyTol cotton event GHB614 genomic DNA and the WT control do not show any hybridization (Figure 3.16).

PT004 probe

No hybridization fragments could be observed in the digested GlyTol cotton event GHB614 samples or in the WT control sample. The expected 1532 bp and 9131 bp fragments are visible in the positive control samples (Figure 3.17).

PT005 probe

The positive controls show the expected hybridization fragment of 9131 bp. GlyTol cotton event GHB614 genomic DNA and the WT control do not show any hybridization fragments (Figure 3.18).

With the performed Southern blot analysis, using overlapping probes covering the complete pTEM2 vector backbone sequences, the absence of vector backbone sequences in *Gossypium hirsutum* GlyTol cotton event GHB614 samples is demonstrated.

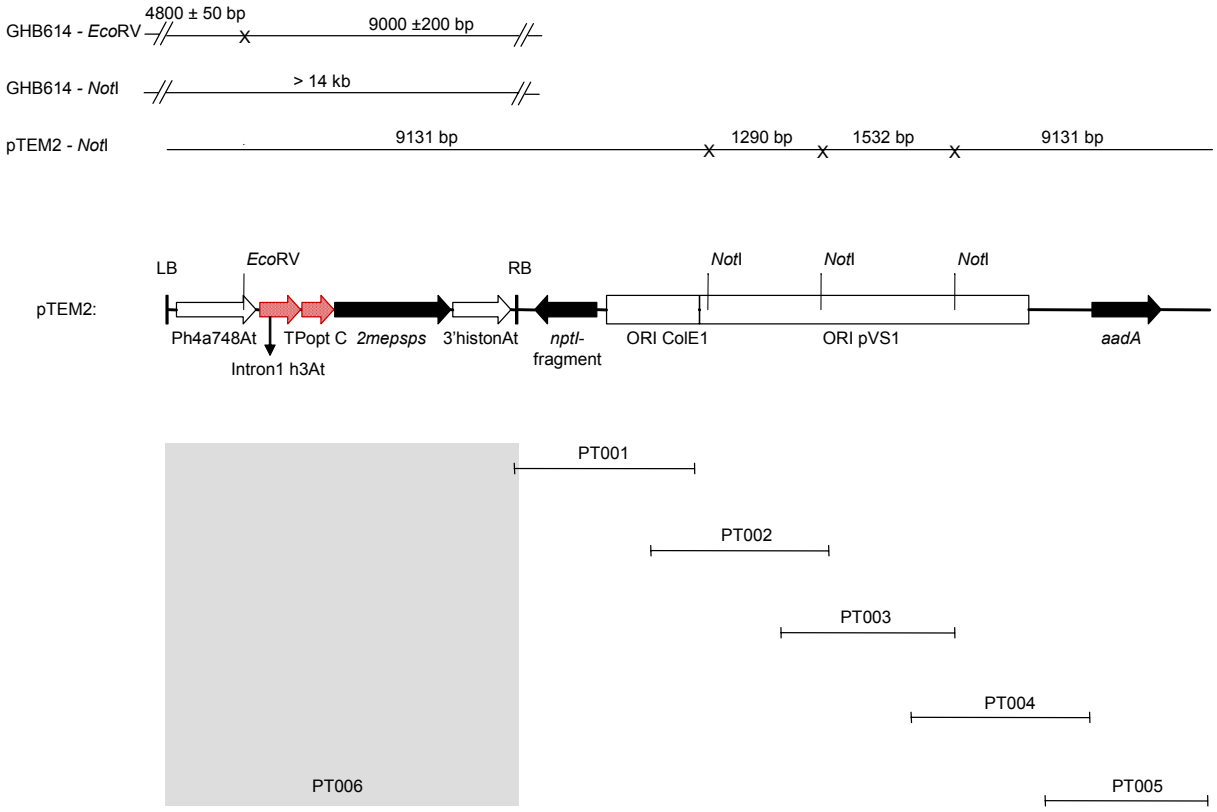


Figure 3.13. Schematic drawing of pTEM2 with indication of relevant restriction sites and position of the probes used

Table 3.3. Summary of Hybridization Results – Demonstration of the Absence of Vector Sequences in GlyTol cotton event GHB614

Probes	*Position in Vector*	*GlyTol cotton GHB614/ EcoRV*	*GlyTol cotton GHB614/ NotI*	*Coker 312/ NotI*	*Coker 312 wild type + pTEM2 (1 copy)*
PT001	3992 → 6050	--	--	--	9100 bp
PT002	5551 → 7589	--	--	--	1250, 1550, 9100 bp
PT003	7044 → 9031	--	--	--	1250; 1550 bp
PT004	8531 → 10575	--	--	--	1550, 9100 bp
PT005	10068 → 11928	--	--	--	9100 bp
PT006	1 → 4032	4800, 9000 bp	>14000 bp	--	9100 bp

"--" = no hybridization

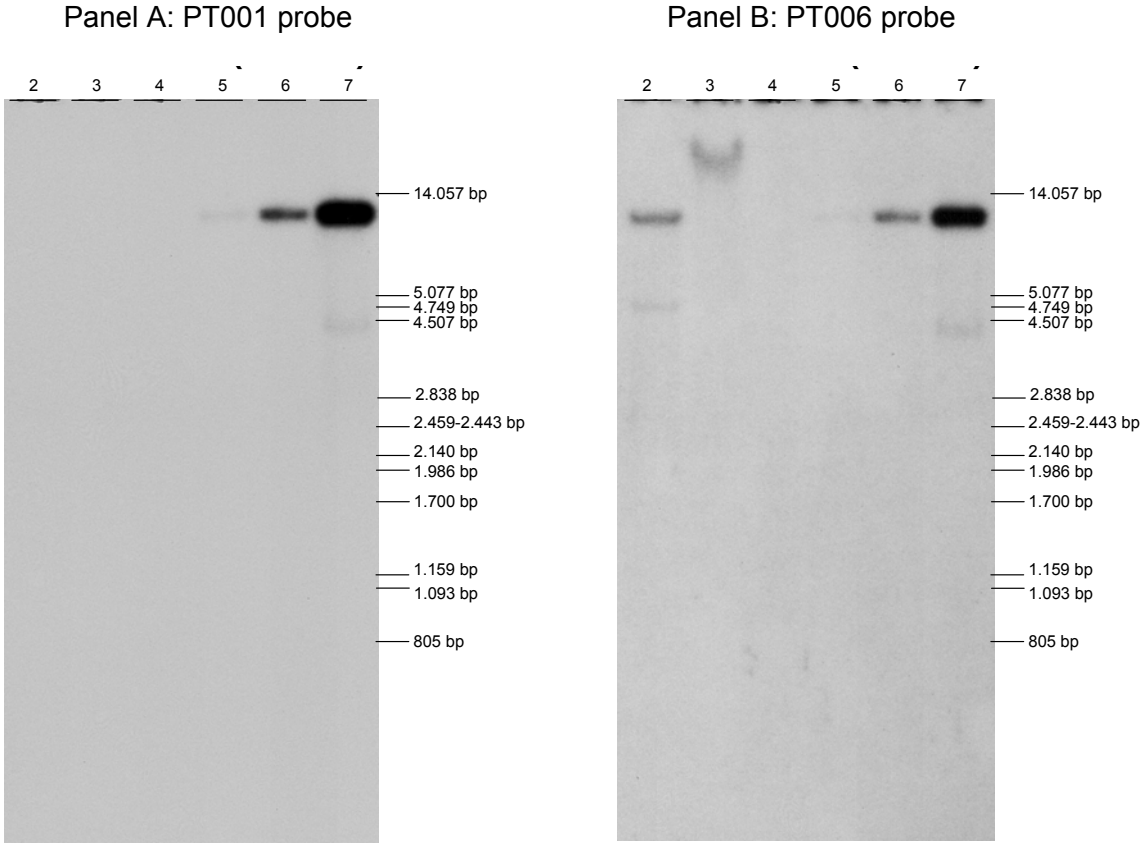


Figure 3.14. Southern blot analysis of GlyTol cotton event GHB614 - Demonstration of the absence of vector backbone sequences - PT001 probe and T-DNA probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed sequentially with the vector backbone probe (PT001: 2059 bp fragment of pTEM2, panel A) and with the T-DNA probe (PT006: 4032 bp fragment of pTEM2, panel B).

