Bayer CropScience

Petition for Determination of Nonregulated Status for Insect-Resistant and Glufosinate Ammonium-Tolerant cotton:

TwinLink™ cotton (events T304-40 x GHB119)

OECD Unique Identifier BCS-GHØØ4-7 x BCS-GHØØ5-8

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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SUMMARY

Bayer CropScience LP (BCS) is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for TwinLink™ cotton. TwinLink cotton was obtained by the traditional breeding cross of events T304-40 and GHB119. BCS requests a determination from APHIS that TwinLink cotton, its parents, and any progeny derived from crosses of TwinLink cotton with traditional cotton varieties, and any progeny derived from crosses of TwinLink 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. TwinLink cotton (events T304-40 x GHB119) is considered a regulated article because it contains sequences from the plant pests, *Agrobacterium tumefaciens*, Subterranean Clover Stunt Virus, Cauliflower Mosaic Virus (CaMV) and Cassava Vein Mosaic Virus (CsVMV).

TwinLink cotton contains the stably integrated genes *cry*1Ab, *cry*2Ae and *bar*, which encode respectively the Cry1Ab, Cry2Ae and PAT proteins. The genes were introduced by *Agrobacterium*-mediated gene transfer. Southern blot analyses show TwinLink cotton contains one complete copy of the *cry*1Ab and *cry*2Ae genes, and 2 copies of the *bar* gene.

Bayer CropScience (BCS) has developed cotton (*Gossypium hirsutum*) plants that express two insecticidal proteins, Cry1Ab and Cry2Ae, from a common soil bacterium, *Bacillus thuringiensis (B.t.)*. The Cry1Ab and Cry2Ae protein are effective in controlling lepidopteran larvae such as bollworm (CBW, *Helicoverpa zea*), tobacco budworm (TBW, *Heliothis virenscens*) larvae and fall armyworm (FAW, *Spodoptera frugiperda*) which are common pests of cotton. These pests cause severe economic damage to the cotton crop if not controlled. If controlled by chemical pesticides, there is the need for large input annually to control these pests. Small scale field trial experiments of cotton expressing the Cry1Ab and Cry2Ae proteins, conducted under notifications and/or permits granted by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), US EPA, Argentina and Spain, have shown the plant's ability to protect itself against these pests.

Transgenic cotton plants expressing the Cry1Ab and Cry2Ae proteins will provide an excellent addition to growers' options for insect control that reduces or eliminates the need for other insecticide inputs and fits well within an integrated pest management program. Cry1Ab is a protein familiar to the Agencies, but is has not been used in commercial cotton. Cry2Ae is a *B.t.* insecticidal protein with many of the common characteristics of this group that is familiar to the agency, but has not been used in commercial cotton.

In addition to the Cry1Ab and Cry2Ae proteins, TwinLink cotton also contains the PAT (phosphinothricin-acetyl-transferase) enzyme, encoded by the *bar* gene. This is the same protein that is in Bayer CropScience LLCotton25 and confers to the plant tolerance to the herbicide Glufosinate Ammonium.

TwinLink cotton has been developed by BCS as an alternative insect resistant and herbicide tolerant cotton product.

TwinLink cotton has been field tested by BCS beginning in 2005 in adapted growing regions of the United States and winter nursery. These tests have occurred at 66 sites under field release authorizations granted by USDA APHIS (USDA authorizations: 05-035-12n; 07-044-102n; 07-044-103n; 07-044-104n; 07-059-101n; 07-059-104n; 07-065-117n; 07-065-118n; 07-065-119n; 08-022-101n; 08-036-127n) Data collected from these field trials and laboratory analyses presented herein demonstrate that TwinLink 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 TwinLink cotton, and any progeny derived from crosses of TwinLink cotton with traditional cotton varieties, and any progeny derived from crosses of TwinLink 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

A	acre
a.i.	active ingredient
AMS	Agricultural Marketing Services
ANOVA	ANalysis Of VAriance
APHIS	Animal and Plant Health Inspection Service
bar	Phosphinothricin acetyltransferase gene
BBMV	Brush Border Membrane Vesicles
BCS	Bayer CropScience
BLASTn	Basic Local Alignment Search Tool comparing nucleotide sequences
BLASTx	BLAST search of protein databases using a translated nucleotide query
bp	base pairs
Bt	Bacillus thuringiensis
BXN	Bromoxynil-tolerant
CaMV	Cauliflower Mosaic Virus
CBW	Cotton Bollworm
COA	Certificate of Analysis
CsVMV	Cassava Vein Mosaic Virus
cm	centimeter
dm	dry matter
DMPT	Demethylphosphinothricin
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ELS	Extra Long Staple
EPA	Environmental Protection Agency
ESA	Endngered Species Act
EU	European Union
FAW	Fall Armyworm
FDA	Food and Drug Administration
FGENESH	Find GENES using Hidden markov model
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
FW	fresh weight
FWS	Fish and Wildlife Service
g	gram
GetORF	EMBOSS database for ORFs
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
ID	identification
Kb	Kilobases
kDa	kiloDalton
kg	kilogram
L	liter
LB	Left Border
lb	pound (1 pound = 0,454 kg)
LC/MS	Liquid Chromatography/Mass Spectroscopy
LOD	Limit of Detection
LOQ	Limit of Quantification
L-PPT	Phosphinothricin
m	meter
M	million
mg	milligram
mL	milliter
mm	millimeter
mM	millimolar
µL	microliter
µM	micromolar
MS	Mass Spectrometry
MSMA	Monosodium acid methanearsoate
µg	microgram
NA	Not Applicable
ng	nanogram
ND	Not Detectable: Below the limit of detection
nm	nanometer
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
ORF	Open Reading Frame
PAT	Phosphinothricin-acetyl-transferase
PBW	Pink bollworm
PCR	Polymerase Chain Reaction
pg	picogram
ppb	parts per billion
RAC	Raw Agricultural Product
RB	Right Border
RBS	Ribosome Binding Site
RR	Roundup Ready
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SIM	Selected Ion Monitoring
TAE	Tris Acetate EDTA
T1, T2, etc	generations after T0 (transformation)
TBW	Tobacco Budworm
T-DNA	transfer DNA from Agrobacterium
TEP	Total Extractable Protein
TEP	Total Extractable Protein
™	Trademark
TwinLink	insect resistant and glufosinate - ammonium tolerant cotton
US/USA	United States of America
USDA	United States Department of Agriculture
WT	Wild type
zPCR	zygosity PCR

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I. RATIONALE

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

The United States Department of Agriculture (USDA) 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 the 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 TwinLink cotton

Cotton is an important fiber crop globally and in the South Plains of USA is the largest contiguous cotton-growing region in the world. The USA, China and India rank as the top three cotton producing countries (USDA-FAS, 2008; Table 1). According to USDA-NASS data, cotton production in the US was 12.73M acres in 2006 and was substantially lower in 2007 and 2008, most likely due to the increased value of corn and interest in biofuel production (USDA-NASS - http://www.nass.usda.gov/QuickStats/index2.jsp). In 2008, 7.78M acres of cotton was harvested as compared to a 20-year high of approximately 16M acres in 1995, yet the need for a high yielding crop of high value cotton fiber at the lowest production cost is ever more important. The cotton industry relies heavily on pest control measures for successful cotton fiber production, both to control weeds and lepidopteran insects, which account for the largest cotton insect pest problem.

To that end, cotton growers in the USA have readily adopted genetically improved cotton, primarily due to reductions in pest management costs, greater yield capture, decreased pesticide costs and overall enhanced grower returns. Ninety-one percent of pesticide reduction was derived from grower adoption of herbicide tolerant crops, mainly corn, cotton and soybeans, with herbicide tolerant cotton accounting for 22% of the total pesticide reduction (Johnson *et al.,* 2007). In 2006, 86% of all cotton in the USA expressed herbicide tolerant traits and 57% expressed insect control traits.

C. Adoption of Bt cotton

Reduction in the levels of insecticide use due to the adoption of Bt cotton have been described in terms of lower numbers and total quantity of chemical insecticide sprays. In Arizona, 5.4 fewer insecticide applications per crop per season was reported by Carriere *et al.* (2001), while a total reduction of 8.7 million and 15 million sprays for the US due to the adoption of Bt cotton in the 1998 and 1999 seasons, respectively (Carpenter and Gianessi, 2001). More recently, Johnson *et al.* (2007) reported a total reduction of 2.8M pounds of insecticide (~2.5% of the total US pesticide reduction) during the 2006 growing season due to insect resistant Bt cotton. On average, profitability levels increased by about $48/acre in 2006 for dual gene Bt cotton (Brookes and Barfoot, 2008) based on technology costs, reduction in insecticide use and

improved yield capture. The total net value for US growers by using Bt cotton in 2006 was $315.5M (Johnson *et al.,* 2007).

Bayer CropScience (BCS) has developed a combined-trait cotton known as TwinLink cotton using conventional breeding techniques by crossing BCS' Cry1Ab Cotton (Event T304-40) with BCS' Cry2Ae Cotton (Event GHB119) to assist growers in controlling lepidopteran pests of cotton. TwinLink cotton will also express a herbicide tolerant trait, based on LibertyLink technology, that can tolerate glufosinate ammonium containing herbicides. Field trial evaluations of small-scale plots infested both naturally and artificially; indicate minimal damage of TwinLink cotton with infestations of cotton bollworm (CBW, *Helicoverpa zea*), tobacco budworm (TBW, *Heliothis virenscens*), pink bollworm (PBW, *Plutella gossypiella*), and fall armyworm (FAW, *Spodoptera frugiperda*). TwinLink not only demonstrates expanded target pest control by the expression of two Cry proteins unique to cotton, but also offers growers a superior resistance management tool for comprehensive product durability.

D. Benefits of Bt cotton

Bacillus thuringiensis (*Bt*) is a bacterium that occurs naturally in the soil and on plants. *Bt* products have an excellent safety record and can be used on crops until close to the day of harvest (EPA, 2005). *Bt* genes have also been transferred to plants for the production of insect-protected crops, such as TwinLink cotton. Mammalian safety of pesticidal proteins incorporated into plants is described in the EPA Biopesticides Registration Documents (2007 - http://www.epa.gov/pesticides/biopesticides/pips/index.htm) and in various publications. The results of these studies support the lack of mammalian toxicity or allergenic potential for Cry1Ab and Cry2Ae. Taken together there will not be a significant risk of toxic or allergenic effects to humans or other animals if exposed to the Cry1Ab or the Cry2Ae proteins as expressed in TwinLink cotton.

In summary, based upon the data provided within this application or from previously cited data, TwinLink cotton will provide growers:

1. Improved insect efficacy for major pests of cotton, independent of weather conditions
2. Combined Bt trait product for excellent insect resistance management
3. Herbicide tolerance based on LibertyLink technology providing growers with additional weed control options
4. Excellent human, animal, non-target organism and environmental safety profile
5. Reduced pesticide use
6. Enhanced yield capture
7. Decreased pest management costs

Leading to an overall enhancement of grower financial returns.

E. Submissions to other regulatory agencies

Food and Drug Administration

TwinLink cotton (events T304-40 x GHB119) 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 TwinLink 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 tolerance exemption is in place for Cry1Ab protein in all crops (40 CFR Part 180, §180.1173), as well as for the PAT protein (40 CFR Part 180, §180.1151). A temporary exemption from the requirement of a tolerance has been issued for Cry2Ae (40 CFR Part 174 §174.530). EPA Section 3 registration applications will be submitted for Cry1Ab cotton, Cry2Ae cotton and TwinLink cotton.

Foreign Governments

BCS intends to submit dossiers to the proper regulatory authorities of foreign governments request for the importation of US Cottonseed and to have regulatory processes in place. These may include submissions to the relevant Regulatory Authorities in Canada, Mexico, EU, Japan, among others. TwinLink 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, US, 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	*1,000 Metric Tons*				
	2004/05	*2005/06*	*2006/07*	*2007/08*	*Est Nov 2008/09*
China Peoples Republic of	6,597	6,183	7,729	8,056	7,947
India	4,137	4,148	4,746	5,356	5,443
United States	5,062	5,201	4,700	4,182	2,945
Pakistan	2,425	2,213	2,155	1,938	1,960
Brazil	1,285	1,023	1,524	1,602	1,361
Uzbekistan Republic of	1,132	1,208	1,165	1,197	1,110
Turkey	904	773	827	675	501
Other	4,898	4,632	3,713	3,238	3,307
Total	26,440	25,382	26,560	26,245	24,574

Source: USDA-Foreign Agriculture Service (http://www.fas.usda.gov/cotton/circular/Current.asp). 2008 estimates

In the US for the 2007 production year, cotton was grown on 9.4 million acres, the major producing states being Texas (4.9 million acres), Georgia (1.0 million acres), Arkansas (0.9 million acres), Mississippi (0.7 million acres), North Carolina and Tennessee (0.5 million acres). The world total planted area in 2007-2008 was 33.2 million hectares, for a production of 121 million bales (USDA-FAS, 2008).

The total production of cotton as an oilseed is estimated to be 46.1 million metric tons in 2007/2008 out of a world total of 391.4 million tons. Cottonseed oil, with a production estimated at 5 million tons in 2007/08, accounts only for 3.9% of total world oil production. With 1.6 million tons for that same year, China is by far the most important producer (http://www.fas.usda.gov/oilseeds/circular/Current.asp - USDA-FAS, 2008).

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, Puerto Rico, the Virgin Islands and south of Tampa (Florida Route 60) in Florida. 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 TwinLink traits (Cry1Ab, Cry2Ae and PAT proteins) 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 TwinLink 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 glufosinate ammonium, 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 that are not tolerated by the modified crop. For example, TwinLink cotton volunteers could easily be controlled by using any number of targeted and broad-specturm herbicides used to control broadleaf weeds in agricultural systems. Potential volunteer cotton plants with the TwinLink traits 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 TwinLink traits, which emerges within conventional or glufosinate susceptible cotton varieties can be controlled with products such as flumioxazin during pre-plant burndown, pendimethlin, and paraquat. Expression of the Bt proteins, Cry1Ab and Cry2Ae, within the TwinLink cotton product, raises no additional concerns with regards to weediness.

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 (Kareiva *et al*., 1994) and described in USDA petition for GlyTol cotton 06-332-01p. 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. 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 and their results 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.

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 TwinLink

cotton to *G. thurberi* is not a concern.

Gossypium 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 glufosinate ammonium is not used in natural non-agricultural areas.

G. Characteristics of the recipient plant

TwinLink cotton (events T304-40 x GHB119) has its origin in the varieties Coker 315 and Coker 312. The variety Coker 315 (PVP 8000087) and the variety Coker 312 (PVP 7200100) are both US Protected Varieties of SEEDCO Corporation, Texas and were developed from a cross of Coker 100 X D&PL-15 and selected through successive generations of line selection.

H. Cotton as a test system in this petition

During the development of TwinLink cotton, the events T304-40 and GHB119 were carried in their Coker genetic background for purposes of equivalence testing. At the same time, the Cry1Ab and Cry2Ae traits were introgressed into commercial and/or advanced breeding varieties to evaluate performance and equivalence with the corresponding counterpart. The individual events were then combined by a conventional breeding cross. Each trial/test in the development of this product used an appropriate control.

III. THE TRANSFORMATION SYSTEM

A. Description of the transformation system

T304-40 and GHB119 cotton were both transformed by *Agrobacterium*-mediated gene transfer of the T-DNAs from pTDL008 and pTEM12 respectively. Each T_0 plant was crossed with conventional cotton in order to obtain homozygous and stable lines (BC_3F_3), and the resulting lines were crossed to obtain TwinLink.

Agrobacterium-mediated gene transfer of a plasmid 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 Tables 2 and 3, are also inserted in the individual events. Even though some of the regulatory elements used in the transformation process were derived from *A. tumefaciens*, a known plant pathogen, the genes that cause crown gall disease were removed, and therefore not incorporated into the recipient plant (Deblaere *et al*., 1985).

The Cry1Ab and Cry2Ae proteins, from *Bacillus thuringiensis* subsp. *berliner* and *B. thuringiensis* subsp. *dakota* respectively, are effective in controlling lepidopteran larvae such as bollworm (CBW, *Helicoverpa zea*) and tobacco budworm (TBW, *Heliothis virenscens*) larvae, which are common pests of cotton.

The *bar* gene is a common genetic element used in several transformations of agricultural crops as a selectable marker, as a means to confer tolerance to the herbicide glufosinate ammonium.

B. Parent lines

Coker 312 and Coker 315 are older commercial varieties of upland cotton (*Gossypium hirsutum*) which are no longer commercially cultivated. However Coker 312 and Coker 315 are well suited for transformation because of their capacity for regeneration from tissue culture.

C. Construction of the plasmids used for transformation

1. Plasmid pTDL008

a. Nature and source of the vector

The vector pTDL008 is a derivative of the vector pGSV20 in which the *bar* gene cassette coding for the phosphinothricin acetyltransferase protein of *Streptomyces hygroscopicus* (described by Thompson *et al*., 1987) was inserted together with the *cry1Ab* gene cassette encoding a fragment of the Cry1Ab crystal protein of *Bacillus thuringiensis* subsp. *berliner* (described by Höfte *et al*., 1986).

A map of plasmid pTDL008 is provided in Figure 1 and the genetic elements borne by this plasmid are described in Table 2.

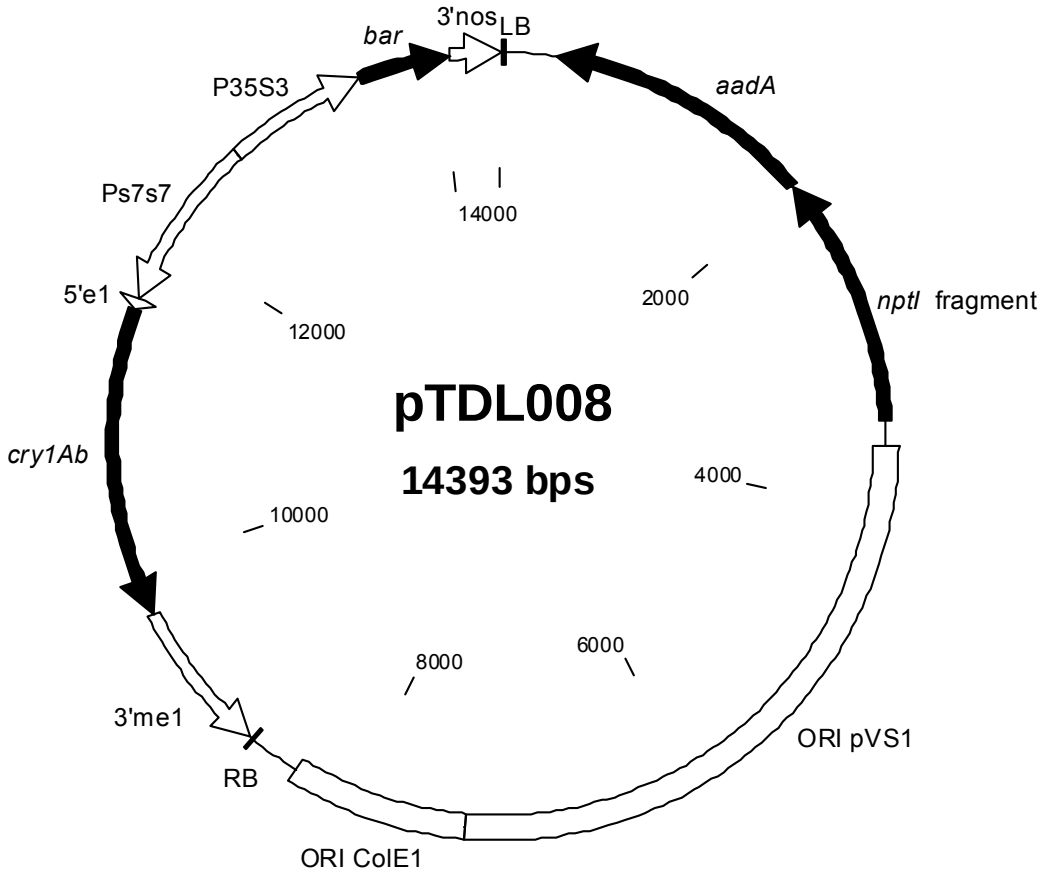


Figure 1 Vector map of pTDL008

b. The genetic elements intended to be inserted into the plant genome

The sequences comprised between base pairs 8767 and 37, also referred to as the transfer-DNA (T-DNA), are intended to be inserted into the plant genome upon transformation. The sequences comprised between base pairs 38 and 8766 compose the vector backbone and are not intended to be inserted into the plant genome. Their absence in T304-40 was experimentally confirmed (Sections IV.D and IV.E).

2. Plasmid pTEM12

a. Nature and source of the vector

The vector pTEM12 (Figure 2) is derived from pGSC1700 (Cornelissen and Vandewiele, 1989). The vector backbone contains the following genetic elements:

1. 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* (ORIpVS1).
2. a selectable marker gene conferring resistance to streptomycin and spectinomycin (*aadA*) for propagation and selection of the plasmid in *Escherichia coli* and *Agrobacterium tumefaciens*.
3. 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 in event GHB119 was experimentally confirmed (Sections IV.D and IV.E).

The genetic elements of plasmid pTEM12 are represented in Figure 2 and their description is provided in Table 3.

b. The genetic elements intended to be inserted into the plant genome

The sequence from bp 1 to bp 4345 of the pTEM12 plasmid is intended to be inserted into the plant genome. The genetic elements in this region are described in Table 3.

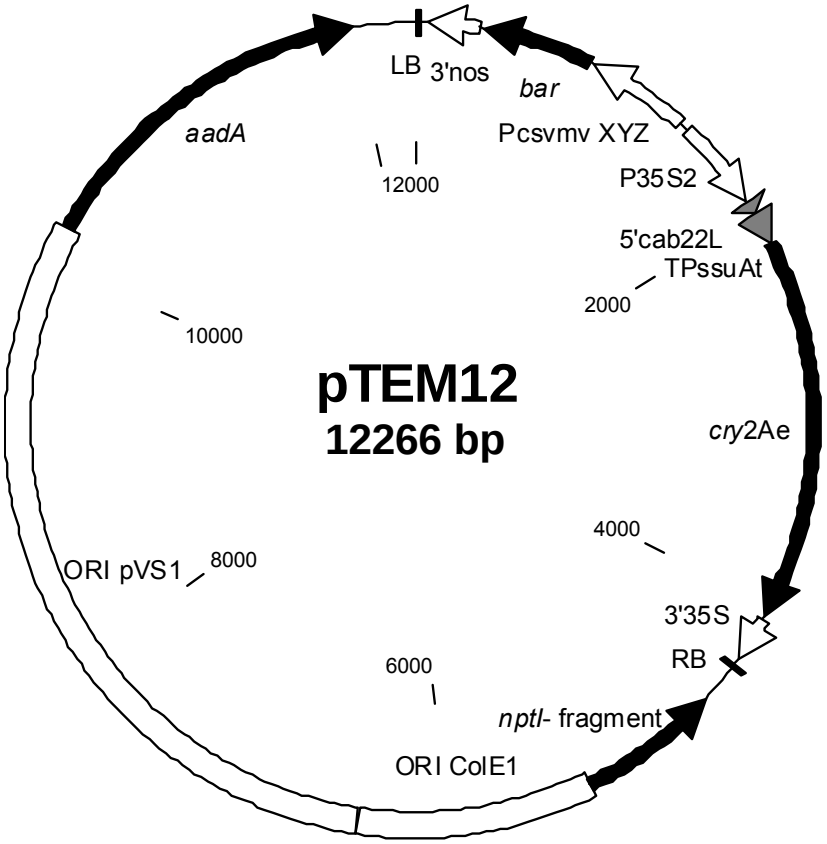


Figure 2 Vector map of pTEM12

D. Open reading frames and associated regulatory regions

1. Plasmid pTDL008

Only the sequence from bp 8767 to bp 37 of the pTDL008 plasmid is intended to be inserted into the plant genome. Please refer to Table 2 below.

Table 2 Genetic elements of vector pTDL008

Nt Positions	*Orientation*	*Origin*
8767-8791		**RB:** right border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
8792-9728	Counter clockwise	**3´me1:** sequence including the 3' untranslated region of the NADP-malic enzyme gene of *Flaveria bidentis* (yellowtop) (Marshall *et al*., 1996).
9729-11582	Counter clockwise	***cry1Ab*:** a sequence encoding the Cry1Ab crystal protein of *Bacillus thuringiensis berliner* 1715 (Höfte *et al*., 1986).
11583-11643	Counter clockwise	**5'e1:** sequence including the leader sequence of the tapetum specific E1 gene (*GE1*) of *Oryza sativa* (rice) (Michiels *et al*., 1992).
11644-12685	Counter clockwise	**Ps7s7:** sequence including the duplicated promoter region derived from subterranean clover stunt virus genome segment 7 (Boevink *et al*., 1995).
12686-13543	Clockwise	**P35S3:** sequence including the promoter region of the Cauliflower Mosaic Virus 35S transcript (Odell *et al*., 1985)
13544-14095	Clockwise	***bar*:** the coding sequence of the phosphinothricin acetyltransferase gene of *Streptomyces hygroscopicus* as described by Thompson *et al*. (1987).
14096-14393 1-12	Clockwise	**3´nos:** sequence including the 3' untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (Depicker *et al*., 1982)
13-37		**LB:** left border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
38-342		Residual plasmid sequences of pTiAch5 flanking the left border repeat (Zhu *et al*., 2000)
343-1965	Counter clockwise	***aadA*:** fragment including the aminoglycoside adenyltransferase gene of *Escherichia coli* as described by Fling *et al*. (1985)
1966-3486	Counter clockwise	Fragment of the neomycine phosphotransferase coding sequence of the *nptI* gene from transposon Tn*903* (Oka *et al*., 1981)
3487-3632		Fragment including the residual sequences upstream of the aminoglycoside adenyltransferase gene of *Escherichia coli* as described by Fling *et al*. (1985)
3633-7403		**ORI pVS1:** fragment including the origin of replication from the *Pseudomonas* plasmid pVS1 for replication in *Agrobacterium tumefaciens* (Hajdukiewicz *et al*., 1994)
7404-8576		**ORI ColE1:** fragment including the origin of replication from the plasmid pBR322 for replication in *Escherichia coli* (Bolivar *et al*., 1977)
8577-8766		Residual plasmid sequences of pTiAch5 flanking the right border repeat (Zhu *et al*., 2000)

2. Plasmid pTEM2

The sequence from bp 1 to bp 4345 of the pTEM12 plasmid is intended to be inserted into the plant genome. Please refer to Table 3 below.

Table 3 Genetic elements of vector pTEM12

Nt Positions	*Orientation*	*Origin*
1-25		**LB:** left border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
26-335	Counter clockwise	**3'nos:** sequence including the 3' untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (Depicker *et al.*, 1982)
336-887	Counter clockwise	**bar:** the coding sequence of the phosphinothricin acetyltransferase gene of *Streptomyces hygroscopicus* as described by Thompson *et al.* (1987)
888-1423	Counter clockwise	**Pcsvmv XYZ:** sequence including the promoter region of the Cassava Vein Mosaic Virus (Verdaguer *et al.*, 1996)
1424-1920	Clockwise	**P35S2:** sequence including the promoter region of the Cauliflower Mosaic Virus 35S transcript (Odell *et al.*, 1985)
1921-1990	Clockwise	**5'cab22L:** sequence including the leader sequence of the chlorophyl a/b binding protein gene from *Petunia hybrida* (Harpster *et al.*, 1988)
1991-2155	Clockwise	**TPssuAt:** coding sequence of the transit peptide of the ribulose-1,5-biphosphate carboxylase small subunit gene ats1A of *Arabidopsis thaliana*, as described by De Almeida *et al.* (1989)
2156-4051	Clockwise	***cry2Ae*:** the coding sequence of an insecticidal protein gene of *Bacillus thuringiensis*, adapted to cotton codon usage
4052-4320	Clockwise	**3'35S:** sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (Sanfaçon *et al.*,1991)
4321-4345		**RB:** right border repeat from the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
4346-4537		Residual plasmid sequences of pTiAch5 flanking the right border repeat (Zhu *et al.*, 2000)
4538-5248		Fragment of the neomycine phosphotransferase coding sequence of the *nptI* gene from transposon Tn*903* (Oka *et al.*, 1981)
5249-6421		**ORI ColE1:** fragment including the origin of replication from the plasmid pBR322 for replication in *Escherichia coli* (Bolivar *et al.*,1977)
6422-10192		**ORI pVS1:** fragment including the origin of replication from the *Pseudomonas* plasmid pVS1 for replication in *Agrobacterium tumefaciens* (Hajdukiewicz *et al.*, 1994)
10193-11961	Clockwise	***aadA*:** fragment including the aminoglycoside adenyltransferase gene of *Escherichia coli* as described by Fling *et al*. (1985)
11962-12266		Residual plasmid sequences of pTiAch5 flanking the left border repeat (Zhu *et al.*,2000)

E. Deduced amino acid sequences

```
  1  MDPERRPADI RRATEADMPA VCTIVNHYIE TSTVNFRTEP QEPQEWTDDL VRLRERYPWL
 61  VAEVDGEVAG IAYAGPWKAR NAYDWTAEST VYVSPRHQRT GLGSTLYTHL LKSLEAQGFK
121  SVVAVIGLPN DPSVRMHEAL GYAPRGMLRA AGFKHGNWHD VGFWQLDFSL PVPPRPVLPV
181  TEI
```

Figure 3 **Deduced amino acid sequence of the PAT protein encoded from the *bar* gene**

```
  1  MADNNPNINE CIPYNCLSNP EVEVLGGERI ETGYTPIDIS LSLTQFLLSE FVPGAGFVLG
 61  LVDIIWGIFG PSQWDAFLVQ IEQLINQRIE EFARNQAISR LEGLSNLYQI YAESFREWEA
121  DPTNPALREE MRIQFNDMNS ALTTAIPLFA VQNYQVPLLS VYVQAANLHL SVLRDVSVFG
181  QRWGFDAATI NSRYNDLTRL IGNYTDHAVR WYNTGLERVW GPDSRDWIRY NQFRRELTLT
241  VLDIVSLFPN YDSRTYPIRT VSQLTREIYT NPVLENFDGS FRGSAQGIEG SIRSPHLMDI
301  LNSITIYTDA HRGEYYWSGH QIMASPVGFS GPEFTFPLYG TMGNAAPQQR IVAQLGQGVY
361  RTLSSTLYRR PFNIGINNQQ LSVLDGTEFA YGTSSNLPSA VYRKSGTVDS LDEIPPQNNN
421  VPPRQGFSHR LSHVSMFRSG FSNSSVSIIR APMFSWIHRS AEFNNIIPSS QITQIPLTKS
481  TNLGSGTSVV KGPGFTGGDI LRRTSPGQIS TLRVNITAPL SQRYRVRIRY ASTTNLQFHT
541  SIDGRPINQG NFSATMSSGS NLQSGSFRTV GFTTPFNFSN GSSVFTLSAH VFNSGNEVYI
601  DRIEFVPAEV TFEAEYD  617
```

Figure 4 **Deduced amino acid sequence of the Cry1Ab protein from *Bacillus thuringiensis* subsp*. berliner***

```
  1  NNVLNNGRTT ICDAYNVVAH DPFSFEHKSL DTIRKEWMEW KRTDHSLYVA PIVGTVSSFL
 61  LKKVGSLIGK RILSELWGLI FPSGSTNLMQ DILRETEQFL NQRLNTDTLA RVNAELEGLQ
121  ANIREFNQQV DNFLNPTQNP VPLSITSSVN TMQQLFLNRL PQFRVQGYQL LLLPLFAQAA
181  NMHLSFIRDV VLNADEWGIS AATLRTYQNY LKNYTTEYSN YCINTYQTAF RGLNTRLHDM
241  LEFRTYMFLN VFEYVSIWSL FKYQSLLVSS GANLYASGSG PQQTQSFTSQ DWPFLYSLFQ
301  VNSNYVLNGF SGARLTQTFP NIGGLPGTTT THALLAARVN YSGGVSSGDI GAVFNQNFSC
361  STFLPPLLTP FVRSWLDSGS DRGGVNTVTN WQTESFESTL GLRCGAFTAR GNSNYFPDYF
421  IRNISGVPLV VRNEDLRRPL HYNEIRNIES PSGTPGGLRA YMVSVHNRKN NIYAVHENGT
481  MIHLAPEDYT GFTISPIHAT QVNNQTRTFI SEKFGNQGDS LRFEQSNTTA RYTLRGNGNS
541  YNLYLRVSSL GNSTIRVTIN GRVYTASNVN TTTNNDGVND NGARFLDINM GNVVASDNTN
601  VPLDINVTFN SGTQFELMNI MFVPTNLPPI Y 631
```

Figure 5 **Deduced amino acid sequence of the Cry2Ae protein from *Bacillus thuringiensis* subsp*. dakota***

IV. CHARACTERIZATION OF TWINLINK COTTON (EVENTS T304-40 x GHB119)

A. Description, history and Mendelian inheritance

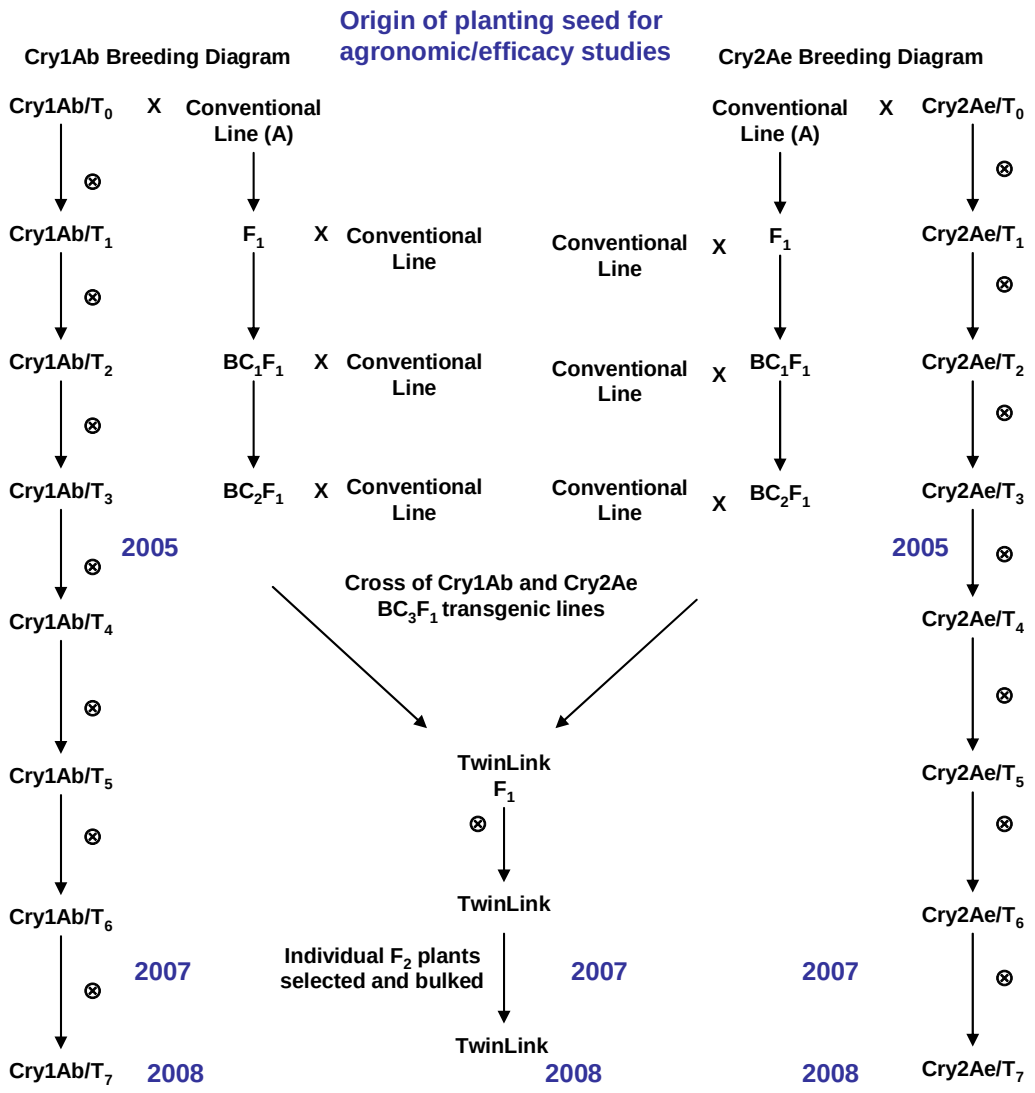
T_1 seed of transformation events T304-40 and GHB119 was harvested from self-pollinated T_0 plants surviving a glufosinate-ammonium herbicide greenhouse screen. T_1 plants were selected for survival following glufosinate-ammonium herbicide application, and at each generation, plants were sprayed with glufosinate-ammonium to eliminate those not expressing the *bar* gene. Homozygous T_3 plants were identified by planting 25 seed and spraying with glufosinate-ammonium 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.

The source of planting seed for the field studies are indicated in Figure 6.
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 (2005). Selfed T_6 and T_7 seed were used for the agronomic studies in 2007 and 2008, respectively. The TwinLink planting seed, individual F_2 plants, identified by PCR to carry both events were identified, carried to maturity and seed harvested (2007) and selfed to produce the planting seed for field studies in 2008.

The source of the samples for the DNA analysis and stability studies are indicated in Figure 6.
Generation BC2F4 (homozygous) was used for detailed insert characterization and protein expression levels. Generations T3, T4, T5, T6 and BC_2F_2 were used for molecular stability analyses. Generation T5 was used for seed composition analysis. Generations T5 and BC_2F_3 were used for replicated agronomic field tests. Generation T7 was used for analyses on absence/presence of vector backbone sequences.

During the development of TwinLink cotton (events T304-40 x GHB119), each locus was bred into selected cotton varieties. Herbicide tolerance screenings were conducted in the greenhouse using glufosinate ammonium at the recommended rate for field use. Plants were scored as tolerant (alive, no damage) or susceptible (damaged severely and dead or dying) 5-7 days post-glufosinate ammonium application. Polymerase chain reaction (PCR) testing was used to verify the transformation events.

To demonstrate the independent inheritance of the two events in TwinLink, the results of an F_2 progeny screen are described. The two events were back-crossed into a conventional breeding line. At the BC_2F_1 generation, the two events were combined and the progeny of this F_1 were analyzed by PCR designed to identity the presence of two events. The plants identified as containing both events (genotype T304-40/GHB119) were carried to maturity and seed harvested for further study. The expected ratio for two independently inhereted loci was achieved in this population of 80 plants. (Table 4) These results complement the DNA analysis characterizing the individual events and demonstrating insert stability (Section IV.C).



Notes

-At each generation, plants were sprayed with glufosinate to eliminate those not expressing the *bar* gene

-⊗ = self-cross

- Homozygous T3 plants were identified by planting 25 seed, spraying with glufosinate to identify segregating seed lots. Homozygosity analysis was also performed to identify homozygous plants.
- Selfed T3 homozygous seed (no segregation for resistance) was used to produce homozygous T4 seed and was the source of the lines that were used in early event agronomic and stability studies.
- Generation BC2F4 (homozygous) was used for detailed insert characterization and protein expression levels.
- Generations T3, T4, T5, T6 and BC2F2 were used for molecular stability analyses.
- Generation T5 was used for seed composition analysis.
- Generations T5 and BC2F3 were used for replicated agronomic field tests.
- Generation T7 was used for analyses on absence/presence of vector backbone sequences.

Figure 6 Breeding tree for the development of TwinLink cotton

Table 4 Segregation Analysis of the Independent Events in TwinLink cotton.

Events T304-40 and GHB119 were independently back-crossed into a conventional BCS breeding line. At the BC_2F_1 generation, the two events were combined and the progeny of this F_1 were analyzed by homozygosity PCR developed to identify the zygosity of each F_2 plant. The individual plants were scored for presence or absence of the events. Four possible genotypes were identified; WT (wild type)/WT, or T304-40/WT, GHB119/WT and T304-40/GHB119.

Parents of the F_1 and zygosity of the TwinLink events	***Generation***	***Ratio***	***Observed*** [a]	***Expected***	***χ^2 [b] calculated***
			WT/WT: T304-40/WT: GHB119/WT: T304-40/GHB119		
Hemizygous T304-40 (*cry1Ab/bar*) plant crossed with Hemizygous GHB119 (*cry2Ae/bar*) plant	F_1	1:1:1:1	19:15:25:21	20:20:20:20	2.6

[a] Tested by homozygosity PCR

[b] Assumes a one locus model for each gene. There was no significant difference (p=0.05) for the χ square goodness-of-fit test for the hypothesis of one locus for each gene. To reject the null hypothesis, the χ square value must be greater than 7.815, with three degrees of freedom.

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

1. T304-40

Genomic DNA isolated from cotton event T304-40 and control FiberMax 966 plants was subjected to Southern blot analysis using the different components of the transgene cassette (5'e1-Ps7s7 promoter, *cry1Ab* gene, 3'me1 terminator, P35S3 promoter, *bar* gene and 3'nos terminator) as well as the complete T-DNA fragment, as probes. The resulting DNA fragments were separated by agarose gel electrophoresis, transferred to a membrane and sequentially hybridized with six different probes, each representing a fragment of the transforming gene cassette, or the complete T-DNA probe.

The expected and observed hybridization fragments, as well as the hybridization strategy, are shown in Appendix 3. Results of this analysis (see Figure 3.2, Appendix 3) demonstrated that the inserted transgenic sequence in the cotton event T304-40 consists of one nearly complete copy of the T-DNA flanked by an inverted incomplete copy of the *cry1Ab* gene cassette and one additional 3'me1 terminator.

2. GHB119

Genomic DNA isolated from cotton event GHB119 and control FiberMax 966 plants was subjected to Southern blot analysis using the different components of the transgene cassette (3'nos terminator, *bar* gene, PCsVMV promoter, P35S2-5'cab22L promoter, TPssuAt-*cry2Ae* gene plus transit peptide and 3'35S-RB terminator plus right border) as well as the complete T-DNA fragment, as probes. The resulting DNA fragments were separated by agarose gel electrophoresis, transferred to a membrane and sequentially hybridized with six different probes, each representing a fragment of the transforming gene cassette, or the complete T-DNA probe.

The expected and observed hybridization fragments, as well as the hybridization strategy, are shown in Appendix 3. Results of this analysis (see Figure 3.4, Appendix 3) demonstrated that a single copy of the T-DNA is integrated in cotton event GHB119 and that the transferred DNA in the plant corresponds to the DNA configuration as originally designed in the transformation vector.

C. Stability of the inserted DNA sequence

1. T304-40

To demonstrate the structural stability of cotton event T304-40, genomic DNA was prepared from several individual plants of different generations, different genetic backgrounds and from plants grown from seeds harvested at three different locations. The isolated DNA was digested with the restriction enzyme *Eco*RV, which has two recognition sites in the integrated DNA fragment. Probing *Eco*RV digested genomic T304-40 DNA with the *cry1Ab* probe showed all three expected fragments in all tested samples. Two of these fragments represent the junctions between the transgenic sequences and the plant DNA sequences and one represents an internal fragment. The hybridization results of the stability analysis over four different genetic backgrounds are presented in Figure 3.5 (Appendix 3), over four different generations in Figure 3.6 (Appendix 3) and over different environmental conditions in Figure 3.7 (Appendix 3).

The obtained results demonstrate the stability of the cotton event T304-40 at the genomic level over different generations, in different backgrounds and in plants grown from seeds harvested in different environments. Segregation data further confirm the stability of the insert, and show that it segregates as one dominant Mendelian locus (see Section IV.A).

2. GHB119

The structural stability of cotton event GHB119 over different generations and in different backgrounds was tested by means of Southern blot analysis. Genomic DNA was prepared from several individual plants of three consecutive generations and two different genetic backgrounds. The impact of environment was assessed by analyzing the progeny of plants cultivated at 6 different field locations. The isolated DNA was digested with the restriction enzyme *Eco*RV, which has two recognition sites in the transforming DNA. Hybridization of these samples with the T-DNA probe revealed the expected profile in all tested samples (Figure 3.8 and Figure 3.9, Appendix 3). This demonstrates the stability of cotton event GHB119 at the genomic level in different generations, genetic backgrounds and growing environments. Segregation data further confirm the stability of the insert, and show that it segregates as one dominant Mendelian locus (see Section IV.A).

3. TwinLink cotton

The genetic stability of the inserted DNAs in TwinLink cotton was equally tested. Genomic DNA was isolated from 20 individual TwinLink plants and digested with the restriction enzyme *Eco*RV. *Eco*RV recognizes the inserted DNA of each event, thus generating restriction profiles that are characteristic for each parent (T304-40 and GHB119) and TwinLink.

Probing DNA digests of the parents and the TwinLink cross with labeled DNA probes containing the *cry1Ab* and *cry2Ae* genes, respectively, revealed the expected restriction fragments in all tested samples (Figures 3.10 and 3.11, Appendix 3). This demonstrates that the conventional breeding cross of events T304-40 and GHB119 has not affected the stability of the inserts, or created a rearrangement in the DNA structure.

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

The *bar* gene was used as the selectable marker for both events, T304-40 and GHB119, therefore the same gene of interest acts as a marker. No other marker genes were present.

Cotton event T304-40 contains no vector backbone sequences as evidenced by using seven overlapping probes covering the complete pTDL008 vector backbone sequences (including *aadA*, ORI pSV1 and ORI ColE1). See Figure 3.13, Appendix 3.

Southern blot analysis using five overlapping vector backbone probes covering the complete vector backbone sequence of the pTEM12 transformation vector confirmed the absence of vector backbone sequences as well as of the bacterial origin of replication, in the genome of cotton event GHB119. See Figure 3.15, Appendix 3.

No bacterial origin of replication was transferred with the *Agrobacterium* mediated transformation system. The inserted DNAs within cotton events T304-40 and GHB119 do not

add a bacterial origin of replication to the wild type cotton genome as a result of the transformation.

E. Absence of remaining parts of the vectors

The Southern blot analyses using overlapping probes covering the complete pTDL008 or pTEM12 vector backbone sequences demonstrate the absence of vector backbone sequences in cotton events T304-40 and GHB119. Please refer to Section IV.D above.