Lane 2: GlyTol cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol cotton event GHB614 – *Not*I digested
Lane 4: Negative control (variety Coker 312) – *Not*I digested
Lane 5: Negative control (variety Coker 312) – *Not*I digested + 0.1 copy pTEM2 - *Not*I digested
Lane 6: Negative control (variety Coker 312) – *Not*I digested + 1 copy pTEM2 - *Not*I digested
Lane 7: Negative control (variety Coker 312) – *Not*I digested + 10 copies pTEM2 - *Not*I digested
Lane 8: MWM Phage Lambda – *Pst*I digested

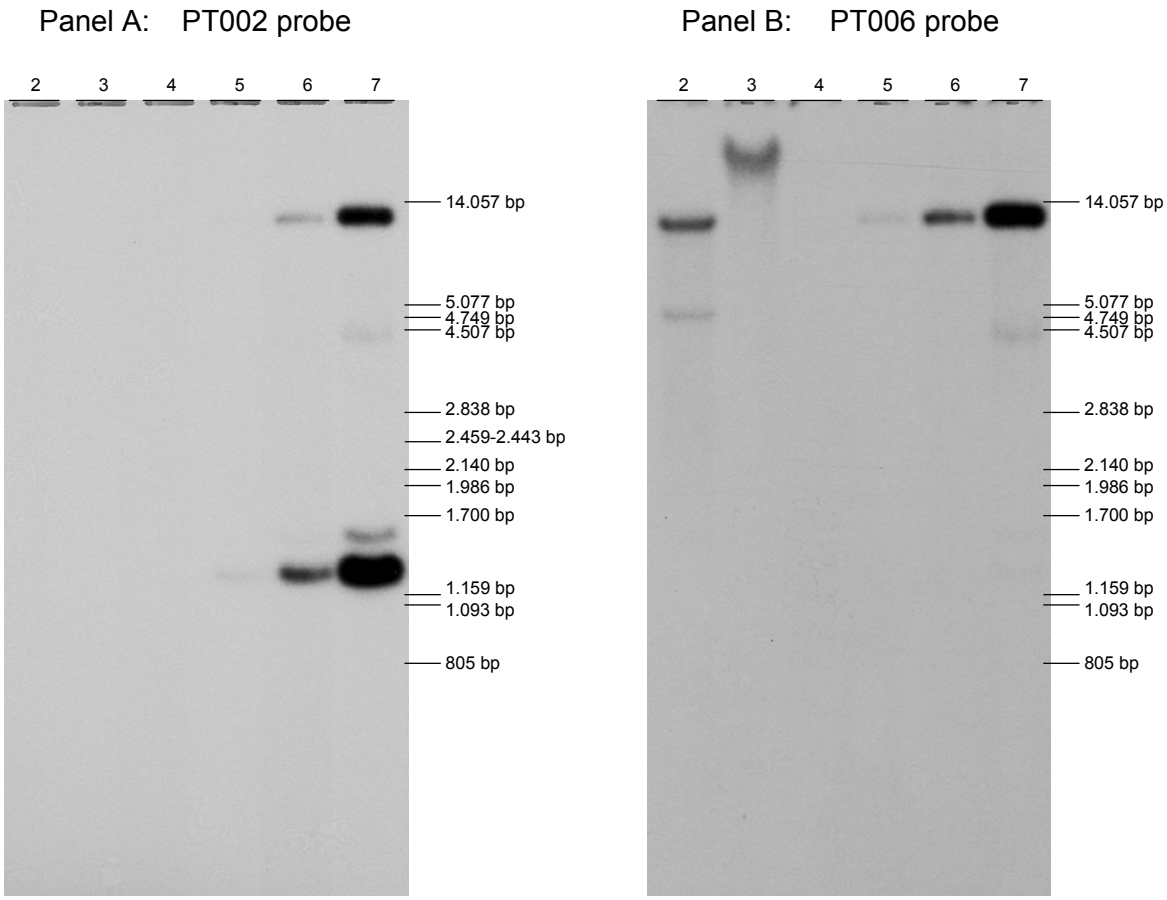


Figure 3.15. Southern blot analysis of GlyTol cotton event GHB614 - Demonstration of the absence of vector backbone sequences - PT002 probe and T-DNA probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed sequentially with the vector backbone probe (PT002: 2039 bp fragment of pTEM2, panel A) and with the T-DNA probe (PT006: 4032 bp fragment of pTEM2, panel B).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol cotton event GHB614 – *Not*I digested
Lane 4: Negative control (variety Coker 312) – *Not*I digested
Lane 5: Negative control (variety Coker 312) – *Not*I digested + 0.1 copy pTEM2 - *Not*I digested
Lane 6: Negative control (variety Coker 312) – *Not*I digested + 1 copy pTEM2 - *Not*I digested
Lane 7: Negative control (variety Coker 312) – *Not*I digested + 10 copies pTEM2 - *Not*I digested
Lane 8: MWM Phage Lambda – *Pst*I digested

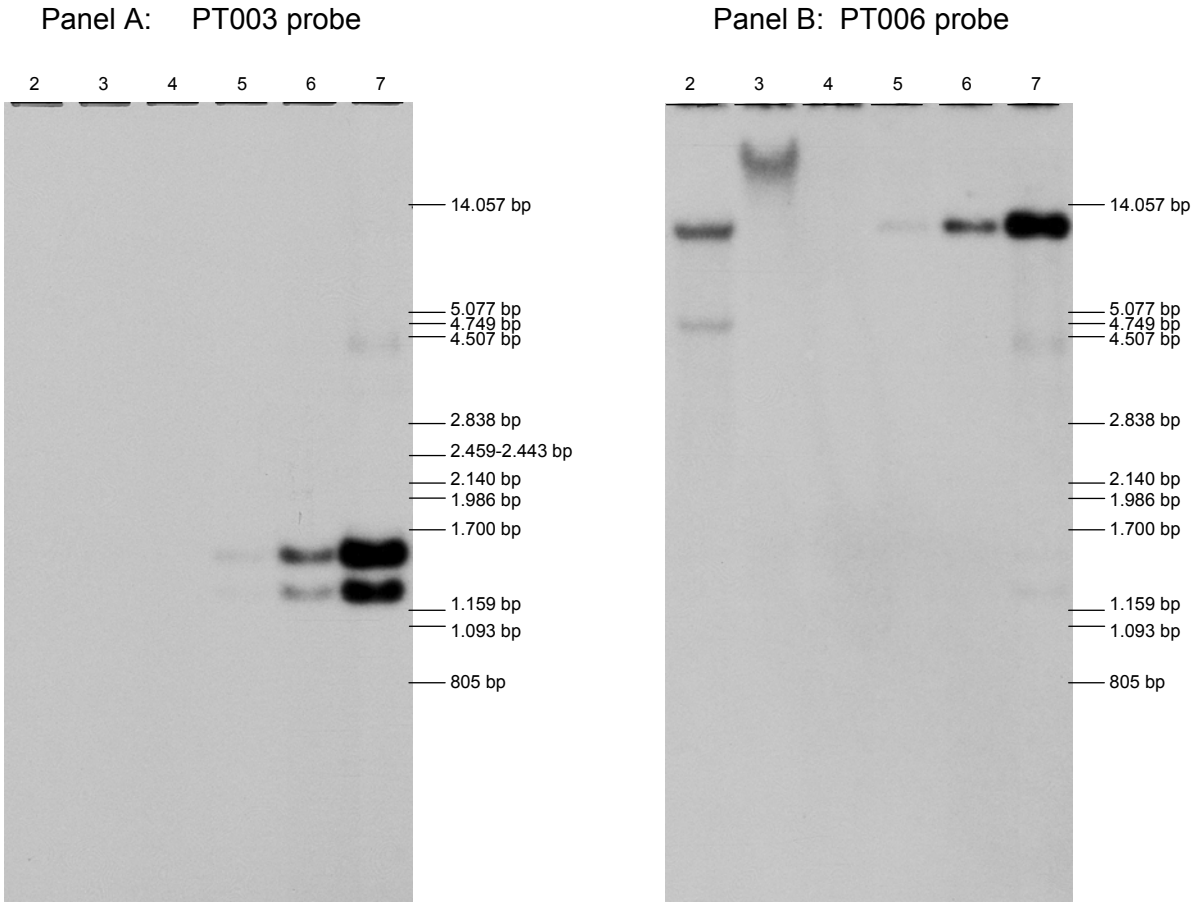


Figure 3.16. Southern blot analysis of GlyTol cotton event GHB614 - Demonstration of the absence of vector backbone sequences - PT003 probe and T-DNA probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed sequentially with the vector backbone probe (PT003: 1988 bp fragment of pTEM2, panel A) and with the T-DNA probe (PT006: 4032 bp fragment of pTEM2, panel B).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol cotton event GHB614 – *Not*I digested
Lane 4: Negative control (variety Coker 312) – *Not*I digested
Lane 5: Negative control (variety Coker 312) – *Not*I digested + 0.1 copy pTEM2 - *Not*I digested
Lane 6: Negative control (variety Coker 312) – *Not*I digested + 1 copy pTEM2 - *Not*I digested
Lane 7: Negative control (variety Coker 312) – *Not*I digested + 10 copies pTEM2 - *Not*I digested
Lane 8: MWM Phage Lambda – *Pst*I digested

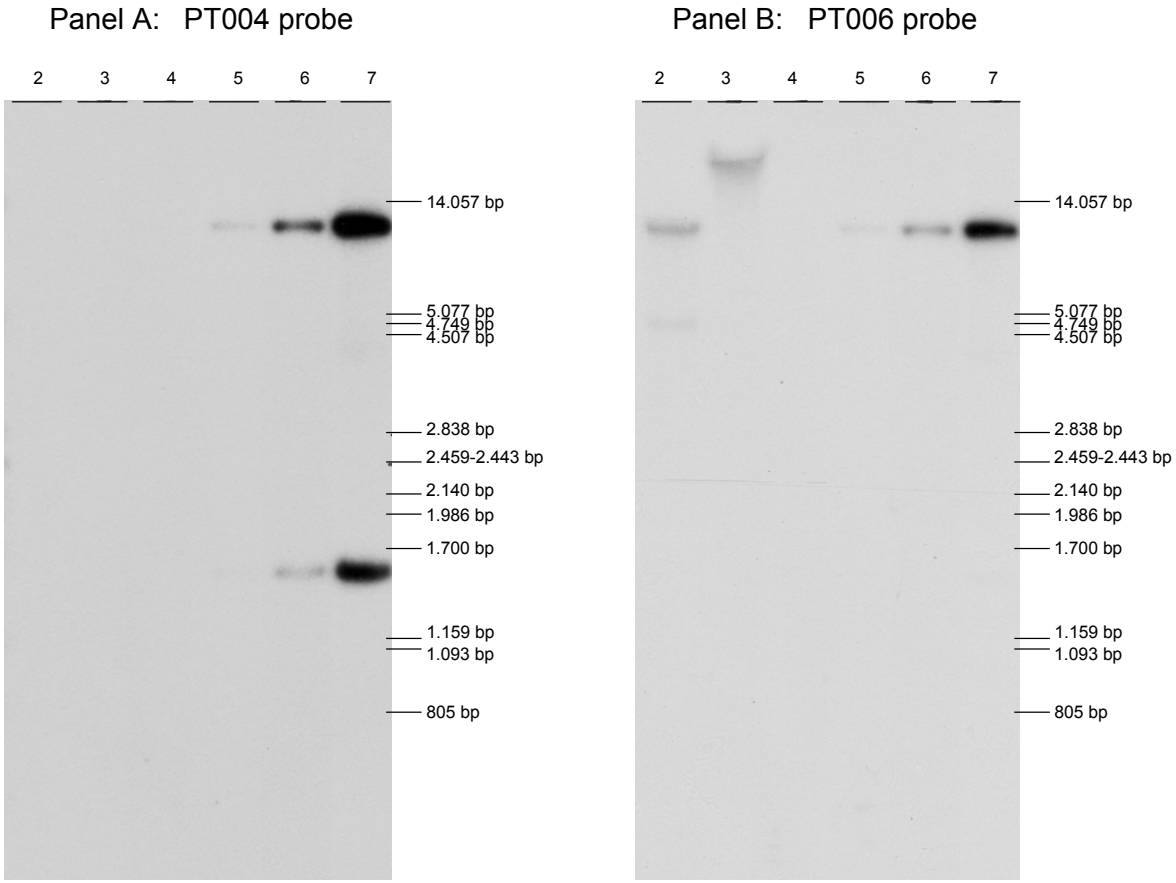


Figure 3.17. Southern blot analysis of GlyTol cotton event GHB614 - Demonstration of the absence of vector backbone sequences - PT004 probe and T-DNA probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed sequentially with the vector backbone probe (PT004: 2045 bp fragment of pTEM2, panel A) and with the T-DNA probe (PT006: 4032 bp fragment of pTEM2, panel B).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol cotton event GHB614 – *Not*I digested
Lane 4: Negative control (variety Coker 312) – *Not*I digested
Lane 5: Negative control (variety Coker 312) – *Not*I digested + 0.1 copy pTEM2 - *Not*I digested
Lane 6: Negative control (variety Coker 312) – *Not*I digested + 1 copy pTEM2 - *Not*I digested
Lane 7: Negative control (variety Coker 312) – *Not*I digested + 10 copies pTEM2 - *Not*I digested
Lane 8: MWM Phage Lambda – *Pst*I digested