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

1. T304-40

Right and left border integration fragment
Southern blot analysis demonstrated that the inserted transgenic sequence in cotton event T304-40 consists of one nearly complete copy of the T-DNA flanked by an inverted incomplete copy of the *cry1Ab* gene cassette and one additional 3'me1 terminator. 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.

A PCR fragment of the pre-insertion locus of cotton event T304-40 was generated using genomic DNA from wild type cotton plants as template and a pair of primers specific for the 5' and 3' flanking sequence. 837 bp were determined at the pre-integration locus, including 480 bp of 5-prime flanking sequence, 325 bp of 3-prime flanking sequence and a target site deletion of 32 bp.

Between the sequences annotated as 5' and 3' flanking sequences at the pre-insertion locus, 32 base pairs can be detected which are present in the pre-insertion locus, but not at the transgenic locus of cotton event T304-40, and were deleted during transformation. A schematic overview of the T304-40 transgenic locus and pre-insertion locus is presented in Appendix 3, Figure 3.16.

BLASTn similarity search, and open reading frame search
Cotton plants transformed using *Agrobacterium*-mediated transformation inserting the T-DNA from vector pTDL008 into the cotton genome generated the cotton event T304-40. Due to the insertion of the *cry1Ab - bar* gene cassette in cotton, a 5-prime and a 3-prime junction, where cotton genomic DNA and inserted T-DNA are fused, were 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. No ORF was found, spanning the 5-prime junction or the 3-prime junction. One putative promoter was found in the 5' flanking sequence. As there are no genes found further downstream, this predicted promoter is most probably not biologically active.

It can be concluded that, using the current databases and tools, no known genes were

interrupted or influenced due to the insertion of transgenic DNA in the pre-insertion locus of T304-40.

A bioinformatic analysis was performed to detect the possible presence of expression due to the formation of newly created open reading frames (ORFs) in the 5', 3'and internal junction regions of cotton event T304-40. Two newly created chimeric ORFs were identified; however as these ORFs lack the necessary regulatory elements, the probability of expression of newly created proteins due to the insertion of DNA containing the *cry1Ab-bar* gene construct is highly unlikely.

2. GHB119

Right and left border integration fragment
Southern blot analysis demonstrated that a single copy of the T-DNA is integrated in cotton event GHB119 and that the transferred DNA in the plant corresponds to the DNA configuration as originally designed in the transformation vector.

A PCR fragment of the pre-insertion locus of cotton event GHB119 was generated using genomic DNA from wild type cotton plants as template and a pair of primers specific for the 5' and 3' flanking sequences. Analysis of the sequence obtained from this fragment shows that 684 bp of the pre-insertion locus were determined, including 367 bp 5' flanking sequences, 309 bp 3' flanking sequences and 8 bp of the target site deletion. Bioinformatic analyses of the pre-insertion locus sequence demonstrate that the characterized 5' and 3' flanking sequences are of cotton plant origin.

The DNA of the pre-insertion locus of cotton event GHB119 was sequenced and subjected to bioinformatic tools in order to identify endogenous cotton genes and/or regulatory elements that may be influenced by the insertion of the transgenic DNA fragment. Homology was found with cotton genomic DNA but no function could be assigned to these sequences. Homology was also found with repetitive sequences indicating that the insertion of the GHB119 T-DNA took place in a region containing repetitive elements. In order to identify the presence of known functional genes in the pre-insertion locus of event GHB119, a BLASTx similarity search was performed, but no known proteins were found.

1. Using the prediction tools GetORF and FGENESH, no genes or ORFs were predicted that could be disrupted by the introduction of the transgenic DNA in the genome.
2. The surrounding sequence of the pre-insertion locus was subjected to a homology analysis comparing it with regulatory elements involved in transcription. Two promoter sequences were predicted (Figure 3.19, Appendix 3). Since no known endogenous genes were found or predicted, it is highly unlikely that these predicted promoters are biologically active.

These bioinformatic analyses on the pre-insertion locus of cotton event GHB119, using the current databases and bioinformatic tools, allow us to conclude that the probability that a functional gene was interrupted is highly unlikely.

BLASTn similarity search, and open reading frame search
A bioinformatic analysis was performed to detect the possible presence of cryptic expression due to the formation of newly created open reading frames (ORFs) in the 5' or 3' junction regions in event GHB119. These junctions are formed by the insertion of a *cry2Ae - bar* gene construct into the cotton genome.

Several *in silico* tools were used to look for ORFs and regulatory elements taking into account the current scientific knowledge of gene expression. Three newly created chimeric ORFs were identified. The surrounding nucleotides of the three ORFs were also subjected to a homology analysis, comparing them with regulatory elements important for transcription and translation. Three newly created chimeric ORFs were identified. As not all regulatory elements were present in the DNA sequence at the 5' or 3' end of the newly created ORFs, the probability of expression of newly created proteins due to the insertion of DNA containing the *cry2Ae - bar* gene construct, is highly unlikely.

V. THE Cry1Ab, Cry2Ae AND PAT PROTEINS

A. History and background

1. Cry proteins

i. *Bacillus thuringiensis* history of safe use

Bacillus thuringiensis (*Bt*) is a Gram-positive, spore forming, rod-shaped bacterium present in soils, grain, on leaf surfaces, and in water. Not only is it found in various natural environments, but also in many animals including wild mammals (*e.g.* voles, deers, rodents, insectivore mammals) and probably humans, as well as in food (*e.g.* pasta, bread and processed food that contains flour) (OECD, 2007).

Bt strains are generally classified as non-pathogenic bacteria in several national classifications for microorganisms. It has been rarely classified as an opportunistic pathogen.

Due to its insecticidal properties, microbial preparations of *Bt* isolates have been used as biopesticides since 1961 in commercial agriculture, forestry and mosquito control (OECD, 2007). More than 100 microbial *Bt* products have been registered so far (USEPA, 2007). *Bt* sprayed crops that have been studied thus far are not toxic to humans or mammals (Betz *et al*., 2000). Instead, their insecticidal activities are very specific to the groups of pest insects of field and vegetable crops.

The insecticidal effect of *Bt* bacteria is largely attributed to proteins called crystal (Cry) delta-endotoxins. Since cloning of the first *cry* gene in 1981 (Schnepf and Whiteley, 1981), a total of 314 different *cry* genes had been identified by 2005 (OECD, 2007).

Results from numerous animal and human epidemiological studies together, over the years, indicate that biopesticides pose minimal risks to humans and animals, and certainly there is no evidence that *Bt* bacterial biopesticides caused any kind of significant infection or outbreaks of food poisoning (McClintock *et al*., 1995; Betz *et al*., 2000; Rosenquist *et al*., 2005).

In conclusion, *Bt* bacterial biopesticides have a long history of safe use.

ii. Cry1Ab protein familiarity

Over the past 50 years, current use of *Bt* pesticides, including those expressing Cry1Ab, is estimated to be several tons annually. Moreover, Cry1Ab protein is expressed in a number of genetically modified crops that have been approved since 1995 and are currently commercialized. No records of allergenicity in humans and mammals were found associated with *Bt* bacteria (OECD, 2007). In addition, microbial *Bt* biopesticides, including those containing the Cry1Ab protein, have shown no toxic effects in several mammalian toxicity studies (Betz *et al*., 2000). Therefore, Cry1Ab has had a long history of safe use for 50 years.

More specifically, the safety profile of the Cry1Ab protein is based on a number of studies that have been submitted to regulatory agencies to support product registration or published in peer-reviewed journals (summarized by Delaney *et al*., 2008). All these studies concluded that the Cry1Ab protein exhibited no adverse potentials for allergenicity or toxicity in mammals. Mainly,

Cry1Ab does not possess characteristics typical of known toxins and allergens. This includes that Cry1Ab originates from a non-pathogenic and non-allergenic organism. It has a known mode of action and has no protein sequence homology with known toxins and allergens. It is rapidly degraded by acid and/or enzymatic hydrolysis when exposed to simulated human gastric fluids. The lack of allergenicity was further confirmed by skin prick tests in two sensitive groups, children with food and inhalant allergy and individuals with asthma-rhinitis (Batista *et al*., 2005). The purified Cry1Ab protein is also devoid of toxic effects at high doses (up to 4000 mg/kg body weight).

In addition, a number of studies with Cry1Ab-incorporated GM crops support the notion that the Cry1Ab protein and the GM crops are non-toxic. Cry1Ab-incorporated GM crops were as safe and nutritious as their non-GM counterparts in rat or chicken feeding studies and no deleterious effects were observed (Wang *et al*., 2002; Brake *et al.*, 2003; Hammond *et al.*, 2006; Schroder *et al.*, 2007).

In conclusion, Cry1Ab has been safely consumed by humans and animals for 50 years, without any records of safety issues.

iii. Cry2Ae protein familiarity

As summarized in the previous sections, Cry proteins have a good safety profile, as evidenced by the extensive use of *Bt* biopesticides, including those expressing the Cry2Ae protein, with excellent safety records.

Among the Cry proteins, Cry2Ae is a protein from the Cry2-type family. Although a number of data support the safety of the *Bt* organisms expressing Cry2Ae, no data have been published on safety assessment of the Cry2Ae protein itself. However, Cry2Ae has 86% identity with Cry2Ab, which has a safe profile.

A number of studies with the Cry2Ab protein have been submitted to regulatory agencies to support product registration. Assessment of those studies by various agencies concluded that the Cry2Ab protein exhibited no adverse potentials of allergenicity or toxicity (US-EPA, 2002; US-FDA, 2002; CFIA, 2004; OECD, 2007).

In addition, studies performed using Cry2Ab expressing GM crops support their safety profile. Short-term feeding studies on poultry, pigs, calves, catfish, quail and cattle demonstrated that no deleterious effects are associated with Cry2Ab expressing corn and that this GM crop is as nutritious and safe as its parental crop control (Hamilton *et al.*, 2004).

2. The PAT protein

PAT protein and PAT expressing cotton LLCotton25 have been approved in the US in 2003 (BNF 00086, US-FDA, 2003).

i. *Streptomyces hygroscopicus* history of safe use

S. hygroscopicus history of safe use data are included in the peer-reviewed scientific journal published by Herouet *et al.* (2005). This organism is a common soil saprophytic bacterial species of *Streptomyces*. Human exposure to *Streptomyces* is not new and these bacteria are widespread in nature worldwide (Kutzner, 1981). Furthermore, it is very likely that this exposure is frequent through the consumption of roots and vegetables. Nevertheless, there is no

evidence of adverse effects related to the exposure to this bacterial species.

ii. PAT protein familiarity

Safety data focused on PAT proteins have been extensively reviewed by international scientific peer-reviewed journals (Herouet *et al.*, 2005). PAT protein is expressed in a number of GM crops that have been approved by regulatory agencies and are consumed by humans and animals since 1995, with excellent safety records.

B. Characterization of the Cry1Ab, Cry2Ae and PAT proteins

1. Biochemistry and mode of action

i. Cry1Ab and Cry2Ae proteins

The insecticidal mode of action for Cry proteins has been studied extensively over the last 20 years. Prior to this, it was known that Cry proteins were not contact poisons, but rather insecticidal proteins that act on the midgut and must be ingested to be cleaved by midgut proteases to be effective against pest insects. It is now well-understood that the insecticidal mode of action is a multi-complex process, which includes a series of critical steps such as crystal solubilization, protoxin proteolysis, peritrophic membrane transport, brush border membrane binding, and pore formation (Aronson and Shai, 2001; Whalon and Wingerd, 2003).

The midgut environment of a lepidopteran larva is both alkaline (pH 8-10) and probably reductive, enabling the Cry proteins to be dissolved (Tojo and Aizawa, 1983; Ogiwara *et al.*, 1992). Once dissolved, the specific larval midgut proteases begin to cleave the inactive protoxin to a biologically active toxin. The requirement of alkaline pH and specific larval gut proteases for activation is an important feature in insect-specific *Bt* activity. Since mammals and other non-target pests (including most other insects) are unable to dissolve *Bt* crystals, their passage through the digestive system of non-target organs occurs most likely in the unaltered, and therefore non-toxic, form.

The toxic fragment of the protein then interacts with specific high affinity receptors on the microvilli of the target's larval midgut (stomach) epithelium, in particular brush border membrane vesicles (BBMV). The surface glycosylation on these receptors is very important for interaction with toxic the fragment of Cry proteins. Invertebrates, but not vertebrates, were shown to have these glycosylations (Griffitts *et al.*, 2005).

Following insertion of at least part of the Cry proteins into the cell membrane, and subsequent oligomerization, a cation-selective channel is formed in the columnar cells of the midgut epithelium (Chen *et al.*, 1995). These pore formation and subsequent destruction of the cell lead to disruption of the integrity of the gut epithelium, starvation, and insect death (Knowles and Dow, 1993).

This overview of the mode of action accounts for the specificity of the Cry protein toxicity, as Cry proteins must be ingested to have an active insecticidal effect, they must be dissolved under alkaline conditions specific to insect gut, they must be cleaved by specific larval midgut proteases to be active, and they must bind to glycoprotein or glycolipid receptors that are not present in the gut of vertebrate species.

In conclusion, there is no mechanistic evidence that Cry proteins pose risks for humans or mammals.

Cry1Ab protein
Cry1Ab protein as expressed in cotton has 617 amino acids. The deduced molecular weight is 69 kDa. The binding of the Cry1Ab toxin to brush border membranes of *Manduca sexta* was tested in buffers of varying pH (Van Rie *et al.*, 1989). No gross changes in affinity or binding site concentration occurred in the pH range 7.4-10.0, though the binding was somewhat reduced at pH 10.0. Heat sensitivity of the Cry1Ab protein was tested by incubating the protein at 45°C and 60°C and assaying the residual activity at various time intervals. The Cry1Ab protein appears to be deactivated by higher temperature. At 45°C the protein loses little activity. However, the Cry1Ab protein is deactivated after 120 minutes at 60°C.

Cry2Ae protein
Cry2Ae protein as expressed in cotton has 631 amino acids. The deduced molecular weight is 71 kDa. Heat sensitivity of the Cry2Ae protein was tested by incubating the protein at 45°C and 60°C and assaying the residual activity at various time intervals. The Cry2Ae protein loses insecticidal potential when exposed to higher temperature. At 45°C the protein lost little activity. However, Cry2Ae is deactivated after 240 minutes at 60°C.

ii. PAT protein

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

2. Protein safety

In order to assess any potential adverse effects to humans or animals resulting from environmental release of the crops containing the Cry1Ab, Cry2Ae and PAT proteins, 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 these proteins, including homology searches of the amino acid sequences with comparison to all known allergens and toxins from large public databases, an *in vitro* digestibility assay of the proteins, and an acute toxicity test in the mouse. Moreover, publicly available review documents issued by regulatory authorities, indicating that similar protein family members are safe, have been used for supporting this safety assessment. The results of studies conducted by BCS are consistent with the published information, confirming that the crops containing these proteins can be safely used as food or feed.

Lack of allergenic potential

- The Cry1Ab, Cry2Ae and PAT proteins have no amino acid sequence similarity to known

allergens, as demonstrated by overall amino acid and epitope homology searches;

- As expected, the Cry1Ab, Cry2Ae and PAT proteins only have similarities with other Cry or PAT proteins;
- The Cry1Ab, Cry2Ae and PAT proteins do not share epitopes with known allergens;
- The Cry1Ab, Cry2Ae and PAT proteins are not glycosylated;
- The Cry1Ab, Cry2Ae and PAT proteins are not heat stable;
- The Cry1Ab, Cry2Ae and PAT proteins are degraded by human simulated gastric and intestinal fluids. This minimizes the likelihood that these proteins could survive in the human digestive tract and be absorbed.

Lack of toxic potential

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

In conclusion, it is considered that the *cry*1Ab*, cry2Ae* and *bar* genes as well as the Cry1Ab, Cry2Ae and PAT proteins are not toxic for mammals and do not possess any of the characteristics associated with food allergens. Therefore, no effects on animal and human health are to be expected by consumption of the *cry1Ab*, *cry2Ae* or *bar* genes and the Cry1Ab, Cry2Ae or PAT proteins.

VI. EXPRESSION OF THE INSERTED SEQUENCES

Several studies were performed to quantify the Cry1Ab, Cry2Ae, and PAT/*bar* proteins in tissues of TwinLink cotton. The levels of the proteins in TwinLink cotton were determined by validated enzyme-linked immunosorbent assays (ELISA's).

A. Protein amounts in fuzzy seed of TwinLink cotton

Seven trials were conducted in 2007; 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 two times with Ignite® 280 SL glufosinate-ammonium herbicide at 0.53 lb ai/A, and the other plots were untreated. Samples of ginned cottonseed (fuzzy seed) were collected and shipped frozen to Bayer CropScience for ELISA determination of the amounts of Cry1Ab, Cry2Ae, and PAT/*bar* proteins in the raw agricultural commodity. Results are summarized in Table 5 and Table 6 and provided in detail in Appendix 2. The subject proteins were detected in all kernel and lint coat fractions from transgenic TwinLink cotton. As expected, the kernel fraction contained most of the analyte.

The amount of each protein in fuzzy seed was calculated based on the amount of analyte present in the lint coat and kernel fractions. The amount of analyte measured in lint coat and kernel, then calculated in fuzzy seed, and the average as a percent of total crude protein on a fresh weight basis are given in Appendix 2, Tables 2.5, 2.7 and 2.9. The measured and calculated amounts of analyte on a dry weight basis are given in Appendix 2, Tables 2.6, 2.8 and 2.10. Ranges in values are also provided for fuzzy seed and the amounts of analyte as a percent of the total crude protein.

On a fresh weight basis, the average Cry1Ab protein content from all test sites ranged from 0.226 µg/g to 1.64 µg /g fresh weight in unsprayed fuzzy seed with an average value of 1.16 µg/g ± 0.482 µg/g. The amount of Cry1Ab determined to be in the sprayed transgenic fuzzy seed ranged from 0.962 to 1.77 µg/g with an average value of 1.38 ± 0.301 µg/g. The Cry1Ab protein comprised an average of 0.000561 ± 0.000206 % of the total crude protein in unsprayed fuzzy seed. For sprayed fuzzy seed, the Cry1Ab protein comprised an average of 0.000694 ± 0.000153 % of the total crude protein.

On a dry weight basis, the average Cry1Ab protein content from all test sites ranged from 0.258 µg/g to 1.81 µg/g dry weight in unsprayed fuzzy seed with an average value of 1.29 ± 0.520 µg/g. The amount of Cry1Ab determined to be in the sprayed transgenic fuzzy seed ranged from 1.09 µg/g to 1.96 µg/g with an average value of 1.54 ± 0.318 µg/g. The Cry1Ab protein comprised an average of 0.000561 ± 0.000206 % of the total crude protein in unsprayed fuzzy seed. For sprayed fuzzy seed, the Cry1Ab protein comprised an average of 0.000694 ± 0.000153% of the total crude protein.

On a fresh weight basis, the average Cry2Ae protein content from all test sites ranged from 4.69 µg/g to 11.7 µg/g fresh weight in unsprayed fuzzy seed with an average value of 8.34 ± 2.55 µg/g. The amount of Cry2Ae determined to be in the sprayed transgenic fuzzy seed ranged from 6.01 µg/g to 16.2 µg/g with an average value of 9.51 ± 3.56 µg/g. The Cry2Ae protein comprised an average of 0.00420 ± 0.00129 % of the total crude protein in unsprayed fuzzy

seed. For sprayed fuzzy seed, the Cry2Ae protein comprised an average of 0.00485 ± 0.00204% of the total crude protein.

On a dry weight basis, the average Cry2Ae protein content from all test sites ranged from 5.34 µg/g to 13.4 µg/g dry weight in unsprayed fuzzy seed with an average value of 9.35 ± 2.93 µg/g. The amount of Cry2Ae determined to be in the sprayed transgenic fuzzy seed ranged from 6.67 µg/g to 18.5 µg/g with an average value of 10.7 ± 4.17 µg/g. The Cry2Ae protein comprised an average of 0.00420 ± 0.00129 % of the total crude protein in unsprayed fuzzy seed. For sprayed fuzzy seed, the Cry2Ae protein comprised an average of 0.00485 ± 0.00204% of the total crude protein.

On a fresh weight basis, the average PAT/*bar* protein content from all test sites ranged from 115 µg/g to 188 µg/g fresh weight in unsprayed fuzzy seed with an average value of 145 ± 28.0 µg/g. The amount of PAT/*bar* determined to be in the sprayed transgenic fuzzy seed ranged from 118 µg/g to 172 µg/g with an average value of 145 ± 22.2 µg/g. The PAT/*bar* protein comprised an average of 0.0728 ± 0.0121 % of the total crude protein in unsprayed fuzzy seed. For sprayed fuzzy seed, the PAT/*bar* protein comprised an average of 0.0736 ± 0.0151% of the total crude protein.

On a dry weight basis, the average PAT/*bar* protein content from all test sites ranged from 125 µg/g to 213 µg/g dry weight in unsprayed fuzzy seed with an average value of 163 ± 32.4 µg/g. The amount of PAT/*bar* determined to be in the sprayed transgenic fuzzy seed ranged from 129 µg/g to 196 µg/g with an average value of 163 ± 27.4 µg/g. The PAT/*bar* protein comprised an average of 0.0728 ± 0.0121 % of the total crude protein in unsprayed fuzzy seed. For sprayed fuzzy seed, the PAT/*bar* protein comprised an average of 0.0736 ± 0.0151% of the total crude protein.

B. Cry1Ab, Cry2Ae, and PAT protein content in plant parts and during the life cycle

Additional data on protein content on plant parts and during the life cycle of the plant were analyzed and submitted as part of the Section 3 Registration package to USEPA. This information may be found in the Cry1Ab cotton, the Cry2Ae cotton and the TwinLink cotton Confidential Statement of Formula documents.

C. Expression of other parts of the insert

There is no expression of other genes (coding sequences) of the inserts since the inserted sequences consist only of the *cry1Ab* - *bar* cassette in T304-40 and *cry2Ae* - *bar* cassette in GHB119. The absence of any additional DNA from the vectors used for the transformation has been documented in Section IV.E.

Table 5 Fresh Weight average quantities of Cry1Ab, Cry2Ae and PAT proteins in raw agricultural commodities of TwinLink cotton as detected by ELISA

*Protein **	*Average protein contents (µg/g sample) ± SD*		*Average protein content (as % of total crude protein) ± SD*	
	No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Cry1Ab				
Range in values	0.226 – 1.64	0.962 – 1.77	0.000147 – 0.000751	0.000455 – 0.000871
Average ± SD	1.16 ± 0.482	1.38 ± 0.301	0.00056 ± 0.000206	0.000694 ± 0.000153
Cry2Ae				
Range in values	4.69 – 11.7	6.01 – 16.2	0.00248 – 0.00601	0.00283 – 0.00870
Average ± SD	8.34 ± 2.55	9.51 ± 3.56	0.00420 ± 0.00129	0.00485 ± 0.00204
PAT/*bar*				
Range in values	115 – 188	118 – 172	0.0505 – 0.0896	0.0512 – 0.0923
Average ± SD	145 ± 28.0	145 ± 22.2	0.0728 ± 0.0121	0.0736 ± 0.0151

* Data based on 7 sites and 12 measurements (2 sample extracts assayed in duplicate from three replicate plots).

Table 6 Dry Weight average quantities of Cry1Ab, Cry2Ae and PAT proteins in raw agricultural commodities of TwinLink cotton as detected by ELISA

*Protein **	*Average protein contents (µg/g sample) ± SD*		*Average protein content (as % of total crude protein) ± SD*	
	No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Cry1Ab				
Range in values	0.258 – 1.81	1.09 – 1.96	0.000147 – 0.000751	0.000455 – 0.000871
Average ± SD	1.29 ± 0.520	1.54 ± 0.318	0.000561 ± 0.000206	0.000694 ± 0.000153
Cry2Ae				
Range in values	5.34 – 13.4	6.67 – 18.5	0.00248 – 0.00601	0.00283 – 0.00870
Average ± SD	9.35 ± 2.93	10.7 ± 4.17	0.00420 ± 0.00129	0.00485 ± 0.00204
PAT/*bar*				
Range in values	125 – 213	129 – 196	0.0505 – 0.0896	0.0512 – 0.0923
Average ± SD	163 ± 32.4	163 ± 27.4	0.0728 ± 0.0121	0.0736 ± 0.0151

* Data based on 7 sites and 12 measurements (2 sample extracts assayed in duplicate from three replicate plots).

D. Verification of the biochemical and functional equivalence of Cry1ab, Cry2Ae and PAT/bar proteins produced in TwinLink cotton and in *E. coli*

Studies on potential toxicology and allergenicity for food, feed and the environment were conducted using Cry1Ab, Cry2Ae and PAT/bar proteins expressed in *E. coli*. The results of these experiments are used to show safety of the same protein produced in TwinLink cotton. 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 Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from *E. coli* were compared to the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves, using the 6 following criteria and associated methods.

1. Molecular weight (SDS-PAGE)

The Cry1Ab, Cry2Ae and PAT/*bar* proteins from *E. coli* and *Bt.* and the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves were analyzed by SDS-PAGE. The proteins from the plant and the corresponding protein from *E. coli* and *Bt.* were denatured and analyzed by electrophoresis on a denaturing polyacrylamide gel where mobility can be correlated to molecular weight. Molecular weight markers on the gel are comprised of a series of recombinant proteins of known molecular weight. The gel was then stained with Pierce Imperial Stain to visualize the protein bands.

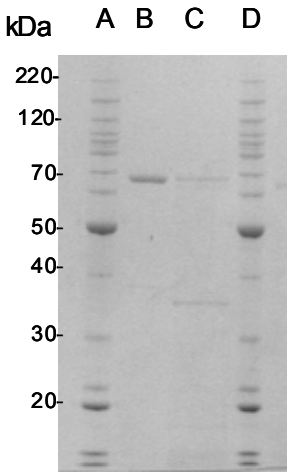
Figure 7, Panel A shows the Pierce Imperial stained gel of Cry1Ab. The electrophoretic mobilities of the Cry1Ab protein produced in *E. coli* and isolated from TwinLink cotton leaves are indistinguishable. The Cry1Ab from TwinLink has other lower molecular weight bands that are attributed to degradation from the isolation procedure. The Cry1Ab proteins from *E. coli* and TwinLink cotton have measured electrophoretic mobilities of 24.5 mm. The electrophoretic mobility of each standard protein was plotted versus its respective molecular weight and an approximate molecular weight of 66 kDa was determined for Cry1Ab. The theoretical molecular weight calculated from the amino acid sequence deduced from the DNA sequence is approximately 69.5 kDa.

Figure 8, Panel A shows the Pierce Imperial stained gel of Cry2Ae. The electrophoretic mobilities of the Cry2Ae proteins produced in *Bt.* or isolated from TwinLink cotton leaves are indistinguishable. The Cry2Ae protein isolated from TwinLink has other higher molecular weight bands in the gel, in which one is believed to be a protein dimer and the other is not related to the protein. The Cry2Ae proteins from *Bt*. and TwinLink have measured electrophoretic mobilities of 33.5 mm. The electrophoretic mobility of each standard protein was plotted versus its respective molecular weight and an approximate molecular weight of 65 kDa was determined for Cry2Ae. The theoretical molecular weight calculated from the amino acid sequence deduced from the DNA sequence is approximately 70.9 kDa.

Figure 9, Panel A shows the Pierce Imperial stained gel of PAT/*bar*. The electrophoretic mobilities of the PAT/*bar* proteins produced in *E. coli.* or isolated from TwinLink cotton leaves are indistinguishable. The PAT/*bar* proteins from *E. coli* and TwinLink have measured electrophoretic mobilities of 76.5 mm. The electrophoretic mobility of each standard protein was

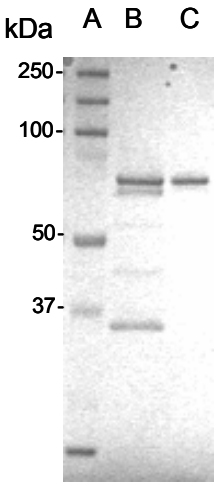
plotted versus its respective molecular weight and an approximate molecular weight of 21.9 kDa was determined for PAT/*bar*. The theoretical molecular weight calculated from the amino acid sequence deduced from the DNA sequence is approximately 21 kDa.

Panel A
Cry1Ab SDS-PAGE



Lanes:
A- BenchMark Protein Ladder
B- Cry1Ab Thrombin Standard
C- Cry1Ab Isolated from TwinLink
D- BenchMark Protein Ladder

Panel B
Cry1Ab Western Blot



Lanes:
A- Kaleidoscope Marker
B- Cry1Ab Isolated from TwinLink Cotton Leaves
C- Cry1Ab Thrombin Standard

Figure 7 Comparison of the Cry1Ab protein from *E. coli* to the Cry1Ab protein isolated from leaves of transgenic TwinLink cotton.

Panel A shows the SDS-PAGE gel stained with Pierce Imperial Protein Stain. Lanes A and D contain molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel. Lane B contains the Cry1Ab Thrombin protein standard produced in *E. coli.* Lane C contains the Cry1Ab protein isolated from TwinLink cotton leaves. Panel B shows the western blot. Lane A contains the molecular weight markers of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the western blot. Lane B contains the Cry1Ab protein isolated from TwinLink cotton leaves. Lane C, contains the Cry1Ab Thrombin protein standard from *E. coli.*

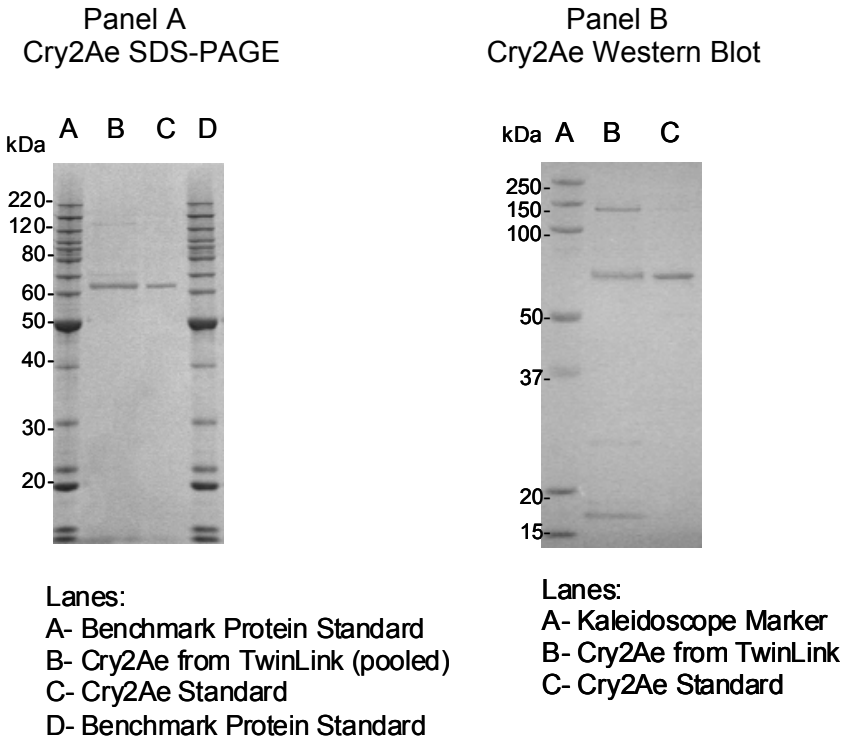
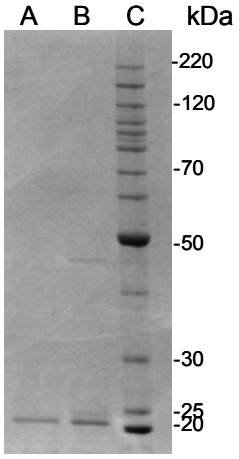


Figure 8 Comparison of the Cry2Ae protein from *Bt.* to the Cry2Ae protein isolated from leaves of transgenic TwinLink cotton.

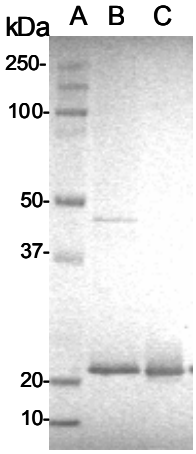
Panel A shows the SDS-PAGE gel stained with Pierce Imperial Protein Stain. Lane A and D contains molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel. Lane B contains the Cry2Ae protein isolated from TwinLink cotton leaves. Lane C contains the Cry2Ae protein standard from *Bt*. Panel B shows the western blot for Cry2Ae. Lane A contains the molecular weight markers of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the western blot. Lane B contains the Cry2Ae protein isolated from TwinLink cotton leaves. Lane C, contains the Cry2Ae protein standard from *E. coli.*

Panel A
PAT/*bar* SDS-PAGE



Lanes:
A- PAT/*bar* isolated from TwinLink
B- PAT/*bar* Standard
C- BenchMark Protein Ladder

Panel B
PAT/*bar* Western Blot



Lanes:
A- Kaleidoscope Marker
B- PAT/*bar* isolated from TwinLink Cotton Leaves
C- PAT/*bar* Standard

Figure 9 Comparison of the PAT/*bar* protein from *E. coli* to the PAT/*bar* protein isolated from leaves of transgenic TwinLink cotton.

Panel A shows the SDS-PAGE gel stained with Pierce Imperial Protein Stain. Lane A contains the PAT/*bar* protein isolated from TwinLink cotton leaves. Lane B contains the PAT/*bar* protein from *E. coli.* Lane C contains molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, and 20 kDa. Underlined molecular weights are shown on the gel. Panel B shows the western blot. Lane A contains the molecular weight markers of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the western blot. Lane B contains the PAT/*bar* protein isolated from TwinLink cotton leaves. Lane C contains the PAT/*bar* protein standard from *E. coli.*

2. Immuno-reactivity (Western Blot)

The Cry1Ab western blot results are shown in Figure 7, Panel B. These results show that the electrophoretic mobilities of the Cry1Ab proteins produced from *E. coli* or isolated from TwinLink cotton leaves are indistinguishable and both proteins are immuno-reactive. The Cry1Ab protein isolated from TwinLink cotton appears to be partially degraded based on the multiple low molecular weight bands beneath the Cry1Ab band in the western. The Cry1Ab protein degradation is believed to come from the isolation process.

The Cry2Ae western blot results are shown in Figure 8, Panel B. These results show that the electrophoretic mobilities of the Cry2Ae proteins produced from *Bt.* or isolated from TwinLink cotton leaves are indistinguishable and both proteins are immuno-reactive. A higher molecular weight band is seen in the western blot for Cry2Ae isolated from TwinLink cotton leaves and a

faint band can be seen for the Cry2Ae protein from *Bt.* This higher molecular weight band is believed to be a dimer of the Cry2Ae protein. Some lower molecular weight bands were also seen for the Cry2Ae protein isolated from TwinLink cotton. These lower molecular weight bands are believed to be degraded Cry2Ae formed as a result of the protein isolation procedure.

The PAT/*bar* western blot results are shown in Figure 9, Panel B. These results show that the electrophoretic mobilities of the PAT/*bar* proteins produced from *E. coli.* or isolated from TwinLink cotton leaves are indistinguishable and both proteins are immuno-reactive. An additional band in the western, possibly caused by a PAT/*bar* protein dimer, was detected in the PAT/*bar* protein sample isolated from TwinLink cotton leaves.

3. N-terminal sequencing

The theoretical N-terminal sequence of the first five amino acids of the Cry1Ab protein deduced from the DNA sequence of the gene in *E. coli* and TwinLink cotton leaves is: methionine, aspartic acid, asparagine, asparagine and proline. An N-terminal sequence was not determined by Edman Degradation because the concentration of the Cry1Ab protein isolated from TwinLink cotton leaves was too low. However, the N-terminal peptide for the Cry1Ab protein isolated from TwinLink was detected in the selected ion mass spectrometry analysis of the protein, indicating that the protein is not modified.

The theoretical N-terminal sequence of the first five amino acids of the Cry2Ae protein deduced from the DNA sequence of the gene in *Bt.* and TwinLink cotton leaves is: asparagine, asparagine, valine, leucine and asparagine. The Cry2Ae protein isolated from TwinLink cotton leaves gave the following sequence for the first five amino acids by Edman Degradation: ?-asparagine-valine-leucine-asparagine. The first amino acid which is asparagine was not detected or could not be distinguished from the second amino acid which is also asparagine. Since the selected ion monitoring mass spectrometry analysis of the Cry2Ae protein isolated from TwinLink cotton was detected, the peptide is not blocked or modified.

The following N-terminal sequence was obtained by Edman Degradation for the Cry2Ae protein from *Bt.*: methionine, asparagine, asparagine, valine and leucine. The asparagine to leucine sequence matches the N-terminal peptide sequence for Cry2Ae. Since the full scan and selected ion monitoring mass spectrometry analysis of the Cry2Ae bacterial N-terminal peptide was detected, indicating that the peptide is not modified and does not contain a methionine amino acid, the identification of a methionine on the N-terminal sequence by Edman Degradation may be caused by contamination in the N-terminal sequencing analysis.

The theoretical N-terminal sequence of the first five amino acids of the PAT/*bar* protein deduced from the DNA sequence of the gene in *E. coli* and TwinLink cotton leaves is: methionine, aspartic acid, proline, glutamic acid and arginine. The following N-terminal sequence was obtained for the PAT/*bar* protein from *E. coli.*: methionine, aspartic acid, proline, glutamic acid and arginine. This sequence is an exact match to the sequence deduced from the DNA sequence of the PAT/*bar* gene for residues 1-5. The PAT/*bar* protein isolated from TwinLink cotton leaves gave a sequence by Edman Degradation of: ?-aspartic-methionine-proline-glutamic acid. Since the PAT/*bar* N-terminal peptide was not detected by selected ion monitoring mass spectrometry indicating that the peptide is modified, not detecting the first amino acid and having an incorrect assignment for the third by Edman Degradation confirms

that the peptide is modified. Post-translational modifications, such as removal of a methionine are often found in proteins from both prokaryotic and eukaryotic organisms (Bradshaw *et al.*, 1998).

4. Peptide profile (LC-MS)

i. Cry1Ab Protein

Peptides from a trypsin digest of the Cry1Ab protein from *E. coli* and the Cry1Ab protein isolated from TwinLink cotton were analyzed by LC/MS using the selected ion monitoring method developed for the analysis. The method was developed based on the protein sequence of the Cry1Ab protein from *E. coli.* The expected peptides from the trypsin digest of the Cry1Ab protein from *E. coli* were identified from their full scan spectra. The most abundant ion for each peptide was chosen for selected ion monitoring. The peptides at positions 116 to 127, EWEADPTNPALR, 128 to 131, EEMR, 450 to 458, APMFSWIHR and 459 to 478, SAEFNNIIPSSQITQIPLTK could not be detected by full scan mass spectrometry, which resulted in a peak for that peptide not being assigned and not detected by selected ion monitoring, Table 7. The peptide not being detected by full scan mass spectrometry could be caused by a missed cleavage.

A 91% sequence coverage of the Cry1Ab protein from *E. coli* was obtained by full scan and selected ion monitoring combined, and 80% sequence coverage was obtained by the selected ion monitoring method, Tables 8 and 9. The analysis of the tryptic digest of the Cry1Ab protein isolated from TwinLink cotton leaves by the selected ion monitoring method obtained 74% sequence coverage of the protein, Tables 8 and 9. The N-terminal peptide, MDNNPNINECIPYNCLSNPEVEVLGGER, of the Cry1Ab protein isolated from TwinLink cotton leaves was detected demonstrating that the N-terminal is not modified. The peptides at positions 100 to 115, LEGLSNLYQIYAESFR, and 602 to 620, IEFVPAEVTFEAEYDLVPR were not detected in the Cry1Ab protein isolated from TwinLink cotton leaves; but were detected in the Cry1Ab protein from *E. coli.* These peptides were not detected possibly because of a missed cleavage, modification, or the sample concentration was too low. The selected ion monitoring results for the Cry1Ab protein demonstrate that 81% of the Cry1Ab protein isolated from TwinLink cotton leaves is identical to the Cry1Ab protein from *E. coli.,* Table 8.

The Cry1Ab protein has sites with a potential for N-glycosylation. N-glycosylated proteins are glycosylated on the asparagine residue and have a tripeptide asparagine-X-serine/threonine sequence, where X is any amino acid except proline. However, the presence of this sequence does not indicate that the protein will be glycosylated. The Cry1Ab protein sequence has 5 tryptic peptides with the N-X-S/T sequence. The selected ion monitoring analysis results of these 5 tryptic peptides from the bacterial standard Cry1Ab protein and the Cry1Ab protein isolated from TwinLink cotton leaves is shown in Table 9. All the Cry1Ab tryptic peptide residues containing the N-X-S/T sequence were detected in both the bacterial standard and Cry1Ab protein isolated from TwinLink cotton. The glycostain analysis results of the Cry1Ab protein isolated from TwinLink and bacterial standard also indicates that the protein is not glycosylated.

Table 7 Cry1Ab isolated from TwinLink cotton trypsin digest peptide map using Electrospray LC/MS

Cry1Ab residue number	*Theoretical mass [M+H]*	*91%cov. (full scan and SIM) 80 % cov, SIM Cry1Ab Std*	*74% cov, SIM Cry1Ab TwinLink*	*Mol. Wt. by Mass Spec.*
1 to 28	3135	1568 [M+2H]	1568 [M+2H]	3134
29 to 87	6569	1643 [M+4H]	1643 [M+4H]	6568
88 to 93	765	*766[a] [M+H]*	*NA**	765
94 to 99	689	689 [M+H]	689 [M+H]	688
100 to 115	1904	952 [M+2H]	ND	951
116 to 127	1400	ND	NA	ND
128 to 131	565	ND	NA	ND
132 to 173	4675	1559 [M+3H]	1559 [M+3H]	4674
174 to 181	908	*908 [M+H]*	NA*	907
182 to 192	1238	1238 [M+H]	1238 [M+H]	1237
193 to 198	782	*782 [M+H]*	*NA**	781
199 to 209	1259	1259 [M+H]	1259 [M+H]	1258
210 to 217	1039	*1039 [M+H]*	*NA**	1038
218 to 224	817	*817 [M+H]*	*NA**	816
225 to 228	590	*589[a] [M+H]*	*NA**	588
229 to 233	728	*728 [M+H]*	*NA**	727
234 to 234	175	NA	NA	NA
235 to 253	2197	1099 [M+2H]	1099 [M+2H]	2196
254 to 258	650	*649[a] [M+H]*	*NA**	648
259 to 265	805	*805 [M+H]*	*NA**	804
266 to 281	1902	1902 [M+H]	1902 [M+H]	1901
282 to 292	1075	1075 [M+H]	1075 [M+H]	1074
293 to 311	2199	2199 [M+H]	2199 [M+H]	2198
312 to 349	4182	2091 [M+2H]	2091 [M+2H]	4180
350 to 360	1204	1204 [M+H]	1204 [M+H]	1203
361 to 368	941	*941 [M+H]*	*NA**	940
369 to 402	3731	1866 [M+2H]	1866 [M+2H]	3730
403 to 403	147	NA	NA	NA
404 to 423	2150	2150 [M+H]	2150 [M+H]	2149
424 to 429	732	732 [M+H]	732 [M+H]	731
430 to 437	977	*977 [M+H]*	*NA**	976
438 to 449	1254	1254 [M+H]	1254 [M+H]	1253
450 to 458	1145	ND	NA	ND
459 to 478	2202	ND	NA	ND
479 to 490	1150	1150 [M+H]	1150 [M+H]	1149
491 to 501	1090	1090 [M+H]	1090 [M+H]	1089
502 to 502	175	NA	NA	NA
503 to 512	1060	1060 [M+H]	1060 [M+H]	1059
513 to 522	1099	1099 [M+H]	1099 [M+H]	1098
523 to 524	338	NA	NA	NA
525 to 526	274	NA	NA	NA
527 to 528	288	NA	NA	NA
529 to 567	4181	1394 [M+3H]	1394 [M+3H]	4179
568 to 601	3714	1239 [M+3H]	1239 [M+3H]	3714
602 to 620	2226	1113 [M+2H]	ND	2224

= Mass Spectrometer is a unit resolution instrument (uncertainty is ± 1 amu) resulting in some ions being off by 1 amu in the full scan spectrum.
*NA** = Not analyzed due to software and sample limitations. NA = Not analyzed. ND = Not Detected
Data obtained from Cry1Ab protein isolated from TwinLink on 3/12/2008.

Table 8 Amino acid coverage of Cry1Ab from E. coli and TwinLink Cotton Leaves

Calculation of % Amino Acid Coverage	*Number of amino acids not detected or analyzed*		*Residue Number*
	E. coli Cry1Ab	*TwinLink Cry1Ab*	
	0	6	88-93
	0	16	100-115
	12	12	116-127
	4	4	128-131
	0	8	174-181
	0	6	193-198
	0	8	210-217
	0	7	218-224
	0	4	225-228
	0	5	229-233
	1	1	234-234
	0	5	254-258
	0	7	259-265
	0	8	361-368
	1	1	403-403
	0	8	430-437
	9	9	450-458
	20	20	459-478
	1	1	502-502
	2	2	523-524
	2	2	525-526
	2	2	527-528
	0	19	602-620
Total	54	161	
Total number of Amino Acids	620	620	
% Amino Acid Not Detected or Analyzed	8.7	26	NA[a]
% Amino Acid Sequence Coverage	91	74	
% Amino Acid Coverage of Cry1Ab from *E. coli.*	100	81	

[a] NA = Not Applicable

Table 9 Selected Ion Monitoring mass spectrometry results of the Cry1Ab sequence residues with the Asparagine-X-Serine/Threonine sequence

Cry1Ab residue number	*Theoretical mass [M+H]*	*Cry1Ab Std*	*Cry1Ab TwinLink*
199 to 209	1259	1259 [M+H]	1259 [M+H]
438 to 449	1254	1254 [M+H]	1254 [M+H]
513 to 522	1099	1099 [M+H]	1099 [M+H]
529 to 567	4181	1394 [M+3H]	1394 [M+3H]
568 to 601	3714	1239 [M+3H]	1239 [M+3H]

ii. Cry2Ae Protein

Peptides from a trypsin digest of the Cry2Ae protein from *Bt.* and the Cry2Ae protein isolated from TwinLink cotton were analyzed by LC/MS using the selected ion monitoring method developed for the analysis. The method was developed based on the protein sequence of the Cry2Ae protein from *Bt.* The expected peptides from the trypsin digest of the Cry2Ae protein from *Bt.* were identified from their full scan spectra. The most abundant ion for each peptide was chosen for selected ion monitoring. The peptides at positions

315 to 338 = LTQTFPNIGGLPGTTTTHALLAAR
433 to 437 = NEDLR
438 to 446 = RPLHYNEIR
447 to 459 = NIESPSGTPGGLR
460 to 468 = AYMVSVHNR
508 to 513 = TFISEK
514 to 522 = FGNQGDSLR
532 to 535 = YTLR
536 to 546 = GNGNSYNLYLR
557 to 562 = VTINGR

could not be detected by full scan mass spectrometry which resulted in a peak for that peptide not being assigned and not detected by selected ion monitoring, Table 10. The peptides not being detected by full scan mass spectrometry could be caused by a missed cleavage or poor ionization under the conditions of analysis.