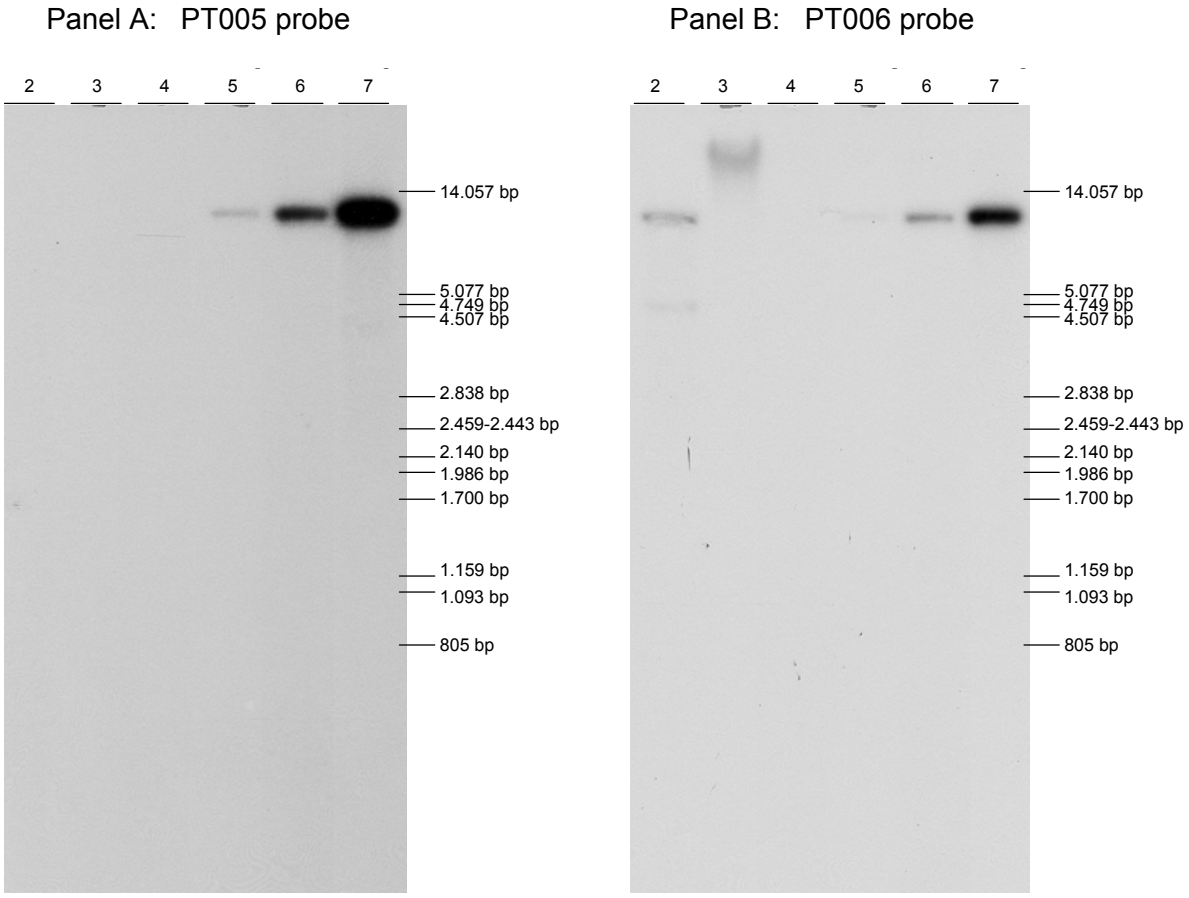


Figure 3.18. Southern blot analysis of GlyTol cotton event GHB614 - Demonstration of the absence of vector backbone sequences - PT005 probe and T-DNA probe

Genomic DNA was isolated from GlyTol cotton event GHB614 plants and from the non-transgenic counterpart Coker 312. DNAs (10 µg) were digested with different restriction enzymes and probed sequentially with the vector backbone probe (PT005: 1861 bp fragment of pTEM2, panel A) and with the T-DNA probe (PT006: 4032 bp fragment of pTEM2, panel B).

Lane 1: MWM Phage Lambda – *Pst*I digested
Lane 2: GlyTol cotton event GHB614 – *Eco*RV digested
Lane 3: GlyTol cotton event GHB614 – *Not*I digested
Lane 4: Negative control (variety Coker 312) – *Not*I digested
Lane 5: Negative control (variety Coker 312) – *Not*I digested + 0.1 copy pTEM2 - *Not*I digested
Lane 6: Negative control (variety Coker 312) – *Not*I digested + 1 copy pTEM2 - *Not*I digested
Lane 7: Negative control (variety Coker 312) – *Not*I digested + 10 copies pTEM2 - *Not*I digested
Lane 8: MWM Phage Lambda – *Pst*I digested

Appendix on protein equivalence

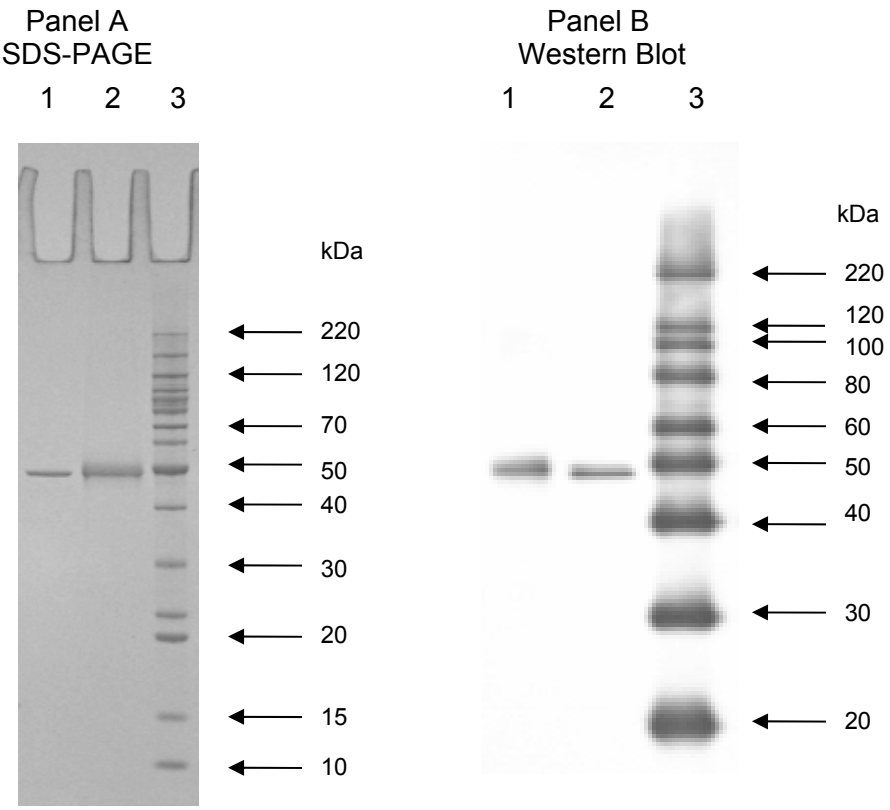


Figure 3.19. Comparison of the 2mEPSPS protein from *E. coli* with the 2mEPSPS protein isolated from leaves of GlyTol cotton event GHB614.