A 80% sequence coverage of the Cry2Ae protein from *Bt.* was obtained by full scan and selected ion monitoring combined and 68% sequence coverage was obtained by the selected ion monitoring method, Tables 11 and 12. The analysis of the tryptic digest of the Cry2Ae protein isolated from TwinLink cotton leaves by the selected ion monitoring method obtained 46% sequence coverage of the protein, Tables 11 and 12. The N-terminal peptide, NNVLNNGR, of the Cry2Ae protein isolated from TwinLink cotton leaves was detected demonstrating that the N-terminal is not modified. The N-terminal sequencing analysis also confirmed the NVLN portion of the N-terminal peptide. The peptides at positions

339 to 373 = V**NYS**GGVSSGDIGAVFNQ**NFS**CSTFLPPLLTPFVR
374 to 382 = SWLDSGSDR
383 to 403 = GGVNTVTNWQTESFESTLGLR
563 to 584 = VYTASNV**NTT**TNNDGVNDNGAR
585 to 631 = FLDINMGNVVASDNTNVPLDI**NVT**FNSGTQFELMNIMFVPTNLPPIY

were not detected in the Cry2Ae protein isolated from TwinLink cotton leaves; but were detected in the Cry2Ae protein from *Bt*. These peptides were not detected possibly because of a missed cleavage, non-glycosylated modifications, or the sample concentration was too low. The selected ion monitoring results for the Cry2Ae protein demonstrate that 58% of the Cry2Ae protein isolated from TwinLink cotton leaves is identical to the Cry2Ae protein from *Bt.,* Table 11.

The Cry2Ae protein also has sites with a potential for N-glycosylation. The Cry2Ae protein sequence has 9 tryptic peptides with the N-X-S/T sequence. The selected ion monitoring analysis results of these 9 tryptic peptides from the bacterial standard Cry2Ae protein and the Cry2Ae protein isolated from TwinLink cotton leaves is shown in Table 12. Cry2Ae tryptic peptide residues 339 to 373, 563 to 584, and 585 to 631, shown above with the N-X-S/T sequence in bold, were not detected by selected ion monitoring. Since a glycostain analysis of the Cry2Ae protein isolated from TwinLink indicates that the protein is not glycosylated, not detecting a peak for these residues can be attributed to a non-glycosylated modification, or low sample concentration.

Table 10 Cry2Ae isolated from TwinLink cotton trypsin digest peptide map using Electrospray LC/MS

Cry2Ae residue number	*Theoretical mass [M+H]*	*80%cov. (full scan and SIM) 68 % cov, SIM Cry2Ae Std*	*46% cov, SIM Cry2Ae TwinLink*	*Mol. Wt. by Mass Spec.*
1 to 8	901	901 [M+H]	901 [M+H]	900
9 to 28	2296	1149 [M+2H]	1149 [M+2H]	2296
29 to 34	705	*704[a] [M+H]*	NA*	703
35 to 35	147	NA	NA	NA
36 to 41	909	908[b] [M+H]	908[b] [M+H]	907
42 to 42	175	NA	NA	NA
43 to 62	2149	*1075 [M+2H]*	NA*	2148
63 to 63	147	NA	NA	NA
64 to 70	674	*674 [M+H]*	NA*	673
71 to 71	175	NA	NA	NA
72 to 94	2605	1302[a] [M+2H]	1302[a] [M+2H]	2602
95 to 103	1165	*1166[a] [M+H]*	NA*	1165
104 to 111	904	904 [M+H]	904[M+H]	903
112 to 124	1428	*1429[a] [M+H]*	NA*	1428
125 to 159	4037	1346[b] [M+3H]	1346[b] [M+3H]	4035
160 to 164	661	661 [M+H]	661 [M+H]	660
165 to 188	2745	*916 [M+3H]*	NA*	2745
189 to 205	1831	NA	NA	NA
206 to 212	930	929[a] [M+H]	929[a] [M+H]	928
213 to 231	2354	1177 [M+2H]	1177 [M+2H]	2352
232 to 236	561	560[a] [M+H]	560[a] [M+H]	559
237 to 244	1061	*1061 [M+H]*	NA*	1060
245 to 262	2289	1144[a] [M+2H]	1144[a] [M+2H]	2286
263 to 314	5685	1422 [M+4H]	1422 [M+4H]	5684
315 to 338	2453	ND	NA	ND
339 to 373	3693	1848 [M+2H]	ND	3694
374 to 382	1023	1022[a] [M+H]	ND	1021
383 to 403	2297	1149[a] [M+2H]	ND	2296
404 to 410	726	726 [M+H]	726 [M+H]	725
411 to 422	1494	1493 [M+H]	1493 [M+H]	1492
423 to 432	1054	1054 [M+H]	1054 [M+H]	1053
433 to 437	647	ND	NA	ND
438 to 446	1198	ND	NA	ND
447 to 459	1258	ND	NA	ND
460 to 468	1077	ND	NA	ND
469 to 469	147	NA	NA	NA
470 to 507	4270	856 [M+5H]	856 [M+5H]	4275
508 to 513	725	ND	NA	ND
514 to 522	994	ND	NA	ND
523 to 531	1054	1054[M+H]	1054[M+H]	1053
532 to 535	553	ND	NA	ND
536 to 546	1271	ND	NA	ND
547 to 556	1034	1034[b] [M+H]	1034[b] [M+H]	1033
557 to 562	660	ND	NA	ND
563 to 584	2298	1151[a] [M+2H]	ND	2300
585 to 631	5233	1309 [M+4H]	ND	5232

[a] = Mass Spectrometer is a unit resolution instrument (uncertainty is ± 1 amu) resulting in some ions being off by 1 amu in the full scan spectrum.
[b] = Selected ion monitoring (SIM) mass is 1 amu off from full scan spectrum because SIM method was developed from an earlier full scan spectrum. NA* = Not analyzed due to software and sample limitations. NA = Not analyzed. ND = Not Detected

Table 11 Amino acid Coverage of Cry2Ae from Bt. and TwinLink Cotton Leaves

Calculation of % Amino Acid Coverage	*Number of amino acids not detected or analyzed*		*Residue number*
	Bt. Cry2Ae	*TwinLink Cry2Ae*	
	0	6	29-34
	1	1	35-35
	1	1	42-42
	0	20	43-62
	1	1	63-63
	0	7	64-70
	1	1	71-71
	0	9	95-103
	0	13	112-124
	0	24	165-188
	17	17	189-205
	8	8	237-244
	24	24	315-338
	0	35	339-373
	0	9	374-382
	0	21	383-403
	5	5	433-437
	9	9	438-446
	13	13	447-459
	9	9	460-468
	1	1	469-469
	6	6	508-513
	9	9	514-522
	4	4	532-535
	11	11	536-546
	6	6	557-562
	0	22	563-584
	0	47	585-631
Total	126	339	
Total number of Amino Acids	631	631	
% Amino Acid Not Detected or Analyzed	20	54	NA[a]
% Amino Acid Sequence Coverage	80	46	
% Amino Acid Coverage of Cry2Ae from *Bt.*	100	58	

[a] NA = Not Applicable

Table 12 Selected Ion Monitoring mass spectrometry results of the Cry2Ae sequence residues with the Asparagine-X-Serine/Threonine sequence

Cry2Ae residue number	Theoretical mass [M+H]	Cry2Ae Std	Cry2Ae TwinLink
125 to 159	4037	1346 [b] [M+3H]	1346[b] [M+3H]
213 to 231	2354	1177 [M+2H]	1177 [M+2H]
339 to 373	3693	1848 [M+2H]	ND
423 to 432	1054	1054 [M+H]	1054 [M+H]
470 to 507	4270	856 [M+5H]	856 [M+5H]
523 to 531	1054	1054[M+H]	1054 [M+H]
547 to 556	1034	1034 [b] [M+H]	103[b] [M+H]
563 to 584	2298	1151 [a] [M+2H]	ND
585 to 631	5233	1309 [M+4H]	ND

[a] = Mass Spectrometer is a unit resolution instrument (uncertainty is ± 1 amu) resulting in some ions being off by 1 amu in the full scan spectrum.
[b] = Selected ion monitoring (SIM) mass is 1 amu off from full scan spectrum because SIM method was developed from an earlier full scan spectrum.
ND = Not Detected due to low sample concentration or a non-glycosylated modification because glycostain analysis indicated that the protein was not glycosylated.

iii. PAT/*bar* Protein

Peptides from a trypsin digest of the PAT/*bar* protein from *E. coli* and the PAT/*bar* protein isolated from TwinLink cotton were analyzed by LC/MS using the selected ion monitoring method previously developed for the analysis of cotton. The method previously developed was based on the protein sequence of the PAT/*bar* protein from *E. coli.* The expected peptides from the trypsin digest of the PAT/*bar* protein from *E. coli* were identified from their full scan spectra. The most abundant ion for each peptide was chosen for selected ion monitoring.

A 96% sequence coverage of the PAT/*bar* protein from *E. coli.* was obtained by selected ion monitoring, Tables 13 and 14. The analysis of the peptides from the tryptic digest of the PAT/*bar* protein isolated from TwinLink cotton leaves by the selected ion monitoring method obtained 81% sequence coverage of the protein, Tables 13 and 14. The N-terminal peptide, MDPER, of the PAT/*bar* protein isolated from TwinLink cotton leaves was not detected indicating that the N-terminal peptide may be modified. Post-translational modifications, such as removal of a methionine are often found in proteins from both prokaryotic and eukaryotic organisms (Bradshaw *et al.*, 1998). The peptides at positions 97 to 99, HQR, 136 to 145, MHEALGYAPR, 146 to 149, GMLR and 150 to 154, AAGFK were not detected in the PAT/*bar* protein isolated from TwinLink cotton leaves; but were detected in the PAT/*bar* protein from *E. coli.* These peptides were not detected possibly because of a missed cleavage, modification, or the sample concentration was too low. The selected ion monitoring results for the PAT/*bar* protein demonstrate that 84% of the PAT/*bar* protein isolated from TwinLink cotton leaves is identical to the PAT/*bar* protein from *E. coli.,* Table 14.

Table 13 PAT/*bar* isolated from TwinLink cotton trypsin digest peptide map using Electrospray LC/MS

PAT/bar residue number	*Theoretical mass [M+H]*	*96 % cov, SIM PAT/bar Std*	*81 % cov, SIM PAT/bar TwinLink*	*Mol. Wt. by Mass Spec.*
1 to 5	647	648 [M+H]	ND	647
6 to 11	727	728 [M+H]	728 [M+H]	727
12 to 12	175	NA*	NA*	NA
13 to 37	2782	930 [M+3H]	930 [M+3H]	2787
38 to 52	1842	923 [M+2H]	923 [M+2H]	1844
53 to 54	288	NA*	NA*	NA
55 to 56	304	NA*	NA*	NA
57 to 78	2391	1198 [M+2H]	1198 [M+2H]	2394
79 to 80	246	NA*	NA*	NA
81 to 96	1859	931 [M+2H]	931 [M+2H]	1860
97 to 99	440	222 [M+2H]	ND	442
100 to 112	1404	703 [M+2H]	703 [M+2H]	1404
113 to 120	879	880 [M+H]	880 [M+H]	879
121 to 135	1523	763 [M+2H]	763 [M+2H]	1524
136 to 145	1145	1146 [M+H]	ND	1145
146 to 149	476	477 [M+H]	ND	476
150 to 154	493	494 [M+H]	ND	493
155 to 183	3353	843** [M+4H]	843** [M+4H]	3368

NA* = Two amino acid masses were not analyzed because it was not indicative of only the PAT/bar protein.
ND = Not Detected
** The peptide is oxidized, possibly on a tryptophan residue.

Table 14 Amino acid coverage of PAT/*bar* from *E. coli* and TwinLink cotton leaves

Calculation of % Amino Acid Coverage	*Number of amino acids not detected or analyzed*		*Residue number*
	E. coli PAT/bar	*TwinLink PAT/bar*	
	0	5	1-5
	1	1	12-12
	2	2	53-54
	2	2	55-56
	2	2	79-80
	0	3	97-99
	0	10	136-145
	0	4	146-149
	0	5	150-154
Total	7	34	
Total number of Amino Acids	183	183	
% Amino Acid Not Detected or Analyzed	4	19	NA[a]
% Amino Acid Sequence Coverage	96	81	
% Amino Acid Coverage of PAT/*bar* from *E. coli.*	100	84	

[a] NA = Not Applicable

5. Biological activity

PAT/*bar* proteins from *E. coli* or isolated from TwinLink cotton leaves, was shown to generate free Coenzyme A sulfhydryl groups during the transfer of the acetyl group to phosphinothricin, resulting in an increase of the absorption at 412 nm by more than 10%. This increase in absorption indicated that both the PAT/*bar* proteins from *E. coli* and TwinLink cotton leaves are biologically active.

The activity of the Cry2Ae protein isolated from TwinLink cotton was determined by feeding the isolated Cry2Ae protein to *Helicoverpa zea* larvae, at the first larvae stage. The Cry2Ae protein standard was also analyzed for comparison. The insects fed the isolated Cry2Ae protein from TwinLink cotton lost approximately the same amount of weight as the insects fed the Cry2Ae protein standard. Therefore, the Cry2Ae protein isolated from TwinLink cotton leaves has the same activity as the Cry2Ae protein standard from Bt.

Activity determination could not be done with Cry1Ab protein isolated from TwinLink cotton because the protein concentration was too low.

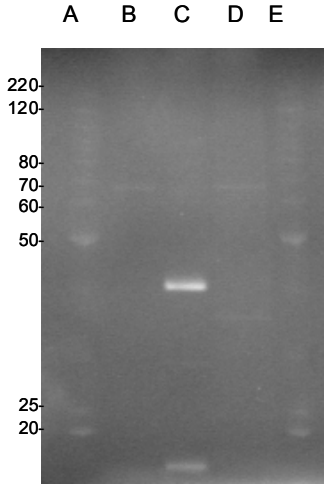
6. Expected and unexpected post-translational modifications

i. Cry1Ab protein

The glyco-staining results for the Cry1Ab protein are shown in Figure 10. The N-glycosylated proteins for Avidin and alpha acidic glycoprotein in lane C are seen as bright bands in the gel. The Cry1Ab protein from *E. coli.* and the Cry1Ab protein isolated from TwinLink cotton leaves in lanes B and D are not bright bands. This indicates that the Cry1Ab proteins are not glycosylated. The mass spectrometry data also confirmed that the Cry1Ab proteins are not glycosylated by detecting the peptides with a potential site for glycosylation.

ii. Cry2Ae and PAT/bar proteins

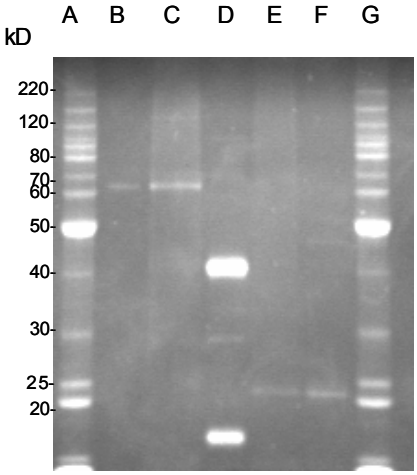
The glycol-staining results for the Cry2Ae and PAT/*bar* proteins are shown in Figure 11. The N-glycosylated proteins for Avidin and alpha acidic glycoprotein in lane D are seen as bright bands in the gel. The Cry2Ae protein from *Bt.* and the Cry2Ae protein isolated from TwinLink cotton leaves in lanes B and C are not detected as bright bands indicating that the proteins are not glycosylated. The PAT/*bar* protein from *E. coli.* and the PAT/*bar* protein isolated from TwinLink cotton leaves in lanes F and E are not seen as bright bands indicating also that the proteins are not glycosylated.



Lanes:
A- BenchMark Protein Ladder
B- Cry1Ab Thrombin Protein Standard
C- BTS4 N-Glycosylated Protein Standard
D- Cry1Ab Isolated from TwinLink Cotton Leaves
E- BenchMark Protein Ladder

Figure 10 GlycoStain of the Cry1Ab protein from *E. coli* and the Cry1Ab protein isolated from leaves of transgenic TwinLink Cotton.

Glycoprofile™ III Fluorescent Glycoprotein analysis of the Cry1Ab protein from *E. coli.* and the Cry1Ab protein isolated from TwinLink cotton leaves. Lanes A and E contain molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the stained gel. Lane B contains the Cry1Ab protein from *E. coli.* Lane C contains the BTS#4 Glycoprotein Standard mix of Phosphorylase B, alpha-Acidic glycoprotein, Carbonic anhydrase and Avidin. Lane D contains Cry1Ab isolated from TwinLink cotton leaves.



Lanes:
A- BenchMark Protein Ladder
B- Cry2Ae Protein Standard
C- Cry2Ae Isolated from TwinLink Cotton Leaves
D- BTS4 N-Glycosylated Protein Marker
E- PAT/bar Isolated from TwinLink Cotton Leaves
F- PAT/bar Protein Standard
G- BenchMark Protein Ladder

Figure 11 GlycoStain of the Cry2Ae and PAT/*bar* proteins from *Bt.* and *E. coli* and the Cry2Ae and PAT/*bar* proteins isolated from leaves of transgenic TwinLink Cotton.

Glycoprofile™ III Fluorescent Glycoprotein analysis of the Cry2Ae protein from *Bt.* and the PAT/*bar* protein from *E. coli.* and the Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves. Lanes A and G contain molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the stained gel. Lane B contains the Cry2Ae protein from *Bt..* Lane C contains the Cry2Ae protein isolated from TwinLink cotton leaves. Lane D contains the BTS#4 Glycoprotein Standard mix of Phosphorylase B, alpha-Acidic glycoprotein, Carbonic anhydrase and Avidin. Lane E contains the PAT/*bar* protein isolated from TwinLink cotton leaves. Lane F contains the PAT/*bar* protein from *E. coli.*

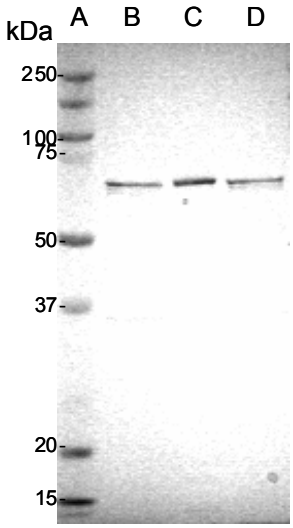
Conclusion

The results from the six analytical tests indicate that the Cry1Ab, Cry2Ae and PAT/*bar* proteins from *E. coli* and *Bt.* are equivalent to the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves. The molecular weights, mobilities, immuno-reactivities, activities for Cry2Ae and PAT/*bar*, as well as 81% of Cry1Ab, 58% of Cry2Ae and 84% of PAT/*bar* protein sequence coverages, are identical. These data demonstrate that the safety data obtained for the Cry1Ab, Cry2Ae and PAT/*bar* proteins produced in *E. coli* and *Bt.* can be used to support the safety of the Cry1Ab, Cry2Ae and PAT/*bar* proteins produced in TwinLink cotton.

Equivalence of Proteins Expressed in Seed

To complement the equivalence evaluation of proteins produced in cotton leaves, an immuno-reactivity study (Western blot) was completed for TwinLink cotton seed. The Western blots indicate that the proteins have the same molecular weights, mobilities and immuno-reactivities. These Western blot studies indicate that the Cry1Ab, Cry2Ae and PAT/*bar* proteins produced in the seed of individual events (T304-40 and GHB119) and TwinLink cotton are the same as the Cry1Ab, Cry2Ae and PAT/*bar* proteins produced from *Escherichia coli* and *Bacillus thuringiensis.*

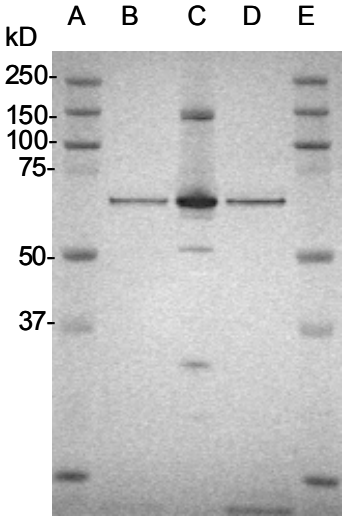
The results of the Cry1Ab western blot show that the molecular weights, electrophoretic mobilities and immuno-reactivities of the Cry1Ab proteins produced in *E. coli* or isolated from event T304-40 and TwinLink cotton seed are indistinguishable (Figure 12).



Lanes:
A- Kaleidoscope Protein Standard
B- Cry1Ab isolated from TwinLink cotton seed
C- Cry1Ab Thrombin Standard
D- Cry1Ab isolated from T304-40 cotton seed

Figure 12 Comparison of the Cry1Ab protein from *E. coli* to the Cry1Ab protein isolated from ground seed of TwinLink cotton.

Lane A contains molecular weight markers of 250, 150, 100, 75, 50, 37, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel. Lane B contains the Cry1Ab protein isolated from TwinLink cotton seed. Lane C contains the Cry1Ab Thrombin protein standard form *E. coli.* Lane D contains the Cry1Ab protein isolated from event T304-40 cotton seed.



Lanes:
A- Kaleidoscope Protein Std
B- Cry2Ae from TwinLink seed
C- Cry2Ae Standard
D- Cry2Ae from GHB119 seed
E- Kaleidoscope Protein Std

Figure 13 Comparison of the Cry2Ae protein from *Bt.* to the Cry2Ae protein isolated from ground seed of TwinLink cotton.

Lanes A and E contain molecular weight markers of 250, 150, 100, 75, 50, 37, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel. Lane B contains the Cry2Ae protein isolated from TwinLink cotton seed. Lane C contains the Cry2Ae protein standard from *Bt*. Lane D contains the Cry2Ae protein isolated from event GHB119 cotton seed.

The results of the Cry2Ae western blot show that the molecular weights, electrophoretic mobilities and immuno-reactivities of the Cry2Ae proteins produced from *Bt.* or isolated from event GHB119 and TwinLink cotton seed are indistinguishable. The western blot result for the Cry2Ae protein produced from *Bt.* in lane C had a higher molecular weight band believed to be a Cry2Ae protein dimer. Some lower molecular weight bands were also seen for the Cry2Ae protein standard indicative of protein degradation (Figure 13).

The results of the PAT/*bar* western blot demonstrate that the molecular weights and electrophoretic mobilities of the PAT/*bar* proteins produced from *E. coli.* or isolated from events T304-40, GHB119 and TwinLink cotton seed are indistinguishable and all four proteins are immunoreactive. Faint bands of protein degradation from the protein isolation process can be seen for the PAT/*bar* proteins isolated from TwinLink, GHB119 and T304-40 (Figure 14).

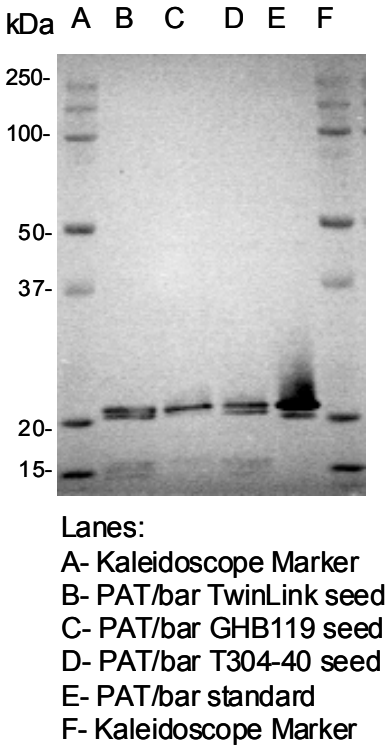


Figure 14 Comparison of the PAT/*bar* protein from E. *coli* to the PAT/*bar* proteins isolated from ground seed of TwinLink cotton.

Lanes A and F contain molecular weight markers of 250, 150, 100, 75, 50, 37, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel. Lane B contains the PAT/bar protein isolated from TwinLink cotton seed. Lane C contains the PAT/bar protein isolated from event GHB119. Lane D contains the PAT/bar protein isolated from event T304-40 cotton seed. Lane E contains the PAT/*bar* protein from *E. coli.*

Conclusion

The results from the western blot analyses indicate that the Cry1Ab, Cry2Ae and PAT/*bar* proteins from *E. coli.* and *Bt.* are equivalent to the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from seed of the individual events (T304-40 and GHB119) and from TwinLink cotton. The molecular weights, mobilities and immuno-reactivities are identical. These data demonstrate that the safety data obtained for the Cry1Ab, Cry2Ae and PAT/*bar* proteins produced in *E. coli* and *Bt.* can be used to support the safety of the Cry1Ab, Cry2Ae and PAT/*bar* proteins produced in TwinLink cotton seed.

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 cotton 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 laboratories, 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 strip (LFS). This method allows qualitative detection of the introduced protein, and can be performed under field and/or laboratory 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 TwinLink cotton can be provided upon request, and upon agreement with BCS.

VIII. AGRONOMIC AND PHENOTYPIC EVALUATION

A. Agronomic performance and evaluation

TwinLink cotton, comprised of transformation events T304-40 and GHB119, was derived by transformation of upland Coker cotton varieties to express the Cry1Ab and Cry2Ae proteins, which are derived from the soil bacterium *Bacillus thuringiensis*. These Cry protein events were selected based on demonstrated production of protein toxins selective to *lepidopteran* pests (*i.e.* Cotton Bollworm, Tobacco Budworm, Armyworm and Pink Bollworm). Bayer CropScience plans to commercialize both events as a combined insect-resistant trait under the trade name TwinLink. Cry proteins are widely used as plant incorporated insecticides in US cotton. In 2008, 69% of US cotton acreage was planted in Bt cotton. Today, Bt cotton is grown in every cotton production region of the US (Table 15) and in the Southwest, represents more than half the area in production. TwinLink cotton and its parents also produce the PAT protein that confers tolerance to the herbicide glufosinate ammonium.

Table 15 Bt cotton production in the United States by Region

Region	*Total acres per region*	*% of Bt cotton planted*
Southeast (AL, GA, NC, SC, VA, FL)	1,947,000	21
Mid-South (MS, LA, MO, AR, TN)	1,900,000	20
Southwest (TX, OK, KS)	5,121,000	54
West (CA, NM, AZ)	446,000	4

Source: USDA Agricultural Marketing Service (http://www.ams.usda.gov/), 2008

TwinLink cotton was evaluated by comparison to its non-transgenic parent though-out the US cotton belt. Agronomic performance trials were conducted in a manner representative of standard agricultural practices within the respective testing regions. Conventional insecticide applications were made in accordance with university extension recommendations to determine trait effectiveness. Thus, comparisons of agronomic performance can be made with conventional and transgenic production practices.

The findings of these tests show:

1. The agronomic performance of TwinLink cotton was equal to or better than that of its non-transgenic counterpart both when sprayed and unsprayed for lepidopteran pests with conventional insecticides.

2. No significant differences were observed in leaf, flower, plant, or boll morphology between TwinLink and the conventional parental pedigree.

3. Laboratory and field experimentation show significant reduction in lepidopteran plant damage and larval survival due to expression of Cry proteins from the component events T304-40 and GHB119 and TwinLink.

4. The agronomic performance of TwinLink cotton treated with the herbicide glufosinate ammonium was equal to or better than that of its unsprayed TwinLink cotton and its unsprayed non-transgenic counterpart.

5. Evaluation of the agronomic performance of component events T304-40 and GHB119, and TwinLink cotton has identified neither safety nor environmental concerns.

B. History of field activities

TwinLink cotton events T304-40 and GHB119 were regenerated *via* tissue culture after transformation of the individual plant cells. In 2005, T_3 seed harvested from T_2 plants was imported for planting in the first field evaluation in Mississippi. T_3 plants were evaluated for agronomic equivalence and line selection. Selected lines of the two events were increased in 2006. Breeding activities to move the events into FiberMax germplasm and to combine the events for TwinLink progressed in the greenhouse. Sufficient seed was produced to conduct the 2007 field trials. Seed harvested from US seed increases in 2007 was used in 2008 field trials. Fields were routinely sprayed with insecticide to prevent possible pollen transfer by insects. Table 16 presents a summary of the field trials and associated authorization permits.

Table 16 Summary of field activities under USDA permits for TwinLink events

Notification Number	*Year*	*Number of Locations*	*Locations*	*Termination Report*
05-035-12n	2005	1	MS	1
07-059-104n	2007	10	LA(2), MS(2), TX(6)	2
07-065-119n	2007	1	NC	8
07-065-118n	2007	1	NC	7
07-065-117n	2007	1	NC	6
07-044-104n	2007	6	LA(1), AZ(1). TX(2), MS(2)	5
07-044-103n	2007	7	LA(1), AZ(1), TX(2), MS(3)	4
07-044-102n	2007	11	LA(2), AZ(1),TX(4), MS(2), NC(1), SC(1)	3
07-059-101n	2007	9	AR(3), GA(2), MS(1), TX(3)	9
08-022-101n	2008	10	TX(2),CA(1),LA(1),MS(3),NC(1),SC(1),AZ(1)	*
08-036-127n	2008	9	TX(2),GA(2),AR(3),AZ(1),MS(1)	*

*Copies of the termination reports for these field trials are provided in Appendix I. Termination reports for the 2008 field trials will be provided to the USDA according to the 6 month post-termination reporting requirement.

C. Agronomic performance of TwinLink cotton (events T304-40 x GHB119)

Replicated field trials were conducted in 2007 and 2008 to compare agronomic performance of the TwinLink cotton events T304-40 and GHB119 with the non-transformed Coker counterpart (see Table 16 above). 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 17.

Table 17 Description of Agronomic Parameters Evaluated

Agronomic Characteristic	*Description*
Boll Type	Visual rating of boll type; 1=loose, 5=intermediate, 9=stormproof.
Boll retention (p1, p2)	Percent bolls retained on plant at first and second position
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 reaction	Visual rating of disease pathogen; 1 = no symptoms 5 = some symptoms apparent, 9 = severe
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 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.
Leaf Uniformity	Consistency of leaf type
Lint Percent	Lint weight divided by seed cotton weight, expressed as a percentage.
Lodging	Visual rating of plant stature; 1=fully upright, no leaning, 5=Leaning 45 degrees from ground, 9=laying on soil surface
Number first position bolls	Total number of bolls per plant set on the first position of fruiting branches
Number second position bolls	Total number of bolls per plant set on the second position of fruiting branches
Number third position bolls	Total number of bolls per plant set on the third position 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.
Number of open Bolls	Total number of bolls mature and open (ready for picking) at a given time point.
Number of Total Bolls	Total number of bolls on an individual cotton plant at a given time point.
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, bolls)	A scale rating of leaf, flower and boll type.
Plant Stand	Evaluation of the germination rates and plant population
Seed index	Average weight in grams of 100 seed, an indication of seed size and maturity.
Sprayed plots	Plots sprayed with selective insecticides for lepidopteran pests
Strain Uniformity	Used to evaluate the uniformity of the event on a 1 to 9 scale. (1 = uniform, 9 = highly variable)
Total number of nodes	Number of reproductive nodes present on the main stem of the plant
Treated plots	TwinLink plots sprayed with glufosinate ammonium herbicide
Unsprayed plots	Plots unsprayed for lepidopteran pests
Yield: Lbs. lint per acre	Productivity expressed as pounds of lint produced per acre

Data from replicated field trials was recorded from 23 locations in 8 states (North Carolina, South Carolina, Georgia, Arkansas, Mississippi, Louisiana, Texas, and Arizona over the 2007 and 2008 growing seasons. Studies were conducted in geographic regions representative of

80% and 87% of the total upland cotton production in the US in 2007 and 2008, respectively. Trials were conducted across the cotton belt to capture the various environmental stresses that upland cotton varieties undergo during the course of a normal production year. These areas represent a large majority of the Bt cotton acreage and target market area for TwinLink cotton.

Findings across locations show that TwinLink cotton is similar for maturity and yield to its conventional variety counterpart. Limited phenotypic differences were recorded between TwinLink and its conventional variety counterpart. These differences can be attributed to premature fruit shed due to lepidopteran damage in the conventional pedigree line. The majority of phenotypic differences recorded can be considered as positive attributes of the TwinLink technology: earlier maturity, increased fruit retention and increased lint yield, due to lepidopteran control.

D. Biotic and abiotic stress characteristics

Field trial managers noted no performance difference of T304-40, GHB119, TwinLink and their non-transgenic parent lines to abiotic and biotic stress. Weed management in all trials was performed in accordance with local University Extension recommendations: weeds were not evaluated in these trials, as all weed populations were controlled using appropriate herbicide applications to eliminate this variable. Summary data (across locations) for the Agronomic Performance evaluations can be found in Appendix 4.

Seedling vigor and plant stand counts were similar for conventional cotton and lines containing the T304-40 and GHB119 event transformations for all the trials planted in optimal conditions and where irrigation was available. Reduced germination was observed in all test plots under less than optimal conditions (marginal moisture, deeper than optimal planting depth, inconsistent soil type).

Data for plant stand were recorded at 13 of the 22 locations over the 2007 and 2008 growing seasons (Table 18). TwinLink stand counts were significantly increased at 9 of the 13 locations compared with its conventional counterpart. Plant stands were within commercially acceptable limits for all trial locations.
[In order to avoid confusion the term “sprayed” refers to insecticide treatments, and “treated” refers to herbicide treatment]

Table 18 Plant stand count data individual locations in 2008.

	Southeast		***Mid-South***		***Texas***	***Arizona***
	Martin, NC	***Sellers, SC***	***Leland, MS***	***Franklin, LA***	***Lubbock, TX***	***Pinal, AZ***
Coker not sprayed	2.73b	2.60a	2.83a	2.31b	1.57a	3.90ab
Coker sprayed	3.53a	2.60a	3.00a	2.88a	1.59a	3.30b
TwinLink not sprayed	2.83b	2.27a	2.75a	2.31b	1.32a	4.49a
TwinLink sprayed	3.68a	2.36a	2.85a	3.03a	1.86a	3.99ab
LSD (0.05)	0.419	0.428	0.644	0.466	0.701	0.906
CV	8.22	10.91	14.1	11.08	27.7	14.45

* Sprayed plots indicate use of selective conventional insecticides to control *lepidopteran* pests.

Disease and lodging ratings were made using a scale of 1 to 9 (1 = normal, 9 = severe) to rate plant pathogen susceptibility or lodging problems. Disease ratings across locations showed no significant differences between transformed plots and their non-transformed counterparts (Table 19).

Table 19 Lodging data by individual location in 2007

	Southeast		***Mid-south***				***Southwest***
	Chula, GA (#1)	***Chula, GA (#2)***	***Tate, MS***	***Jackson, AR***	***Crittenden AR (#1)***	***Crittenden AR (#2)***	***Hockley, TX***
Coker	1	1	1	2	1	1	1
TwinLink *	1	1	1	2	1	1	1
TwinLink treated	1	1	1	2	1	1	1
LSD (0.05)	0	0	0	0	0	0	0
CV	0	0	0	0	0	0	0

* TwinLink plots were either treated or not treated with glufosinate ammonium herbicide to eliminate wild type plants from transformed seed lots.

Leaf, flower and boll morphology data was recorded at three locations in 2008 with no significant difference between TwinLink and the conventional parent (Table 20). Ratings are 1=normal and 2=abnormal.

Table 20 Plant part morphology data in 2008

Comparing conventional insecticide and Bt cotton treatments.

	Sellers, SC			***Leland, MS***			***Lubbock, TX***		
	Leaf	***Flower***	***Boll***	***Leaf***	***Flower***	***Boll***	***Leaf***	***Flower***	***Boll***
Coker unsprayed	1	1	1	1	1	1	1	1	1
Coker sprayed	1	1	1	1	1	1	1	1	1
TwinLink unsprayed	1	1	1	1	1	1	1	1	1
TwinLink sprayed	1	1	1	1	1	1	1	1	1
LSD (0.05)	0	0	0	0	0	0	0	0	0
CV	0	0	0	0	0	0	0	0	0

* Sprayed plots indicate use of selective conventional insecticides to control *lepidopteran* pests.

E. Maturity and plant phenotype characteristics

Plant maturity data were collected in seven locations in 2007 and four locations in 2008. No significant differences were recorded in plant maturity at three of the seven locations, while four of the seven locations in 2007 recorded significant differences in maturity (Table 21, Table 22 and Table 23). Where significant differences in maturity were recorded, there is a clear trend towards increased earliness (shorter period to first flower and greater % open bolls) in the TwinLink plots compared with the Coker plots which were not sprayed with insecticide (Table 23). This would be expected as a result of control of lepidopteran pests and higher fruit

retention in the TwinLink plots relative to Coker. In contrast, no difference was observed in maturity between TwinLink plots treated with glufosinate ammonium herbicide and untreated TwinLink plots (Tables 21 and 22).

Boll type visual assessments were taken at seven locations in 2007 (1-9, 1=loose, 5=intermediate, 9=storm-proof). No significant differences were observed in boll type across the seven locations.

Table 21 Maturity data from individual locations in the Southeast and Texas in 2007

	Chula, GA (Loc #1)		*Chula, GA (Loc #2)*		*Texas*	
	Days to 1st flower	*% open bolls*	*Days to 1st flower*	*% open bolls*	*Days to 1st flower*	*% open bolls*
Coker	55.7a	43b	54.7a	33.3b	73.0a	50b
TwinLink	53.0b	70a	52.7b	69.3a	63.0b	75a
TwinLink treated *	54.0ab	59ab	52.0b	72.0a	63.0b	75a
LSD (0.05)	2.20	22.1	2.00	21.15	0	0
CV	1.79	16.97	1.66	16.03	0	0

* TwinLink plots were either treated or not treated with glufosinate ammonium herbicide to eliminate wild type plants from transformed seed lots.

Table 22 Maturity data from individual locations in the Mid-south in 2007

	Tate, MS		*Jackson, AR*		*Crittenden, AR (#1)*		*Crittenden, AR (#2)*	
	Days to 1st flower	*% open bolls*	*Days to 1st flower*	*% open bolls*	*Days to 1st flower*	*% open bolls*	*Days to 1st flower*	*% open bolls*
Coker	55.0a	50.0a	51.0a	58a	45.0a	50a	45.0a	50a
TwinLink	55.0a	50.0a	49.0b	55a	45.0a	50a	45.0a	50a
TwinLink treated *	55.0a	50.0a	49.0b	57a	45.0a	50a	45.0a	50a
LSD (0.05)	0	0	0	12.2	0	0	0	0
CV	0	0	0	9.53	0	0	0	0

* TwinLink plots were either treated or not treated with glufosinate ammonium herbicide to eliminate wild type plants from transformed seed lots.

Table 23 Maturity data from individual locations in 2008.

	Sellers, SC		*Leland, MS*		*Lubbock, TX*		*Pinal, AZ*
	Days to 1st flower	*Days to 1st boll opening*	*Days to 1st flower*	*Days to 1st boll opening*	*Days to 1st flower*	*Days to 1st boll opening*	*% open bolls*
Coker unsprayed	63.0a	121a	58b	102b	67.0a	128a	55a
Coker sprayed	63.0a	120a	60a	103ab	67.0a	128a	50a
TwinLink unsprayed	63.0a	120a	59ab	103ab	67.0a	128a	58a
TwinLink sprayed	63.0a	120a	58b	104a	67.0a	128a	50a
LSD (0.05)	0	0.8	2.0	2.1	0	0	15.6
CV	0	0.42	2.12	1.27	0	0	18.36

* Sprayed plots indicate use of selective conventional insecticides to control *lepidopteran* pests.

F. Yield and fiber quality

Yield was recorded at all of the 23 locations over the 2007 and 2008 growing seasons (Appendix 4, Tables 4.2, 4.4, 4.6 and 4.10). Yields were within expectations for the Coker varietal background, but significantly lower than would be expected for commercial varieties. TwinLink had significantly greater yield than its conventional counterpart at multiple locations (Appendix 4, Tables 4.2, 4.4, 4.6, 4.8 and 4.10) and at the Sellers location (Table 24). Increases in yield would be expected as a result control of lepidopteran pests and higher fruit retention in the TwinLink plots relative to the Coker plots.

Table 24 Fiber Analysis from Sellers, SC location in 2008

	Coker		*TwinLink*		*Significance*		
	Unsprayed	*Sprayed**	*Unsprayed*	*Sprayed*	*LSD***	*CV (0.05)*	*SIG*
Yield	1227.1b	1326.9ab	1334.6ab	1434.0a	201.81	9.48	ab
Fiber Length	1.27a	1.25ab	1.22b	1.22b	0.031	1.57	ab
Fiber strength	36.0a	35.8ab	33.8c	34.2bc	1.66	2.97	abc
Fiber uniformity %	86.1a	85.3ab	84.8b	84.8b	1.17	0.86	ab
Micronaire	4.9	4.9	4.7	4.7	0.29	3.77	N.S.
% lint	40.8b	41.7ab	41.9ab	42.4a	1.50	2.25	ab

* Sprayed plots indicate use of selective conventional insecticides to control *lepidopteran* pests.
** LSD = 0.05

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 2007 season for fiber length when analyzed across seven locations (Appendix 4, Table 4.2 and Table 4.4, and at the Sellers location in Table 24). No significant differences were observed in 2007 for length uniformity, lint percent, fiber strength, and micronaire.

Fiber quality data is available for only two locations for 2008 at time of dossier preparation. Significant differences were observed at the Sellers, SC location (Table 24), but trends were not consistent across locations.

During equivalence evaluation of the transformed trait (see Section VIII.G) no significant differences were recorded in fiber characteristics between the T304-40, GHB119, and TwinLink lines compared to their conventional counterpart. Backcrossing reduces the influence of the Coker background in which the transformation took place. Therefore, it was determined that the differences in fiber characteristics recorded in the 2007 and 2008 trials were associated with the somaclonal variation in the Coker background, and the relative early stage of backcrossing (BC_2F_3) rather than the T304-40 or GHB119 traits *per se*. These differences would be expected to be overcome with further backcrossing in elite germplasm backgrounds.

G. Equivalence between Coker-derived and commercial varieties

Somaclonal variation can occur when transformed plant cells are stressed by the surrounding environment, which can result in differences between a regenerated Coker plant and the original plant from which the tissue was taken. Somaclonal variation can lead to variances in the plant which are not linked to the transgene event and are removed by backcrossing. Commercial

germplasm was backcrossed with the T_0 generation of the respective Coker transformant and then progressed to the BC_2F_3 generation for evaluation along with the Coker variety (see Breeding diagram in Appendix 2).

H. Composition analysis

A study was conducted to obtain composition analysis data on RAC (cottonseed) samples of TwinLink cotton and its non-transgenic counterpart. The nutritional components of cottonseed were compared for the TwinLink cotton and the non-transgenic counterpart Coker 315.

Cotton plants were grown in the field by Bayer CropScience in 2007. Seven field trials were conducted in EPA Regions II, IV, and VIII in Georgia, Arkansas, Mississippi and Texas, all important cotton growing regions of the Southern United States. The plants in this study were grown under conditions typical of production practices. There were six TwinLink cotton transgenic plots and three non-transgenic plots at each test site. Three of the TwinLink cotton transgenic plots were sprayed two times with glufosinate-ammonium herbicide at a target rate of 0.53 lb ai/A, and three transgenic plots were non-treated. Three replicate non-transgenic samples and six replicate transgenic samples (3 non-treated and 3 treated with glufosinate-ammonium herbicide) of fuzzy seed were collected from each of the field test sites and shipped frozen to Bayer CropScience BioAnalytics laboratory, Research Triangle Park, North Carolina. The fuzzy seeds were sub-sampled and shipped frozen to the analytical facility, Covance Laboratories, Inc., Madison, Wisconsin for composition analyses.

Composition data were obtained for 63 samples (9 samples from each of 7 field trials) of ginned cottonseed (also known as fuzzy seed).
There were 21 samples from each of three groups:

1. Non-transgenic, non-tolerant Coker 315 cotton
2. TwinLink cotton from combined-trait transgenic events T304-40 and GHB119 that was not treated with glufosinate herbicide
3. TwinLink cotton from combined-trait transgenic events T304-40 and GHB119 that was treated two times with glufosinate herbicide.

Chemical analysis of the samples included the nutritional factors proximate, minerals, vitamins, amino acids, and fatty acids (cottonseed oil). Antinutritional factors of cotton include the gossypols, phytic acid, and the cyclic fatty acids. The mean values and the literature standards are provided in Tables 25-29.

Data were obtained from the transgenic non-treated, transgenic treated and non-transgenic samples and are presented on a fresh weight basis. The data are also presented on a dry weight basis by correcting the fresh weight values for the moisture content determined for each sample. Mean values and standard deviations were calculated for the dry matter data. Fatty acid data are presented on a fresh weight basis and additionally, together with the cyclopropenoid fatty acid data, as relative quantities of the total sum of fatty acids.

Table 25 Proximate and fibre compounds in fuzzy seed of TwinLink Cotton and the non-transgenic counterpart Coker 315 compared to commercial cotton varieties (Reference ranges)

Parameter	*Non-Transgenic*	*Transgenic Non-treated*	*Transgenic Treated* [a]	*Reference ranges* [c]
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	
Moisture % fw	11.13 ± 2.17	10.70 ± 2.32	10.72 ± 2.43	4.0 - 15.9
Fat % dm	23.30 ± 2.29	22.38 ± 2.40	22.38 ± 2.29	11.8 - 36.3
Protein % dm	18.40 ± 2.40	18.53 ± 2.38	18.76 ± 1.83	11.7 - 34.2
Ash % dm	3.94 ± 0.33	4.01 ± 0.22	4.04 ± 0.30	3.2 - 5.0
Total carb. % dm [b]	54.38 ± 2.37	55.10 ± 2.63	54.82 ± 2.27	36.4 - 74.4
ADF % dm	39.64 ± 2.64	39.53 ± 2.89	38.92 ± 2.60	29.0 - 66.9
NDF % dm	46.05 ± 3.77	46.44 ± 3.75	47.24 ± 3.05	38.1 - 71.4

[a] Treated with glufosinate ammonium
[b] Total carbohydrates calculated as 100% - (protein %dm + fat %dm + ash %dm)
[c] References: Amann, 1999. Berberich *et al*., 1996. Bertrand *et al*., 2005. Calhoun *et al*., 1995. ILSI, 2007. Lundquist, 1995. Nida et al*., 1996.* OECD, 2004. USCA, 1982.

Table 26 Minerals and Vitamin E in fuzzy seed of TwinLink Cotton and the non-transgenic counterpart Coker 315 compared to commercial cotton varieties (Reference ranges)

Parameter	*Non-Transgenic*	*Transgenic Non-treated*	*Transgenic Treated* [a]	*Reference ranges*[b]
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	
Calcium (% dm)	0.126 ± 0.014	0.136 ± 0.023	0.133 ± 0.021	0.09 - 0.33
Phosphor (% dm)	0.569 ± 0.100	0.574 ± 0.075	0.578 ± 0.071	0.31 - 0.86
Potassium (% dm)	1.12 ± 0.06	1.14 ± 0.06	1.14 ± 0.07	0.96 - 1.42
Magnesium (% dm)	0.349 ± 0.036	0.365 ± 0.031	0.364 ± 0.028	0.27 - 0.49
Iron (mg/kg dm)	45.2 ± 11.2	43.8 ± 9.7	43.3 ± 7.5	23.2 - 160
Zinc (mg/kg dm)	33.7 ± 4.3	34.7 ± 4.8	35.0 ± 4.3	17.8 - 63.0
Alpha Tocopherol (mg/kg dm)	122 ± 14	130 ± 13	132 ± 9	16 - 245

[a] Treated with glufosinate ammonium
[b] References: Calhoun et al., 1995. ILSI, 2007. Lundquist, 1995. OECD, 2004. USCA, 1982. FAO/WHO, 2001

Table 27 Anti-nutrients in fuzzy seed of TwinLink Cotton and the non-transgenic counterpart Coker 315 compared to commercial cotton varieties (Reference ranges)

Parameter	*Non-Transgenic*	*Transgenic Non-treated*	*Transgenic Treated* [a]	*Reference ranges* [b]
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	
Free gossypol (% dm)	0.709 ± 0.128	0.754 ± 0.139	0.715 ± 0.146	0.23 - 1.40
Total gossypol (% dm)	0.799 ± 0.103	0.835 ± 0.114	0.801 ± 0.096	0.46 - 1.99
Phytic acid (% dm)	1.62 ± 0.47	1.60 ± 0.31	1.55 ± 0.37	0.85 - 2.57
Malvalic acid (% rel.)	0.467 ± 0.075	0.473 ± 0.106	0.474 ± 0.094	0.17 - 1.5
Sterculic acid (% rel.)	0.308 ± 0.063	< 0.10 - 0.389	0.314 ± 0.080	0.12 - 0.92
Dihydrosterculic acid (% rel.)	0.145 ± 0.015	< 0.10 - 0.155	0.135 ± 0.012	0.11 - 0.50

[a] Treated with glufosinate ammonium
[b] References: Berberich *et al*. 1996. Calhoun *et al*., 1995. ILSI, 2007. Nida *et al*., 1996. OECD, 2004. Phelps *et al*., 1965. Wozenski and Woodburn, 1975.

Table 28 Total Amino Acids in fuzzy seed of TwinLink Cotton and the non-transgenic counterpart Coker 315 compared to commercial cotton varieties (Reference ranges)

Parameter	*% dry matter*			
	Non-Transgenic	*Transgenic Non-treated*	*Transgenic Treated* [a]	*Reference ranges* [b]
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	
Alanine	0.79 ± 0.09	0.76 ± 0.07	0.77 ± 0.10	0.42 - 1.51
Arginine	2.40 ± 0.31	2.28 ± 0.26	2.32 ± 0.34	1.05 - 4.40
Aspartic acid	1.92 ± 0.24	1.84 ± 0.19	1.86 ± 0.27	1.00 - 3.55
Cysteine	0.36 ± 0.04	0.35 ± 0.04	0.34 ± 0.05	0.16 - 0.86
Glutamic acid	3.96 ± 0.51	3.74 ± 0.43	3.82 ± 0.57	1.96 - 8.16
Glycine	0.85 ± 0.10	0.82 ± 0.07	0.83 ± 0.11	0.44 - 1.58
Histidine	0.57 ± 0.07	0.54 ± 0.06	0.55 ± 0.08	0.31 - 1.03
Isoleucine	0.66 ± 0.08	0.63 ± 0.06	0.65 ± 0.09	0.35 - 1.17
Leucine	1.19 ± 0.15	1.14 ± 0.11	1.16 ± 0.16	0.63 - 2.23
Lysine	0.94 ± 0.10	0.90 ± 0.07	0.91 ± 0.11	0.52 - 1.65
Methionine	0.32 ± 0.04	0.31 ± 0.03	0.30 ± 0.04	0.15 - 0.54
Phenylalanine	1.11 ± 0.14	1.06 ± 0.11	1.08 ± 0.15	0.54 – 2.03
Proline	0.74 ± 0.09	0.70 ± 0.07	0.71 ± 0.10	0.41 - 1.39
Serine	0.88 ± 0.12	0.84 ± 0.09	0.86 ± 0.12	0.50 - 1.63
Threonine	0.65 ± 0.08	0.63 ± 0.07	0.65 ± 0.08	0.34 - 1.21
Tryptophan	0.20 ± 0.02	0.19 ± 0.02	0.19 ± 0.03	0.10 - 0.49
Tyrosine	0.55 ± 0.07	0.53 ± 0.06	0.53 ± 0.07	0.32 - 1.17
Valine	0.93 ± 0.11	0.89 ± 0.08	0.91 ± 0.12	0.45 - 1.67

[a] Treated with glufosinate ammonium
[b] References: Bertrand. *et al*. 2005. ILSI, 2007. Lawhon. *et al*., 1977. OECD, 2004.

Table 29 Total Fatty Acids in fuzzy seed of TwinLink Cotton and the non-transgenic counterpart Coker 315 compared to commercial cotton varieties (Reference ranges)

Parameter	*% relative*			
	Non-Transgenic	*Transgenic Non-treated*	*Transgenic Treated* [a]	*Reference ranges* [b]
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	
Saturated				
C12:0 (lauric acid)	< 0.10	< 0.10 - 0.21	< 0.10	0 - 0.20
C14:0 (myristic)	0.66 ± 0.11	0.62 ± 0.10	0.63 ± 0.10	0.53 - 1.17
C16:0 (palmitic)	23.19 ± 0.77	23.27 ± 0.56	23.32 ± 0.62	21.1 - 29.9
C17:0 (margaric acid)	< 0.10 - 0.13	< 0.10 - 0.11	< 0.10 - 0.11	ND
C18:0 (stearic)	2.44 ± 0.08	2.52 ± 0.13	2.52 ± 0.13	2.15 - 3.4
C20:0 (arachidic)	0.27 ± 0.02	0.26 ± 0.01	0.26 ± 0.02	0 - 0.48
C22:0 (behenic)	< 0.10 - 0.22	< 0.10 - 0.21	< 0.10 - 0.17	0 - 0.27
C24:0 (lignoceric)	< 0.10 - 0.18	< 0.10 - 0.15	< 0.10	0 - 0.30
Sum Saturated	26.56 - 27.09	26.67 - 27.35	26.73 - 27.01	23.78 - 35.52
Mono-unsaturated				
C16:1 (palmitoleic)	0.46 ± 0.03	0.47 ± 0.02	0.47 ± 0.03	0.46 - 0.86
C18:1 (oleic)	14.70 ± 0.78	15.95 ± 0.40	15.95 ± 0.45	13.4 - 22.0
Sum Mono-unsaturated	15.16	16.42	16.42	13.86 - 22.86
Poly-unsaturated				
C18:2 (linoleic)	57.90 ± 1.45	56.53 ± 0.86	56.52 ± 1.01	36.3 - 64.0
C18:3 (alpha linolenic)	0.20 ± 0.03	0.20 ± 0.02	0.19 ± 0.03	< 0.10 - 0.62
Sum Poly-unsaturated	58.10	56.73	56.71	36.3 - 64.62
Sum of total fatty acids	***99.82 - 100.4***	***99.82 - 100.5***	***99.86 - 100.1***	-

ND No data available
[a] Treated with glufosinate ammonium
[b] References: Berberich *et al.* 1996 (values for Coker 312). Bertrand et al. 2005. ILSI, 2007. Nida *et al*., 1996. OECD, 2004.

I. Conclusions for agronomic evaluation of TwinLink cotton

Results across locations show that TwinLink and the conventional parent lines are similar for yield, plant morphology, and fiber quality.

Seedling vigor and plant stand counts were similar for conventional cotton and lines containing the T304-40 and GHB119 event transformations for all the trials planted in optimal conditions and where irrigation was available. Reduced germination was observed in all test plots under less than optimal conditions.

No significant differences were found when evaluating plant responses to biotic and abiotic stresses in 2007. In a minority of trials TwinLink cotton plants demonstrate a potential for increased lodging due to heavy fruit load. Differences in lodging seen in the data appear not to be a deleterious result of the transformation, but rather an indirect effect of higher fruit retention as a result of reduced lepidopteran damage.

IX. ENVIRONMENTAL SAFETY/IMPACT OF NON CONTAINED USE OF TWINLINK COTTON

A. Potential for gene transfer / out crossing

1. 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 TwinLink trait if that cross would occur.

2. 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; Van Deynze *et al.,* 2005).

Outcrossing potential to wild/weedy relatives
The potential for outcrossing can be defined as the ability of gene escape to wild cotton relatives. Previously the USDA stated in the environmental assessment document of LLCotton25 cotton that "gene flow from genetically engineered cotton into wild cotton relatives is not likely, and if it occurs, would not lead to increased weediness" (USDA, 2003). *G. tomentosum,* found only in Hawaii, is the only species capable of crossing with domesticated cotton that will produce fertile offspring. Outcrossing to *G. tomentosum* is unlikely as there is neither selective advantage nor 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.

3. Potential of horizontal gene flow from TwinLink 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 TwinLink 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, 2003). Previous findings by the USDA of similar herbicide-tolerant cotton during environmental assessment expected no change in weediness potential, and an example is glufosinate-tolerant cotton (LibertyLink), commercially sold today. The largest concern is that of volunteer plants that could become weedy in subsequent years. Volunteers are also limited by the geography in which they may exist as cotton does not survive as a perennial where freezing temperatures are reached during the winter. Volunteers can easily be controlled by crop rotation, tillage and/or pre- or post-emergence herbicides. For example, glufosinate-tolerant cotton volunteers could easily be controlled by using the herbicide glyphosate.

There is limited probability that TwinLink cotton or any *Gossypium* species containing TwinLink cotton would become a weed problem. In the comparative studies presented in this petition there were no consistent significant differences in phenotypic or plant morphological characteristics between the transgenic TwinLink cotton and the conventional counterpart line Coker that would impact plant pest or noxious weed potential. Based on these data there was no evidence to suggest that TwinLink 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

An assessment of the risk to non-target species has been performed for Twinlink cotton and for the Cry1Ab protein and Cry2Ae protein produced by TwinLink cotton. Each assessment comprised: hazard identification, exposure assessment, testing of species potentially exposed and, if necessary, dose-response evaluation for affected species.

The mode of action of Cry proteins in target organisms is well understood and is mediated by binding proteins in the gut, which have considerable inter-species variability. *Bacillus thuringiensis* (Bt) Cry proteins (including several Cry1 and Cry2 proteins) have been extensively studied in laboratory assays and field tests, and cotton and corn expressing these proteins have been grown on significant acreage in the US for several years with no reports of significant adverse effects. All these studies indicate that Cry proteins pose no unacceptable risk for any organisms except the narrowly-targeted pest species and very close relatives (USEPA, 2001). BCS has generated additional data on the *Gossypium hirsutum* transformation events T304-40 and GHB119 and the Cry1Ab and Cry2Ae proteins encoded in these events and the results are

summarized in this chapter. There is much detailed information about the specificity of Cry1Ab and Cry2 proteins to lepidopteran species (*e.g.* Hoffmann *et al.*1988). Cry1Ab and Cry2Ae are both narrowly targeted to lepidopteran insects. Whilst there is no evidence of toxicity to other non-target organisms, it is prudent to identify those organisms most likely to be exposed to cotton plants or plant material, and assess their sensitivity to the Cry proteins produced by TwinLink cotton.

The main routes of exposure to non-target organisms are:

- Grazing by herbivorous insects or other animals
- Predation on insects that have fed on Bt cotton
- Consumption of cotton seeds (birds, mammals)
- Pollen transfer
- Leaf fall
- Plowing in of senescent plants

1. Cry1Ab and Cry2Ae cotton

For most species, testing was carried out using purified protein expressed in the bacterium *E. coli*. This purified protein could be incorporated into the appropriate diet for the non-target organism, and in this way, the organisms could be treated with a dose significantly higher than the expected environmental concentration (EEC). The basis for concluding 'no risk' to a target species is the presence of evidence that there are no significant effects at the maximum expected environmental concentration (EEC).

Table 30 Summary of Non-Target Organism Data of the Cry1Ab protein

Organism	*Lifestage*	*Endpoint(s)*	*Result*
Mammal (*Mus musculus)*	Young adult	Mortality, body weight, clinical signs	NOEC[1] 2000 mg/kg
Honeybee	larva	Mortality, development, Adult emergence, behavior	NOEC 20 µg/g
Ladybug (*Coleomegilla maculata*)	larva	Mortality, development, Adult emergence, behavior	NOEC 10 µg/g
Lacewing (*Chrysoperla carnea)*	larva	mortality	NOEC 29 µg/g[3]
Springtail (*Folsomia candida*)	larva	Mortality, reproduction	NOEC 4.5 µg/g[2]
Daphnia	immature	Mortality, development, reproduction	NOEC 48 µg/g[3]

[1]NOEC = No Effect Concentration
[2]This is a conservative estimate based on ELISA analysis of diet samples. Actual exposure was probably significantly higher.
[3] Preliminary results

Table 31 Summary of Non-Target Organism Data of the Cry2Ae protein

Organism	*Lifestage*	*Endpoint(s)*	*Result*
Mammal (*Mus musculus)*	Young adult	Mortality, body weight, clinical signs	NOEC[1] 2000 mg/kg
Honeybee	larva	Mortality, development, Adult emergence, behavior	NOEC 50 µg/g
Ladybug (*Coleomegilla maculata*)	larva	Mortality, development, Adult emergence, behavior	NOEC 64 µg/g
Lacewing (*Chrysoperla carnea)*	larva	mortality	NOEC 27 µg/g[3]
Springtail (*Folsomia candida*)	larva	Mortality, reproduction	No mortality at 44 µg/g.
Earthworm	adult	Mortality	NOEC 100 mg/kg soil
Daphnia	immature	Mortality, development, reproduction	NOEC 48 µg/g[3]

[1]NOEC = No Effect Concentration
[3] Preliminary results

The conclusion from the risk assessments for the two individual proteins is that Cry1Ab and Cry2Ae pose minimal risk to any species except the target Lepidoptera and very close relatives. There was no indication of risk to birds, mammalian wildlife, aquatic organisms, soil-dwelling organisms or several species of non-target insects.

2. Protein degradation in soils

An aerobic soil degradation study using the most sensitive target insect, *Heliothis virescens*, to determine the DT_{50} (time for 50% of initial concentration of bioactive material to dissipate) was conducted to ascertain the environmental risk assessment of the individual Cry1Ab and Cry2Ae proteins produced from *Escherichia coli* and *Bacillus thuringiensis* respectively. A 30 day aerobic soil degradation study was performed with the proteins produced from bacteria in three US soils at a concentration of a) Cry1Ab 15 µg/g, which represents fifteen times the 1 µg/g concentration expressed in cotton leaves and b) Cry2Ae 50 µg/g, which represents five times the 10 µg/g concentration expressed in cotton leaves. The three US soils from Proctor (Arkansas), Senatobia (Mississippi) and East Bernard (Texas) selected for the study are from areas where transgenic cotton is grown. The insect bioassay monitored growth inhibition of larval *Heliothis virescens*. A dose response curve run concurrently with the insect bioassay was used to determine the Cry1Ab and Cry2Ae protein concentration in the soil. The DT_{50} was determined from the soil protein concentration first-order exponential regression curve. An average DT_{50} of 3.6 days for Cry1Ab and 3.4 days for Cry2Ae for all three soils was obtained from the insect assay data.

3. TwinLink cotton

A *B. thuringiensis* strain expressing more than one type of crystal protein might possibly be expected to have synergistic or additive effects on the intended target pest insect. Binding studies demonstrate that Cry1Ab and Cry2Ae bind to distinct receptor sites in the insect gut. Comparison of insect efficacy data on Cry1Ab cotton (event T304-40), Cry2Ae cotton (event GHB119), and TwinLink cotton (event T304-40 x event GHB119) indicates an additive effect of the two insect control proteins and not any synergy of those two proteins.

Since no significant effects were seen with either protein on non-target organisms, an effect from a combination of the two proteins is unlikely. However, in order to investigate whether there was an unexpected interaction, three species were selected for testing with TwinLink plant material. This approach has previously been used in the assessment of non-target effects of cotton expressing more than one insecticidal protein (USEPA 2005, 2008)

1) Birds

The possibility of intrinsic toxicity of the cotton plant to birds was addressed via a broiler feeding study using meal prepared from plant material from the TwinLink™ cotton plant, which expresses both Cry1Ab and Cry2Ae protein. The birds were fed with a diet containing 10% TwinLink cotton seed, and the two control groups were fed on diet containing either non-transgenic "near isogenic" cottonseed, or commercial cottonseed. Birds were 1 day old at start of study, and were housed 10 per pen (replicate), 14 replicates per group.

Following 42 days of daily exposure to TwinLink cottonseed meal at 10% in diet, there were no negative effects detected in feed consumption, feed conversion ratio, survival, body weight gain, or in weight of chilled carcass, leg, thigh, wing or breast between ROSS #308 broiler chickens fed with the genetically modified cottonseed, and the two control groups.

This study indicates that TwinLink cotton poses minimal risk to birds.

2) Bees

Cotton is wind pollinated, and bees are not therefore brought in to cotton for pollination purposes. However, honey bees and other wild pollinators will visit cotton on occasion, and may consume cotton nectar. Some pollen may also be brought back to the hive, where it may be fed to developing larvae. A recent meta-analysis of bee studies (Duan *et al.* 2008) indicated that Bt proteins pose minimal risk to bees. However, bee populations are the subject of intense scrutiny at the moment, due to significant unexplained declines, and the intrinsic toxicity of Cry1Ab protein and Cry2Ae protein have therefore been investigated. Neither showed any effect on bee larval development or adult emergence. The honey bee larval assay provides a useful test system for investigating any possible harmful effect of TwinLink pollen.

Pollen was collected from field-grown TwinLink cotton and the non-transgenic near-isoline, Coker 315 and shipped on dry ice to the laboratory in California for testing. Honeybee larvae were treated with 2mg of pollen and 10 µL 30% sucrose solution. Honey bee larvae were treated in brood frames selected from outdoor beehives and brought into the laboratory just prior to treatment. The test treatment consisted of 4 replicates of 20 larvae treated with TwinLink pollen, and the control treatment consisted of 4 replicates of 20 larvae treated with Coker 315 pollen. A fourth set of 4 replicates were treated with a reference substance, potassium arsenate (2,000 µg/mL) which is known to act as a stomach poison. This treatment was included to confirm exposure of the larvae.

Treated frames were returned to the hives, and checked at Day 6 for capping. This gives an overall measure of larval survival and development, since if larvae do not develop normally, the cells will not be capped by the nurse bees. At Day 13, frames were brought into the laboratory and the test area was covered with an adult emergence cage. Frames were kept in a growth chamber at 29.9 to 32.3 degrees centigrade and 56 to 74 percent for relative humidity.

Data recorded Day 6 revealed the percent survival from dosing to capping was 88.8% for larvae treated with the TwinLink pollen at 2 mg/cell and 86.3% for larvae treated with Coker 315 pollen at 2 mg/cell. Comparatively, the potassium arsenate treatments at 2,000 µg/ml had a reduced survival rate of 0.0%, none of the larvae survived to capping.

Frames were checked for adult emergence twice daily until Day 15. The percent larval survival from dosing to adult emergence was identical to the survival from dosing to capping (88.8% for TwinLink, 86.3% for Coker 315). There were no obvious effects on behavior of morphology of the emerged adults.

This data indicates that TwinLink cotton poses minimal risk to honeybees.

c) Collembola

Soil dwelling organisms could be exposed to Cry1Ab or Cry2Ae from TwinLink cotton through falling leaves or incorporation of senescent plant material. Exposure levels will be rather low, however, as both proteins break down rapidly in soil (DT_{50} for Cry1Ab is 3.6 days, DT_{50} for Cry2Ae is 3.4 days).

This study was conducted using leaf material from greenhouse-grown TwinLink cotton and a near-isoline, Coker 315. The leaves were collected and rapidly lyophilized.

The study was started with 12 day old larvae which were maintained in 4 ounce glass arenas containing a substrate of plaster of Paris and activated charcoal mixed in a ratio of 8:1 by weight. Collembola were fed a diet prepared by mixing dry Brewer's yeast (human consumption quality) with the lyophilized plant material. TwinLink cotton leaf material was added to the diet at 5, 20 and 50% of the total diet. There were four replicate arenas for the test and four control arenas treated with diet mixed with Coker 315 leaf material. Aliquots of diet were kept in the fridge, and fresh diet was added to the arenas every four days.

Percent mortality of adult organisms and the number of offspring produced during the exposure period were determined at test termination (day 28).

Results

There was no significant difference in mortality or reproduction between the larvae treated with TwinLink cotton and those treated with Coker 315 cotton.

Conclusion

TwinLink cotton leaf material had no effect on Collembola even when incorporated into the diet at 50%. This represents a much higher exposure than would normally be found in a cotton field, and indicates that TwinLink cotton does not pose any risk to Collembola.

4. Summary of assessment of effect on non-target organisms

Insects are the organisms most likely to have significant exposure to Twinlink cotton either by direct feeding on plants or pollen, or by feeding on other insects which have fed on the cotton plants. Cry proteins have been extensively studied for many years on many species of insects. Additional data was generated by Bayer CropScience on the effects of Cry1Ab, Cry2Ae and TwinLink cotton on sensitive insect species representative of insects found in cotton plants. No effects were observed on the predaceous insect *Coleomegilla maculata* (ladybug), or on the honey bee, which might be exposed to pollen from TwinLink plants.

There is also some possibility of exposure to soil-dwelling organisms to plant material. This exposure will be limited, as the Cry proteins have a very short half-life in soil. The half-lives for both Cry1Ab and Cry2Ae protein were measured in soils typical of cotton-growing regions, and the mean half-life was approximately three days for both proteins. Both proteins were, however, tested against the springtail, *Folsomia candida,* since springtails play an important role in the breakdown of plant material in the soil. Neither protein had a significant effect on this collembolan (springtail) species at environmentally relevant concentrations. An additional study using leaf material from TwinLink cotton also indicated no significant effect. Data had previously been generated indicating that Cry1Ab poses minimal risk to earthworm, and data for Cry2Ae was generated by Bayer CropScience to confirm that it too poses minimal risk.

Some exposure to birds and wildlife in cotton fields might also occur, but studies indicate that Cry1Ab or Cry2Ae are not intrinsically toxic to birds or mammals.

The risk of exposure to aquatic organisms is extremely small. There is a slight chance of exposure via pollen drifting to streams or rivers, but cotton pollen is heavy and sticky and will not drift far from the cotton field. Expression levels of the Cry proteins in pollen are low and any exposure will be minimal.

With reference to the herbicide tolerance of TwinLink cotton, 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 and Towery, 2004). 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.

It can be concluded that the TwinLink proteins pose minimal risk to non-target organisms.

D. Endangered species considerations

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

It is not anticipated that the use of TwinLink cotton will impact any current listed species of concern. Of the total 747 plants listed as endangered, 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 TwinLink 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 TwinLink cotton through sexual reproduction.

There are no reports of threatened or endangered species feeding on cotton plants; therefore, such species would not be exposed to cotton tissue containing these proteins. Cry1Ab and Cry2Ae protein is highly specific to Lepidoptera, so the only organisms which might be considered to be 'at risk' from TwinLink cotton would be endangered lepidopteran insects. While there are endangered lepidopteran species in cotton growing counties (*e.g.* Kern Primrose Sphinx moth, Saint Francis' Satyr butterfly) the larvae are highly unlikely to be exposed to Cry2Ae proteins because their habitats do not overlap with cotton fields and their larvae do not feed on cotton and will not be exposed to Cry1Ab or Cry2Ae protein in pollen. The amount of pollen that would drift from these cotton plants onto plants fed upon by endangered/threatened species, would be very small, and the expression level of both Cry1Ab and Cry2Ae in pollen is very low. It is also therefore highly unlikely that TwinLink could outcross to any wild or weedy relatives of cotton.

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

E. Effects on current agricultural practices in cotton

TwinLink cotton is an alternative cotton product that contains insect resistant and herbicide tolerant traits. Agricultural practices that are today used for both products are expected to be maintained when incorporating TwinLink to the farmer's choice of cotton products. Adoption and use of genetically modified cotton can provide positive impacts on agricultural practices. These positive impacts have been detailed in a study by Brookes and Barfoot (Brookes and Barfoot 2006) and include:

Herbicide tolerant crops

• Increased management flexibility that comes from a combination of the ease of use associated with broad-spectrum, post-emergent herbicides;

• Compared to conventional crops, where post-emergent herbicide application may result in 'knock-back' (some risk of crop damage from the herbicide), this problem is less likely to occur in GM HT crops;

• Facilitation of adoption of no/reduced tillage practices with resultant savings in time and equipment usage;
• Improved weed control has reduced harvesting costs – cleaner crops have resulted in reduced times for harvesting;
• Elimination of potential damage caused by soil-incorporated residual herbicides in follow-on crops.

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

Genetically modified herbicide tolerant cotton was first grown commercially in the US in 1997 and by 2006, was planted on 65% of the total cotton plantings. Genetically modified insect-resistant cotton has been grown commercially in the US since 1996 and by 2006, was used in 57% (3.5 million ha) of total cotton plantings (Brookes and Barfoot 2006). Table 32 describes percentages of the total cotton planted and their composition based on trait in 2007.

Table 32 Genetically engineered (GE) upland cotton varieties by State and United States, 2000-2008

	Insect-resistant (Bt) only									*Herbicide-tolerant only*								
State	*2000*	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*	*2008*	*2000*	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*	*2008*
	Percent of all upland cotton planted									*Percent of all upland cotton planted*								
AL 1/						10	10	10	18						28	25	25	15
AR	33	21	27	24	34	42	28	32	30	23	29	37	25	15	12	21	16	4
CA	3	11	6	9	6	8	9	4	7	17	27	26	27	39	40	40	51	45
GA	18	13	8	14	13	29	19	17	19	32	43	55	32	23	11	13	10	5
LA	37	30	27	30	26	21	13	17	19	13	14	9	15	7	10	13	11	6
MS	29	10	19	15	16	14	7	16	19	13	15	22	16	23	23	22	19	13
MO 1/						20	32	13	12						59	40	63	68
NC	11	9	14	16	18	17	19	13	19	29	37	27	29	27	24	19	16	14
TN 1/						13	16	10	10						8	10	17	14
TX	7	8	7	8	10	14	18	16	16	33	35	40	39	40	35	34	36	31
Other	17	18	19	18	22	18	21	27	22	21	33	35	32	24	26	24	20	20
U.S.	15	13	13	14	16	18	18	17	18	26	32	36	32	30	27	26	28	23
	Stacked gene varieties									*All GE varieties*								
State	*2000*	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*	*2008*	*2000*	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*	*2008*
	Percent of all upland cotton planted									*Percent of all upland cotton planted*								
AL 1/						54	60	60	65						92	95	95	98
AR	14	28	26	46	45	42	45	47	64	70	78	90	95	94	96	94	95	98
CA	4	2	1	3	7	5	8	6	8	24	40	33	39	52	53	57	61	60
GA	32	29	30	47	58	55	64	68	73	82	85	93	93	94	95	96	95	97
LA	30	47	49	46	60	64	68	68	73	80	91	85	91	93	95	94	96	98
MS	36	61	47	61	58	59	69	62	66	78	86	88	92	97	96	98	97	98
MO 1/						16	25	23	19						95	97	99	99
NC	36	38	45	48	46	54	60	64	62	76	84	86	93	91	95	98	93	95
TN 1/						75	67	71	73						96	93	98	97
TX	6	6	4	6	8	14	18	28	31	46	49	51	53	58	63	70	80	78
Other	36	33	32	38	45	46	45	42	48	74	84	86	88	91	88	90	89	90
U.S.	20	24	22	27	30	34	39	42	45	61	69	71	73	76	79	83	87	86

1/ Estimates published individually beginning in 2005. Source : 2007-2008: U.S. Dept. of Agriculture, National Agricultural Statistics Service (NASS), Acreage. June 30, 2008.

1. Insect resistant cotton

As described in Section I of this petition, TwinLink cotton is an alternative for control of common cotton lepidopteran pests. Heliothine pests (budworm/bollworm) infested about 83 percent of the U.S. cotton crop in 2005, second only to thrips, which were found in 92 percent of the U.S. crop. Heliothine damage to the crop resulted in the loss of 520,000 bales of cotton in the United States.

Oklahoma reported the largest loss to insects, 13.3 percent, representing 63,900 bales of cotton. Arizona was second with 11.98 percent loss, followed by South Carolina, 8 percent, Alabama, 7.2 percent, North Carolina, 6.8 percent, California, 6.6 percent and Virginia, 5.6 percent. All other states reported a less than 5 percent loss.

The largest number of bales lost by state was in Texas, 392,800 bales, followed by Mississippi, 168,000 bales, California, 160,000 bales, and Arkansas, 132,000 bales. The biggest cost plus loss state was not surprising, Texas at $222 million, followed by Arkansas, $165 million, Mississippi, $162 million, and Georgia, $102 million.

The bollworm/budworm complex retained its position as the nation's No. 1 pest in cotton production in 2007, reducing yields by 0.91 percent, a slight increase over 2006, according to Williams, 2008. Total losses from all insect pests in 2007 were 3.61 percent. According to the report, losses below 5 percent "continue to reflect the outstanding contribution technology has made to managing pest complexes which long have plagued cotton producers."

Bollworms were the dominant species (92 percent) of the heliothine complex in 2007. Heliothine damages resulted in the loss of 229,000 bales of cotton. South Carolina (4.5 percent) and Alabama (2.88 percent) reported the highest loss to heliothines, with Georgia (1.58 percent), Florida (1.28 percent) and North Carolina (1.04 percent) rounding out the top five. Williams (2008) reported that Texas, which lost 78,000 bales, was the only state to report losses greater than 50,000 bales.

Bt cotton acreage decreased in 2007 to 7.1 million acres, although this was more reflective of the overall decrease in cotton acres, according to the report. Bt cotton comprised 65 percent of total acreage. Heliothine were sprayed with insecticide on 2.21 million Bt cotton acres in 2007. Foliar insecticide applications represent 47 percent of the cost of insect management in cotton.

2. Glufosinate-ammonium tolerant cotton

A detailed evaluation of glufosinate ammonium tolerant cotton was submitted in the LLCotton25 USDA Petition for determination of non-regulated status. The same arguments are applicable for the evaluation of TwinLink cotton. USDA responded in their Environmental Assessment and Finding of No Significant Impact (2003).

Glufosinate-ammonium may positively impact current agronomic practices in cotton by 1) offering broad spectrum, post-emergence weed control with a wide application window, which allows treatment only when weeds reach economical thresholds; 2) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 3) providing a new herbicidal mode of action that allows for improved weed shift and resistance management; 4) decreasing cultivation needs; 5) allowing the application of less total pounds of active ingredient than used presently, and, 6) providing a more profitable and sustainable cotton system for cotton growers.

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°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.

The total acreage planted with upland cotton in the US is shown in Table 33, 97% of these acres received herbicide application for weed control (USDA, NASS 2008). Many acres are often treated multiple times using herbicide tolerant cotton during pre-plant burndown and at least one application post-emergence.

Table 33 Agricultural Chemical Usage 2007 - Cotton Summary

State	*Planted Acreage*	*Herbicide*	*Herbicide*	*Insecticide 1/*	*Insecticide 1/*
	1000 acres	*Percent*	*1000 lbs.*	*Percent*	*1000 lbs.*
AL	400	98	941	55	88
AR	860	97	2399	92	1092
CA	455	90	565	90	506
GA	1030	100	3163	85	956
LA	335	98	992	99	562
MS	660	100	2132	97	1231
MO	380	100	995	83	270
NC	500	100	1479	79	300
SC	180	100	535	92	85
TN	515	100	1482	94	228
TX	4925	96	11532	43	2624
Total	10240	97	26214	66	7943

Released May 21, 2008, by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, U.S. Department of Agriculture

1/ Total Applied excludes Bt's (*Bacillus thuringiensis*) and other biologicals.

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 34 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).

Conventional methods of weed 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 use 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 glufosinate ammonium) 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
TwinLink 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 monocotyledonous crops such as corn in rotational systems. Additionally, other herbicides, such as glyphosate (RoundUp®) 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 glufosinate 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.

There are no reported glufosinate-tolerant weeds.

The Herbicide Glufosinate-ammonium and Current Uses
Glufosinate ammonium is currently registered under various trade names for control of weeds in cotton and other crops, and as Ignite®/Liberty® for use on LibertyLink® corn, cotton, soy, rice and canola varieties. It acts by inhibiting the enzyme glutamine synthase which causes a toxic buildup of ammonia within the weed. Glufosinate-ammonium is a nonselective herbicide for both non-crop and crop uses. It is highly biodegradable, has no residual activity, and has very low toxicity for humans. Glufosinate-ammonium is an ecologically sound herbicide that degrades rapidly in microbially active soils and also readily binds to soil particles. Glufosinate-ammonium poses less risk of adverse effects of drift to non-target areas than current market standards. Glufosinate-ammonium and its short-lived metabolites have not been found to accumulate in the environment. Soil microorganisms, bees, earthworms, birds and mammals are unaffected by glufosinate-ammonium. This herbicide has a different mode of action than the other major herbicides used in cotton that it is intended to replace or supplement, and unlike the other herbicides, there are no weed biotypes with resistance reported to this class of herbicide in an international survey of herbicide resistant weeds (Heap, 2002). Glufosinate ammonium is registered by EPA for use in cotton.

Table 35 Important Weeds Labeled for Control by Ignite® Herbicide in Cotton

Provided are the common and scientific names.

Grass weeds	
Barnyardgrass	*Echinochloa crus-galli*
Crabgrass, large	*Digitaria sangulinalis*
Johnsongrass	*Sorghum halepense*
Texas Panicum	*Panicum texanum*
Watergrass	*Echinochloa oryzoides*
Broadleaf weeds	
Pigweed species	*Amaranthus spp.*
Sicklepod	*Cassia obtusifolia*
Cocklebur, common	*Xanthium strumarium*
Prickly Sida	*Sida spinosa*
Black nightshade	*Solanum nigrum*
Morning glory species	*Ipomoea spp.*
Sunflower	*Helanthus annuus*

TwinLink Cotton and Glufosinate-ammonium may positively impact current agronomic practices in cotton by 1) offering broad spectrum, post-emergence weed control with a wide application window, which allows treatment only when weeds reach economical thresholds; 2) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 3) providing an effective herbicidal mode of action that allows for improved weed shift and resistance management; 4) decreasing cultivation needs; 5) allowing the application of less total pounds of active ingredient than used presently, and, 6) providing a more profitable and sustainable cotton system for cotton growers.

F. Summary of Environmental Safety / Impact on non-contained use of TwinLink cotton

TwinLink cotton was evaluated for agronomic impacts during seed germination and dormancy studies, protein safety assessment, composition analysis, and agronomic performance evaluation. These assessments of TwinLink cotton and the Cry1Ab, Cry2Ae and PAT proteins 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 TwinLink cotton 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 TwinLink cotton. The limited range of movement of cotton pollen described in Section II coupled with the low acute oral toxicity of the three expressed proteins 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. Therefore the agronomic consequences of introduction of TwinLink 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 TwinLink cotton 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.

G. Insect Resistance Management (IRM) Plan

A comprehensive insect resistance management program is described in the EPA Section 3 Registration application for TwinLink Cotton. The IRM program provides a full characterization of the TwinLink product, demonstrating that it expresses a combination of two Bt proteins, Cry1Ab and Cry2Ae, at a high dose level for all major target pests. Binding data confirm the literature results that these two proteins bind to unique binding sites in the insect midgut. Computer modeling results show that TwinLink cotton will have long-term durability in the marketplace. A plan for monitoring of insect susceptibility is also provided.

X. STATEMENT OF GROUNDS UNFAVORABLE

Data generated from agronomic tests and molecular characterization indicate that no unfavorable grounds are associated with TwinLink cotton.

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

XI. REFERENCES

Amann M. M. 1999. Identification of Nutrients and Anti-nutrients to Analyze in Cotton-derived Foods for Human and Animal Consumption. Environ Arlington, VA, USA.

Aronson, A.I. and Shai, Y. 2001. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. FEMS Microbiol. Lett. 195:1-8.

Arumuganathan K., Earle E.D. 1991. Nuclear DNA Content of Some Important Plant Species. Plant Molecular Biology Reporter, 9 (3), 208-218.

Batista R., Nunes B., Carmo M., Cardoso C., Jose H.S., de Almeida A.B., Manique A., Bento L., Ricardo C.P., Oliveira M.M. 2005. Lack of detectable allergenicity of transgenic maize and soya samples. J. Allergy Clin. Immunol. 116:403-410.

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

Berger L.A., Vaissière B.E., Moffett J.O., Merritt, S.J. 1988. Bombus spp. (Hymenoptera: Apidae) as pollinators of male-sterile Upland cotton on the Texas High Plains. Environ. Entomol. 17. 789-794.

Bertrand, J.A., Sudduth T.Q., Condon A., Jenkins T.C., Calhoun C. 2005. Nutrient Content of Whole cottonseed. J. Dairy Sci. 88:1470-1477.

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

Boevink P., Chu P.W.G., Keese P. (1995). Sequence of subterranean clover stunt virus DNA: Affinities with the geminiviruses. Virology 207: 354-361.

Bolivar, F, Rodrigues, R.L, Greene, P.J, Betlach, M.C., Haymeker, H.L., Boyer, H.W., Crosa J., and Talkow, S. 1977. Construction and Characterization of New Cloning Vehicles. II. A Multipurpose Cloning System. *Gene* 2:95-113.

Bradford, M.M. 1976. A refined and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248.

Bradshaw R.A., Brickey W.W., Walker K.W. 1998. N-terminal processing: the methionine aminopeptidase and N-acetyl transferase families. Trends Biochem. Sci. 23: 263-267.

Brake J., Faust M.A., and Stein J. 2003. Evaluation of transgenic event Bt11 hybrid corn in broiler chickens. Poult. Sci. 82:551-559.

Brookes G, Barfoot P. 2008. GM crops: global socio-economic and environmental impacts 1996-2006. PG Economics Ltd, UK. 118 pages.

Bryson, C.T., Salisbury, C., and McCloskey, W.B. 1999. Weeds and their control. In Cotton: Origin, History, Technology, and Production. C.W. Smith and J. Cothern, (eds.). John Wiley & Sons, Inc., New York: 617-658.

Calhoun M.C., Kuhlmann S.W., Baldwin B.C. 1995. cotton feed composition and gossypol availability and toxicity. In Proc. 2nd National Alternative Feeds Symp., Wooster, OH, Sept. 24-26, 1995. 125-145.

Canadian Food Inspection Agency (CFIA). 2004. Decision Document DD2003-45 - Determination of the safety of Monsanto's insect resistant Bollgard II™ cotton (*Gossypium hirsutum* L.). CFIA, Plant Products Directorate, Plant Biosafety Office. June 6, 2004.

Carpenter, J.E., and L.P. Gianessi. 2001. Agricultural Biotechnology: Updated benefit estimates. National Center for Food and Agricultural Policy. http://www.ncfap.org/pesticid1.htm

Carrière, Y. and T.J. Dennehy, B. Pedersen, S. Haller, C. Ellers-Kirk, L. Antilla, Y.B. Liu, E. Willott, B. Tabashnik. 2001. Large-scale management of insect resistance to transgenic cotton in Arizona: can transgenic insecticidal crops be sustained? Journal of Economic Entomology 94(2): 315-325.

Chen X.J., Curtiss A., Alcantara E., Dean D.H. 1995. Mutations in domain I of *Bacillus thuringiensis* delta-endotoxin Cry1Ab reduce the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles. J. Biol. Chem. 270:6412-6419.

Cornelissen M., Vandewiele M. 1989. Nuclear transcriptional activity of the tobacco plasmid psbA promoter. *Nucleic Acids Research*. 17 (1) 19-29.

Crockett L. 1977. Wildly Successful Plants: North American Weeds. University of Hawaii Press, Honolulu, Hawaii.

De Almeida E.R.P., Gosselé V., Muller C.G., Dockx J., Reynaerts A., Botterman J, Krebbers E., Timko M.P. 1989. Transgenic expression of two marker genes under the control of an Arabidopsis rbcs promoter: sequence encoding the Rubisco transit peptide increase expression levels. Molec. Gen. Genet. 218:78-86.

Deblaere R., Bytebier B., De Greve H., Deboeck F., Schell J., Van Montagu M., Leemans J. 1985. Efficient octopine Ti-plasmid derived vectors for Agrobacterium mediated gene transfer to plants. *Nucleic Acid Research*. 13. 4777-4788.

Delaney B., Astwood J.D., Cunny H., Conn R.E., Herouet-Guicheney C., Macintosh S., Meyer L.S., Privalle L., Gao Y., Mattsson J., Levine, M. 2008. Evaluation of protein safety in the context of agricultural biotechnology. Food Chem. Toxicol. 46 Suppl 2:S71-97.

Depicker A., Stachel S., Dhaese P., Zambryski P., Goodman H.M. 1982. Nopaline Synthase: transcript mapping and DNA sequence. J. Molec. Appl. Genet. 1:561-573.

Devore, J., Peck, R. 1986. Statistics. West Publishing Company, St. Paul.

D'Halluin, K., De Block, M., Denecke, J., Janssens, J., Leemans, J., Reynaerts, A., Botterman, J. 1992. The bar Gene as Selectable and Screenable Marker in Plant Engineering. Methods in Enzymology, 216, 415-426.

Duan, J.J., Marvier, M., Huesing, J., Dively, G., Huang, Z.Y. 2008. A Meta-Analysis of Effects of Bt Crops on Honey Bees (Hymenoptera: Apidae). PLoSONE 3(1): e1415. http://www.plosone.org/article/info:doi/10.1371/journal.pone.0001415

Endrizzi, J.E., Turcotte, E.I. and Kohel, R.J. 1984. cotton, Agronomy No. 24, p 82-129, Soil Science Society of America, Inc. (Kohel, R.J. and C.F. Lewis, eds) Wisconsin, USA.

EPA- see United States Environmental Protection Agency.

Fawcett, R., Towery, D. 2004. Conservation Tillage and Plant Biotechnology: How New Technologies Can Improve the Environment By Reducing the Need to Plow. Conservation Technology Information Center. 1220 Potter Drive, Suite 170, West Lafayette, IN 47906. www.ctic.purdue.edu.

FAO/WHO Food Standards. 2001. Named Vegetable Oils. Codex Stan 210 from Codex Alimentarius vol. 8. http://codexalimentarius.net/standard-list accessed December 5, 2002.

Ferrell, J.A., G.E. MacDonald, and B.J. Brecke. 2006. Weed management in cotton. Available at http://edis.ifas.ufl.edu/WG003. Univ. of Florida IFAS, Gainesville.

Fling M.E., Kopf J., Richards C. 1985. Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3"(9)-O-nucleotidyltransferase. Nucleic Acids Research. 13(19): 7095-7106.

Fryxell P.A. 1979. The natural history of the cotton tribe (*Malvaceae*, tribe *Gossypieae*), Texas A&M University Press. College Station and London.

Fryxell PA. 1992. A revised taxonomic interpretation of *Gossypium* L. (Malvaceae). Rheedea 2: 108-165.

Gianessi L.P., Silvers C.S., Sankula S., Carpenter J.E. 2002. Plant Biotechnology: Current and Potential Impact For Improving Pest Management In US Agriculture. An Analysis of 40 Case Studies. Herbicide Tolerant Cotton. National Center for Food and Agricultural Policy. 1616 P Street, NW. Washington, DC 20036. 61 pages.

Griffitts J.S., Haslam S.M., Yang T., Garczynski S.F., Mulloy B., Morris H., Cremer P.S., Dell A., Adang M.J., Aroian R.V. 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science. 307:922-925.

Hajdukiewicz P., Svab Z., Maliga P. 1994. The small, versatile *pPZP* family of *Agrobacteium binary* vectors for plant transformation. Plant Molecular Biology. 25:989-994.

Hamilton K.A., Pyla P.D., Breeze M., Olson T., Li M., Robinson E., Gallagher S.P., Sorbet R., Chen Y. 2004. Bollgard II cotton: compositional analysis and feeding studies of cottonseed from insect-protected cotton (*Gossypium hirsutum L.*) producing the Cry1Ac and Cry2Ab2 proteins. J. Agric. Food Chem. 52:6969-6976.

Hammond B.G., Dudek R., Lemen J.K. and Nemeth M.A. 2006. Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. Food Chem. Toxicol. 44:1092-1099.

Harpster M.H., Townsend J.A., Jones J.D.G., Bedbrook J., Dunsmuir P. 1988. Relative strengths of the 35S Cauliflower Mosaic Virus, 1', 2' and nopaline synthase promoters in transformed tobacco, sugarbeet and oilseed rape callus tissue. Molec. Gen Genet. 212:182-190.