Panel A shows the SDS-PAGE gel stained with Coomassie brilliant blue. Lane 1 contains approximately 300 ng of 2mEPSPS protein produced in *E. coli*. Lane 2 contains approximately 1200 ng 2mEPSPS protein from GlyTol cotton leaves. Lane 3 contains molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Only the underlined molecular weights are marked by arrows.
Panel B shows a western blot. Lane 1 contains approximately 5 ng of 2mEPSPS protein from GlyTol cotton. Lane 2 contains approximately 5 ng of the 2mEPSPS protein from *E. coli*. Lane 3 contains molecular weight markers of 220, 120, 100, 80, 60, 50, 40, 30, and 20 kDa.

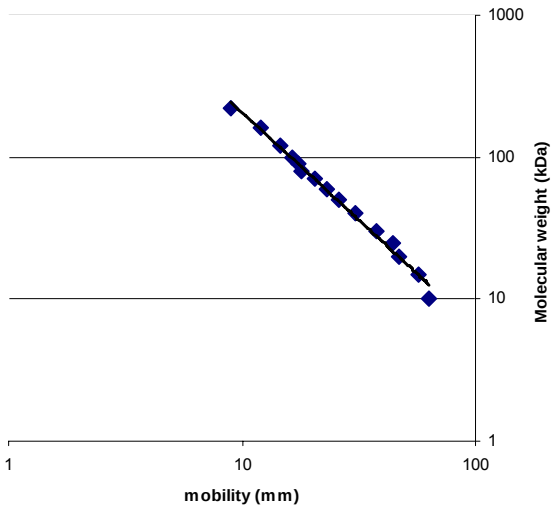


Figure 3.20. Standard curve of electrophoretic mobility versus molecular weight.

The logarithm of the molecular weight of the protein standards for the SDS-PAGE gel (Figure 3.19, Panel A) was plotted against the logarithm of their respective mobilities. Excel Trend function determined that $y = 6865.6x^{-1.519}$. The R^2 value for this curve was 0.9911. The equation defining this curve was used to calculate a molecular weight of approximately 39 kDa for cotton event GHB614. When only the markers from 25 through 80 kDa were used, the equation of the line was $y = 3944.5x^{-1.3408}$ with an R^2 value of 0.9984. The molecular weight calculated using this equation was approximately 42 kDa. This is close to the molecular weight of approximately 47 kDa calculated from the amino acid sequence.

Table 3.4. Masses of the tryptic peptides calculated from the selected ion monitored for the 2mEPSPS protein from *E. coli* and GlyTol cotton

2mEPSPS residue number	Theoretical mass (m/z)	Mass + charge of ion selected for monitoring	Calculated masses [a] of 2mEPSPS peptides from *E. coli*	Calculated masses [a] of 2mEPSPS peptides from GHB614 cotton leaf
1 to 13	1399.7	1399 [M+H]	ND [c]	NA [b]
14 to 20	733.8	734 [M+H]	733	ND
21 to 25	501.6	502 [M+H]	501	501
26 to 30	576.6	577 [M+H]	576	576
31 to 61	3342.9	1672 [M+2H]	3342	3342
62 to 71	1033	1033 [M+H]	1032	1032
72 to 74	289	289 [M+H]	288	288
75 to 75	175	NA[b]	NA[b]	NA[b]
76 to 84	790	790 [M+H]	789	789
85 to 91	805.9	807 [M+H]	806	806
92 to 106	1648.9	1648 [M+H]	1647	1647
107 to 128	2105	1053 [M+2H]	2104	2104
129 to 130	306	NA[b]	NA[b]	NA[b]
131 to 142	1296	1296 [M+H]	1295	1295
143 to 160	1907	954 [M+2H]	1906	1906
161 to 171	969	969 [M+H]	968	968
172 to 173	246	NA[b]	NA[b]	NA[b]
174 to 204	3219.8	1610[M+2H]	3218	3218
205 to 216	1435.8	1436 [M+H]	1435	1435
217 to 220	548.7	548 [M+H]	547	547
221 to 224	450.6	450 [M+H]	449	449
225 to 233	1103	1103 [M+H]	1102	1102
234 to 237	570.7	571 [M+H]	570	570
238 to 241	389	389 [M+H]	388	388
242 to 243	310	NA[b]	NA[b]	NA[b]
244 to 246	331	NA[b]	NA[b]	NA [b]
247 to 286	3870	1291 [M+3H]	3870	3870
287 to 297	1226	1226 [M+H]	1225	1225
298 to 312	1631.8	1631 [M+H]	1630	1630
313 to 317	605.6	606 [M+H]	605	605
318 to 318	147	NA [b]	NA [b]	NA [b]
319 to 321	397	NA [b]	NA [b]	NA [b]
322 to 329	905	905 [M+H]	904	904
330 to 351	2260.7	1131[M+2H]	2260	2260
352 to 357	733.8	734 [M+H]	733	733
358 to 359	246	NA [b]	NA [b]	NA [b]
360 to 363	534	534 [M+H]	533	533
364 to 368	589.8	589 [M+H]	588	588
369 to 373	591.7	592 [M+H]	591	591
374 to 392	2019	1010 [M+2H]	2018	2018
393 to 405	1533.6	1534 [M+H]	1533	1533
406 to 423	1882	1882 [M+H]	1881	1881
424 to 429	648.7	649 [M+H]	648	648
430 to 430	147	NA [b]	NA [b]	NA [b]
431 to 444	1679.9	1680 [M+H]	1679	1679
445 to 445	133	NA [b]	NA [b]	NA [b]

[a] The mass spectrometer measures mass (m) divided by charge (z) with unit resolution. Mass (m) includes the mass of the peptide + the number of positive charges. For example, the ion detected for peptide 31 – 61 containing 2 protons was 1672. Thus its calculated mass is 2(1672) - 2 or 3342. The uncertainty in the calculated mass is obtained from the average mass determination uncertainty for the peptide using all the charge states detected in the full scan spectrum.

[b] NA – not analyzed. No attempt was made to detect digestion products of 1 or 2 amino acids. Some tripeptides were not analyzed due to instrument limitations.

[c] ND – not detected.

Table 3.5. Amino acid coverage of 2mEPSPS from *E. coli* and GlyTol cotton

Calculation of % Amino Acid Coverage	Number of Amino Acids Not Detected		Residue Number
	E. coli 2mEPSPS	GlyTol 2mEPSPS	
	13	13	1-13
	0	7	14-20
	1	1	75-75
	2	2	129-130
	2	2	172-173
	2	2	242-243
	3	3	244-246
	1	1	318-318
	3	3	319 - 321
	2	2	358-359
	1	1	430-430
	1	1	445-445
Total	18	25	NA [a]
Total number of Amino Acids	445	445	
% Amino Acid Not Detected	4.0	5.6	
% Amino Acid Coverage	93.0	91.5	