Herouet C., Esdaile D.J., Mallyon B.A., Debruyne E., Schulz A., Currier T., Hendrickx K., van der Klis R.J., Rouan D. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the pat and bar sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regul Toxicol Pharmacol. 41:134-149.

Höfte H., de Greve H., Seurinck J., Jansens S., Mahillon J., Ampe C., Vandekerckhove J., Vanderbruggen H., van Montagu M., Zabeau M.,Vaeck M. 1986. Structural and functional analysis of a cloned delta endotoxin of Bacillus thuringiensis berliner 1715. Eur. J. Biochem. 161:273-280.

Hofmann, C., Vanderbruggen, H. V., Höfte, H., Van Rie, J., Jansens, S., and Van Mellaert, H. 1988a. Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA 85:7844-7848

Holm L., Pancho J.V., Herbarger J.P., Plucknett D.L. 1979. A geographical Atlas of World Weeds. John Wiley and Sons, NY. 391 pp.

ILSI. 2007. ILSI Crop Composition database. Accessed March 13, 2007. http://www.cropcomposition.org/cgi-perl/search_ora.cgi.

Itoh Y., Watson J.M., Haas D., Leisinger T. 1984. Genetic and molecular characterization of the Pseudomonas plasmid pVS1. Plasmid, 11, 206.

Jenkins J.N. 1993. Cotton. *In* Traditional crop breeding practices: an historical review to serve as a baseline for assessing the role of modern biotechnology. OECD.

Jenkins N., Parekh R.B., James D.C. 1996. Getting the glycosylation right: implications for the biotechnology industry. Nat. Biotechnol. 14:975-981.

Johnson, S.R., Strom, S., Grillo, K. 2007. Quantification of the Impacts on US Agriculture of Biotechnology-Derived Crops Planted in 2006. National Center for Food and Agricultural Policy, accessed at: http://ncfap.org/index.html

Kareiva P., Morris W., Jacobi C. 1994. Studying and managing the risk of cross-fertilization between transgenic crops and their wild relatives. Molec. Ecology 3. 15-21.

Knowles B.H., Dow J.A.T. 1993. The crystal *∂*-endotoxins of *Bacillus thuringiensis*: Models for their mechanism of action on the insect gut. BioEssays. 469-476.

Kützner H.J. 1981. The family Streptomycetaceae (Chapter 156). *In*: The prokaryotes: a handbook on habitats, isolation and identification of bacteria. Starr M.P., Stolp H., Trüper H.G., Ballows A., Schlegel H.G., Eds. Pages 2028-2090.

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

Lundquist R. 1995. Current Uses of Traditional Co-Products. Wooster, OH: September 24-26, Proceedings of the 2nd National Alternative Feeds Symposium. 95-104.

Marshall J.S., Stubbs,J.D., Taylor W.C. 1996. Two genes encode highly similar choroplastic NADP-malic enzymes in Flaveria. Plant Physiol. 111:1251-1261.

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

McGregor, S.E. 1976. Insect Pollination of Cultivated Crop Plants, Agricultural Handbook No. 496, Unites States Department of Agriculture Research Service, Washington, DC.

Meredith W.R., Bridge R.R. 1973. Natural crossing in cotton *Gossypium hirsutum* L. in the delta of Mississippi. Crop Sci. 13. 551-552.

Michiels F., Morioka S., Scheirlinck T., Komari T. 1992. Stamen-specific promoters from rice. Patent Application WO92/13956A1 (20AUG-1992). PLANT GENETIC SYSTEMS N.V. (BE).

Moffett J.O., Stith L.S., Burkhart C.C., Shipman, C.W. 1976. Honey bee visits to cotton flowers. Environ. Entomol. 4. 203-206.

Muenscher W.C. 1980. Weeds. Second Edition. Cornell University Press, Ithaca and London.

Nida D.L., Patzer S., Harvey P., Stipanovic R., Wood R., Fuchs, R.L. 1996. Glyphosate-tolerant cotton: the composition of the cottonseed is equivalent to that of conventional cottonseed. J. Agric. Food Chem. 44. 1967-1974.

Niles, G.A. and Feaster, C.V. 1984. Cotton, Agronomy No. 24, p 205, Soil Science Society of America, Inc., (Kohel, R.J. and C.F. Lewis, eds) Wisconsin, USA

Odell J.T., Nagy F., Chua N.-H. 1985. Identification of DNA sequences required for activity of the Cauliflower Mosaic Virus 35S promoter. Nature. 313:810-812.

Ogiwara K., Indrasith L.S., Asano S., Hori H. 1992. Processing of delta-endotoxin from *Bacillus thuringiensis* subsp. kurstaki HD-1 and HD-73 by gut juices of various insect larvae. J. Invertebr. Pathol. 60:121-126.

Oka A., Sugisaki H., Takanami M. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. Journal of Molecular Biology, 147, 217-226.

Organization for Economic Co-operation and Development (OECD). 2004. Consensus Document on Compositional Considerations for New Varieties of cotton (Gossypium hirsutum and Gossypium barbadense): Key Food and Feed Nutrients and Anti-Nutrients. ENV/JM/FOOD(2004)16. 18-Aug-2004.

Organization for Economic Co-operation and Development (OECD). 2007. Consensus document on safety information on transgenic plants expressing *Bacillus thuringiensis*-derived insect control protein. 109 pages.

Phelps R.A., Shenstone F.S., Kemmerer R.J., Evans, R.J. 1965. A review of cyclopropenoid compounds: biological effects of some derivatives. Poult. Sci. 44. 358-394.

Rosenquist H., Smidt L., Andersen S.R., Jensen G.B., Wilcks A. 2005. Occurrence and significance of Bacillus cereus and Bacillus thuringiensis in ready-to-eat food. FEMS Microbiol. Lett. 250:129-136.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanfaçon H., Brodman P., Hohn T. 1991. A dissection of the Cauliflower Mosaic Virus polyadenylation signal. Genes and Development. 5:141-149.

Sankula S., Marmon G., Blumenthal E. 2005. Biotechnology-Derived Crops Planted in 2004 - Impacts on US Agriculture. National Center for Food and Agricultural Policy. 1616 P-street, NW. Washington, DC 20036. www.ncfap.org.

Schnepf H.E., Whiteley H.R. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 78:2893-2897.

Schroder M., Poulsen M., Wilcks A., Kroghsbo S., Miller A., Frenzel T., Danier J., Rychlik M., Emami K., Gatehouse A., Shu Q., Engel K.H., Altosaar I., Knudsen I. 2007. A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. Food Chem. Toxicol. 45:339-349.

Sedmak J.J., Grossberg S. E. 1977. A rapid, sensitive and versatile assay for protein using Coomassic brilliant blue G250. Anal. Biochem. 79, 544.

Thompson C., Van Montagu M., Leemans J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO Journal. 6(9). 2513-2518.

Tojo A., Aizawa K. 1983. Dissolution and degradation of Bacillus thuringiensis delta-endotoxin by gut juice protease of the silkworm Bombyx mori. Appl. Environ. Microbiol. 45:576-580.

USCA. 1982. United States – Canadian Tables of Feed Composition. 1982. 3rd Revision. National Academy Press, Washington D.C.

USDA. 2003. Aventis CropScience USA LP Petition 02-042-01p to USDA/APHIS Seeking a Determination of Non-regulated Status for Glufosinate-ammonium Herbicide-tolerant Cotton Transformation Event LLCotton25. Environmental Assessment and Finding of No Significant Impact.

USDA-FAS. 2006. Cotton: world markets and trade. United States Department of Agriculture. Foreign Agricultural Service. Circular Series FOP 9-06. September 2006. 26 pages

USDA-NASS. http://www.nass.usda.gov/QuickStats/index2.jsp#top.

United States Environmental Protection Agency (USEPA,) (2001). Biopesticides Registration Action Document (BRAD) – *Bacillus thuringiensis* Plant-Incorporated Protectants, October 15 2001. U.S. Environmental Protection Agency, Washington, D.C. http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm

United States Environmental Protection Agency (USEPA). 2002. Biopesticides Registration Action Document: *Bacillus thuringiensis* Cry2Ab2 protein and its genetic material necessary for its production in cotton. 74 pages.

United States Environmental Protection Agency (USEPA). 2005. Bt Cry1F/Cry1Ac WideStrike® Cotton Registration Action Document. http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006512-006513.pdf

United States Environmental Protection Agency (USEPA). 2007. Biopesticide active ingredients and products containing them. 39 pages.

United States Environmental Protection Agency (USEPA) 2008 Bacillus thuringiensis modified Cry1Ab (SYN-IR67B-1) and Vip3Aa19 (SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006529.pdf

United States Food and Drug Administration (US-FDA). 1992. Statement of Policy: Foods Derived From New Plant Varieties. http://www.cfsan.fda.gov/~lrd/biotechm.html#reg

United States Food and Drug Administration (US-FDA). 2002. Biotechnology Consultation: BNF No. 000074. Insect-Protected Bollgard II Cotton Line 15985. July 16, 2002. 7 pages.

United States Food and Drug Administration (US-FDA). 2003. Biotechnology Consultation: Note to the File BFN No. 000086. Glufosinate Tolerant Cotton Transformation Event LLCotton25. June 05, 2003. http://www.cfsan.fda.gov/~rdb/bnfm086.html (Accessed on November 11, 2008). 6 pages.

Vaissière B. 1990. Pollen dispersal and carryover in Upland cotton, Gossypium hirsutum L. Study report for the Department of Entomology, Texas A & M University, College Station, TX. USA.

Van Deynze A.E., Sundstrom F.J., Bradford K.J. 2005. Pollen-mediated gene flow in California cotton depends on pollinator activity. Crop Science, 45: 1565-1570.

Van Rie J., Jansens S., Hofte H., Degheele D., Van Mellaert H. 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. 186:239-247.

Verdaguer B., De Kochko A., Beachy R.N., Fauquet C. 1996. Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter. Plant Mol. Biol. 31:1129-1139.

Wang Z.-H., Wang Y., Cui H.-R., Xia Y.-W., Altosaar I., and Shu Q.-Y. 2002. Toxicological evaluation of transgenic rice flour with a synthetic *cry1Ab* gene from *Bacillus thuringiensis*. J. Science Food Agric. 82:738-744.

Whalon M.E., Wingerd B.A. 2003. Bt: mode of action and use. Arch. Insect Biochem. Physiol. 54:200-211.

Williams, M.R. 2008. Cotton Loss Estimates, 2007, presented at the 2008 Beltwide Cotton Conferences. January 8-11, 2008. Nashville, TN

Wozenski J., Woodburn, M. 1975. Phytic acid (myoinositol hexaphosphate) and phytase activity in four cottonseed protein products. Cereal Chemistry 52. 665-669.

Xin Z.Y., Brettell R.I.S., Cheng Z.M., Waterhause P.M., Appels R., Banks P.M., Zhou G.H., Chen X., Larkin P.J. 1988. Characterization of a potential source of barley yellow dwarf virus resistance for wheat. Genome. 30: 250-257.

Zambryski P. 1988. Basic processes underlying Agrobacterium-mediated DNA transfer to plant cells. Ann. Rev. Genet. 22: 1-30.

Zhu J., Oger P.M., Schrammeijer B., Hooykaas P.J.J., Farrand S.K., Winans S.C. 2000. The Bases of Crown Gall Tumorigenesis. Journal of Bacteriology. 182 (14):3885-3895.

Appendix 1. FIELD TRIALS TERMINATION REPORTS 2005-2007

Bayer CropScience

Termination Report 1 05-035-12n

USDA Field Termination Report

USDA Notification Number: 05-035-12n

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

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

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

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

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Oktibbeha Co., MS	0.98	6/16/05	11/2/05	40 ft. isolation buffer

Purpose of Release: This trial was established to evaluate the performance of Plant Incorporated Protectant traits in various cotton varieties.

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

Plant Disposition: Plants were harvested on 11/2/05 to evaluate yield and were then destroyed in field. No seed was retained after the completion of the trial, and cooperator indicated that all seed provided was utilized in the creation of the plots.

Volunteer Monitoring: Volunteer monitoring has been discontinued due to the replant of regulated cotton in the same trial area on 5/19/06.

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 with lacewings, assassin bugs, lady beetles, and various coleopteran species observed.

Weather Synopsis:	Cooperator reported that the growing season had above average moisture, with above average temperatures on average.
Containment Measures:	Plot was contained utilizing a 40 ft. isolation border which encompassed this field site.

Bayer CropScience

Termination Report 2 07-059-104n

USDA Field Termination Report

USDA Notification Number: 07-059-104n

Applicant Internal Number: IR-7J-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Lines Planted: GEM1

Regulated Article: *Gossypium hirsutum*; cotton

Section I. Site Release Information:

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

County / State	Acreage Planted	Date Planted	Date Terminated
1 St. Landry / LA (1)	1.28	6/24/07	11/13/2007
2 St. Landry / LA (2)	1.28	6/24/07	11/13/2007
3 Tate / MS (1)	0.72	5/25/07	10/9/2007
4 Tate / MS (2)	0.80	5/16/07	11/02/2007
5 Hockley / TX (1)	0.96	6/4/07	11/15/2007
6 Hockley / TX (2)	0.96	6/5/07	11/29/2007
7 Uvalde / TX (1)	0.81	5/7/07	10/25/2007
8 Uvalde / TX (2)	2.40	5/7/07	10/26/2007
9 Wharton / TX (1)	1.10	6/5/07	11/1/2007
10 Wharton / TX (2)	3.47	6/8/07	11/16/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. St. Landry Co., LA (1) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

2. St. Landry Co., LA (2) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

3. Tate Co., MS (1) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

4. Tate Co., MS (2) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.

e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

5. Hockley Co., TX (1) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

6. Hockley Co., TX (2) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

7. Uvalde Co., TX (1) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

8. Uvalde Co., TX (2) :
   a) Cooperator noted no unusual plant growth characteristics.

b) No observed differences in plant disease.
c) No unusual differences in insect pest or beneficial insect's populations.
d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

9. Wharton Co., TX (1) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

10. Wharton Co., TX (2) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 3 07-044-102n

USDA Field Termination Report

USDA Notification Number: 07-044-102n

Applicant Internal Number: IR-7D-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum*; cotton

Lines: GEM1

Section I. Site Release Information:

Trials were conducted at eleven (11) locations:

County / State	Acreage Planted	Date Planted	Date Terminated
1 Franklin / LA	0.293	6/7/2007	11/16/2007
2 Pinal / AZ	0.363	5/31/2007	12/19/2007
3 Hidalgo / TX	0..333	4/17/2007	8/30/2007
4 Oktibbeha / MS	0.263	5/23/2007	11/5/2007
5 Madison / LA	1.50	5/12/07	11/5/2007
6 Washington / MS	2.10	5/17/07 & 5/29/07	11/5/2007
7 Halifax / NC	0.67	5/20/07 & 6/14/07	6/12/07 & 11/15/07
8 Dillon / SC	0.67	5/22/07	10/8/2007
9 Lubbock / TX	0.49	5/26/07	11/13/2007
10 Uvalde / TX	0.95	5/7/07	10/25/2007
11 Wharton / TX	1.02	5/16/07	11/2/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics

f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Franklin Co., LA
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

2. Pinal Co., AZ :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

3. Hidalgo Co., TX :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

4. Oktibbeeha Co., MS :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.

c) No unusual differences in insect pest or beneficial insect's populations.
d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

5. Madison Co., LA :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

6. Washington Co., MS :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

7. Halifax Co., NC replant :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.

g) This test was destroyed by an overspray of roundup on 6/12/2007 and replanted on 6/14/2007 the subsequent test was planted to late for reliable yield data so it was terminated without harvesting on 11/15/2007. Trial was successfully conducted without any environmental releases.

8. Dillon Co., SC :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

9. Lubbock Co., TX :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

10. Uvalde Co., TX :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

11. Wharton Co., TX :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.

d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 4 07-044-103n

USDA Field Termination Report

USDA Notification Number: 07-044-103n

Applicant Internal Number: IR-7E-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum;* cotton

Lines: GEM2

Section I. Site Release Information:

Trials were conducted at seven (7) locations:

County / State	Acreage Planted	Date Planted	Date Terminated
1 Franklin / LA	0.293	6/7/2007	11/16/2007
2 Pinal / AZ	0.363	5/31/2007	12/19/2007
3 Hidalgo / TX	0.333	4/17/2007	8/30/2007
4 Oktibbeha / MS	0.263	5/23/2007	11/5/2007
5 Washington / MS (1)	0.10	5/17/07 & 5/29/07	11/5/2007
6 Washington / MS (2)	0.94	6/15/2007	11/01/2007
7 Lubbock / TX	0.17	5/26/07	11/13/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Franklin Co., LA
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
    f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
    g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

2. Pinal Co., AZ :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
    f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
    g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

3. Hidalgo Co., TX :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
    f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
    g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

4. Oktibbeeha Co., MS :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.

f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

5. Washington Co., MS 1 :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

6. Washington Co., MS 2:
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

7. Lubbock Co., TX :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 5 07-044-104n

USDA Field Termination Report

USDA Notification Number: 07-044-104n

Applicant Internal Number: IR-7F-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum*; cotton

Lines: TwinLink

Section I. Site Release Information:

Trials were conducted at six (6) locations:

County / State	Acreage Planted	Date Planted	Date Terminated
1 Franklin / LA	0.293	6/7/2007	11/16/2007
2 Pinal / AZ	0.363	5/31/2007	12/19/2007
3 Hidalgo / TX	0.333	4/17/2007	8/30/2007
4 Oktibbeha / MS	0.263	5/23/2007	11/5/2007
5 Washington / MS	0.10	5/17/07 & 5/29/07	11/5/2007
6 Lubbock / TX	0.17	5/26/07	11/13/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Franklin Co., LA

a) Cooperator noted no unusual plant growth characteristics.
b) No observed differences in plant disease.
c) No unusual differences in insect pest or beneficial insect's populations.
d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

2. Pinal Co., AZ :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

3. Hidalgo Co., TX :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

4. Oktibbeeha Co., MS :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.

g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

5. Washington Co., MS :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

6. Lubbock Co., TX :
   h) Cooperator noted no unusual plant growth characteristics.
   i) No observed differences in plant disease.
   j) No unusual differences in insect pest or beneficial insect's populations.
   k) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   l) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   m) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   n) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 6 07-065-117n

USDA Field Termination Report

USDA Notification Number: 07-065-117n

Applicant Internal Number: IR-7K-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Lines Planted: GEM1 (T304-40)

Regulated Article: *Gossypium hirsutum*; cotton

Section I. Site Release Information:

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

County / State	Acreage Planted	Date Planted	Date Terminated
1 Martin / NC	0.137	5/24/07	10/17/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Martin Co., NC :
   a) No observed differences between transgenic and non-transgenic agronomic growth.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.

e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) Early season Deer feeding controlled by electric fence (USDA letter 7/24/2007). Trial was successfully conducted without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 7 07-065-118n

USDA Field Termination Report

USDA Notification Number: 07-065-118n

Applicant Internal Number: IR-7L-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Lines Planted: GEM2 (GHB119)

Regulated Article: *Gossypium hirsutum*; cotton

Section I. Site Release Information:

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

County / State	Acreage Planted	Date Planted	Date Terminated
1 Martin / NC	0.137	5/24/07	10/17/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Martin Co., NC :
   a) No observed differences between transgenic and non-transgenic agronomic growth.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.

e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) Early season Deer feeding controlled by electric fence (USDA letter 7/24/2007). Trial was successfully conducted without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 8 07-065-119n

USDA Field Termination Report

USDA Notification Number: 07-065-119n

Applicant Internal Number: IR-7M-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Lines Planted: TwinLink (GHB119/T304-40)

Regulated Article: *Gossypium hirsutum;* cotton

Section I. Site Release Information:

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

County / State	Acreage Planted	Date Planted	Date Terminated
1 Martin / NC	0.137	5/24/07	10/17/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Martin Co., NC :
   a) No observed differences between transgenic and non-transgenic agronomic growth.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.

e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) Early season Deer feeding controlled by electric fence (USDA letter 7/24/2007). Trial was successfully conducted without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Bayer CropScience

Termination Report 9 07-059-101n

USDA Field Termination Report

USDA Notification Number: 07-059-101n

Applicant Internal Number: IR-7I-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Lines Planted: TwinLink

Regulated Article: *Gossypium hirsutum*; cotton

Section I. Site Release Information:

Trials utilizing this trait were conducted at nine (9) locations:

County / State	Acreage Planted	Date Planted	Date Terminated
1 Crittenden / AR (1)	0.29	6/4/07	10/20/2007
2 Crittenden / AR (2)	0.29	6/4/07	10/20/2007
3 Jackson / AR	0..29	6/5/07	11/14/2007
4 Tift / GA (1)	0.29	6/13/07	11/30/2007
5 Tift / GA (2)	0.28	6/15/07	11/29/2007
6 Tate / MS	0.20	6/5/07	10/21/2007
7 Hockley / TX (1)	2.02	6/9/07	12/7/2007
8 Hockley / TX (2)	0.27	6/4/07	11/16/2007
9 Wharton / TX	0.29	6/11/07	8/14/2007

Section II. List of characteristic observations between the transgenic & non-transgenic plants, insects and environment:

a	Plant Growth Characteristics
b	Disease Differences
c	Insect Populations
d	Adverse Effects on Non-Target Insects or Environment
e	Weediness Characteristics
f	Weather Synopsis
g	Plot Damage

Section III. Summarized results from the BCS regulatory In-Season Observation forms:

1. Crittenden Co., AR (1) :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
    f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
    g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

2. Crittenden Co., AR (2) :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
    f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
    g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

3. Jackson Co., AR :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
    f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
    g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

4. Tift Co., GA (1) :
    a) Cooperator noted no unusual plant growth characteristics.
    b) No observed differences in plant disease.
    c) No unusual differences in insect pest or beneficial insect's populations.
    d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
    e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.

f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

5. Tift Co., GA (2) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

6. Tate Co., MS :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

7. Hockley Co., TX (1) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.
   d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
   e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
   f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
   g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

8. Hockley Co., TX (2) :
   a) Cooperator noted no unusual plant growth characteristics.
   b) No observed differences in plant disease.
   c) No unusual differences in insect pest or beneficial insect's populations.

d) There was no indication of any adverse effects to non-target insect populations, beneficial insects or the environment.
e) There were no indications of increased weediness characteristics in either the transgenic or non-transgenic plots.
f) Weather was reported as normal, with expected variations in rainfall and temperatures during the growing season for this location.
g) No plot damage occurred. Trial was successfully conducted without any environmental releases or compliance incidents.

9. Wharton Co., TX :
This test was terminated early, August 14, 2007 following flood damage. The potential compliance incident was reported to USDA/BRS and resolved. Volunteer monitoring is scheduled to end September 2008.

Section IV. Plant Disposition:

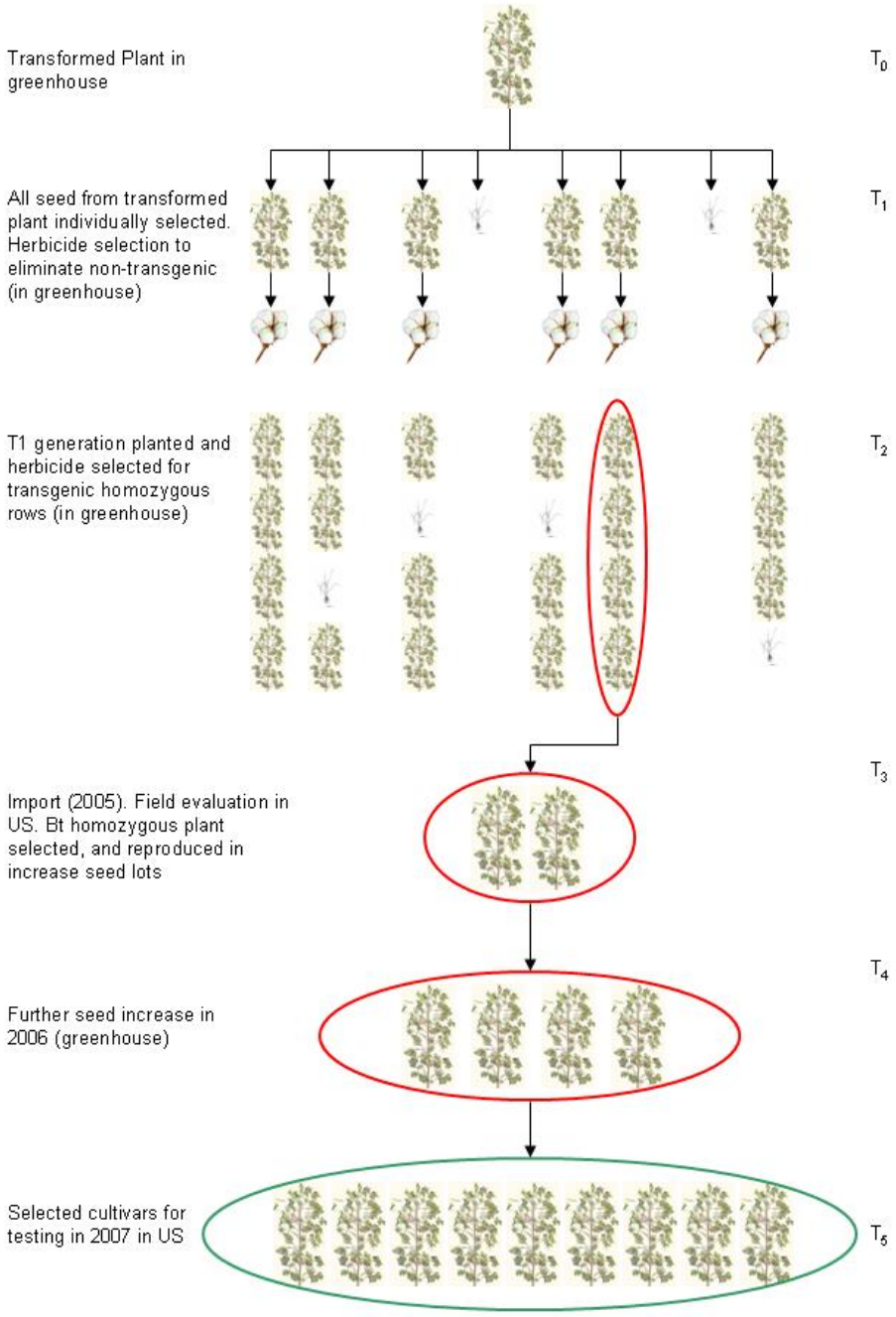
All plot locations were harvested and all remaining harvested material that was not sent to a Bayer CropScience Facility or other Laboratories for analysis were destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed by deep burial within the plot.

Section V. Volunteer Monitoring:

Volunteer monitoring is ongoing and is scheduled for completion one year from harvest.

Appendix 2. MATERIALS AND METHODS - PRODUCT CHARACTERIZATION

Breeding diagram for each parent of TwinLink cotton used in agronomic evaluation



Materials and methods for molecular characterization – DNA tests

Materials

DNA for the analyses was isolated from leaves of T304-40, GHB119, TwinLink, and the conventional control produced in the greenhouse. The references included the plasmids (pTDL008 for T304-40 and pTEM12 for GHB119) that were used in their respective transformations. For Southern blot analysis of cotton genomic DNA, digested plasmid DNA (approximately 0.1, 1, 10 genomic copies equivalent) was mixed with genomic DNA of the conventional control and separated by electrophoresis on agarose gels. Standard DNA molecular weight markers, such as phage Lambda DNA digested with *Pst*I and/or *Eco*RI and/or *Hind*III were used for size estimation of the DNA fragments.

Identity of the materials

The identity of the greenhouse produced material was confirmed by PCR analysis prior to use, to confirm the presence or absence of T304-40 or GHB119, 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 pTDL008 or pTEM12, as appropriate. Concentration of the different DNA preparations was determined by spectrophotometry using a Nanodrop ND-8000, or by fluorescence using the Quant-iT™ PicoGreen® dsDNA Reagent.

Approximately 10 µg of genomic DNA 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 either 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 plasmid DNA 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. 32 pg of pTDL008, and ca. 27 pg of pTEM12 plasmid DNA [*Gossypium hirsutum* genome size: 4.5x10^{9}bp (Arumuganathan and Earle, 1991); pTDL008 = 14393 bp; pTEM12 = 12266 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. Standard DNA molecular weight markers, such as phage Lambda DNA digested with *Pst*I and/or *Eco*RI and/or *Hind*III were used for size estimation of the DNA fragments.

PCR preparation of DNA probes

The DNA templates 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 GE Healthcare. Unincorporated nucleotides were removed by separation on a micro Bio-Spin-30 column from Bio-Rad.

Materials and methods for protein characterization tests

Studies on potential toxicology and allergenicity for food, feed and the environment are conducted with Cry1Ab, Cry2Ae and PAT/*bar* proteins expressed in *Bacillus thuringiensis (Bt.)* and *E. coli*. 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.. Six analytical tests show that the proteins produced in *Bt*. and *E. coli* are representative of Cry1Ab, Cry2Ae and PAT/*bar* proteins produced in TwinLink cotton.

Materials

The plant-produced Cry1Ab, Cry2Ae and PAT/*bar* proteins were isolated from greenhouse-grown TwinLink plants. 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 columns used for purification were purchased from Pierce (Rockford, IL, product number 44894), and prepared using a covalently attached monoclonal antibodies specific for each protein.

The Cry1Ab (BCS reference standard, Batch No. MIN1443, purity 98.59%) and PAT/*bar* (BCS reference standard, Batch N° NB010905P44P2, purity 65.82%) protein reference standards were produced in *E. coli*. The Cry2Ae protein reference standard (BCS reference standard, Batch No. NB170907, purity 94.37%) was produced in *Bacillus thuringiensis*. The protein solutions are stored in an Ultrafreezer.

Analysis by N-terminal sequencing

The affinity purified Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves and the bacterial proteins were loaded onto PVDF membranes and sent to Eurosequence bv (Groningen, the Netherlands) for analysis of the N-terminal amino acid sequence of the proteins by Edman degradation. Prior to analysis, the samples were reduced and alkylated using N-isopropyliodoacetamide.

For an N-terminal sequence the concentration of the Cry1Ab protein isolated from TwinLink cotton leaves was too low for N-terminal sequencing. However, the N-terminal peptide for the Cry1Ab protein isolated from TwinLink was detected in the selected ion mass spectrometry analysis of the protein, indicating that the protein is not modified.

Analysis by SDS-PAGE

The Cry1Ab, Cry2Ae and PAT/*bar* proteins from *E. coli* and *Bt.* and the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves were analyzed by SDS-PAGE. The proteins from the plant and the corresponding protein from *E. coli* and *Bt.* were denatured and analyzed by electrophoresis on a denaturing polyacrylamide gel where mobility can be correlated to molecular weight. SDS-PAGE was performed using an Invitrogen Bis-Tris 10% polyacrylamide gel (InVitrogen, CA, product number NP0301) and a MOPS SDS running buffer according to the manufacturer's instructions. Molecular weight markers on the gel are comprised of a series of recombinant proteins of known molecular weight. The gel was then stained with Pierce Imperial Stain to visualize the protein bands.

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 subject 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 horseradish peroxidase (HRP) linked anti-mouse antibody. All reagents except the monoclonal primary antibodies used for western blotting were obtained from GE Healthcare (NJ) as an ECL Plus luminescent detection kit (product number RPN 2108). Kaleidoscope molecular weight markers from GE Healthcare were used.

Analysis by HPLC/Electrospray Mass Spectrometry

The Cry1Ab, Cry2Ae and PAT/*bar* proteins from *Bt.* and *E. coli* and the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves were denatured for 1 hour at 37 °C in Rapid Gest (Waters Corporation, 1mg/mL) containing 10 mM dithiothreitol and digested with trypsin for 1 hour at 37°C. A selected ion monitoring method was developed for Cry1Ab and Cry2Ae using the peptides generated from the trypsin digest of the Cry1Ab and Cry2Ae proteins from *E. coli.*and *Bt.* A method for PAT/bar was developed earlier and this was used for the analysis of the PAT/bar protein. A full scan mass spectrum was generated for the Cry1Ab and Cry2Ae peptides that were detected in the trypsin digest of the Cry1Ab and Cry2Ae proteins from *E. coli.* and *Bt.* The most abundant ions indicative of each peptide were used for selected ion monitoring (SIM) of the peptides in the sample. The peptides from the Cry1Ab *E. coli.*, Cry2Ae *Bt.* and PAT/*bar E. coli* standard trypsin digest and peptides from the the trypsin digest of the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves were analyzed using the selected ion methods. The presence of a peak for the peptide in the selected ion monitoring chromatogram of the sample at a retention time detected for a peak of the same peptide in the trypsin digest of the Cry1Ab from *E. coli.,* Cry2Ae from *Bt.* and PAT/*bar* from *E. coli* standards confirms the presence of that peptide in the trypsin digest sample of the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton. The limit of detection of a peak is defined as approximately three times signal to noise.

Glycoprotein staining analysis

The Cry1Ab, Cry2Ae and PAT/*bar* proteins from *E. coli* and *Bt.* and the Cry1Ab, Cry2Ae and PAT/*bar* proteins isolated from TwinLink cotton leaves were analyzed by SDS-PAGE. 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. Glyco-Staining was performed using a Sigma GlycoProfile™ III Fluorescent Glycoprotein Detection Kit (Sigma, cat # PP0300) according to the manufacturers instructions.

Analysis of enzymatic activity

The enzymatic activity of the PAT/*bar* protein isolated from TwinLink cotton leaves was determined according to D'Halluin *et al*. (1995). The method measures the activity of PAT/*bar* by the generation of free Coenzyme A sulphydryl groups during the transfer of the acetyl group of Acetyl Coenzyme A to phosphinothricin. The reaction of the reduced Coenzyme A with 5,5-dithiobis(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid which has an absorption at

412 nm. The detection of an absorbance at 412 nm indicates that the PAT/*bar* protein is active.

The activity of the Cry2Ae protein was determined from an insect assay. The Cry2Ae protein isolated from TwinLink cotton and the Cry2Ae protein from *Bt.* were added to Lepidoptera diet and fed to *Helicoverpa zea* larvae, at the first larvae stage. The Cry1Ab protein could not be isolated from TwinLink cotton leaves in enough quantity to perform an insect assay.

Materials and methods for protein levels in seeds

Field design

TwinLink cotton plants containing events T304-40 and GHB119, along with cotton plants representing the non-transgenic (non-transformed) counterpart Coker 315 were field tested by Bayer CropScience in 2007 under USDA APHIS Notification 07-059-101n. Field trials were established in EPA Regions II, IV, and VIII in the following locations: Trial number 02-01-Tift County, Georgia; Trial number 02-02-Tift County, Georgia; Trial 04-03-Jackson County Arkansas; Trial number 04-04-Crittenden County, Arkansas; Trial number 04-06-Tate County, Mississippi; Trial number 08-08-Hockley County, Texas. These areas are typical cotton growing regions of the southeastern United States, and 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. The test plots were randomized at each field location. Seed for planting was supplied for each field trial by Bayer CropScience.

Three of the transgenic event plots in each field trial were sprayed two times with Ignite® 280 SL glufosinate-ammonium herbicide, and the other plots were untreated. The Ignite herbicide contained a labeled concentration of 2.34 pounds per U.S. gallon of glufosinate-ammonium active ingredient (280 grams of active ingredient per liter). Each application of Ignite herbicide was made at a target rate of 29 fluid ounces of product per acre (2.1 liters per hectare), equivalent to 0.53 lb ai/acre (0.60 kg ai/hectare).

Two replicate samples of ginned cottonseed (fuzzy seed) were obtained from each test plot. The samples were shipped frozen to the BioAnalytics 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 Bayer CropScience for the two seed lots used for planting in this study. Identity and purity of the TwinLink cottonseed and the corresponding non-transgenic (Coker 315) cottonseed were confirmed to be acceptable for use.

Sample preparation

A CoA to determine identity of the samples harvested from the seven field sites was prepared by Bayer CropScience. The data certified that the transgenic samples harvested from the field were indeed TwinLink cotton, and that the non-transgenic samples were harvested from non-transgenic plots.

Fuzzy seed was difficult to grind to homogeneity with dry ice due to the residual lint adhering to the seed. 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, identified as lint coat sample. The weights of the kernel and lint coat fractions were recorded for each sample of fuzzy seed that was analyzed in order to reconstruct the amount of protein analyte in the fuzzy seed as it was received from the field.

Each of the frozen kernel samples 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 -20ºC 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 Cry1Ab, Cry2Ae, and PAT/bar proteins were extracted from raw agricultural products of cotton using a buffer described by Xin *et al*., (1988). A representative portion (approximately 0.1 g) of ground sample was mixed with an appropriate amount of extraction buffer (approximately 20 mL) 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 the protein and TEP analyses. Duplicate extracts were prepared for each sample.

Bioassay

All quantitative determinations of the Cry1Ab, Cry2Ae, and PAT/*bar* proteins were conducted at Bayer CropScience, Research Triangle Park, NC. A commercial ELISA (EnviroLogix, MA) specific for each protein was used to measure the amounts of Cry1Ab, Cry2Ae, and PAT/bar. Each ELISA was validated in kernel and lint coat matrices for the each protein.

Validation

A validation was performed for Cry1Ab, Cry2Ae, and PAT/*bar* proteins separately, using non-transgenic kernel and lint coat samples fortified at the concentrations listed in Tables 2.1 through 2.4. 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.

Table 2.1 Validation of Sample Extraction for the Cry1Ab ELISA with Fortified Non-transgenic Controls of Kernel and Lint Coat

Sample ID	*Validation of Cotton Seed Kernel for Cry1Ab (Field Sample ID: 02-01; BTID: 1243A)*			*Validation of Lint Coat for Cry1Ab (Field Sample ID: 02-01; BTID: 1243A)*		
Cry1Ab Fortified (ng/mL)	*Cry1Ab Detected (ng/mL)[a] Mean ± SD*	*% Cry1Ab Recovery Mean ± SD*	*Cry1Ab Recovery % CV*	*Cry1Ab Detected (ng/mL) Mean ± SD*	*% Cry1Ab Recovery Mean ± SD*	*Cry1Ab Recovery % CV*
100	121 ± 17.6	121 ± 17.6	14.6	79.5 ± 5.52	79.5 ± 5.52	6.94
60	50.1 ± 3.47	83.5 ± 5.78	6.92	57.3 ± 3.79	95.4 ± 6.32	6.63
5	5.19 ± 0.630	104 ± 12.6	12.2	4.42 ± 0.169	88.4 ± 3.37	3.81
2.5	2.86 ± 0.381	114 ± 15.2	13.3	1.80 ± 0.069	72.1 ± 2.76	3.82
1.25	1.54 ± 0.126	123 ± 10.1	8.23	1.15 ± 0.038	92.3 ± 3.08	3.34
0.645	0.771 ± 0.086	120 ± 13.4	11.2	0.556 ± 0.121	88.9 ± 19.3	21.8

[a] The Cry1Ab protein detected and its recovery are expressed as the average of 10 data points from duplicate extracts of 5 samples at each fortification level using non-transgenic matrix.

Table 2.2 Validation of Sample Extraction for the Cry2Ae ELISA with Fortified Non-transgenic Controls of Kernel and Lint Coat

Sample ID	*Validation of Cotton Seed Kernel for Cry2Ae (Field Sample ID: 02-01; BTID: 1243A)*			*Validation of Lint Coat for Cry2Ae (Field Sample ID: 02-01; BTID: 1243A)*		
Cry2Ae Fortified (ng/mL)	*Cry2Ae Detected (ng/mL)[a] Mean ± SD*	*% Cry2Ae Recovery Mean ± SD*	*Cry2Ae Recovery % CV*	*Cry2Ae Detected (ng/mL)[a] Mean ± SD*	*% Cry2Ae Recovery Mean ± SD*	*Cry2Ae Recovery % CV*
100	65.0 ± 5.47	65.0 ± 5.47	8.41	99.2 ± 2.45	99.2 ± 2.45	2.47
30	23.8 ± 3.86	79.3 ± 12.9	16.2	27.6 ± 0.47	92.0 ± 1.56	1.69
15	15.9 ± 1.60	106 ± 10.6	10.0	12.3 ± 0.69	81.9 ± 4.61	5.63
10	10.2 ± 0.560	102 ± 5.63	5.53	8.00 ± 0.55	80.0 ± 5.47	6.83
7.5	6.66 ± 0.190	88.7 ± 2.56	2.88	6.74 ± 0.33	89.9 ± 4.34	4.83
3.75	3.30 ± 0.160	88.0 ± 4.26	4.84	3.58 ± 0.35	95.4 ± 9.43	9.88
1.875	1.85 ± 0.120	98.7 ± 6.21	6.29	1.97 ± 0.17	105 ± 9.07	8.65
0.938	0.980 ± 0.250	105 ± 26.9	25.7	1.02 ± 0.06	109 ±6.87	6.32
0.469	0.700 ± 0.120	149 ± 25.4	17.0	0.82 ± 0.26	175 ± 55.7	31.8

[a] The Cry2Ae protein detected and its recovery are expressed as the average of 10 data points from duplicate extracts of 5 samples at each fortification level using non-transgenic matrix.

Table 2.3 Validation of Sample Extraction for the PAT/*bar* ELISA with Fortified Non-transgenic Controls of Kernel and Lint Coat

Sample ID	*Validation of Cotton Seed Kernel for PAT/bar (Field Sample ID: 02-01; BTID: 1243A)*			*Validation of Lint Coat for PAT/bar (Field Sample ID: 02-01; BTID: 1243A)*		
PAT/bar Fortified (ng/mL)	*PAT/bar Detected (ng/mL)[a] Mean ± SD*	*% PAT/bar Recovery Mean ± SD*	*PAT/bar Recovery % CV*	*PAT/bar Detected (ng/mL) Mean ± SD*	*% PAT/bar Recovery Mean ± SD*	*PAT/bar Recovery % CV*
1000	65.0 ± 5.47	65.0 ± 5.47	8.41	99.2 ± 2.45	99.2 ± 2.45	2.47
7.5	23.8 ± 3.86	79.3 ± 12.9	16.2	27.6 ± 0.47	92.0 ± 1.56	1.69
3.75	15.9 ± 1.60	106 ± 10.6	10.0	12.3 ± 0.69	81.9 ± 4.61	5.63
1.875	10.2 ± 0.560	102 ± 5.63	5.53	8.00 ± 0.55	80.0 ± 5.47	6.83
0.938	6.66 ± 0.190	88.7 ± 2.56	2.88	6.74 ± 0.33	89.9 ± 4.34	4.83
0.469	0.700 ± 0.120	149 ± 25.4	17.0	0.82 ± 0.26	175 ± 55.7	31.8

[a] The PAT/*bar* protein detected and its recovery are expressed as the average of 10 data points from duplicate extracts of 5 samples at each fortification level using non-transgenic matrix.

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.

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/20ml extraction buffer. The data are summarized in Table 2.4. An absorbance reading giving rise to a protein analyte concentration above this limit of detection level is assumed to be greater than the zero dose reading.

The limit of quantification (LOQ) is defined as the lowest concentration of the standard that meets the validity 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 calculated LOQs are summarized in Table 2.4 below.

Table 2.4 Limits of Detection and Quantitation of Cry1Ab, Cry2Ae and PAT/*bar* Protein in Raw Agricultural Commodities of TwinLink Cotton as Detected by ELISA.

		LOD		*LOQ*	
		(ng/mL)	*ng/g [a] Sample*	*(ng/mL)*	*ng/g [a] Sample*
Cry1Ab	Kernel	0.0990	19.8	0.645	129
Cry1Ab	Lint Coat	0.0660	13.2	0.645	129
Cry2Ae	Kernel	0.463	92.6	1.88	375
Cry2Ae	Lint Coat	0.503	101	0.938	188
PAT/*bar*	Kernel	0.148	29.6	0.938	188
PAT/*bar*	Lint Coat	0.220	44.0	1.88	375

[a] Calculated based on the extraction of 0.1 g matrix per 20 mL of extraction buffer for kernel and lint coat.

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.

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 the Cry1Ab, Cry2Ae, and PAT/*bar* proteins. 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 1243A), 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.

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 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 BioAnalytics Laboratories, BCS, NC. The calculations and conversions were done as described below.

The values of percent crude protein on both a fresh weight and dry weight basis were used to calculate the Cry1Ab, Cry2Ae, and PAT/*bar* protein concentration as percent of crude protein. The following is the conversion formula used:

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

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:

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

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 each subject protein content in kernel and lint coat fractions at a significance level of 0.05 (α = 5%). 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 (< 0.05) 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

Please refer to tables

Table 2.5 Fresh Weight average quantities of Cry1Ab protein in raw agricultural commodities of TwinLink cotton as detected by ELISA

Sample	*Trial Number*	*[a]Average Cry1Ab content (µg/g sample) ± SD*		*[b]Average Cry1Ab content (as % of total crude protein) ± SD*	
		No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Kernel	B02-01	2.47 ± 0.326	2.49 ± 0.270	NA	NA
	B02-02	2.65 ± 0.396	3.33 ± 0.761	NA	NA
	B04-03	0.467 ± 0.0475	2.77 ± 0.346	NA	NA
	B04-04	1.87 ± 0.0357	1.91 ± 0.610	NA	NA
	B04-05	2.27 ± 0.317	2.22 ± 0.150	NA	NA
	B04-06	3.45 ± 0.0306	3.70 ± 0.878	NA	NA
	B08-08	3.09 ± 0.587	2.94 ± 0.995	NA	NA
Average	NA	2.32 ± 0.844	2.77 ± 1.71	NA	NA
Lint Coat	B02-01	0	0	NA	NA
	B02-02	0	0	NA	NA
	B04-03	0	0	NA	NA
	B04-04	0	0	NA	NA
	B04-05	0	0	NA	NA
	B04-06	0	0	NA	NA
	B08-08	0	0.0565 ± 0.0978	NA	NA
Average	NA	0 ± 0	0.00807 ± 0.0978	NA	NA
Fuzzy Seed	B02-01	1.19	1.22	0.000598	0.000609
	B02-02	1.28	1.62	0.000676	0.000817
	B04-03	0.226	1.38	0.000147	0.000871
	B04-04	0.956	0.962	0.000455	0.000455
	B04-05	1.17	1.09	0.000597	0.000591
	B04-06	1.64	1.77	0.000751	0.000833
	B08-08	1.64	1.60	0.000704	0.000681
Range in Values	NA	0.226 – 1.64	0.962 – 1.77	0.00047 – 0.000751	0.000455 – 0.000871
Average ± SD	NA	1.16 ± 0.482	1.38 ± 0.301	0.00056 ± 0.000206	0.000694 ± 0.000153

[a] Standard Deviation was not calculated for individual 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 Cry1Ab value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the seven sites. Standard deviations for the individual sites were based on 12 measurements (two sample extracts assayed in duplicate from three replicate plots). Standard deviations for the averages of kernel and lint coat are based on 84 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at seven sites). The data for the fuzzy seed were calculated from the amount of Cry1Ab protein present in kernel and lint coat fractions taking into account their respective weights.

[b] Average Cry1Ab as % of crude protein is not applicable (NA) because crude protein determinations were not made on the individual lint coat and kernel; but only on the entire fuzzy seed samples.

Table 2.6 Dry Weight average quantities of Cry1Ab protein in raw agricultural commodities of TwinLink cotton as detected by ELISA

Sample	*Trial Number*	*[a]Average Cry1Ab content (µg/g sample) ± SD*		*[b]Average Cry1Ab content (as % of total crude protein) ± SD*	
		No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Kernel	B02-01	NA	NA	NA	NA
	B02-02	NA	NA	NA	NA
	B04-03	NA	NA	NA	NA
	B04-04	NA	NA	NA	NA
	B04-05	NA	NA	NA	NA
	B04-06	NA	NA	NA	NA
	B08-08	NA	NA	NA	NA
Average	NA	NA	NA	NA	NA
Lint Coat	B02-01	NA	NA	NA	NA
	B02-02	NA	NA	NA	NA
	B04-03	NA	NA	NA	NA
	B04-04	NA	NA	NA	NA
	B04-05	NA	NA	NA	NA
	B04-06	NA	NA	NA	NA
	B08-08	NA	NA	NA	NA
Average	NA	NA	NA	NA	NA
Fuzzy Seed	B02-01	1.34	1.37	0.000598	0.000609
	B02-02	1.46	1.84	0.000676	0.000817
	B04-03	0.258	1.56	0.000147	0.000871
	B04-04	1.08	1.09	0.000455	0.000455
	B04-05	1.33	1.25	0.000597	0.000591
	B04-06	1.81	1.96	0.000751	0.000833
	B08-08	1.74	1.70	0.000704	0.000681
Range in Values	NA	0.258 – 1.81	1.09 – 1.96	0.000147 – 0.000751	0.000455 – 0.000871
Average ± SD	NA	1.29 ± 0.520	1.54 ± 0.318	0.000561 ± 0.000206	0.000694 ± 0.000153

[a] Standard Deviation was not calculated for individual 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 Cry1Ab value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the seven sites. Standard deviations for the individual sites were based on 12 measurements (two sample extracts assayed in duplicate from three replicate plots). Standard deviations for the averages of kernel and lint coat are based on 84 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at seven sites). The data for the fuzzy seed were calculated from the amount of Cry1Ab protein present in kernel and lint coat fractions taking into account their respective weights

[b] Average Cry1Ab as % of crude protein is not applicable (NA) because crude protein determinations were not made on the individual lint coat and kernel; but only on the entire fuzzy seed samples.

Table 2.7 Fresh Weight average quantities of Cry2Ae protein in raw agricultural commodities of TwinLink cotton as detected by ELISA

Sample	*Trial Number*	*[a]Average Cry2Ae content (µg/g sample) ± SD*		*[b]Average Cry2Ae content (as % of total crude protein) ± SD*	
		No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Kernel	B02-01	21.6± 1.96	18.8 ± 2.60	NA	NA
	B02-02	8.37 ± 1.15	11.6 ± 1.78	NA	NA
	B04-03	12.2 ± 1.94	16.4 ± 0.302	NA	NA
	B04-04	17.1 ± 0.467	20.6 ± 6.51	NA	NA
	B04-05	21.6 ± 2.64	31.4 ± 10.0	NA	NA
	B04-06	15.0 ± 0.619	12.3 ± 1.78	NA	NA
	B08-08	12.8 ± 3.28	13.9 ± 5.35	NA	NA
Average	NA	15.5 ± 5.22	17.9 ± 13.6	NA	NA
Lint Coat	B02-01	1.13 ± 0.218	1.16 ± 0.628	NA	NA
	B02-02	1.22 ± 0.756	0.990 ± 0.802	NA	NA
	B04-03	1.21 ± 0.775	0.971 ± 0.168	NA	NA
	B04-04	1.98 ± 0.524	2.83 ± 1.76	NA	NA
	B04-05	1.31 ± 0.407	1.45 ± 0.341	NA	NA
	B04-06	0.464 ± 0.334	0.266 ± 0.240	NA	NA
	B08-08	1.11 ± 0.0350	1.12 ± 1.08	NA	NA
Average	NA	1.20 ± 1.33	1.26 ± 2.35	NA	NA
Fuzzy Seed	B02-01	11.0	9.81	0.00552	0.00490
	B02-02	4.69	6.16	0.00248	0.00311
	B04-03	6.52	8.68	0.00423	0.00546
	B04-04	9.70	11.7	0.00462	0.00555
	B04-05	11.7	16.2	0.00601	0.00870
	B04-06	7.39	6.01	0.00339	0.00283
	B08-08	7.35	7.99	0.00315	0.00339
Range in Values	NA	4.69 – 11.7	6.01 – 16.2	0.00248 – 0.00601	0.00283 – 0.00870
Average ± SD	NA	8.34 ± 2.55	9.51 ± 3.56	0.00420 ± 0.00129	0.00485 ± 0.00204

[a] Standard Deviation was not calculated for individual 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 Cry2Ae value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the seven sites. Standard deviations for the individual sites were based on 12 measurements (two sample extracts assayed in duplicate from three replicate plots). Standard deviations for the averages of kernel and lint coat are based on 84 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at seven sites). The data for the fuzzy seed were calculated from the amount of Cry2Ae protein present in kernel and lint coat fractions taking into account their respective weight.

[b] Average Cry2Ae as % of crude protein is not applicable (NA) because crude protein determinations were not made on the individual lint coat and kernel; but only on the entire fuzzy seed samples.

Table 2.8 Dry Weight average quantities of Cry2Ae protein in raw agricultural commodities of TwinLink cotton as detected by ELISA

Sample	*Trial Number*	*[a]Average Cry2Ae content (µg/g sample) ± SD*		*[b]Average Cry2Ae content (as % of total crude protein) ± SD*	
		No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Kernel	B02-01	NA	NA	NA	NA
	B02-02	NA	NA	NA	NA
	B04-03	NA	NA	NA	NA
	B04-04	NA	NA	NA	NA
	B04-05	NA	NA	NA	NA
	B04-06	NA	NA	NA	NA
	B08-08	NA	NA	NA	NA
Average	NA	NA	NA	NA	NA
Lint Coat	B02-01	NA	NA	NA	NA
	B02-02	NA	NA	NA	NA
	B04-03	NA	NA	NA	NA
	B04-04	NA	NA	NA	NA
	B04-05	NA	NA	NA	NA
	B04-06	NA	NA	NA	NA
	B08-08	NA	NA	NA	NA
Average	NA	NA	NA	NA	NA
Fuzzy Seed	B02-01	12.3	11.0	0.00552	0.00490
	B02-02	5.34	6.99	0.00248	0.00311
	B04-03	7.43	9.84	0.00423	0.00546
	B04-04	11.0	13.4	0.00462	0.00555
	B04-05	13.4	18.5	0.00601	0.00870
	B04-06	8.19	6.67	0.00339	0.00283
	B08-08	7.79	8.48	0.00315	0.00339
Range in Values	NA	5.34 – 13.4	6.67 – 18.5	0.00248 – 0.00601	0.00283 – 0.00870
Average ± SD	NA	9.35 ± 2.93	10.7 ± 4.17	0.00420 ± 0.00129	0.00485 ± 0.00204

[a] Standard Deviation was not calculated for individual 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 Cry2Ae value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the seven sites. Standard deviations for the individual sites were based on 12 measurements (two sample extracts assayed in duplicate from three replicate plots). Standard deviations for the averages of kernel and lint coat are based on 84 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at seven sites). The data for the fuzzy seed were calculated from the amount of Cry2Ae protein present in kernel and lint coat fractions taking into account their respective weights. Average dry weight content of Cry2Ae in kernel and lint coat is not applicable (NA) because moisture determinations were made only on the entire fuzzy seed samples and not the individual lint coat and kernel.

[b] Average Cry2Ae as % of crude protein is not applicable (NA) because crude protein determinations were not made on the individual lint coat and kernel; but only on the entire fuzzy seed samples.

Table 2.9 Fresh Weight average quantities of PAT/*bar* protein in raw agricultural commodities of TwinLink cotton as detected by ELISA

Sample	*Trial Number*	*[a]Average PAT/bar content (µg/g sample) ± SD*		*[b]Average PAT/bar content (as % of crude protein) ± SD*	
		No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Kernel	B02-01	278 ± 24.5	238 ± 7.51	NA	NA
	B02-02	275 ± 40.3	276 ± 33.9	NA	NA
	B04-03	234 ± 19.1	280 ± 23.8	NA	NA
	B04-04	365 ± 42.6	338 ± 73.7	NA	NA
	B04-05	283 ± 15.2	342 ± 19.0	NA	NA
	B04-06	370 ± 22.4	329 ± 50.2	NA	NA
	B08-08	221 ± 11.9	223 ± 98.8	NA	NA
Average	NA	290 ± 72.7	289 ± 141	NA	NA
Lint Coat	B02-01	2.34 ± 0.544	2.67 ± 0.369	NA	NA
	B02-02	4.41 ± 3.17	3.39 ± 1.87	NA	NA
	B04-03	2.28 ± 2.34	2.02 ± 0.915	NA	NA
	B04-04	3.56 ± 1.94	4.71 ± 2.28	NA	NA
	B04-05	3.63 ± 1.47	4.66 ± 3.06	NA	NA
	B04-06	1.65 ± 1.15	2.77 ± 2.65	NA	NA
	B08-08	2.43 ± 0.552	4.09 ± 4.35	NA	NA
Average	NA	2.90 ± 4.83	3.47 ± 6.70	NA	NA
Fuzzy Seed	B02-01	135	118	0.0680	0.0590
	B02-02	136	136	0.0711	0.0684
	B04-03	115	140	0.0742	0.0885
	B04-04	188	172	0.0896	0.0814
	B04-05	147	170	0.0751	0.0923
	B04-06	176	158	0.0809	0.0744
	B08-08	118	121	0.0505	0.0512
Range in Values	NA	115 – 188	118 – 172	0.0515 – 0.0896	0.0512 – 0.0923
Average ± SD	NA	145 ± 28.0	145 ± 22.2	0.0728 ± 0.0121	0.0736 ± 0.0151

[a] Standard Deviation was not calculated for individual 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 PAT/*bar* value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the seven sites. Standard deviations for the individual sites were based on 12 measurements (two sample extracts assayed in duplicate from three replicate plots). Standard deviations for the averages of kernel and lint coat are based on 84 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at seven sites). The data for the fuzzy seed were calculated from the amount of PAT/bar protein present in kernel and lint coat fractions taking into account their respective weights.

[b] Average PAT/*bar* as % of crude protein is not applicable (NA) because crude protein determinations were not made on the individual lint coat and kernel; but only on the entire fuzzy seed samples.

Table 2.10 Dry Weight average quantities of PAT/*bar* protein in raw agricultural commodities of TwinLink cotton as detected by ELISA

Sample	*Trial Number*	*[a]Average PAT/bar content (µg/g sample) ± SD*		*[b]Average PAT/bar content (as % of crude protein) ± SD*	
		No Glufosinate	*Glufosinate Treated*	*No Glufosinate*	*Glufosinate Treated*
Kernel	B02-01	NA	NA	NA	NA
	B02-02	NA	NA	NA	NA
	B04-03	NA	NA	NA	NA
	B04-04	NA	NA	NA	NA
	B04-05	NA	NA	NA	NA
	B04-06	NA	NA	NA	NA
	B08-08	NA	NA	NA	NA
Average	NA	NA	NA	NA	NA
Lint Coat	B02-01	NA	NA	NA	NA
	B02-02	NA	NA	NA	NA
	B04-03	NA	NA	NA	NA
	B04-04	NA	NA	NA	NA
	B04-05	NA	NA	NA	NA
	B04-06	NA	NA	NA	NA
	B08-08	NA	NA	NA	NA
Average	NA	NA	NA	NA	NA
Fuzzy Seed	B02-01	152	132	0.0680	0.0590
	B02-02	154	154	0.0711	0.0684
	B04-03	131	159	0.0742	0.0885
	B04-04	213	196	0.0896	0.0814
	B04-05	168	195	0.0751	0.0923
	B04-06	195	175	0.0809	0.0744
	B08-08	125	129	0.0505	0.0512
Range in Values	NA	125 – 213	129 – 196	0.0505 – 0.0896	0.0512 – 0.0923
Average ± SD	NA	163 ± 32.4	163 ± 27.4	0.0728 ± 0.0121	0.0736 ± 0.0151

[a] Standard Deviation was not calculated for individual 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 PAT/*bar* value of fuzzy seed. This is based only on the calculated average values (kernel + lint coat) obtained at the seven sites. Standard deviations for the individual sites were based on 12 measurements (two sample extracts assayed in duplicate from three replicate plots). Standard deviations for the averages of kernel and lint coat are based on 84 actual measurements (2 sample extracts assayed in duplicate from 3 replicate plots at seven sites). The data for the fuzzy seed were calculated from the amount of PAT/*bar* protein present in kernel and lint coat fractions taking into account their respective weights. Average dry weight content of PAT/*bar* in kernel and lint coat is not applicable (NA) because moisture determinations were made only on the entire fuzzy seed samples and not the individual lint coat and kernel.

[b] Average PAT/*bar* as % of crude protein is not applicable (NA) because crude protein determinations were not made on the individual lint coat and kernel; but only on the entire fuzzy seed samples.

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

This study was carried out to determine the amount of PAT protein, Cry1Ab protein, and Cry2Ae protein in various TwinLink cotton plant matrices produced during the growth of the plant. The matrices included leaves, squares, and grain.

Materials

Transgenic plants were grown under greenhouse conditions and received one spray application of Ignite® herbicide at 0.52 lb glufosinate ai/A. Coker 315 plants were grown as controls and did not receive glufosinate (Ignite herbicide) treatment. Five replicate samples of all matrices were collected for analysis. Samples for each treatment were frozen and analyzed for the PAT/*bar* protein, Cry1Ab protein, and Cry2Ae protein content by ELISA at the BioAnalytics Laboratories, Bayer CropScience, NC.

Sample preparation and protein extraction

The vegetative plant sample consisted of the total amount of each matrix from five plants. Samples for analysis were prepared either by grinding in a Waring Laboratory Blender prechilled with dry ice or by grinding in a mortar and pestle after freezing in liquid nitrogen. Each tissue 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. Grinding with liquid nitrogen involved placing the sample in a mortar surrounded with dry ice. The sample was placed in the mortar and covered with liquid nitrogen. The pestle was then placed in the mortar for cooling. The sample was then ground to a fine powder immediately after the liquid nitrogen dissipated. The liquid nitrogen method was usually used when the amount of available sample was small. 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 extraction of vegetative samples was carried out using a buffer described by Xin *et al*., 1988. A representative portion of ground sample was mixed with the extraction buffer in a 50 mL polypropylene centrifuge tube. Samples were extracted for 30 minutes at approximately 4°C on a shaker (IKA-SCHÜTTLER MTS 4) at 250 rpm and then centrifuged at approximately 18,000 x g for 10 minutes at approximately 4°C. The clear supernatant was then used for Cry1Ab, Cry2Ae, PAT/*bar,* and TEP analyses. Duplicate extracts were prepared for each sample.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

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.

The limit of detection is expressed in the unit of concentration in the extract (ng/mL) and the unit weight (ng/g fresh weight) in the matrix. This relationship is based on the ratio of the weight of the matrix to the volume of the extraction buffer. An ELISA reading resulting in a PAT/*bar*, Cry1Ab, or Cry2Ae protein concentration at or above the limit of detection level is assumed to be greater than the zero dose reading.

The limit of quantitation is given by the lowest concentration of the standard that meets the following two validity criteria: 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.

Table 2.11 Limit of Detection and Quantitation of PAT/bar Protein in Control Cotton Coker 315 Leaves, Squares, and Grain as Detected by ELISA

Matrix	*LOD*		*LOQ*[a]	
	ng/mL Extract	*ng/g Sample*	*ng/mL Extract*	*ng/g Sample*
Leaves	0.002	0.12	0.469	28.1
Squares	0.096	5.76	0.469	28.1
Grain	0.013	0.26	0.469	9.38

[a] Calculated based on the extraction of approximately 0.1 g matrix per 6 mL extraction buffer for leaves and squares and 0.1 g matrix per 2 mL extraction buffer for grain. The data are expressed in ng/mL in the extract and ng/g in the sample.

Table 2.12 Limit of Detection and Quantitation of Cry1Ab Protein in Control Cotton Coker 315 Leaves, Squares, and Grain as Detected by ELISA

Matrix	*LOD*		*LOQ*[a]	
	ng/mL Extract	*ng/g Sample*	*ng/mL Extract*	*ng/g Sample*
Leaves	0.013	0.78	0.078	4.68
Squares	0.025	1.50	0.078	4.68
Grain	0.040	0.80	0.078	1.56

[a] Calculated based on the extraction of approximately 0.1 g matrix per 6 mL extraction buffer for leaves and squares and 0.1 g matrix per 2 mL extraction buffer for grain. The data are expressed in ng/mL in the extract and ng/g in the sample.

Table 2.13 Limit of Detection and Quantitation of Cry2Ae Protein in Control Cotton Coker 315 Leaves, Squares, and Grain as Detected by ELISA

Matrix	*LOD*		*LOQ*[a]	
	ng/mL Extract	*ng/g Sample*	*ng/mL Extract*	*ng/g Sample*
Leaves	0.211	12.7	0.938	56.3
Squares	0.203	12.2	0.469	28.1
Grain	0.183	3.66	0.469	9.38

[a] Calculated based on the extraction of approximately 0.1 g matrix per 6 mL extraction buffer for leaves and squares and 0.1 g matrix per 2 mL extraction buffer for grain. The data are expressed in ng/mL in the extract and ng/g in the sample.

Determination of Total Extractable Protein content

SoftMax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of protein from the Bradford assay (Bradford, 1976). 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 measurement with the lowest CV was used.

Protein analyte content

The amount of PAT/*bar,* Cry1Ab, and Cry2Ae proteins in the extracts were determined by ELISA. The assays used were commercial ELISA kits (catalogue # AP014 for PAT/*bar* protein, AP003 for Cry1Ab protein, and # AP005 for Cry2Ae protein) manufactured by EnviroLogix, Inc. (Portland, ME). All assays resulted in color development in the last step, which was measured in a microplate reader (Molecular Devices THERMOmax) at 450 nm.

Average concentration of Cry1Ab, Cry2Ae, and PAT/bar in leaves, squares, and grain on a fresh and dry weight basis are given in Table 2.14.

Calculations and conversions

SoftMax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of immunoreactive PAT/*bar*, Cry1Ab, and Cry2Ae proteins in the ELISA. The optical density (OD) values were adjusted for the buffer blank and then any background due to the matrix was subtracted, using the average value from 2 wells containing non-transgenic extracts, assayed on the same plate. The optical density corrected for buffer blank and non-transgenic background was converted to the protein analyte concentration using the standard curve also from the same plate. The data point for the dilution falling nearest the center of the standard curve was used. If OD values from different dilutions were equally distant from the midpoint of the Standard Curve, the measurement with the lowest CV were used. To obtain the amount of protein analyte in the matrix, the amount of protein determined from the standard curve was multiplied by the ratio of volume of extraction buffer to grams matrix extracted.

Statistics

Descriptive statistics (mean, standard deviation, and coefficient of variance) were calculated for each tissue and growth stage using Microsoft® Excel 2002. All statistical analyses were done on data with full precision. Results may be rounded to two or three significant numbers.

Table 2.14 Average Analyte Concentrations for Leaves, Squares and Grain on a Fresh Weight and Dry Weight Basis for TwinLink Transgenic Cotton

Matrix	*Average Cry1Ab Protein Content (ug/g fresh weight) ± SD*	*Average Cry2Ae Protein Content (ug/g fresh weight) ± SD*	*Average PAT/bar Protein Content (ug/g fresh weight) ± SD*	*Percent moisture* [a]	*Average Cry1Ab Protein Content (ug/g dry weight) ± SD*	*Average Cry2Ae Protein Content (ug/g dry weight) ± SD*	*Average PAT/bar Protein Content (ug/g dry weight) ± SD*
Leaves	0.42 0.26	7.18 3.83	77.08 38.6	82.6 ± 3.4	2.40 ± 1.51	41.3 ± 22	443 ± 222
Squares	0.27 0.09	1.07 0.32	2.08 5.02	78.1 ± 3.2	1.23 ± 0.41	4.89 ± 1.46	95.9 ± 22.9
Grain	0.39 0.21	0.21 0.19	1.53 0.66	8.56 ±0.46	0.43 ± 0.23	0.23±0.21	1.67 ± 0.72

[a] The values for percent moisture in the samples were obtained from analysis at Covance Laboratories. The complete set of data is given in Appendix 3.

Materials and methods for agronomic equivalence studies

Materials

Materials for agronomy evaluation were created at field sites in 2007 and 2008 in the southeastern, mid-southern, and mid-western regions of the United States. Seven locations in 2007, and two locations in 2008 (only data available at time of dossier preparation) in eight states were used to produce the reference material for fiber analysis and agronomic performance. Agronomic trials consisted of three treatment regimes; two of the transformed cotton and the other the corresponding non-transgenic Coker counterpart. Agronomic field studies utilized an experimental treatment regime which compared the transformed events T304-40 and GHB119 sprayed, and unsprayed with glufosinate ammonium compared to the non-transformed Coker counterpart. Agronomic data was also taken on efficacy trials in 2007 and 2008 (discussed in following section).

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 2007 and 2008 were utilized to characterize and evaluate agronomic performance of the selected events, and develop materials for nutritional and compositional testing. Trials were conducted in three geographic regions of the United States (see xxx).

All trials received similar agronomic treatments for the care and upkeep of the plots. 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 herbicide applications
- Growth regulator application
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants

Trials were performed using a randomized complete block design using four row plots with four replications and either 3,6 or 8 treatments. A total of 22 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, 9 were conducted to evaluate growth habit and agronomics, and six measured impact of abiotic and biotic stress. 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 2.15 Treatment schedule for agronomic field tests in 2007 and 2008

Entry	*Treatment*	*Description*
Coker	Not sprayed	Non-transgenic parent unsprayed
T304-40xGHB119	Sprayed with glufosinate ammonium	Transgenic TwinLink sprayed
T304-40xGHB119	Not sprayed	Transgenic TwinLink sprayed

Plant mapping was conducted on 5 consecutive plants in the plots which were representative of general field conditions. Plant height, number of nodes, first fruiting position, second fruiting position, third fruiting position (one location), and total boll count (including vegetative bolls) were taken as a measure of agronomic performance throughout the year. This data showed the development and potential reproductive success of the cotton plant as an indication of the yield of the plant. Visual ratings of 1-9 (1 = most favorable rating and 9 = least favorable rating) were taken for lodging and boll type. Visual ratings of 1-2 (1=normal and 2=abnormal) were taken for morphology characteristics.

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%. Least significant difference (LSD) and coefficients of variance (CV) are presented in data tables 4.1 - 4.10).

Materials and methods for efficacy studies

Materials

Materials for efficacy evaluation were created at field sites in 2007 and 2008 in the southeastern, mid-southern, and mid-western regions of the United States. Six locations in 2007 and eight locations in 2008 in six states were used to produce the reference material for fiber analysis, agronomic performance, and lepidopteran control efficacy. External university and USDA trials consisted of 8 treatment regimes; 4 genotypes either treated or untreated for lepidopteran pests (Table 2.2). Internal locations had the same treatments in 2007, however, the treated T304-40 and GHB119 were dropped in 2008.

Table 2.16 Treatment schedule for external/university efficacy field tests in 2007 and 2008

Entry	*Treatment*	*Description*
Coker	Unsprayed	No lepidopteran supplemental control
Coker	Sprayed	Lepidopteran supplemental control with conventional insecticides
T304-40	Unsprayed	No lepidopteran supplemental control
T304-40	Sprayed	Lepidopteran supplemental control with conventional insecticides
GHB119	Unsprayed	No lepidopteran supplemental control
GHB119	Sprayed	Lepidopteran supplemental control with conventional insecticides
T304-40xGHB119	Unsprayed	No lepidopteran supplemental control
T304-40xGHB119	Sprayed	Lepidopteran supplemental control with conventional insecticides

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

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 herbicide applications
- Growth regulator application
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants

Trials were performed using a randomized complete block design using two row plots with four replications of four treatments. A total of 8 efficacy parameters were used to measure the amount of plant damage and lepidopteran larval survival. Damage assessments and larval survival were taken on squares, plant terminals, white flowers, and bolls at multiple time frames through fruiting period (n=2-8). An additional 22 agronomic parameters were also evaluated.. Of the 22 potential agronomic parameters observed, six determined yield quality and quantity, 10 were conducted to evaluate growth habit and agronomics, and six were visual measures of plant morphology, biotic and abiotic stress (disease and lodging). These parameters were used to evaluate equivalence in crop development, fiber qualities, and environmental tolerance.

Plant mapping was conducted on 5 consecutive plants in the plots which were representative of general field conditions. Plant height, number of nodes, first fruiting position, second fruiting position, third fruiting position (one location), and total boll count (including vegetative bolls) were taken as a measure of agronomic performance throughout the year. This data showed the development and potential reproductive success of the cotton plant as an indication of the yield of the plant. Visual ratings of 1-9 (1 = most favorable rating and 9 = least favorable rating) were taken for lodging and boll type. Visual ratings of 1-2 (1=normal and 2=abnormal) were taken for morphology characteristics.

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%. Least significant difference (LSD) and coefficients of variance (CV) are presented in data tables 4.1 - 4.10).

Method for agronomic efficacy studies

Artificial Infestations of *Helicoverpa* spp. (Cotton Bollworm) in Bt cotton

Objective: Artificial Infestations

This method increases cotton bollworm (CBW) pressure in experimental field trial plots testing Bt cotton. Timing of infestations will vary depending on location, but should commence at early bloom. Weekly infestations will continue for 6 consecutive weeks.

Materials and Methods

1) The R&D specialist in your region will arrange for egg orders according to trial planting date and supply sufficient corn grits for entire season. Eggs should be kept in temperature controlled environment upon arrival.
2) Preparation of inoculants:
    a. Weigh 25g of corn grits per plot to be infested (ex. 2 untreated, infested plots with 4 replications would be 8 total plots, or 200g of corn grits) into a large Tupperware container.
    b. Gently mix egg shipment, corn grits, and 25mL of water by rotating and swirling container. The total egg count in shipment will be determined prior to arrival at testing location.

Figure 2.1

    c. Obtain total number of application devices needed for all plots (For example above, 8 total containers). An example of an application device is shown in Figure 2.X. Equally divide mixture into application devices.
    d. Incubate mixture 12-24 hours to allow for egg hatching.
3) Once neonates are observed in the mixture, infestation of experimental plots can proceed**.** Place application devices into a cooler with cool packs for transport to the field. Place paper barrier between cool packs and application devices to minimize condensation.
4) Only infest unsprayed plots. Evenly distribute corn grit and neonate mixture to the center two rows of all experimental plots. All infestations should be carried out during the early morning. Avoid infesting plants after 10am when higher temperatures will cause significant insect mortality. Leaves should be slightly damp prior to infestation. Moisture should be present with morning applications, but if not, use a spray bottle to dampen cotton terminals prior to infestation. For the 5th and 6th week infestation, dispense eggs and grits into white flowers.

Damage Assessments after Infestations

Insect damage ratings and larval counts should be conducted prior to the first infestation. Weekly assessments will be conducted 5-7 days after infestations for the subsequent 5-6 weeks. Trial protocols with describe insect and damage assessments needed for trial.

Assessing the plots.

Ensure that you assess the plots prior to each infestation. For the first 4 infestations, concentrate sampling in the top third of the plants. For the 5th and 6th infestation, begin checking for insect and damage lower in the plants and commence boll assessments. Continue to assess the plots for 2 weeks after the final infestation.

5-7 days after each infestation, record larval survival in the unsprayed untreated control (UTC) 'check' plots of Coker conventional cotton. We are aiming for an average of 1 surviving larva per cotton plant. For the 5th and 6th infestation, dispense eggs and grits into white flowers.

Control of Secondary Insect Pests

All plots should be sprayed with selective chemistry for non-lepidopteran pests as required by best local agronomic practices. Conventional insecticides should be applied to 'Sprayed' or 'Treated' plots to minimize lepidopteran damage. Allow 48 hours after an infestation before applying any insecticide sprays.

Method for Terminal Leaf Bioassays of Helicoverpa spp. (Cotton Bollworm) in Bt cotton

Objective: Bioassays

Leaf tissue bioassays will be performed in parallel with artificial infestations of CBW neonates. First in-star CBW larvae will be placed on leaf material taken weekly from experimental plots for six consecutive weeks. Mortality will be compared between transgenic lines and their conventional counterparts to determine Bt efficacy against CBW.

Materials and Methods

1) Each shipment of CBW eggs for field infestations will also have neonates on artificial diet. Neonates on diet will be left overnight to reach the L1 stage.
2) At each sampling date 10-20 of the most recently expanded leaves will be sampled from each plot prior to infestation. Sampled leaves should measure approximately 5 cm across and be removed from the plant with as much petiole attached as possible. Leaves from each replicate are kept separate in labeled paper sacks and placed in a small plastic cooler containing 'blue ice' for transport to the laboratory bioassay the same day.
3) Place individual leaves in a 50 x 9 mm Tight-Fit Lid sealing Petri dish (BD Falcon #351006, VWR International) and infest with a single day old fed L1 larva. Infested larvae should be of consistent size and movement for all leaf samples.
4) Bioassays are kept at a constant temperature (25-30 degrees centigrade) and >50% relative humidity prior to assessment.
5) Five days after exposure (DAE), larvae are prodded with a camel-hair brush and considered alive if coordinated movement is observed. Larvae from each dish are scored according to the criteria in Table 2.2 and Figure 3 :

Sampling and Scoring

Table 2.17 Sampling schedule for bioassays

	Sample number and timing						
Protocol #	***Unsprayed* plots evaluated***	***1 64 DAP***	***2 71 DAP***	***3 78 DAP***	***4 85 DAP***	***5 92 DAP***	***6 99 DAP***
BD-08-NAR-HA8	Coker						
	TwinLink						

* Plots unsprayed for lepidopteran pests – see protocols.

Table 2.18 Scoring criteria for bioassays

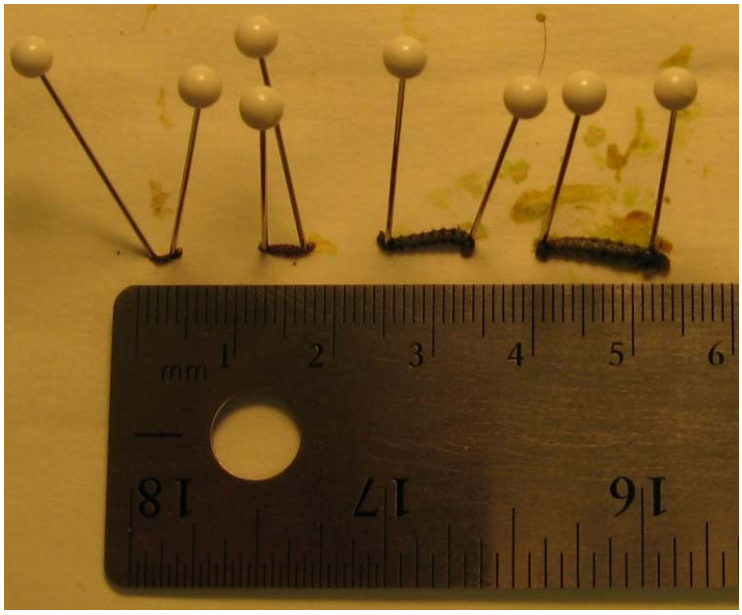
Condition	*Score*
Dead	0
Alive L1	1
Alive L2	2
Alive L3	3

Analysis

Percent survival and development of larvae is analyzed using SCOUT or REML-ANOVA, and means are separated according to Fisher's Protected LSD (Littell *et al.* 1996; PROC MIXED, SAS Institute 2001).

Figure 2.2 Bioassay set up

Figure 2.3 Size comparison for bioassay.



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

TwinLink cotton plants containing events T304-40 and GHB119, along with cotton plants representing the non-transgenic (non-transformed) counterpart Coker 315 were field tested by Bayer CropScience in 2007 under USDA APHIS Notification 07-059-101n. Field trials were established in EPA Regions II, IV, and VIII in the following locations: Trial number 02-01-Tift County, Georgia; Trial number 02-02-Tift County, Georgia; Trial 04-03-Jackson County Arkansas; Trial number 04-04-Crittenden County, Arkansas; Trial number 04-05-Crittenden County, Arkansas;Trial number 04-06-Tate County, Mississippi; Trial number 08-08-Hockley County, Texas. These areas are typical cotton growing regions of the southeastern United States, and 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. The test plots were randomized at each field location. Seed for planting was supplied for each field trial by Bayer CropScience.

Three of the transgenic event plots in each field trial were sprayed two times with Ignite 280 SL glufosinate-ammonium herbicide, and the other plots were untreated. The Ignite 280 SL herbicide contained a labeled concentration of 2.34 pounds per U.S. gallon of glufosinate-ammonium active ingredient (280 grams of active ingredient per liter). Each application of Ignite herbicide was made at a target rate of 29 fluid ounces of product per acre (2.1 liters per hectare), equivalent to 0.53 lb ai/acre (0.60 kg ai/hectare).

Three replicate non-transgenic samples and six replicate transgenic samples (3 untreated and 3 treated with Ignite herbicide) of fuzzy seed were collected from each of the field test sites and shipped frozen to Bayer CropScience BioAnalytics lab, Research Triangle Park, North Carolina. The fuzzy seeds were sub-sampled and shipped frozen to the analytical facility, Covance Laboratories, Inc., Madison, Wisconsin for composition analyses. Shipping and storage of the regulated seed was carried out under applicable USDA regulations and Bayer CropScience guidelines.

Characterization of the material

Certificates of Analysis (COA) were produced by Bayer CropScience for the two seed lots used for planting in this study. Identity and purity of the TwinLink cottonseed and the corresponding non-transgenic (Coker 315) cottonseed were confirmed to be acceptable for use. A COA to determine identity of the samples harvested from the seven field sites was prepared by Bayer CropScience. The data certified that the transgenic samples harvested from the field were indeed TwinLink cotton, and that the non-transgenic samples were harvested from non-transgenic plots.

Analytical procedures

The composition of the cottonseed was determined at Covance Laboratories, Inc. The analyses performed and the methods used are listed in Table 2.19.

Table 2.19 Analyses Performed on TwinLink Cottonseed and Its Non-transgenic Counterpart

Parameter	*Covance Mnemonic*	*Covance Method Reference*
Moisture	M100	AOAC 926.08 and 925.09
Crude protein	PGEN	AOAC 955.04 and 979.09
Crude fat	FAAH	AOAC 922.06 and 954.02
Ash	ASHM	AOAC 923.03
Carbohydrate	CHO	Difference between 100 and the sum of moisture, crude protein, fat, and ash. Agric. Handbook No. 74
Acid Detergent Fiber	ADF	Agric. Handbook No. 379
Neutral Detergent Fiber	NDFE	AACC 32.20 + Agric. Handbook No. 379
Ca, K, Mg, Fe, Zn, P	ICPS	AOAC 984.27 and 985.01
Vitamin E and Tocopherols	TTLC	HPLC method is based on the references below
Amino Acids	TAA5	AOAC 982.30
Fatty Acids	FALC	AOAC 996.06 and AOCS Ce 1-62
Phytic acid	PHYT	HPLC method is based on the references below
Gossypol (total)	GOSS	AOCS Ba 7-58 abd Ba 8-78
Gossypol (free)	GOSF	AOCS Ba 7-58 abd Ba 8-78
Amino Acids	TAA5	AOAC 982.30
Cyclopropenoid Fatty Acids	CPFA	HPLC method is based on the references below

Data were obtained from the transgenic unsprayed, transgenic sprayed and non-transgenic samples and presented on a fresh weight basis. The data were also presented on a dry weight basis by correcting the fresh weight values for the moisture content determined for each sample. Mean values and standard deviations were calculated for the dry matter data. Fatty acid data were presented on a fresh weight basis and additionally, together with the cyclopropenoid fatty acid data, as relative quantities of the total sum of fatty acids.

Appendix 3. CHARACTERIZATION OF TWINLINK COTTON

1. Verification of the insert in Cry1Ab cotton event T304-40

Genomic DNA isolated from Cry1Ab cotton event T304-40 was digested with one of the following restriction enzymes: *Apa*I, *Dra*III, *Eco*RI, *Eco*RV, *Nde*I, *Bgl*I, *Swa*I, *Ssp*I, *Sac*I and *Xba*I. Wild type genomic DNA digested with *Xba*I was used as negative control; wild type genomic DNA digested with *Xba*I and supplemented with an equimolar amount of pTDL008 plasmid DNA digested with *Xba*I was used as positive control. The resulting DNA fragments were analyzed by Southern blot with six different probes, corresponding to the different genetic elements contained in the pTDL008 T-DNA. Probe information is presented in Table 3.1. A schematic overview of the Southern blot strategy is presented in Figure 3.1.

Table 3.2 summarizes the expected and obtained hybridization fragments. An overview of the obtained Southern blot results is presented in Figure 3.2.

The results of the Southern blot analysis demonstrate that the inserted transgenic sequence in Cry1Ab cotton event T304-40 consists of one nearly complete copy of the T-DNA flanked by an inverted incomplete copy of the *cry*1Ab gene cassette and one additional 3'me1 terminator.

Table 3.1 Event T304-40 Insert verification – probe information

Probe template ID	*Genetic element*	*Size probe template*
PT020-1	3'me1 probe	859 bp
PT021-1	*cry*1Ab probe	1822 bp
PT022-1	P35S3 probe	801 bp
PT023-1	*bar* probe	425 bp
PT024-1	3'nos probe	214 bp
PT040-1	5'e1-Ps7s7 probe	1144 bp
PT041-3	T-DNA probe	5050 bp

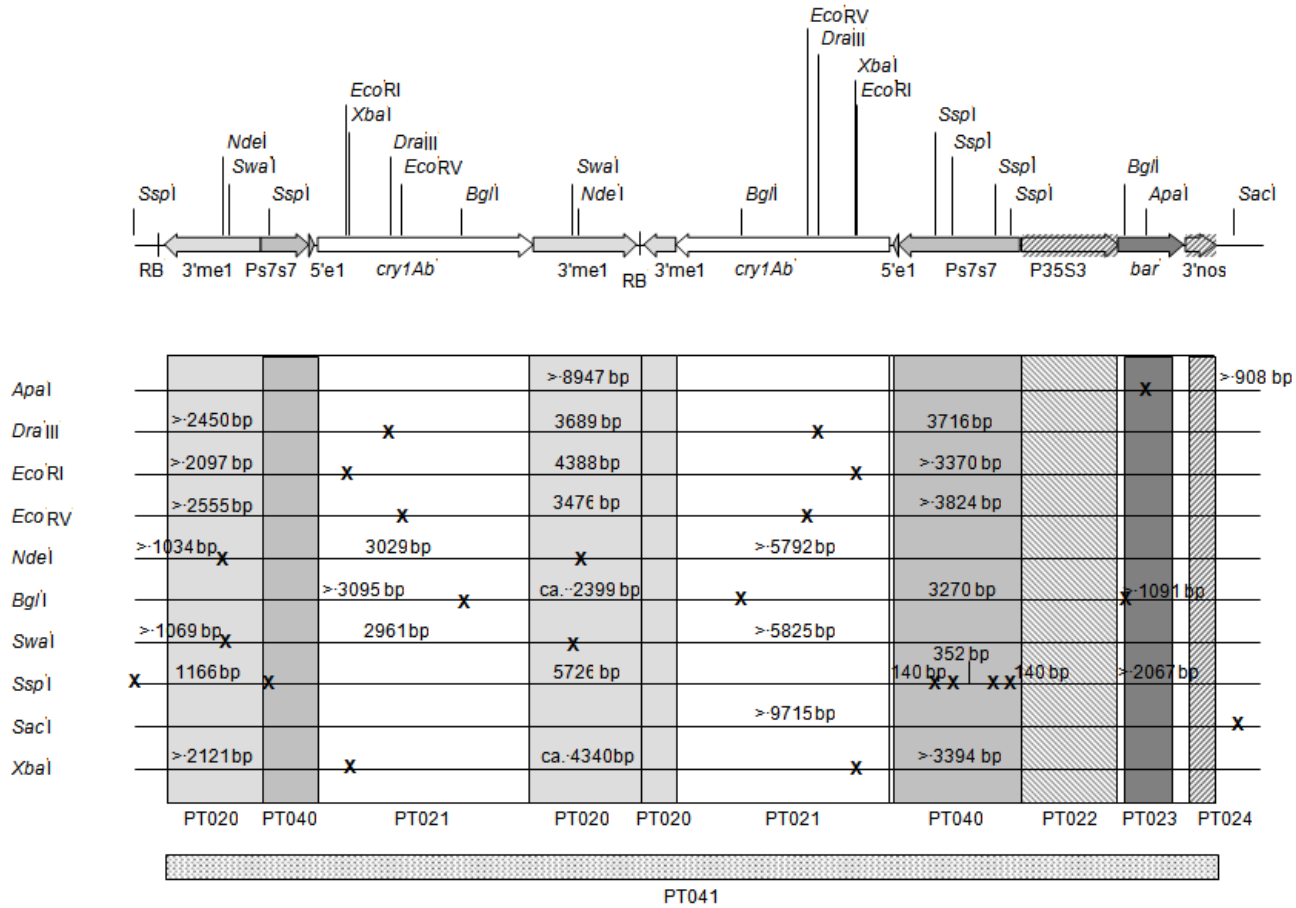


Figure 3.1 Schematic overview of Southern blot strategy for Cry1Ab cotton event T304-40

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Table 3.2 Expected and obtained hybridization fragments – Cry1Ab cotton event T304-40

				PT020: 3'me1		PT021: cry1Ab		PT022: P35S3		PT023: bar		PT024: 3'nos		PT040: 5'e1-Ps7s7		PT041: T-DNA	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
ApaI	5' integration fragm.	> 8947	> 14 Kb	Yes	Yes	Yes	Yes	Yes	No*	Yes	No*	No	No	Yes	Yes	Yes	Yes
	3' integration fragm.	> 908	3400	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	Yes	No*
DraIII	5' integration fragm.	> 2450	2550	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	internal fragment	3689	3689	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	3' integration fragm.	> 3716	> 14 Kb	No	No	Yes	No*	Yes	No*	Yes	No*	Yes	Yes	Yes	Yes	Yes	Yes
EcoRI	5' integration fragm.	> 2097	2850	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	internal fragment	4388	4388	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	3' integration fragm.	> 3370	5100	No	No	Yes	No*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
EcoRV	5' integration fragm.	> 2555	3100	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	internal fragment	3476	3476	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	3' integration fragm.	> 3824	6800	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
NdeI	5' integration fragm.	> 1034	5400	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No*
	internal fragment	3029	3029	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	3' integration fragm.	> 5792	12000	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
BglI	5' integration fragm.	> 3095	> 14 Kb	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	internal fragment	2399	2399	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	internal fragment	3270	3270	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes
	3' integration fragm.	> 1091	1900	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	Yes	No*
SwaI	5' integration fragm.	> 1069	1450	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No*
	internal fragment	2961	2961	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	3' integration fragm.	> 5825	7800	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
SspI	5' integration fragm.	1166	1166	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes	Yes	No*
	internal fragment	5726	5726	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	internal fragment	(140)	Too small	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	No
	internal fragment	352	352	No	No	No	No	No	No	No	No	No	No	Yes	Yes	Yes	No*
	internal fragment	(140)	Too small	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	No
	3' integration fragm.	> 2067	4500	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
SacI	Complete insert	> 9715	> 14 Kb	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
XbaI	5' integration fragm.	> 2121	3400	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	Internal fragment	4340	4340	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	3' integration fragm.	> 3394	5100	No	No	Yes	No*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
WT - *XbaI*	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None
WT + 1 copy pTDL008 - *XbaI*	plasmid fragment	3100	3100	No	No	Yes	No*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	plasmid fragment	11293	11293	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes

(*) Weak or no hybridization can be caused by the small overlap between probe template and fragment, ratio probe length/fragment length, hybridization efficiency and blotting efficiency.