[a] NA = Not Applicable

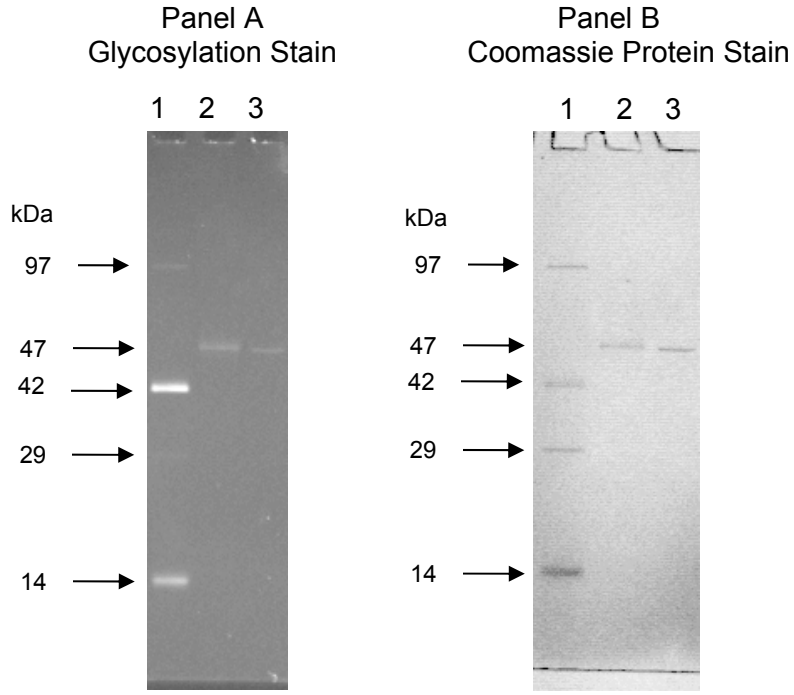


Figure 3.21. Glycoprotein staining of 2mEPSPS proteins from *E. coli* and GlyTol cotton

Panel A. Glycoprotein stain. Lane 1 contains marker proteins indicated by arrows. Markers are phosphorylase B (not glycosylated, MW = 97 kDa), α1-acidic glycoprotein (glycosylated, MW = 42 kDa), carbonic anhydrase (not glycosylated, MW = 29 kDa) and avidin (glycosylated, MW = 14 kDa). Marker proteins were present at approximately 500 ng per band except for avidin which was present at approximately 1000 ng per band. Lane 2 contains approximately 300 ng of 2mEPSPS protein from GlyTol cotton event GHB614. Lane 3 contains approximately 300 ng of 2mEPSPS protein from *E. coli*. Panel B. Coomassie brilliant blue stain. After the glycoprotein stain, the gel in panel A was stained with Coomassie brilliant blue.

Appendix 4. REGIONAL AGRONOMIC DATA

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Table 4.1. Mid-south equivalence data 2005

Mid-South Equivalency Data 2005									
Name	Yield	Fiber length	Fiber uniformity	Fiber strength	Elongation	Micronaire	Lint %	Boll size	% Open bolls
C312 a	519.36	1.2	85.48	30.03	8.4	5.3	36.83	5.22	44.5
GHB614/C312	450.26	1.18	85.5	30.78	8.5	5.45	34.1	5.07	46.75
FM9740 b	391.51	1.04	83.75	32.4	8.35	5.58	37.75	5.62	60.25
GHB614/FM9740	325.4	1.10b	84.73	33.48	8.63	5.38	36.98b	5.06	60.5
LSD (0.01)	114.78	0.04	1.48	1.75	0.37	0.22	1.11	0.6	13.16
CV	11.98	1.8	0.86	2.67	2.05	1.99	1.5	5.52	11.84
SIG	NS	b	NS	NS	NS	NS	b	NS	NS
Name	Stand count	Lodging	Days to bloom	Days to 1st open boll	Plant height	Nodes	Height to node ratio	Seed index	Strain uniformity
C312 a	90	2.75	51.25	102	40.5	17.88	2.27	6.23	5.3
GHB614/C312	102.5	4.75	50.25	99	38.33	17.65	2.17	6.38	4.5
FM9740 b	62.5	6.25	52.75	90	34.78	17.73	1.96	6.23	4.3
GHB614/FM9740	65	6.25	53.75	91	34.33	17.18	2	5.88	4
LSD (0.01)	23	2.69	3.95	6.2	3.47	0.95	0.21	0.48	2.11
CV	13.23	26.08	3.74	3	4.59	2.68	4.75	3.79	24.3
SIG	NS	NS	NS	NS	NS	NS	NS	NS	NS
Name	Seed per boll	Plant type	Pubescence	Disease reaction	Leaf morphology	Flower morphology	Plant morphology		
C312 a	27.6	7.5	1	1	1	1	1		
GHB614/C312	26.8	4.5	1	1	1	1	1		
FM9740 b	26.77	3.25	1	1	1	1	1		
GHB614/FM9740	27.66	3	1	1	1	1	1		
LSD (0.01)	4.19	2.11	N.V.	N.V.	N.V.	N.V.	N.V.		
CV	7.66	17.57	N.V.	N.V.	N.V.	N.V.	N.V.		
SIG	NS	NS							

a and b are treatment regimens and indicate in which treatments significant differences were found

Bayer CropScience

Table 4.2. Southwest equivalence data 2005

Southwest Equivalency Data 2005								
Name	Yield	Fiber length	Fiber uniformity	Fiber strength	Elongation	Micronaire	Lint %	% Open bolls
C312 a	1063.92	1.21	82.55	30.5	5.6	3.13	39.91	21.25
GHB614/C312	888.98	1.17a	83.43	30.1	5.55	3.33	39.02	20
FM9740 b	964.78	1.07	81.68	29.7	5.25	3.58	40.25	22.5
GHB614/FM9740	925.7	1.03b	81.68	29.85	5.28	3.5	40.49	23.75
LSD (0.01)	178.98	0.03	1.2	1.6	0.27	0.31	0.94	7.48
CV	12.27	1.99	0.96	3.52	3.22	6.23	1.55	24.07
SIG	NS	ab	NS	NS	NS	NS	NS	NS
Name	Lodging	Strain uniformity	1st position bolls	Plant height	Nodes	Height to node ratio	Disease reaction	Total boll load
C312 a	1.25	4.75	3.5	33.25	16.25	2.05	2	6.75
GHB614/C312	1.5	4	3.5	32.75	15.5	2.11	2	6.25
FM9740 b	2.5	4.5	3.5	33.25	16.5	2.02	1.5	6
GHB614/FM9740	1.75	7.00b	4.00b	34	17.5	1.94	2	8.00b
LSD (0.01)	0.85	0.79	1	1.05	0.92	0.92	0.81	1.79
CV	31.55	10.9	19.69	4.26	3.93	3.93	28.04	18.14
SIG	NS	b	b	NS	NS	NS	NS	b

a and b are treatment regimens and indicate in which treatments significant differences were found

Table 4.3. Regional plant mapping data (2004 & 2005)

Plant Height (in.)												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	31.75	29.75	34	34.54	59.33	56.17	33.57	34.1	25.75	25.8	28.07	29.51
1X b	31.67	31	32.42	31.96	59.25	59.33	34.45	35.76	24.66	25.93	30.08	29.35
3X c	31.58	31.16	32.66	33.32	60.5	59.17	37.18	35.66	24.68	25.63	29	28.46
LSD (0.01)		0.48		3.59		5.46		4.58		5.26		2.28
CV		14.29		10.09		8.58		10.41		15.7		7.13
SIG		N.S.		N.S.		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Number of Nodes												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	16.08	15.67	15.85	16.38	17.08	16.58	17.01	16.71	16.83	16.83	16.75	16.71
1X b	16.17	15.83	15.29	15.53	16.92	16.75	17.22	17.3	16.2	16.75	17.92	16.91
3X c	16.83	16.33	16.06	16.06	17.58	16.83	17.29	17.07	15.89	17.08	17.47	17.03
LSD (0.01)		5.26		1.25		1.87		1.17		2.27		1.12
CV		29.25		7.27		9.88		5.51		10.13		5.99
SIG		N.S.		N.S.		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Number of first position bolls												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	6.08	6.25	4.51	5.26	5.17	5.92	4.77	4.89	4.18	3.82	4.83	5.04
1X b	6.42	6.58	4.8	4.85	5.42	5.42	4.71	4.66	4.05	4.31	5.26	4.93
3X c	6.83	6.42	4.66	5.11	5.42	6	4.31	4.78	4.15	3.71	5.33	4.42
LSD (0.01)		2.16		0.59		1.21		1.4		0.79		0.92
CV		31.31		10.9		21.99		28.05		17.17		19.43
SIG		N.S.		N.S.		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.3. Regional plant mapping data (2004 & 2005) Continued