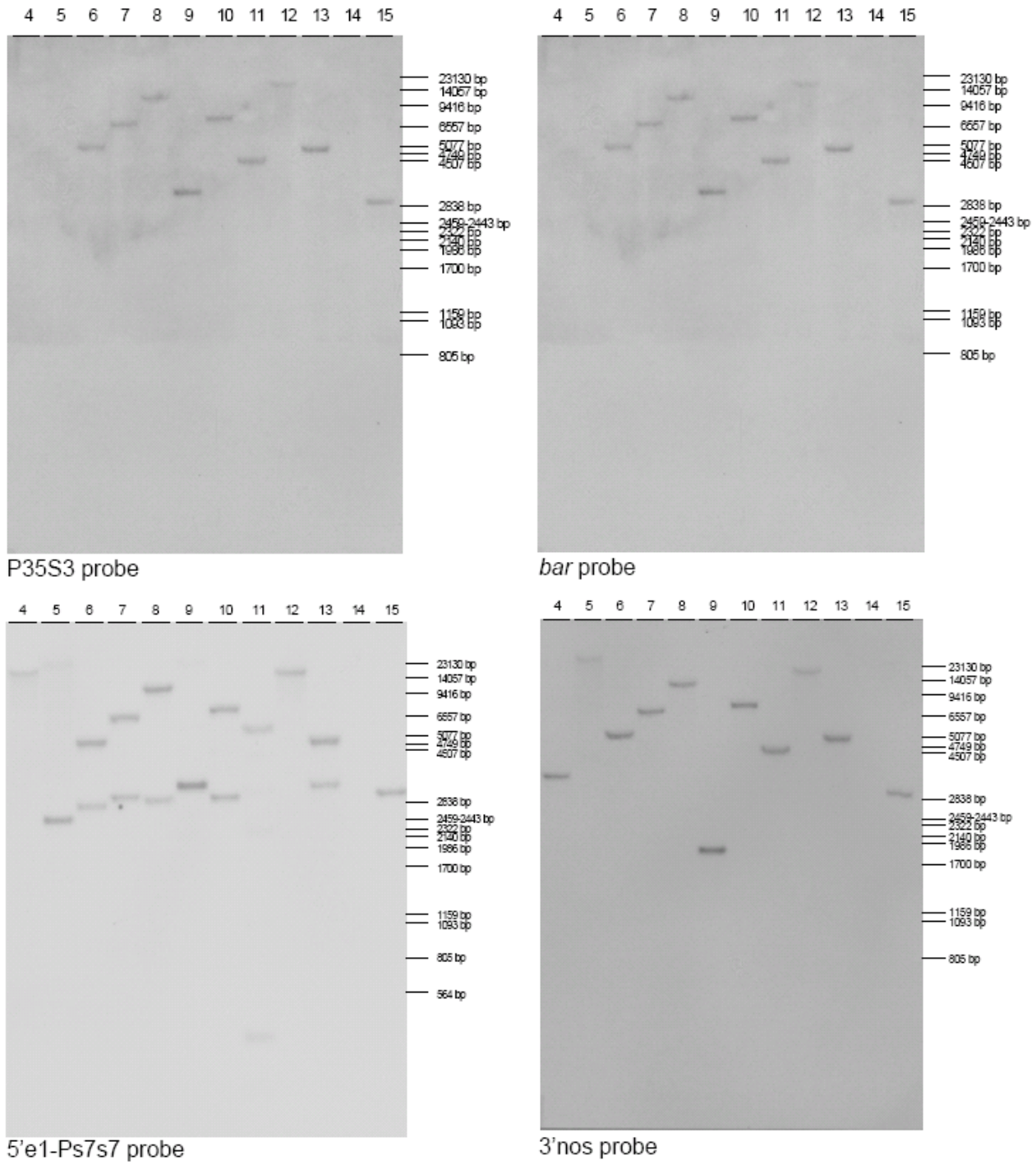
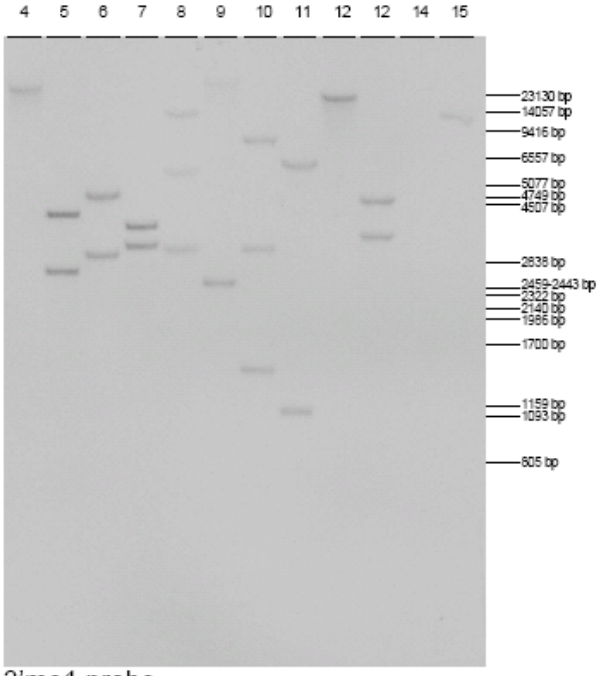
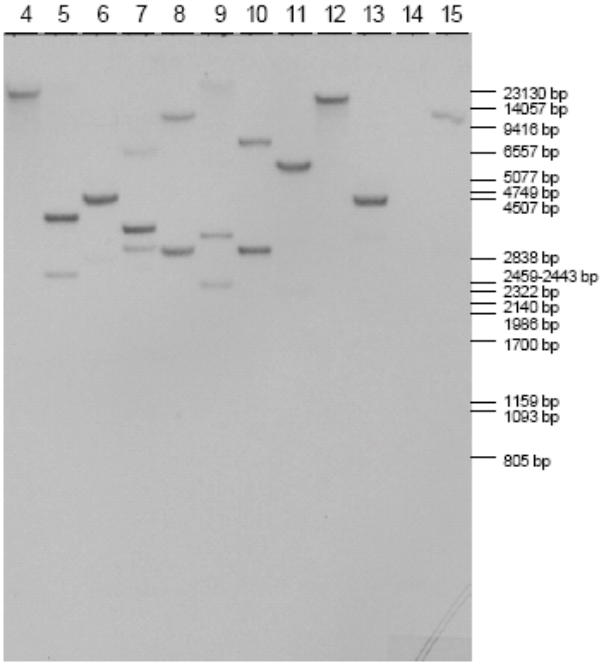


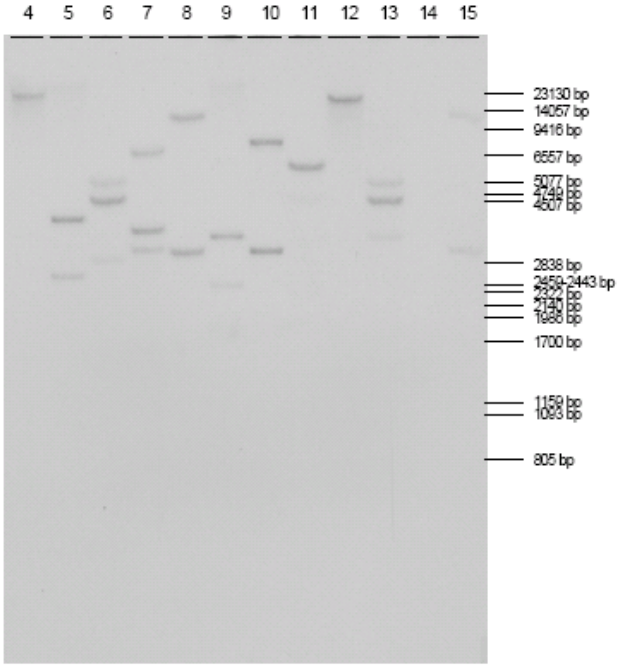
Figure 3.2 Cry1Ab cotton event T304-40 Insert verification – Southern blot results



3'me1 probe



cry1Ab probe



T-DNA probe

Lane 4:	T304-40 – *Apa*I digested
Lane 5:	T304-40 – *Dra*III digested
Lane 6:	T304-40 – *Eco*RI digested
Lane 7:	T304-40 – *Eco*RV digested
Lane 8:	T304-40 – *Nde*I digested
Lane 9:	T304-40 – *Bgl*I digested
Lane 10:	T304-40 – *Swa*I digested
Lane 11:	T304-40 – *Ssp*I digested
Lane 12:	T304-40 – *Sac*I digested
Lane 13:	T304-40 – *Xba*I digested
Lane 14:	Wild type cotton variety FM966 *Xba*I digested
Lane 15:	wild type variety FM966 *Xba*I digested + an equimolar amount of pTDL008 *Xba*I digested

Figure 3.2 (Continued) Cry1Ab cotton event T304-40 Insert verification – Southern blot results

2. Verification of the insert in Cry2Ae cotton event GHB119

Genomic DNA prepared from GHB119 plants was digested with one of the following enzymes or enzyme combinations: *Dra*I, *Eco*RV, *Hind*III, *Nco*I, *Bam*HI, *Eco*RI, *Pst*I, *Ava*I, *Nde*I, *Xho*I, *Eco*RI/*Pst*I and *Eco*RI/*Nde*I. The resulting DNA fragments were analyzed by Southern blot with six different probes, corresponding to the different genetic elements contained in the pTEM12 T-DNA.

Probe information is given in Table 3.3, and a schematic of the Southern blot strategy is presented in Figure 3.3. Table 3.4 summarizes the expected and obtained hybridization fragments and an overview of the Southern Blot results is shown in Figure 3.4.

Hybridization results demonstrated that a single copy of the T-DNA is integrated in event GHB119 and that the configuration of the inserted DNA corresponds to that of the original transformation vector.

Table 3.3 Cry2Ae cotton event GHB119 Insert verification – probe information

Probe template ID	*Description*	*Size probe template*
PT026	T-DNA probe	4325 bp
PT024	3' nos probe	214 bp
PT023	*bar* probe	425 bp
PT044	PCsVMV probe	572 bp
PT045	P35S2-5'cab22L probe	532 bp
PT046	TPssuAt-*cry*2Ae probe	2100 bp
PT047	3'35S-RB probe	264 bp

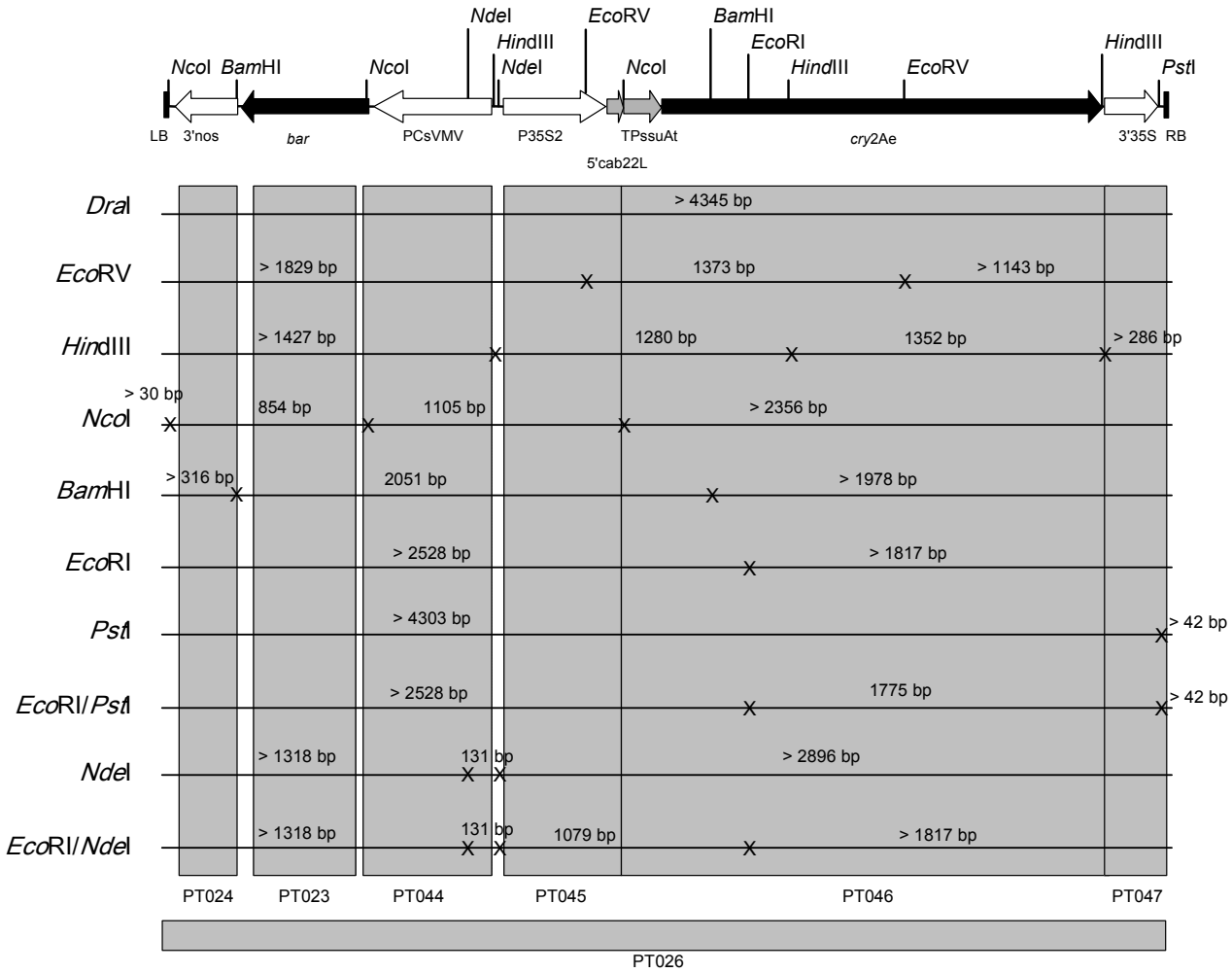


Figure 3.3 Schematic overview of Southern blot strategy for Cry2Ae cotton event GHB119

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Table 3.4 Expected and obtained hybridization fragments – event GHB119

Digest	Description	Expected fragment sizes (bp)	Obtained fragment sizes (bp)	PT024: LB-3'nos		PT023: *bar*		PT044: Pcsvmv		PT045: P35S2-5'cab22L		PT046: TPssuAT-*cry2Ae*		PT047: 3'35S-RB		PT026: T-DNA probe	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
*Dra*I	Complete insert	>4345	4430	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
*Eco*RV	5' integration fragm. internal fragm. 3' integration fragm.	>1829 1373 >1143	3380 1373 8890	Yes No No	Yes No No	Yes No No	Yes No No	Yes No No	Yes No No	Yes Yes No	Yes Yes No	No Yes Yes	No Yes Yes	No No Yes	No No Yes	Yes Yes Yes	Yes Yes Yes
*Hin*dIII	5' integration fragm. internal fragm. internal fragm. 3' integration fragm.	>1427 1280 1352 >286	5150 1280 1352 8830	Yes No No No	Yes No No No	Yes No No No	Yes No No No	Yes No No No	Yes No No No	No Yes No No	No Yes No No	No Yes Yes No	No Yes Yes No	No No No Yes	No No No Yes	Yes Yes Yes Yes	Yes Yes Yes No**
*Nco*I	5' integration fragm. internal fragm. 3' integration fragm.	854 1105 >2356	854 1105 5940	Yes No No	Yes No No	Yes No No	Yes No No	No Yes No	No Yes No	No Yes No	No Yes No	No No Yes	No No Yes	No No Yes	No No Yes	Yes Yes Yes	Yes Yes Yes
*Bam*HI	5' integration fragm. internal fragm. 3' integration fragm.	>316 2051 >1978	3120 2051 7070	Yes No No	Yes No No	No Yes No	No Yes No	No Yes No	No Yes No	No Yes No	No Yes No	No Yes Yes	No Yes Yes	No No Yes	No No Yes	Yes Yes Yes	Yes Yes Yes
*Eco*RI	5' integration fragm. 3' integration fragm.	>2528 >1817	5180 2480	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes Yes	Yes Yes	No Yes	No Yes	Yes Yes	Yes Yes
*Pst*I	5' integration fragm.	>4303	7800	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
*Eco*RI/*Pst*I	5' integration fragm. internal fragm.	>2528 1775	5200 1775	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes Yes	Yes Yes	No Yes	No Yes	Yes Yes	Yes Yes
*Nde*I	5' integration fragm. internal fragm. 3' integration fragm.	>1318 131 >2896	4200 No hybr. 5380	Yes No No	Yes No No	Yes No No	Yes No No	Yes No * No	Yes No No	No No Yes	No No Yes	No No Yes	No No Yes	No No Yes	No No Yes	Yes No * Yes	Yes No Yes
*Eco*RI/*Nde*I	5' integration fragm. internal fragm. internal fragm. 3' integration fragm.	>1318 131 1079 >1817	3960 No hybr. 1079 2450	Ye No No No	Yes No No No	Yes No No No	Yes No No No	Yes No * No No	Yes No No No	No No Yes No	No No Yes No	No No Yes Yes	No No Yes Yes	No No No Yes	No No No Yes	Yes No * Yes Yes	Yes No Yes Yes
WT genomic DNA - EcoRV	Negative control	No fragment.	No fragment.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
WT genomic DNA + pTEM12 - *Eco*RV	Positive control Positive control	10893 1373	10893 1373	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes Yes	Yes Yes	Yes Yes	Yes Yes	Yes No	Yes No	Yes Yes	Yes Yes

(*) The fragment is too small to be visible.
(**) The fragment is very small as compared to the probe size. As a result, this fragment is not visualized.

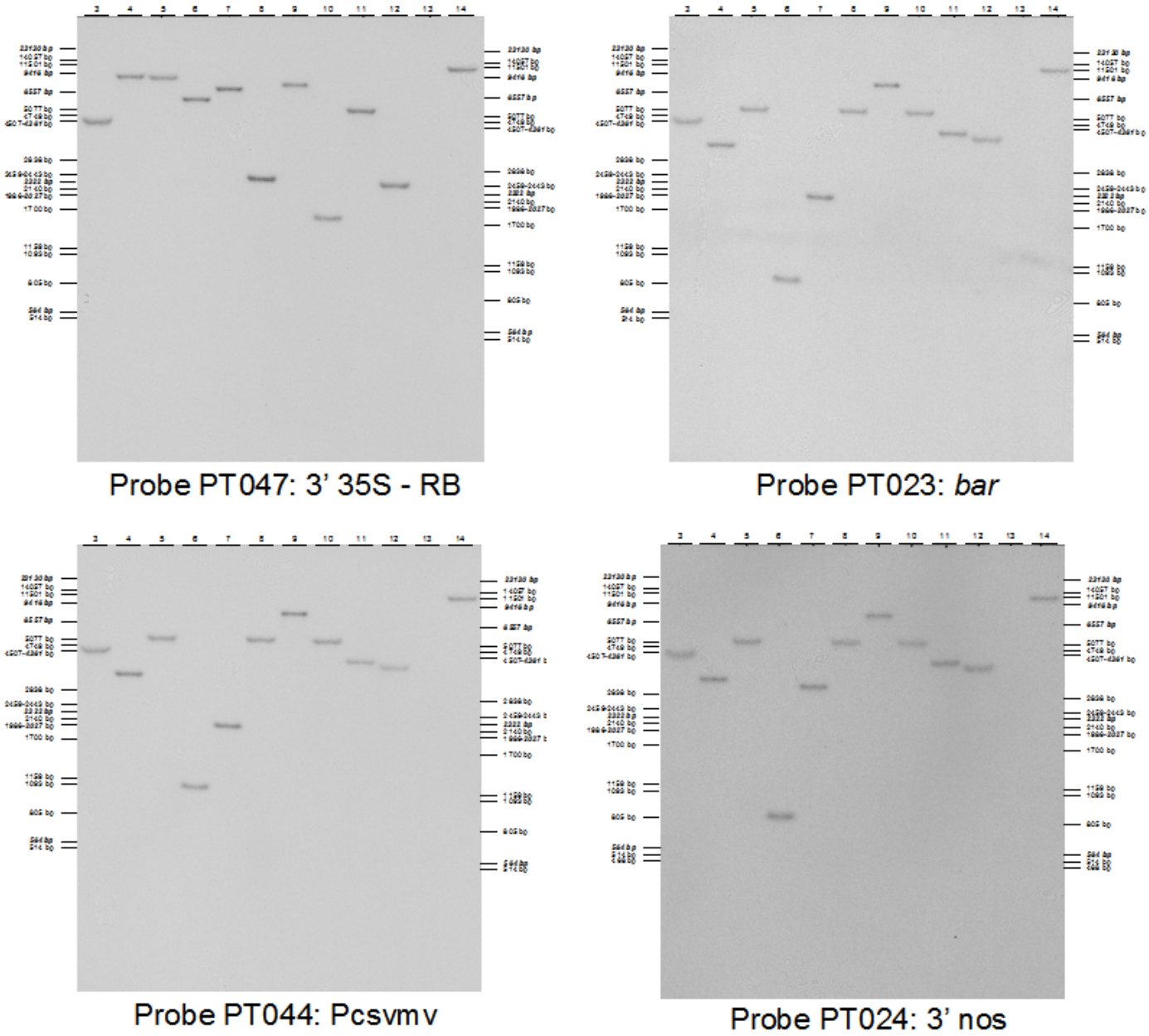
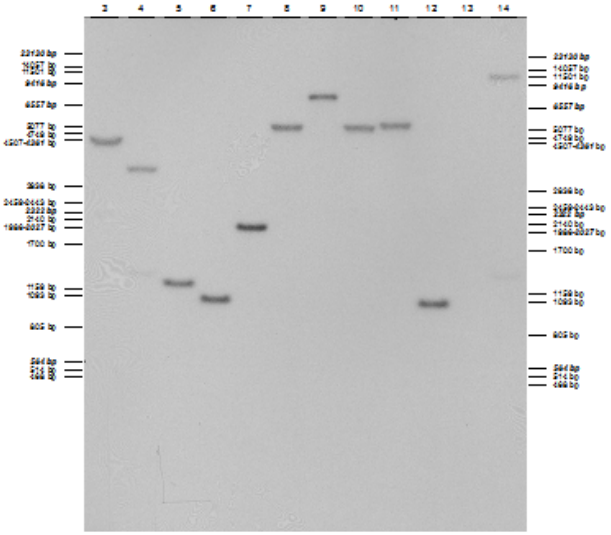
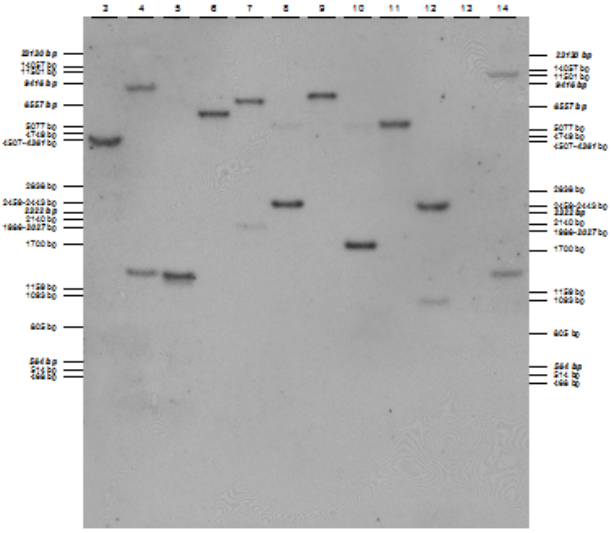


Figure 3.4 Cry2Ae cotton event GHB119 Insert verification - Hybridization results

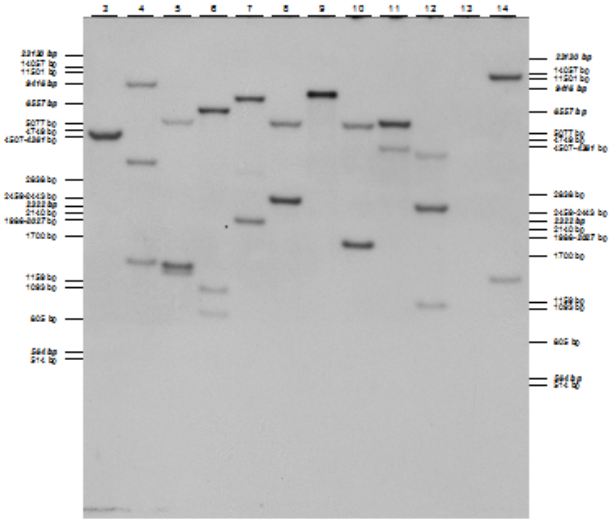
Lane 1 : Phage Lambda - *Pst*I digested
Lane 2 : Phage Lambda - *Hind*III digested
Lane 3 : GHB119 - *Dra*I digested
Lane 4 : GHB119 - *Eco*RV digested
Lane 5 : GHB119 - *Hind*III digested
Lane 6 : GHB119 - *Nco*I digested
Lane 7 : GHB119 - *Bam*HI digested
Lane 8 : GHB119 - *Eco*RI digested
Lane 9 : GHB119 - *Pst*I digested
Lane 10 : GHB119 - *Eco*RI/*Pst*I digested
Lane 11 : GHB119 - *Pst*I digested
Lane 12 : GHB119 - *Nde*I/*Eco*RI digested
Lane 13 : Wild type variety FM966 - *Eco*RV digested
Lane 14 : Wild type variety FM966 - *Eco*RV digested + an equimolar amount of pTEM12 - *Eco*RV digested
Lane 15 : Empty lane
Lane 16 : Phage Lambda - *Pst*I digested
Lane 17 : Phage Lambda - *Hind*III digested



Probe PT045: P35S2–5'cab22L



Probe PT046: TPssuAT-*cry2Ae*



Probe PT026: T-DNA

Lane 1: Phage Lambda – *Pst*I digested
Lane 2: Phage Lambda – *Hind*III digested
Lane 3: GHB119 – *Dra*I digested
Lane 4: GHB119 – *Eco*RV digested
Lane 5: GHB119 – *Hind*III digested
Lane 6: GHB119 – *Nco*I digested
Lane 7: GHB119 – *Bam*HI digested
Lane 8: GHB119 – *Eco*RI digested
Lane 9: GHB119 – *Pst*I digested
Lane 10: GHB119 – *Eco*RI/*Pst*I digested
Lane 11: GHB119 – *Nde*I digested
Lane 12: GHB119 – *Nde*I/*Eco*RI digested
Lane 13: wild type variety FM966 – *Eco*RV digested
Lane 14: wild type variety FM966 – *Eco*RV digested + an equimolar amount of pTEM12 – *Eco*RV digested
Lane 15: Empty lane
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

Figure 3.4 (Continued) Cry2Ae cotton event GHB119 Insert verification - Hybridization results

3. Genetic stability of the inserted DNA in Cry1Ab cotton event T304-40

Southern blot analysis was performed to assess the impact of environmental conditions or different backgrounds on the structural stability of the transgenic sequence and to assess the structural stability over different generations.

To demonstrate the structural stability of cotton event T304-40, genomic DNA was prepared from several individual plants of different generations and different genetic backgrounds, and from plants grown from seeds harvested at three different locations. The isolated DNA was digested with the restriction enzyme *Eco*RV, which has two recognition sites in the integrated DNA fragment. Probing *Eco*RV digested genomic T304-40 DNA with the *cry*1Ab probe (Figure 3.5) showed all three expected fragments in all samples tested. Two of these fragments represent the junctions between the transgenic sequences and the plant DNA sequences and one represents an internal fragment.

Table 3.5 summarizes the expected and obtained hybridization results. The hybridization results of the stability analysis over different genetic backgrounds are presented in Figure 3.5, over different generations in Figure 3.6, and over different environmental conditions in Figure 3.7.

Table 3.5 Expected and obtained hybridization fragments – event T304-40

Samples	*Condition tested*	*Seed lot*	*N° of plants*	*Expected fragment sizes (*)*	*Expected results obtained ?*	*Description*
Test item samples	Background A	32CON0466	20	ca 3100 bp 3476 bp ca 6800 bp	Yes Yes Yes	5' integration fragment Internal fragment 3' integration fragment
	Background C315	32CON0467	20			
	Background B	32CON0468	20			
	Background C	32CON0469	20			
	Location Vinyols	/	11			
	Location Camarles	/	18			
	Location Santa Oliva	/	18			
	Generation F_1	05GAGH000743	15			
	Generation BC_1F_1	05GAGH000742	14			
	Generation BC_2F_2	05GAGH000741	12			
	Generation BC_2F_2	05GAGH000740	18			
DNA negative control	Wild type variety FM966 (Seed lot 04GAGH003113)			No hybridization	Yes	Negative control
DNA positive control	Wild type variety FM966 + an equimolar amount pTDL008			14393bp	Yes	Positive control fragment

(*) Fragment sizes as determined in the detailed insert characterization of T304-40.

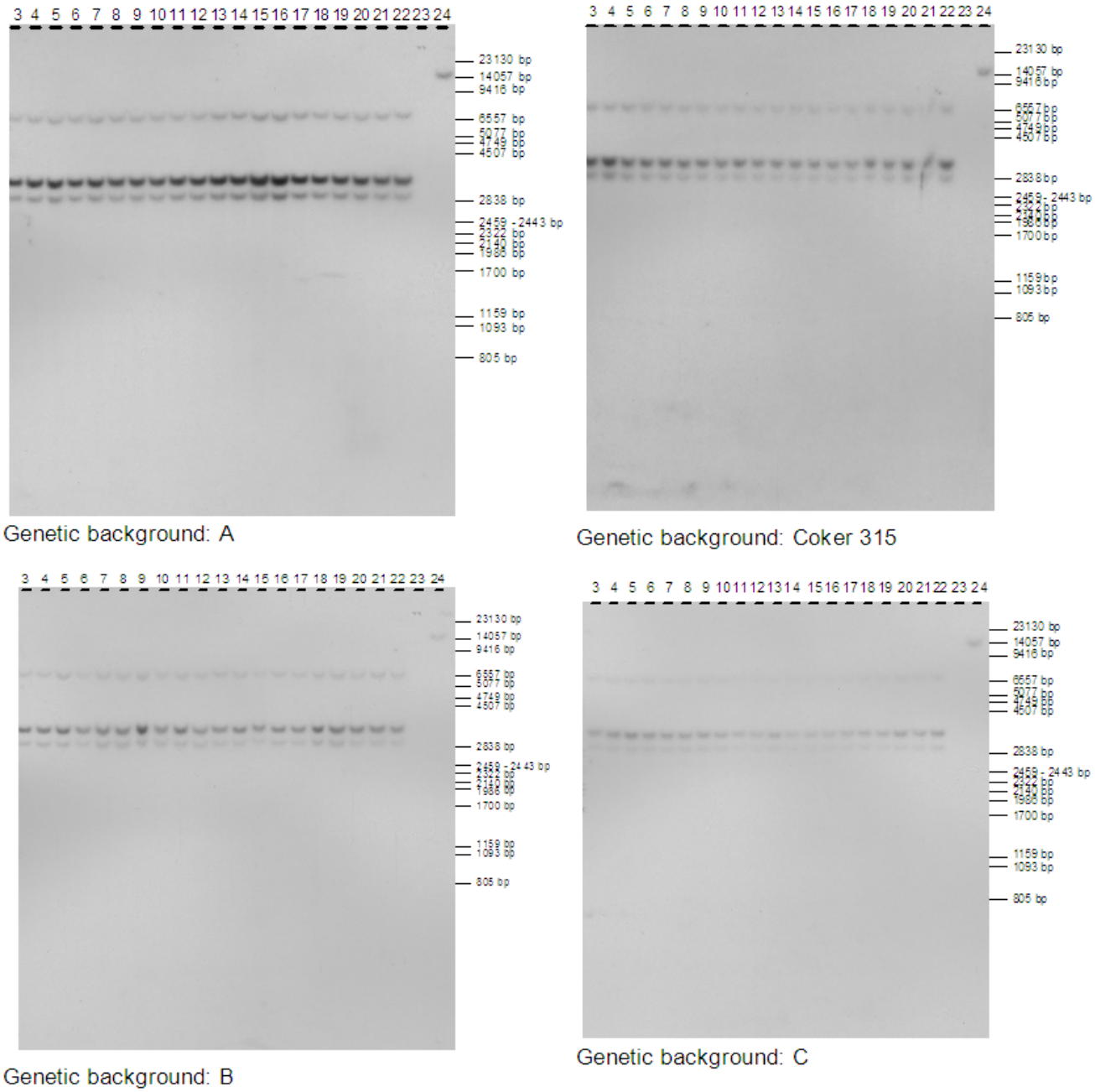


Figure 3.5 Hybridization results of the stability analysis of cotton event T304-40 in different genetic backgrounds

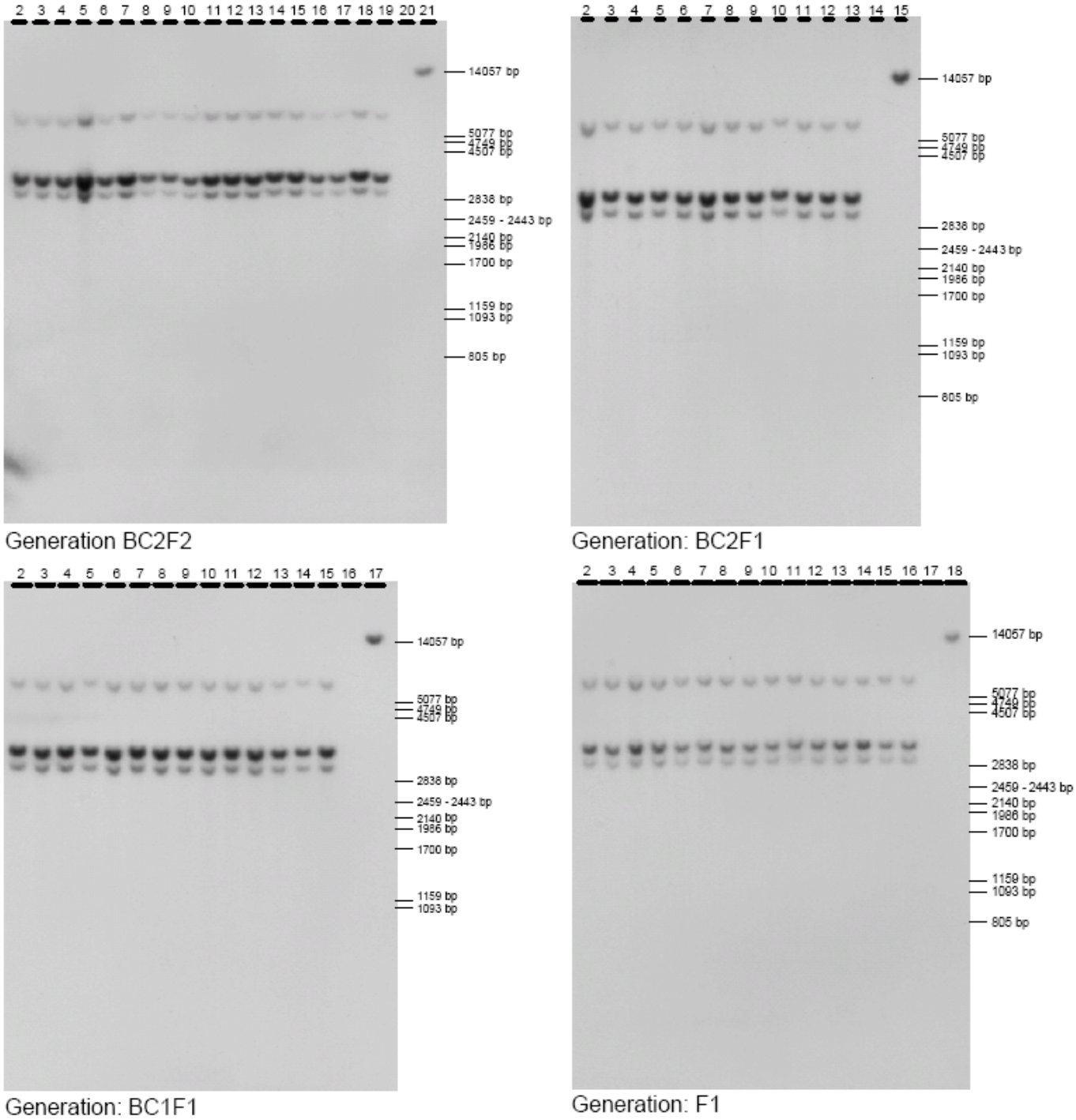
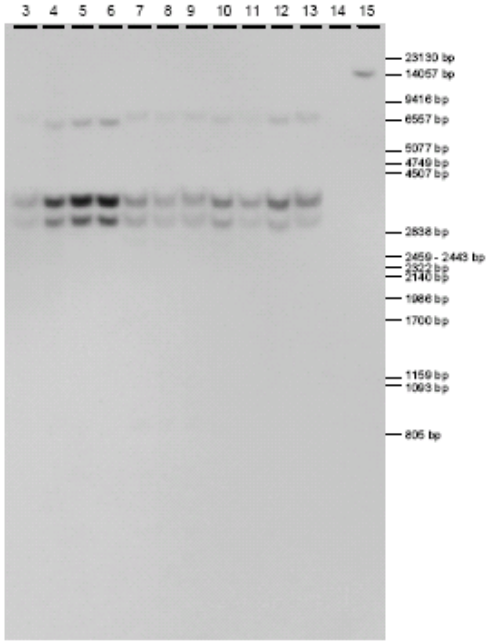
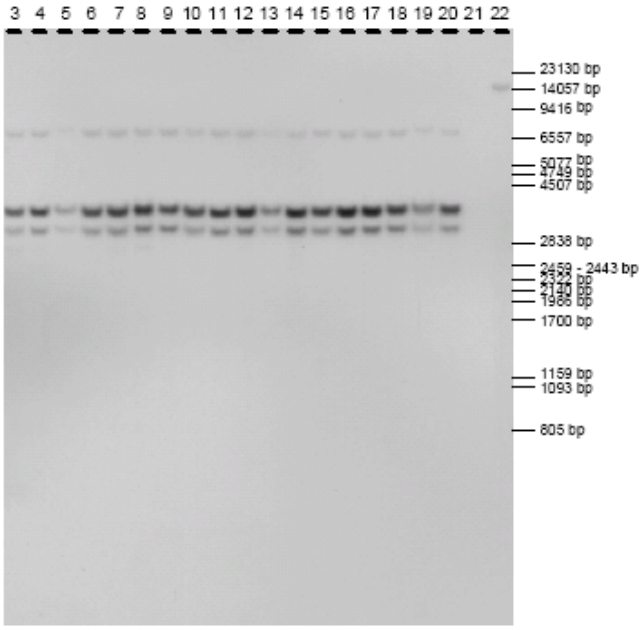


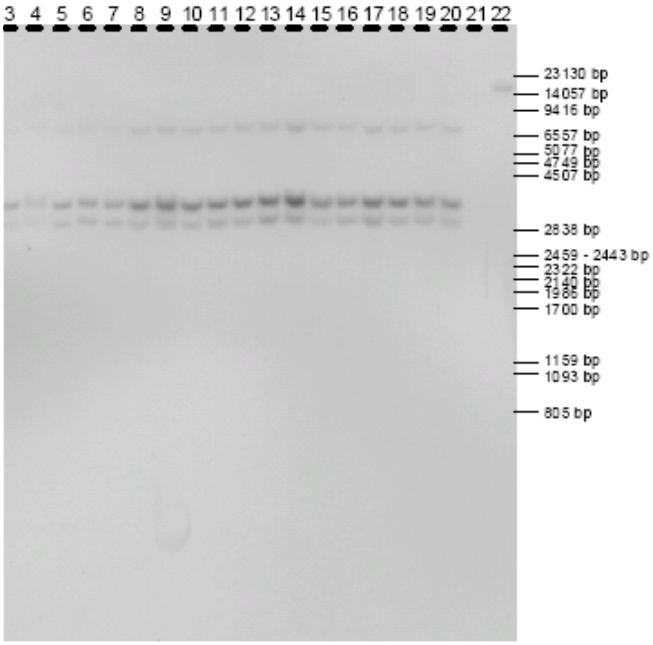
Figure 3.6 Hybridization results of the stability analysis of cotton event T304-40 over different generations



Environment: Vinyols (Spain)



Environment: Santa Oliva (Spain)



Environment: Camarles (Spain)

Figure 3.7 Hybridization results of the stability analysis of cotton event T304-40 over different environmental conditions

4. Genetic stability of the inserted DNA in Cry2Ae cotton event GHB119

The structural stability of Cry2Ae cotton event GHB119 over different generations, in different backgrounds, and over different environmental conditions was tested by means of Southern blot analysis. Genomic DNA was prepared from several individual plants of three consecutive generations and two different genetic backgrounds. The impact of environment was assessed by analyzing the progeny of plants cultivated at six different field locations. The isolated DNA was digested with the restriction enzyme *Eco*RV, which has two recognition sites in the transforming DNA. Hybridization of these samples with the T-DNA probe revealed the expected profile in all tested samples. This demonstrates the stability of cotton event GHB119 at the genomic level in different generations, different genetic backgrounds, and over different environmental conditions.

Hybridization results are shown in Figures 3.8 and 3.9, and are summarized in Tables 3.6 and 3.7.

Table 3.6 GHB119 Structural stability over generations and genetic backgrounds - Expected and obtained hybridization fragments

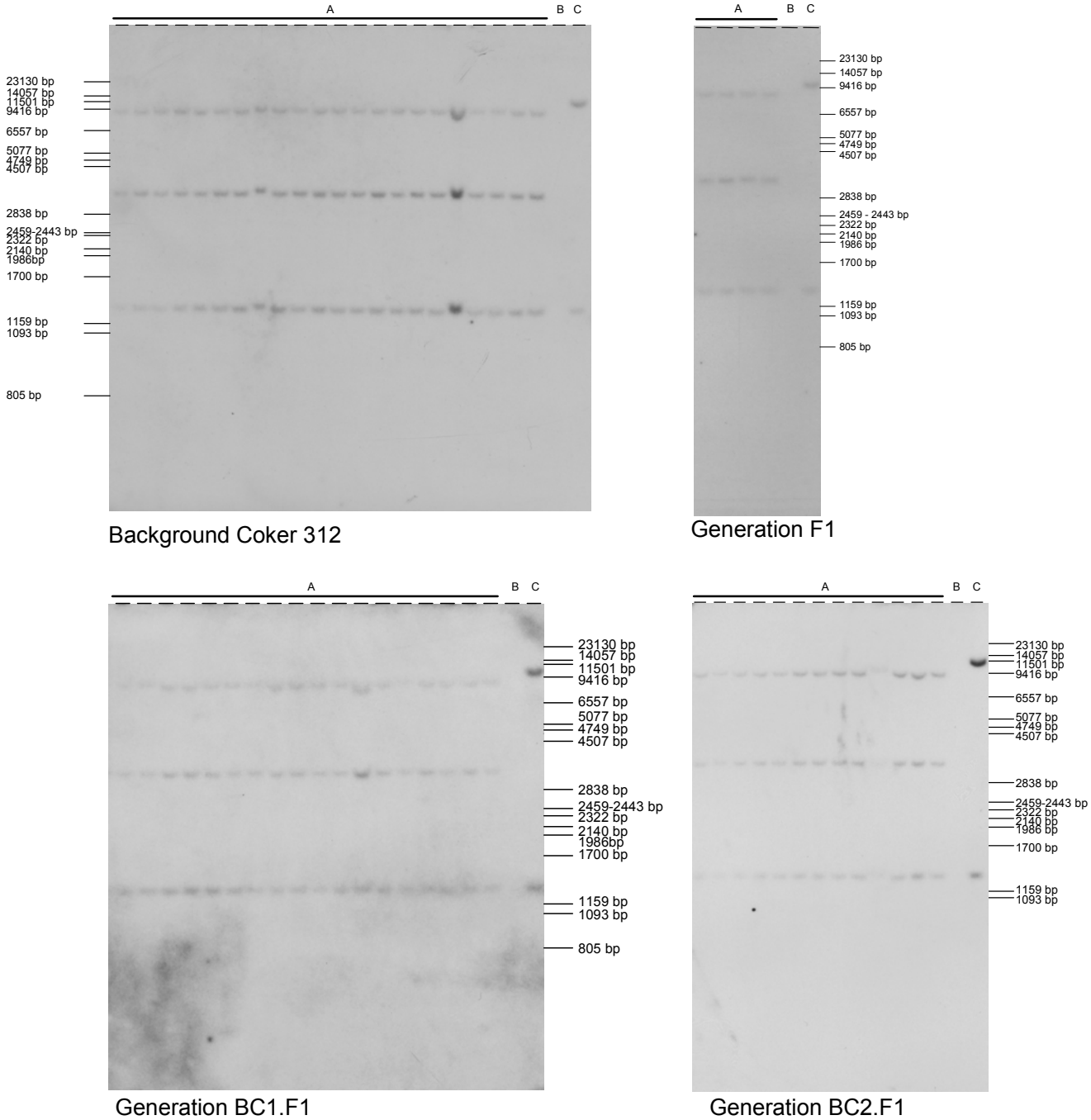
Samples	*Condition tested*	*Seedlot*	*N° of plants*	*Expected fragment sizes (*)*	*Expected fragments obtained ?*	*Description*
Test item samples	Background Coker 312	05GC03	22	ca. 3380 bp	Yes	5'integration fragment
	Generation F_1	05GC16	4			
	Generation BC_1F_1	05GC33	18	1373 bp	Yes	internal fragment
	Generation BC_2F_1	05GC59	13	ca. 8890 bp	Yes	3'integration fragment
DNA negative control	Wild type variety FM966 (Seedlot 04GAGH003113)			No hybridization	Yes	
DNA positive control	Wild type variety FM966 + an equimolar amount pTEM12			1373bp 10893 bp	Yes Yes	Positive control fragments

(*) Fragment sizes as determined in the detailed insert characterization of GHB119

Table 3.7 GHB119 Structural stability over environments - Expected and obtained hybridization fragments

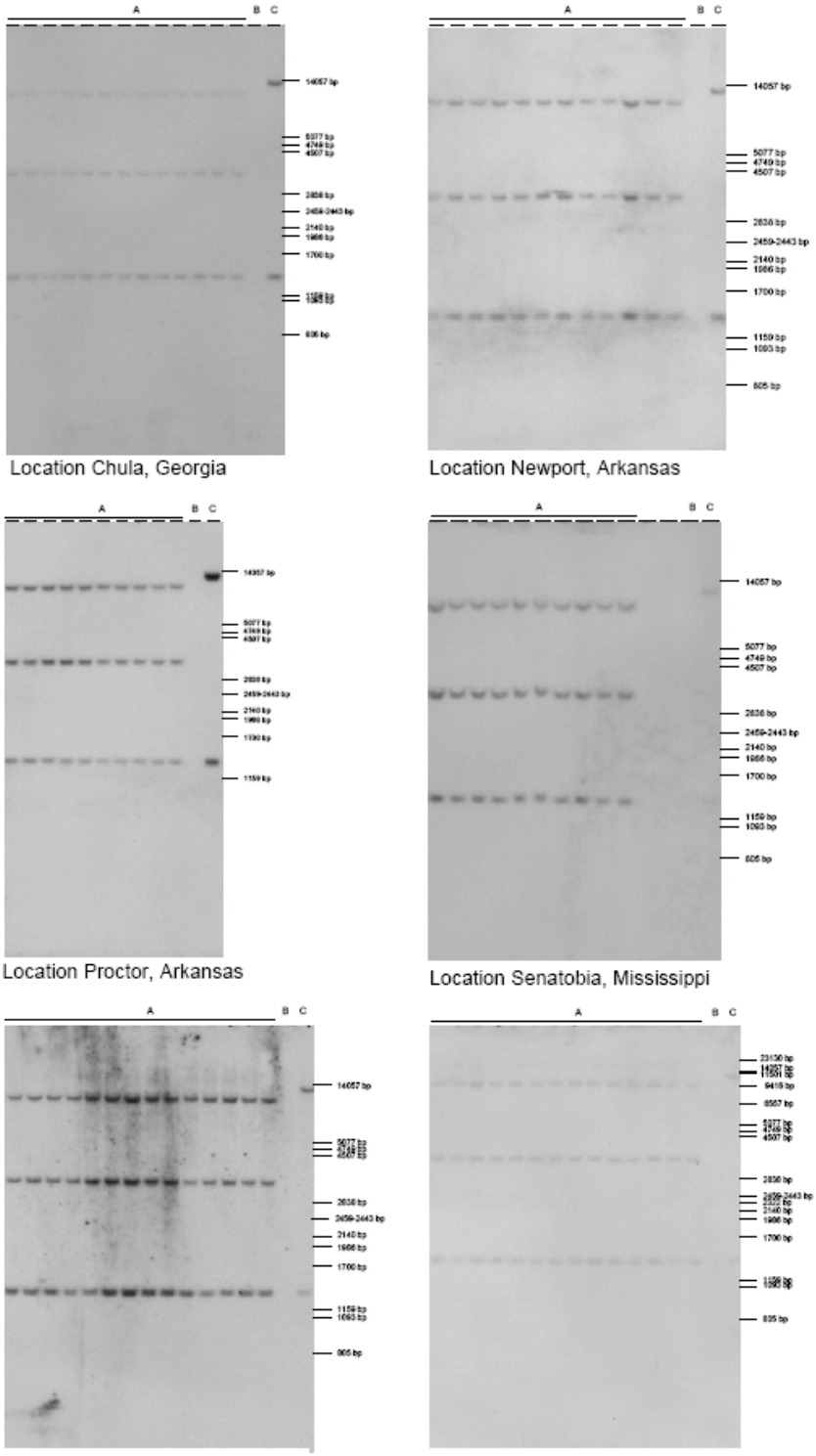
Samples	*Condition tested*	*Seedlot*	*N° of plants*	*Expected fragment sizes (*)*	*Expected fragments obtained ?*	*Description*
Test item samples	Location Chula, Georgia	CY06B001-201-31	13	ca. 3380 bp	Yes	5'integration fragment
	Location Newport, Arkansas	CY06B001-402-32	12			
	Location Proctor, Arkansas	CY06B001-403-33	10	1373 bp	Yes	internal fragment
	Location Senatobia, Mississippi	CY06B001-404-32	10			
	Location East Bernard, Texas	CY06B001-605-33	14	ca. 8890 bp	Yes	3'integration fragment
	Location Levelland, Texas	CY06B001-806-33	14			
DNA negative control	Wild type variety FM966 (Seedlot 04GAGH003113)			No hybridization	Yes	
DNA positive control	Wild type variety FM966 + an equimolar amount pTEM12			1373bp 10893 bp	Yes Yes	Positive control fragments

(*) Fragment sizes as determined in the detailed insert characterization of GHB119



A: GHB119 samples; B: Negative control; C: Positive control

Figure 3.8 Structural stability of cotton event GHB119 over genetic backgrounds and generations - Hybridization results



A: GHB119 samples; B: Negative control; C: Positive control

Figure 3.9 Structural stability of event GHB119 over environments - Hybridization results

5. Genetic Stability of TwinLink cotton (events T304-40 x GHB119)

The genetic stability of the inserted DNA in Cry1Ab cotton event T304-40 and in Cry2Ae cotton event GHB119 cotton was demonstrated in plants grown in different genetic backgrounds, in a single genetic background grown for multiple generations, and in a single genetic background grown in different environments. Southern blot analysis was used to determine whether DNA rearrangements occurred during the production of TwinLink from a conventional breeding cross between T304-40 and GHB119 cotton. T304-40 contains the cry1Ab and bar genes on a single genetic insert. GHB119 contains the cry2Ae and bar genes on a single genetic insert.