Total # of Bolls												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	13	14	N/A	N/A	9.5	9.67	7.69	8.22	5.76	5.6	9.33	9.83
1X b	14.33	14.33	N/A	N/A	10.17	9.92	7.83	8	5.58	6.6	11.15	9.62
3X c	16.58	14.58	N/A	N/A	9.17	9.83	7.81	7.81	5.6	5.46	10.23	8.8
LSD (0.01)		5.66		-		2.26		2.19		1.36		1.74
CV		37.19		-		24.89		26.29		20.79		19
SIG		N.S.		-		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.4. Regional agronomic parameters (2004 and 2005)

Height to Node Ratio												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1.92	2	2.16	2.11	3.53	3.41	1.97	2.04	1.55	1.54	1.69	1.78
1X b	2	2	2.11	2.06	3.53	3.56	2	2.07	1.54	1.55	1.7	1.74
3X c	2.08	2	2.04	2.08	3.48	3.54	2.15	2.09	1.56	1.37	1.66	1.69
LSD (0.01)		0.23		0.2		0.29		0.66		0.27		0.14
CV		14.3		8.88		8.12		7.38		13.35		7.44
SIG		N.S.		N.S.		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Number of Days Until first bloom												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	50.33	52	63.42	65	61.67	60.67	55.92	55.58	64.88	65.25	N/A	N/A
1X b	50.17	52.25	63.92	65.83	60.17	60	55.08	56.25	64.63	65.38	N/A	N/A
3X c	50.08	52.5	62.5	65.58	60.25	57.75	54.67	55.08	64.63	64.63	N/A	N/A
LSD (0.01)		3.17		1.47		2.9		2.01		1.02		/
CV		5.66		2.62		4.53		3.31		1.18		/
SIG		N.S.		bc		N.S.		N.S.		N.S.		

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Seed / Boll												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	26.58	25.75	N/A	N/A	25.75	23.5	23.49	23.33	26.38	25	N/A	N/A
1X b	28.5	25.08	N/A	N/A	24.83	21.83	24.96	23.94	26.5	26	N/A	N/A
3X c	26.5	25.75	N/A	N/A	24	21.08	23.68	24.02	26.75	24	N/A	N/A
LSD (0.01)		2.61		/.		3.69		4.53		2.41		/.
CV		9.03		/		15.15		18.2		7.51		/
SIG		b				N.S.		N.S.		c		

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.4. Regional agronomic parameters (2004 and 2005) Continued

% open bolls												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	N/A	N/A	50	52.19	47.58	53.17	50.8	50.8	39.37	26.88	28.75	35.41
1X b	N/A	N/A	49.17	48.75	54.42	57.83	47.5	50.8	40.62	27.5	27.5	28.75
3X c	N/A	N/A	48.75	48.33	55.08	54.92	47.5	46.7	41.25	28.75	27.91	26.25
LSD (0.01)		/		6.2		16.38		6.33		13.41		8.36
CV		/		14.05		35.46		16.82		41.61		32.8
SIG				N.S.		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Stand count												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	N/A	N/A	4.33	3.42	N/A	N/A	4.63	3.77	N/A	N/A	4.5	5.25
1X b	N/A	N/A	4.58	4.5	N/A	N/A	5.07	3.77	N/A	N/A	4.08	5.5
3X c	N/A	N/A	4.33	3.83	N/A	N/A	5	4.74	N/A	N/A	4.58	5.33
LSD (0.01)		/		0.94		/		1.06		/		0.94
CV		/		18.91		/		21.68		/		21.8
SIG				N.S.				b				c

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Strain uniformity												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	2.75	3.17	N/A	N/A	4	3.33	N/A	N/A	4.75	4.59
1X b	1	1	2.67	3.5	N/A	N/A	3.44	3.33	N/A	N/A	5.08	4.5
3X c	1	1	2.42	3.17	N/A	N/A	3.22	3.89	N/A	N/A	4.75	4.17
LSD (0.01)		N.V.		0.567		/		0.98		/		0.55
CV		N.V.		26.11		/		27.14		/		16.41
SIG				abc				N.S.				N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.4. Regional agronomic parameters (2004 and 2005) Continued

Leaf Morphology												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
1X b	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
3X c	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
LSD (0.01)		N.V.		N.V.		N.V.		N.V.		/		/
CV		N.V.		N.V.		N.V.		N.V.		/		/
SIG												

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Flower Morphology												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
1X b	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
3X c	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
LSD (0.01)		N.V.		N.V.		N.V.		N.V.		/		/
CV		N.V.		N.V.		N.V.		N.V.		/		/
SIG												

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Plant Morphology												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
1X b	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
3X c	1	1	1	1	1	1	1	1	N/A	N/A	N/A	N/A
LSD (0.01)		N.V.		N.V.		N.V.		N.V.		/		/
CV		N.V.		N.V.		N.V.		N.V.		/		/
SIG												

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.5. Regional reproductive success data (2004 & 2005)

Fertility												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1	1	1.06	1.04	1.67	1.67	N/A	N/A	N/A	N/A
1X b	1	1	1	1	1.03	1.03	1.56	1.78	N/A	N/A	N/A	N/A
3X c	1	1	1	1	1.13	1.05	1.56	1.44	N/A	N/A	N/A	N/A
LSD (0.01)		N.V.		N.V.		0.81		0.55		/		/
CV		N.V.		N.V.		49.06		11.57		/		/
SIG						N.S.		N.S.				

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Boll Size												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	5.23	5.13	4.85	4.75	5.25	5.08	4.4	4.25	4.61	4.46	N/A	N/A
1X b	5.53	5.18	4.89	4.72	5.11	4.75	4.68	4.28	4.59	4.52	N/A	N/A
3X c	5.26	5.11	4.71	4.38	4.85	4.42	4.42	4.36	4.61	4.13	N/A	N/A
LSD (0.01)		0.43		0.46		0.87		0.81		0.42		/
CV		7.48		8.93		17.43		17.11		7.32		/
SIG		N.S.		N.S.		N.S.		N.S.		c		

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Seed Index												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	11.68	12.23	10.99	11.47	12.69	13.78	5.77	5.88	9.96	10.54	N/A	N/A
1X b	11.53	12.62	10.5	11.36	12.6	13.7	5.78	5.78	10.16	10.36	N/A	N/A
3X c	12.02	12.26	11.47	11.99	12.27	13.2	5.74	5.8	10.11	10.24	N/A	N/A
LSD (0.01)		1.4		0.57		1.1		1.85		0.78		/
CV		11.28		4.68		8.23		34.46		5.63		/
SIG		N.S.		b		N.S.		N.S.		N.S.		

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.6. Regional abiotic stress data (2004 and 2005)

Chlorosis 1												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1	1	1.42	1.25	1.33	1	1	1	2.08	1
1X b	1	1	1	1	1.42	1.25	1.67	1	1	1	1.67	1
3X c	1	1	1	1	1.33	1.33	1.33	1	1	1.38	1.75	1.33
LSD (0.01)		N.V.		N.V.		0.61		0.27		0.39		0.71
CV		N.V.		N.V.		38.77		22.44		25.38		53.03
SIG						N.S.		abc		N.S.		a

Chlorosis 2												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1	1	1.83	2.08	N/A	N/A	1	1	N/A	N/A
1X b	1	1	1	1	1.92	1.83	N/A	N/A	1	1	N/A	N/A
3X c	1	1	1	1	1.92	2.08	N/A	N/A	1	1	N/A	N/A
LSD (0.01)		N.V.		N.V.		0.83		/		N.V.		/
CV		N.V.		N.V.		36.06		/		N.V.		/
SIG						N.S.						

Chlorosis 3												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1.75	1.92	2.17	2.33	N/A	N/A	1	1	2.33	1.17
1X b	1	1	1.92	1.75	2.67	2	N/A	N/A	1	1	2	1
3X c	1	1	2.25	1.92	2.83	2.25	N/A	N/A	1	1.25	2.08	2.25
LSD (0.01)		N.V.		0.57		0.62		/		0.23		0.67
CV		N.V.		28.43		24.26		/		24.28		40.42
SIG				N.S.		b				c		abc

Table 4.7. Regional biotic stress data table (2004 and 2005)

Lodging												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1	1	1.67	2.42	1	1	2.44	2.67	1	1	1.75	2.42
1X b	1	1	1.67	2.75	1	1	2.56	2.44	1	1	2	2.33
3X c	1	1	2.5	1.75	1	1	2.67	2.56	1	1	1.92	2.33
LSD (0.01)		N.V.		0.93		0.4		0.91		N.V.		0.65
CV		N.V.		41.86		41.68		36.92		N.V.		31.95
SIG				bc		N.S.		N.S.				N.S.

Disease Rating												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	N/A	N/A	N/A	N/A	1	1	1	1	1	1	2.75	2.75
1X b	N/A	N/A	N/A	N/A	1	1	1	1	1	1	2.58	2.83
3X c	N/A	N/A	N/A	N/A	1	1	1	1	1	1	2.75	2.5
LSD (0.01)		/		/		N.V.		N.V.		N.V.		0.63
CV		/		/		N.V.		N.V.		N.V.		24.52
SIG												N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.8. Regional yield data (2004 and 2005)

Yield By Region												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1121.58	994.67	795.93	686.3	1090	1022	548	509	716	625.25	1200	1183
1X b	1129.58	982.33	823.69	733.32	1034	972	561	513	699.75	589.38	1223	1313
3X c	972.92	925.67	806.49	736.22	1022	997	453	561	963.38	545	1253	1172
LSD (0.01)		113.67		94.83		285		169.84		121.95		197.39
CV		10.47		11.59		28.84		31.56		16.24		16.67
SIG		ab		a		N.S.		N.S.		N.S.		N.S.

% Lint												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	40.76	39.27	37.16	35.97	38.45	36.42	37.62	34.82	40.78	40.53	40.68	38.95
1X b	40.7	37.24	38.26	36.14	36.66	36.39	37.95	34.6	41.43	40.45	40.99	39.02
3X c	40.2	38.12	38.63	34.93	39.13	36.55	37.43	35.21	41.63	39.96	39.82	38.8
LSD (0.01)		2.17		1.4		1.38		1.25		2.04		7.47
CV		5.08		3.51		3.39		3.07		22.37		18.44
SIG		b		abc		ac		abc		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.9. Regional fiber quality data (2004 and 2005)

Fiber Length												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	1.23	1.21	1.21	1.21	1.24	1.24	1.16	1.15	1.16	1.16	1.17	1.17
1X b	1.22	1.21	1.21	1.21	1.25	1.25	1.15	1.16	1.16	1.17	1.15	1.2
3X c	1.23	1.22	1.21	1.2	1.24	1.24	1.16	1.16	1.17	1.16	1.17	1.19
LSD (0.01)		0.02		0.02		0.03		0.03		0.05		0.17
CV		1.7		1.78		2.08		2.64		3.12		13.98
SIG		N.S.		N.S.		N.S.		N.S.		N.S.		N.S.

Fiber Strength												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	31.6	32.98	31.6	32.98	30.52	31.78	32.46	34.13	27.06	27.78	28.02	28.73
1X b	31.17	32.63	31.17	32.63	30.34	31.61	32.77	34.09	27.54	28.93	28.54	29.36
3X c	31.63	33.55	31.63	33.55	30.49	31.76	33.39	34.05	27.76	28.54	28.13	29.24
LSD (0.01)		1.29		1.29		3.4		1.37		1.98		4.58
CV		3.69		3.69		5.46		3.72		5.34		15.29
SIG		abc		abc		N.S.		a		N.S.		N.S.

Fiber Uniformity												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	85.83	85.91	85.1	85.79	85.36	85.98	84.29	84.73	91.94	82.95	91.94	82.95
1X b	85.46	85.87	84.88	85.96	85.54	86.48	84.17	84.7	82.14	82.96	82.14	82.96
3X c	85.96	86.29	84.99	85.68	85.45	86.45	84.34	84.62	82.16	82.84	82.16	82.84
LSD (0.01)		0.57		0.79		0.73		1.03		1.87		1.87
CV		0.63		0.87		0.81		1.15		1.76		1.76
SIG		N.S.		b		b		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Table 4.9. Regional fiber quality data (2004 and 2005) continued

Micronaire												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	4.81	4.77	4.8	4.89	4.18	4.39	5.27	5.28	3.6	3.8	3.81	3.42
1X b	4.77	4.88	4.86	4.77	4.36	4.48	5.36	5.22	3.71	3.65	3.5	3.87
3X c	4.72	4.72	4.76	4.83	4.5	4.27	5.18	5.38	3.76	3.76	3.45	3.73
LSD (0.01)		0.24		0.21		0.78		0.39		0.38		0.86
CV		4.95		4.14		6.98		7.2		8.46		22.81
SIG		N.S.		N.S.		N.S.		N.S.		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found

Elongation												
Treatment	Southeast 2004		Southeast 2005		Mid-south 2004		Mid-south 2005		Southwest 2004		Southwest 2005	
	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614	C312	GHB614
Control a	7.93	8.09	8.45	8.78	7.07	7.08	8.33	8.64	5.38	5.65	5.03	5.02
1X b	7.72	8.09	8.39	8.63	7.15	7.13	8.32	8.63	5.3	6.04	5.03	5.33
3X c	7.82	8.3	8.3	8.81	7.2	7.16	8.26	8.51	5.31	5.49	5.06	5.25
LSD (0.01)		0.29		0.26		0.23		0.28		0.74		0.93
CV		3.5		2.89		5.1		3.07		9.92		17.64
SIG		bc		ac		N.S.		ab		N.S.		N.S.

a, b, and c are treatment regimens and indicate in which treatments significant differences were found