Genomic DNA was isolated from 20 individual TwinLink plants. Two separate aliquots of each plant DNA extract were digested with the restriction enzyme EcoRV. EcoRV recognizes the inserted DNA of each event, thus generating restriction profiles that are characteristic for each parent (T304-40 and GHB119) and TwinLink.

DNA from non-transgenic cotton plants was used as a negative control. This DNA was also supplemented with vector DNA containing either the cry1Ab gene or the cry2Ae gene to serve as a positive control on their respective Southern blots. Each blot also contained the positive control DNA from the appropriate parent. The positive control for T304-40 analysis was genomic DNA prepared from WT leaf material mixed with an equimolar amount of pTDL008 plasmid DNA and digested with EcoRV. The positive control for GHB119 analysis was genomic DNA prepared from WT leaf material mixed with an equimolar amount of pTEM12 plasmid DNA and digested with EcoRV. Probing DNA digests of the parents and the cross with labeled DNA probes containing the cry1Ab and cry2Ae genes, respectively, revealed the expected restriction fragments in all tested samples.

The three expected fragments were observed in the DNA from all 20 TwinLink cotton plants digested with EcoRV and hybridized with the cry1Ab probe (Figure 3.10). The EcoRV restriction of TwinLink genomic DNA yields four fragments that can be detected by hybridization with the GHB119 T-DNA probe (Figure 3.11). Three of the fragments are produced by the GHB119 event DNA in TwinLink. A fourth fragment (6200 bp) results from the presence of the T304-40 event in TwinLink cotton. This fourth fragment is not detected in the GHB119 parent DNA (Lane 21). The four expected fragments were observed in the DNA from all 20 TwinLink cotton plants digested with EcoRV and hybridized with the GHB119 T-DNA probe.

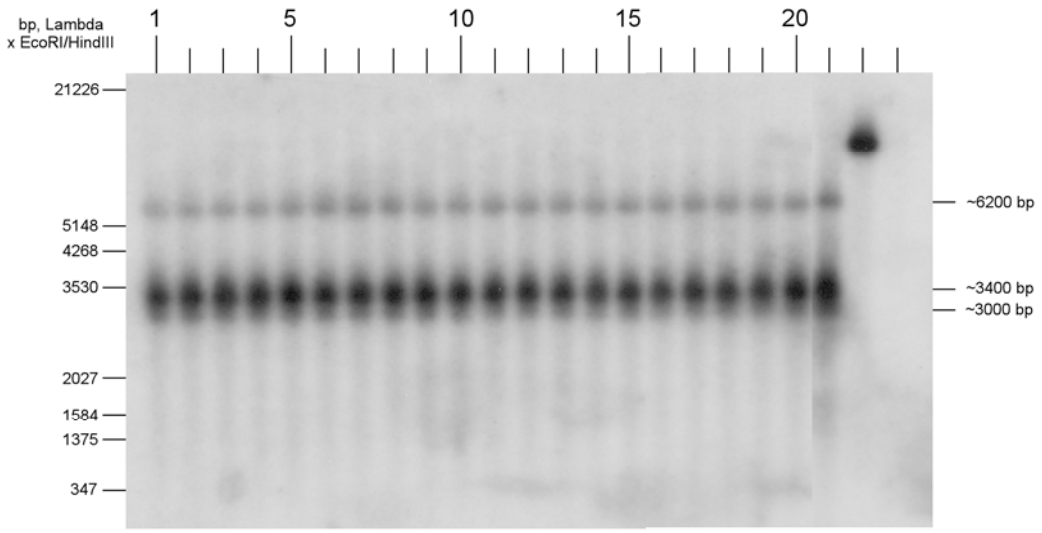


Figure 3.10 Demonstration of the stability of TwinLink cotton for the *cry1Ab* DNA insert.

Genomic DNA was isolated from TwinLink cotton plants and from the non-transgenic counterpart (Coker 315). Genomic DNAs were digested with *Eco*RV and probed with part of the T-DNA (1822 bp fragment of pTLD008).

Lane 1: TwinLink – plant 1 – *Eco*RV
Lane 2: TwinLink – plant 2 – *Eco*RV
Lane 3: TwinLink – plant 3 – *Eco*RV
Lane 4: TwinLink – plant 4 – *Eco*RV
Lane 5: TwinLink – plant 5 – *Eco*RV
Lane 6: TwinLink – plant 6 – *Eco*RV
Lane 7: TwinLink – plant 7 – *Eco*RV
Lane 8: TwinLink – plant 8 – *Eco*RV
Lane 9: TwinLink – plant 9 – *Eco*RV
Lane 10: TwinLink – plant 10 – *Eco*RV
Lane 11: TwinLink – plant 11 – *Eco*RV
Lane 12: TwinLink – plant 12 – *Eco*RV
Lane 13: TwinLink – plant 13 – *Eco*RV
Lane 14: TwinLink – plant 14 – *Eco*RV
Lane 15: TwinLink – plant 15 – *Eco*RV
Lane 16: TwinLink – plant 16 – *Eco*RV
Lane 17: TwinLink – plant 17 – *Eco*RV
Lane 18: TwinLink – plant 18– *Eco*RV
Lane 19: TwinLink – plant 19 – *Eco*RV
Lane 20: TwinLink – plant 20 – *Eco*RV
Lane 21: T304-40 parent – *Eco*RV
Lane 22: WT variety Coker 315 plus 1 copy pTDL0008 – *Eco*RV
Lane 23: WT variety Coker 315 – *Eco*RV

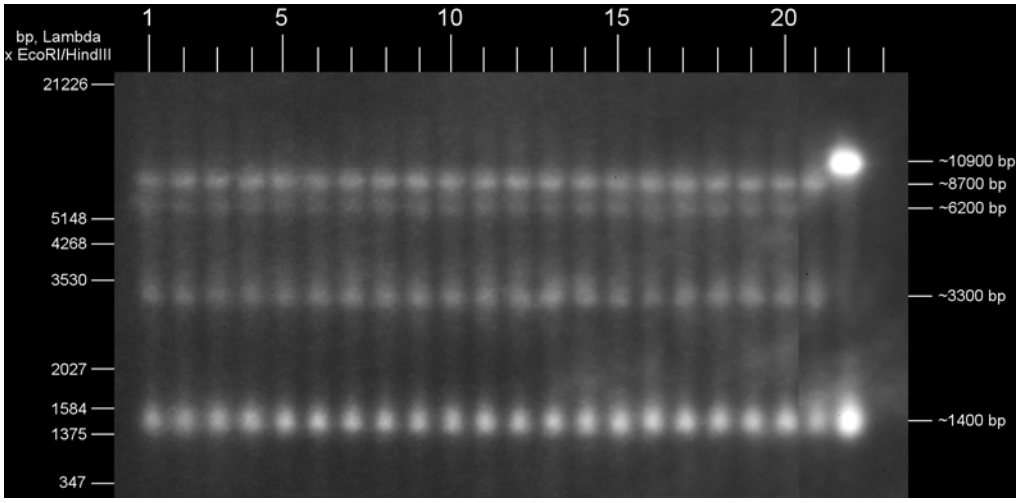


Figure 3.11 Demonstration of the stability of TwinLink cotton for the *cry2Ae* DNA insert.

Genomic DNA was isolated from TwinLink cotton plants and from the non-transgenic counterpart (Coker 315). Genomic DNAs were digested with *Eco*RV and probed with part of the T-DNA (4346 bp fragment of pTEM12).

Lane 1: TwinLink – plant 1 – *Eco*RV
Lane 2: TwinLink – plant 2 – *Eco*RV
Lane 3: TwinLink – plant 3 – *Eco*RV
Lane 4: TwinLink – plant 4 – *Eco*RV
Lane 5: TwinLink – plant 5 – *Eco*RV
Lane 6: TwinLink – plant 6 – *Eco*RV
Lane 7: TwinLink – plant 7 – *Eco*RV
Lane 8: TwinLink – plant 8 – *Eco*RV
Lane 9: TwinLink – plant 9 – *Eco*RV
Lane 10: TwinLink – plant 10 – *Eco*RV
Lane 11: TwinLink – plant 11 – *Eco*RV
Lane 12: TwinLink – plant 12 – *Eco*RV
Lane 13: TwinLink – plant 13 – *Eco*RV
Lane 14: TwinLink – plant 14 – *Eco*RV
Lane 15: TwinLink – plant 15 – *Eco*RV
Lane 16: TwinLink – plant 16 – *Eco*RV
Lane 17: TwinLink – plant 17 – *Eco*RV
Lane 18: TwinLink – plant 18– *Eco*RV
Lane 19: TwinLink – plant 19 – *Eco*RV
Lane 20: TwinLink – plant 20 – *Eco*RV
Lane 21: GHB119 parent – *Eco*RV
Lane 22: WT variety Coker 315 plus 1 copy pTDL0008 – *Eco*RV
Lane 23: WT variety Coker 315 – *Eco*RV

6. Absence of vector backbone in Cry1Ab cotton event T304-40

Genomic DNA isolated from T304-40 plants was digested with either *Eco*RV or *Not*I restriction enzymes and analyzed by Southern blot. Seven overlapping vector backbone probes covering the complete vector backbone sequence were assessed. A positive control hybridization was performed using a T-DNA probe.

A schematic representation of the restriction fragments and hybridization probes is given in Figure 3.12. An overview of the expected and obtained hybridization results is presented in Table 3.8. The obtained results are presented in Figure 3.13.

The absence of hybridization signal with the vector backbone probes demonstrates the absence of vector backbone sequences, while the positive control hybridization assures that the experimental conditions for this Southern blot analysis allowed detection of the integrated DNA fragments.

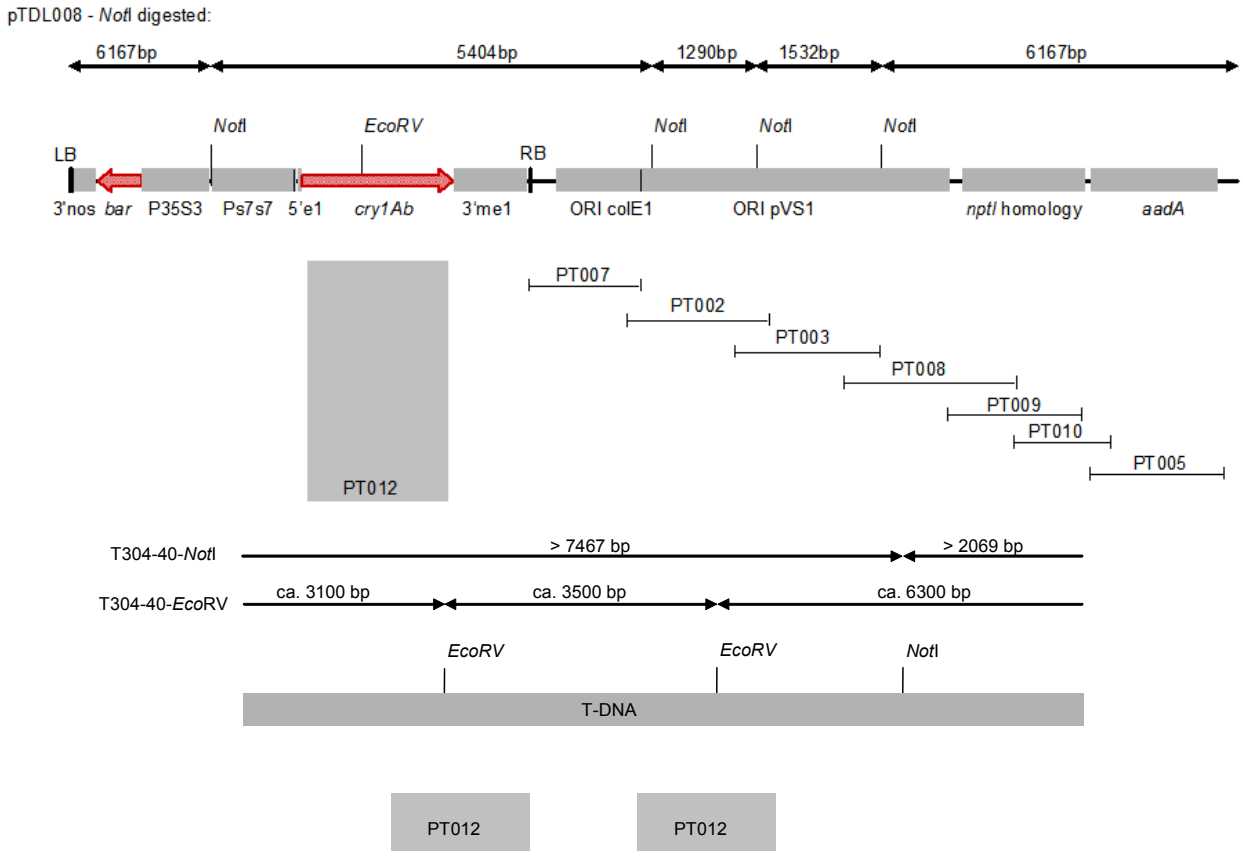


Figure 3.12 Absence of vector backbone in cotton event T304-40 -- Schematic overview of the hybridization strategy

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Table 3.8 Absence of vector backbone in event T304-40 - Expected and obtained hybridization results

Probe template ID	T304-40 - EcoRV		T304-40 - NotI		WT (FM966) - NotI		WT - NotI + 0.1; 1; or 10 copies pTDL008 - NotI			
	Expected hybridization fragments (bp)	Obtained hybridization fragments (bp)	Expected hybridization fragments (bp)	Obtained hybridization fragments (bp)	Expected hybridization fragments (bp)	Obtained hybridization fragments (bp)	Expected hybridization fragments (bp)	Obtained hybridization fragments (bp)		
								0.1 copy	1 copy	10 copies
PT002	none	none	none	none	none	none	1290 (1532)[1] 5404	1290 5404	1290 1532 5404	1290 1532 5404 6167[2]
PT003	none	none	none	none	none	none	1290 1532	1290 1532	1290 1532	1290 1532
PT005	none	none	none	none	none	none	6167	6167	6167	6167
PT007	none	none	none	none	none	none	5404	5404	5404	5404
PT008	none	none	none	none	none	none	1532 6167	1532 6167	1532 6167	1532 5404[2] 6167
PT009	none	none	none	none	none	none	6167	6167	6167	6167
PT010	none	none	none	none	none	none	6167	6167	6167	6167
PT012	ca. 6300 ca. 3500 ca. 3000	6300 ± 200 3500 ± 100 3100 ± 100	> 7467	>14K	none	none	5404	5404	5404	5404

[1] Probably not visible due to limited overlap (85 bp) between PT002 and this vector fragment.
[2] Background hybridization

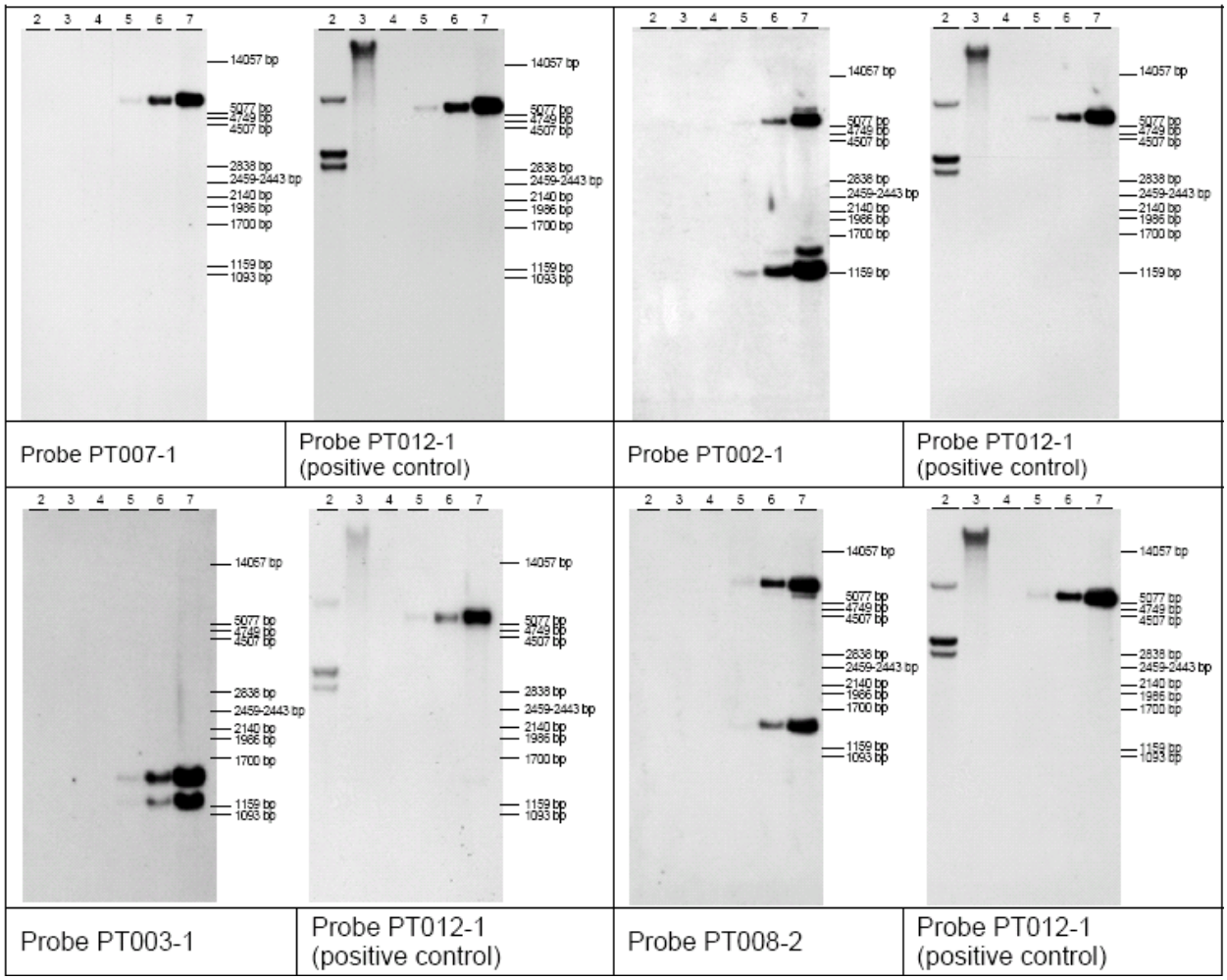


Figure 3.13 Absence of vector backbone in cotton event T304-40– Southern blot results

Lane 1: Phage Lambda – *Pst*I digested (not shown)
Lane 2: Cotton event T304-40– *Eco*RV digested
Lane 3: Cotton event T304-40– *Not*I digested
Lane 4: Non-trangenic control – *Not*I digested
Lane 5: Non-trangenic control + 0.1 copy pTDL008 – *Not*I digested
Lane 6: Non-trangenic control + 1 copy pTDL008 – *Not*I digested
Lane 7: Non-trangenic control + 10 copies pTDL008 – *Not*I digested

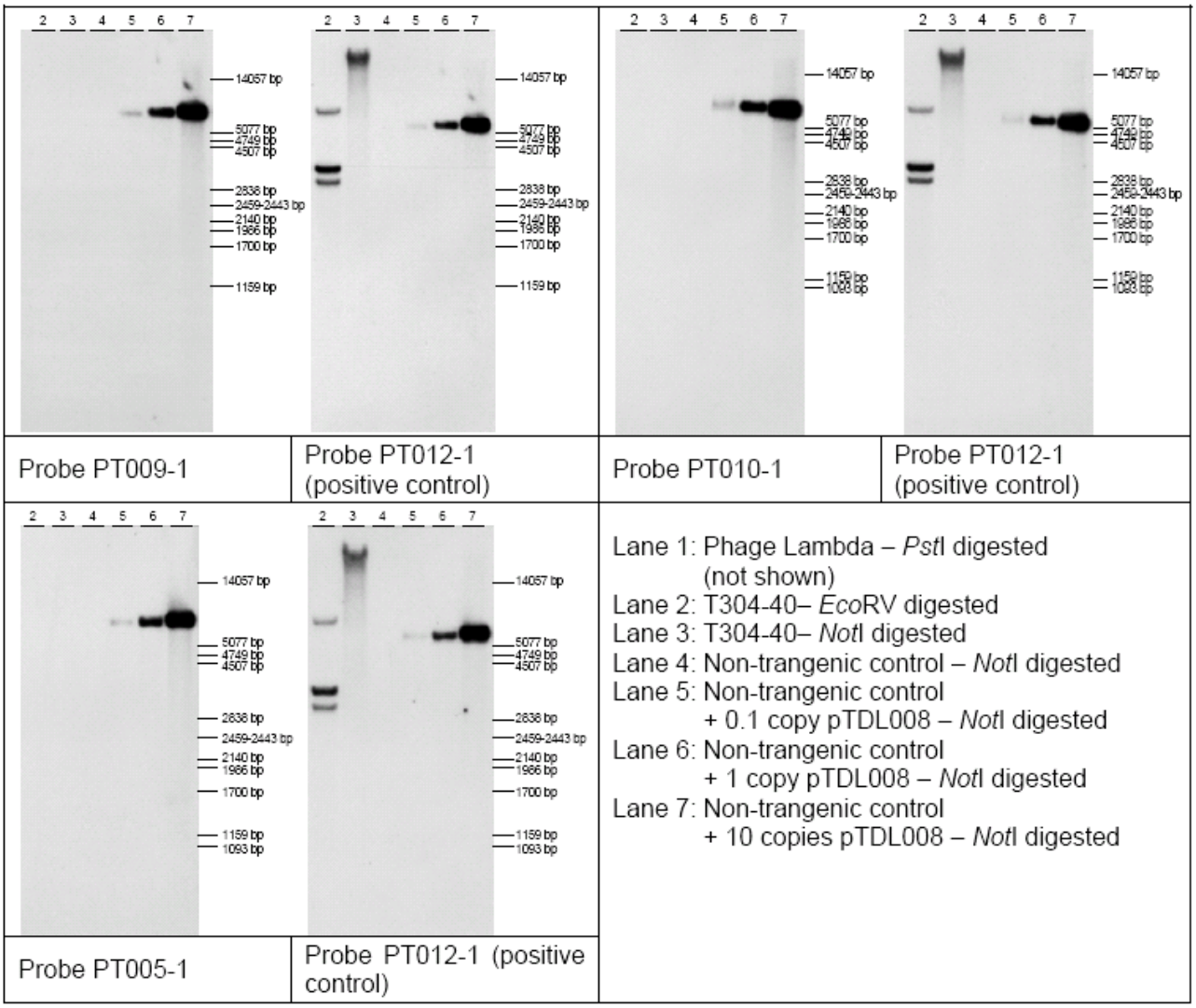


Figure 3.13 (Continued) Absence of vector backbone in cotton event T304-40– Southern blot results

7. Absence of vector backbone in Cry2Ae cotton event GHB119

Genomic DNA isolated from GHB119 plants was digested with either *Eco*RV or *Dra*I restriction enzymes and analyzed by Southern blot using pTEM12 vector backbone probes. Five overlapping vector backbone probes covering the complete vector backbone sequence were assessed. A positive control hybridization was performed using a T-DNA probe. The absence of hybridization signal with the vector backbone probes demonstrates the absence of vector backbone sequences, while the positive control hybridization assures that the experimental conditions for this Southern blot analysis allowed an effective detection of the integrated DNA fragments (Figure 3.15)

A schematic representation of the restriction fragments and hybridization probes is given in Figure 3.14. An overview of the expected and obtained hybridization results is presented in Table 3.9. The obtained results are presented in Figure 3.15.

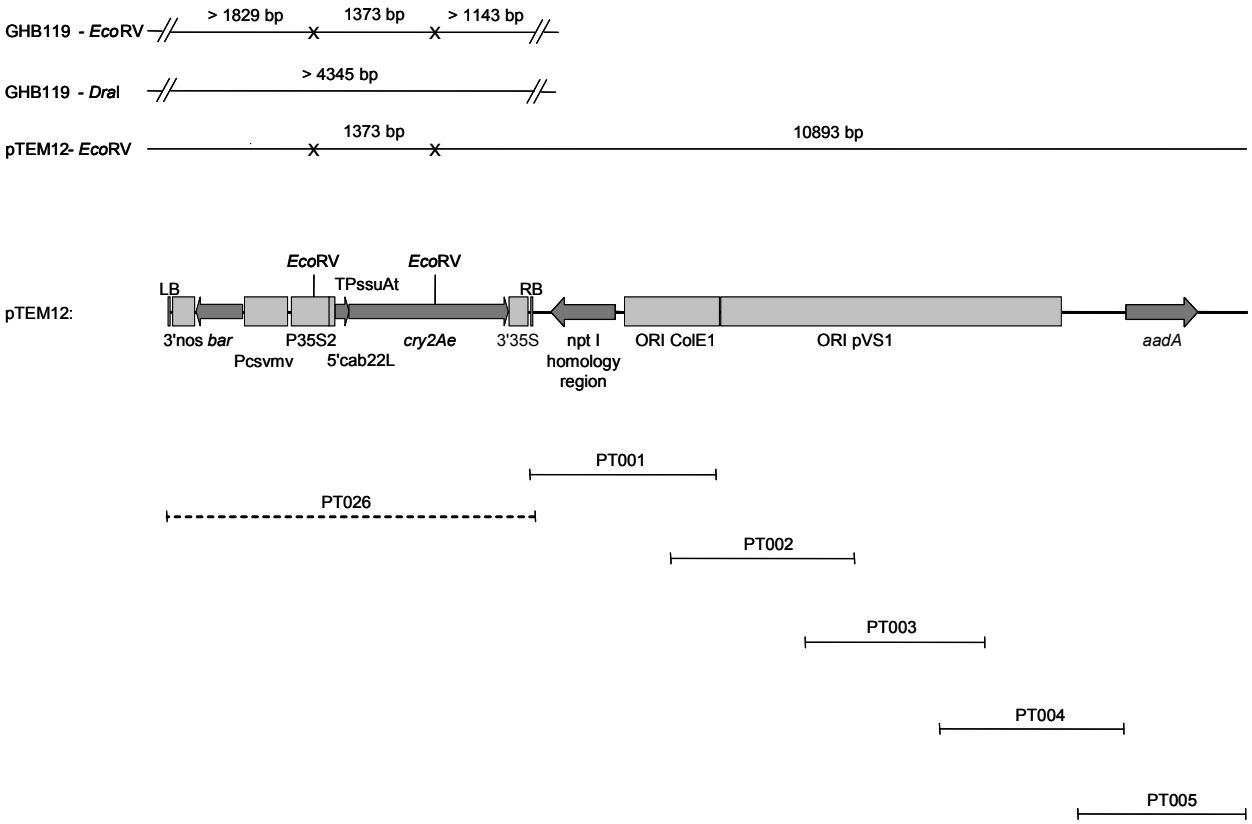


Figure 3.14 Absence of vector backbone in cotton event GHB119 -- Schematic overview of the hybridization strategy

Table 3.9 Absence of vector backbone in event GHB119 -- Expected and obtained hybridization fragments

Probe template ID	*GHB119 - EcoRV*		*GHB119 - DraI*		*WT (FM966) - EcoRV*		*WT (FM966) - EcoRV + 0.1 and 1 copy pTEM12 - EcoRV*		
	Expected hybridization fragments	*Obtained hybridization fragments*	*Expected hybridization fragments*	*Obtained hybridization fragments*	*Expected hybridization fragments*	*Obtained hybridization fragments*	*Expected hybridization fragments*	*Obtained hybridization fragments*	
								0.1 copy	*1 copy*
PT001-2	/	/	/	/	/	/	10893 bp	10893 bp	10893 bp
PT002-2	/	/	/	/	/	/	10893 bp	10893 bp	10893 bp
PT003-2	/	/	/	/	/	/	10893 bp	10893 bp	10893 bp
PT004-3	/	/	/	/	/	/	10893 bp	10893 bp	10893 bp
PT005-2	/	/	/	/	/	/	10893 bp	10893 bp	10893 bp
PT026-2	3380 bp 1373 bp 8890 bp	3380 bp 1373 bp 8890 bp	4430 bp	4430 bp	/	/	10893 bp 1373 bp	10893 bp 1373 bp[3]	10893 bp 1373 bp
	3380 bp 1373 bp 8890 bp	obtained obtained obtained	4430 bp	obtained	/	/	10893 bp 1373 bp	10893 bp not visible	10893 bp obtained

[3] Faint band, not always visible on all blots

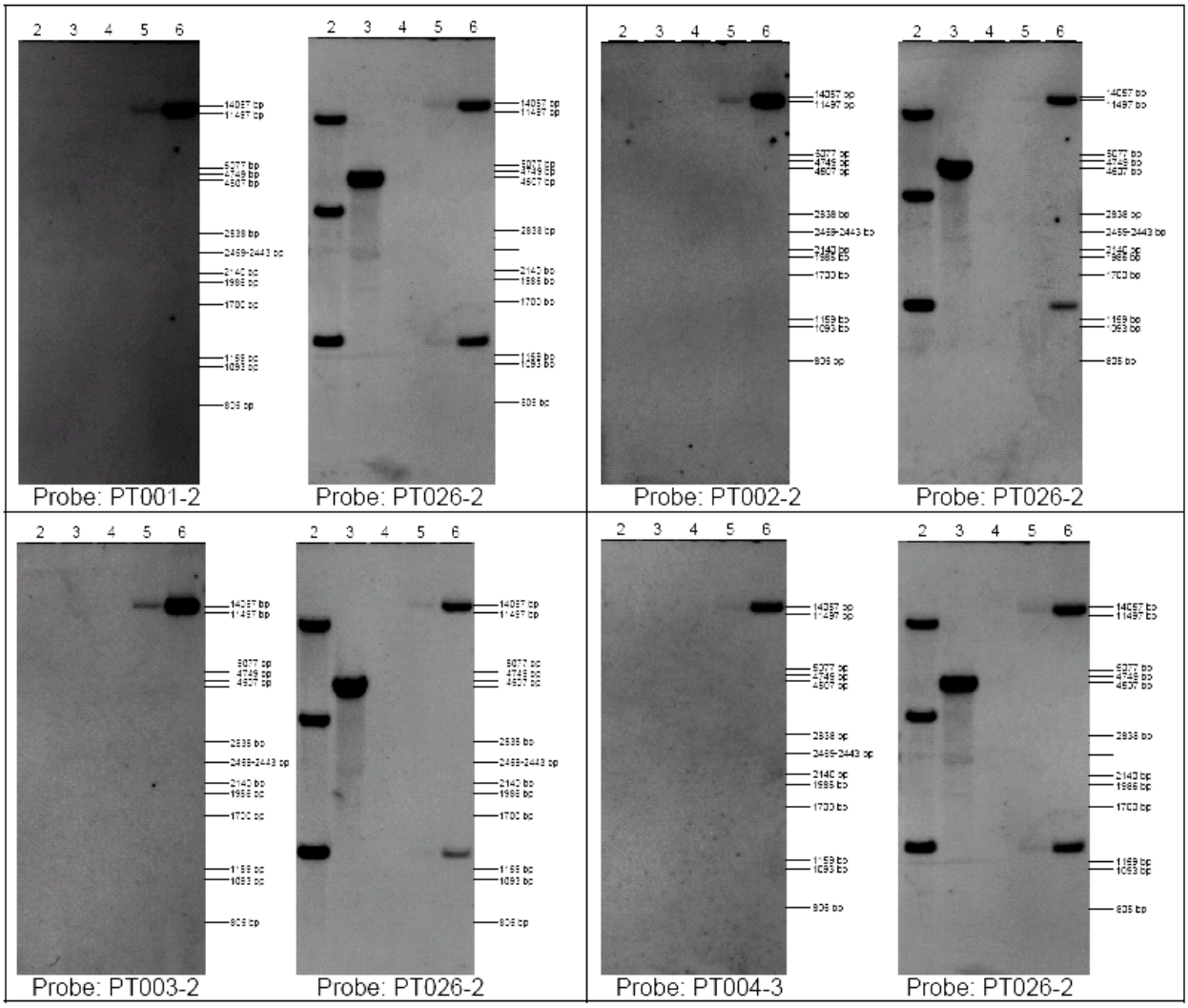
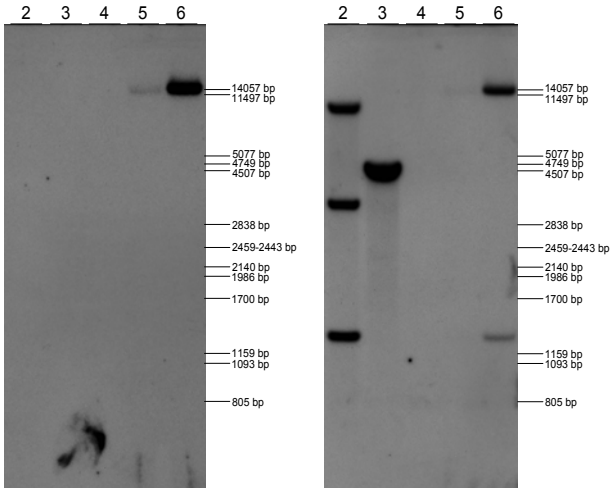


Figure 3.15 Southern blot analysis of GHB119 – Absence of vector backbone

Lane 1: Phage Lambda – *Pst*I digested
Lane 2: Cotton event GHB119 – *Eco*RV digested
Lane 3: Cotton event GHB119 – *Dra*I digested
Lane 4: Wild type cotton variety FM966 – *Eco*RV digested
Lane 5: Wild type cotton variety FM966 – *Eco*RV digested + 0.1 copy pTEM12 – *Eco*RV digested
Lane 6: Wild type cotton variety FM966 – *Eco*RV digested + 1 copy pTEM12 – *Eco*RV digested
Lane 7: Phage Lambda – *Pst*I digested



Probe: PT005-2 Probe: PT026-2

Lane 1: Phage Lambda – *Pst*I digested
Lane 2: Cotton event GHB119 – *Eco*RV digested
Lane 3: Cotton event GHB119 – *Dra*I digested
Lane 4: Wild type cotton variety FM966 *Eco*RV digested
Lane 5: Wild type cotton variety FM966 *Eco*RV digested + 0.1 copy pTEM12 – *Eco*RV digested
Lane 6: Wild type cotton variety FM966 *Eco*RV digested + 1 copy pTEM12 – *Eco*RV digested
Lane 7: Phage Lambda – *Pst*I digested

Figure 3.15 (Continued) Southern blot analysis of cotton event GHB119 – Absence of vector backbone

8. Cry1Ab cotton event T304-40 pre-insertion locus

a. The flanking sequences

The flanking sequences of T304-40 were determined by PCR. Based on this sequence information, a PCR fragment was generated using genomic DNA from wild type cotton plants as template and a pair of primers specific for the 5' and 3' flanking sequence. This fragment (828 bp) corresponds to the region of the original cotton genome where the insertion took place, referred to as the pre-insertion locus. Figure 3.16 gives a schematic representation of the pre-insertion locus and the inserted DNA surrounded by the flanking sequences.

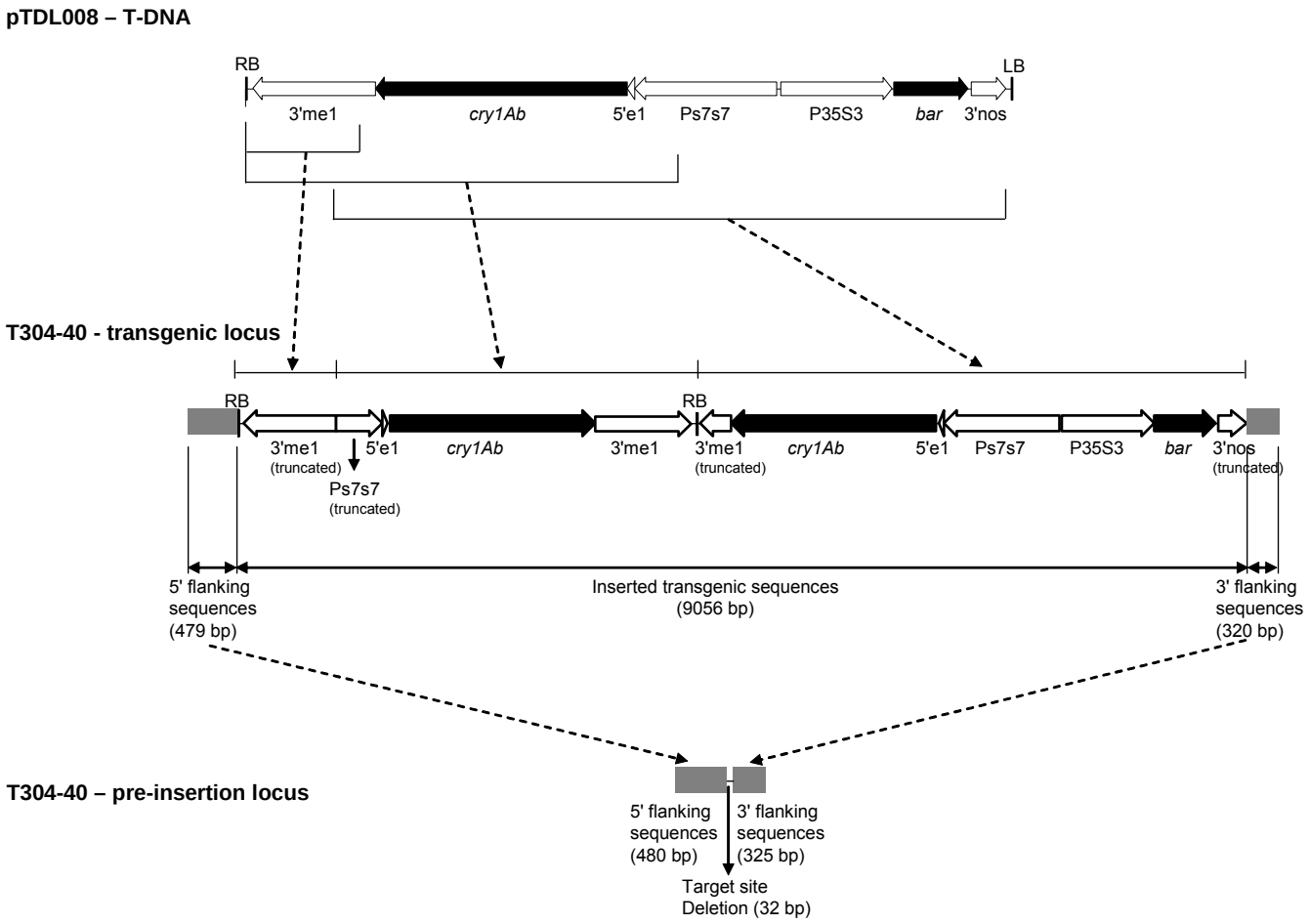


Figure 3.16 Schematic drawing of the T304-40 transgenic locus and pre-insertion locus

b. Identification of potentially disrupted genes at the integration site

The sequence of the pre-insertion locus of cotton event T304-40 was subjected to a bioinformatic analysis to determine the possibility that endogenous genes are interrupted by the insertion of the transgenic sequences.

Homology was found between the cotton genomic DNA and small sequences from the cotton DNA database but no function was assigned to these sequences. Also homology was found with repetitive sequences indicating that the insertion of the T304-40 T-DNA took place in a region containing such elements.

In order to identify the presence of known functional genes in the pre-insertion locus of event T304-40, a BLASTx similarity search was performed, but no known proteins were found.

Also no ORFs or genes were predicted. One putative promoter was found in the 5' flanking sequence. As there are no genes found further downstream, this predicted promoter is most probably not biological active.

It can be concluded that, using the current databases and tools, no known genes were interrupted or influenced due to the insertion of transgenic DNA in the pre-insertion locus of T304-40.

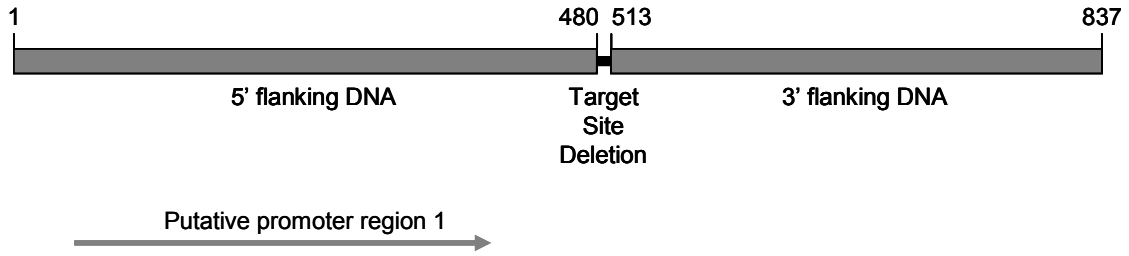


Figure 3.17 Schematic overview of the regulatory element search result on the pre-insertion locus of T304-40

c. Identification of potentially unintended newly-created genes in the transformation event

A bioinformatic analysis was performed to detect the possible presence of cryptic expression due to the formation of newly created open reading frames (ORFs) in the 5', 3'and internal junction regions of cotton event T304-40. These junctions are formed by the T-DNA insertion from the pTDL008 vector, containing the *cry*1Ab and *bar* gene cassettes into the cotton genome. Several *in silico* tools were used to look for ORFs and regulatory elements taking into account the current scientific knowledge on gene expression.

- Two newly created chimeric ORFs were identified (GetORF analysis)

- No putative newly created gene was predicted (FGENESH analysis)
- Eleven promoters and promoter-like regions were identified (TSSP analysis)
- None of the two ORFs will potentially be transcribed by any of the predicted promoters
- None of the two ORFs possesses a highly conserved RBS region, leading most probably to absence of translation

Based on this analysis, the probability of newly created protein expression in the cotton event T304-40 is highly unlikely.

9 Cry2Ae cotton event GHB119 pre-insertion locus

a. The flanking sequences

The flanking sequences of GHB119 insert were determined by PCR. Based on this sequence information, a PCR fragment was generated from non-transgenic cotton using a pair of primers specific for the 5' and 3' flanking sequence. This fragment (684 basepairs) corresponds to the region of the original cotton genome where the insertion took place, referred to as the pre-insertion locus. Figure 3.18 shows a schematic representation of the pre-insertion locus and the inserted DNA surrounded by the flanking sequences.

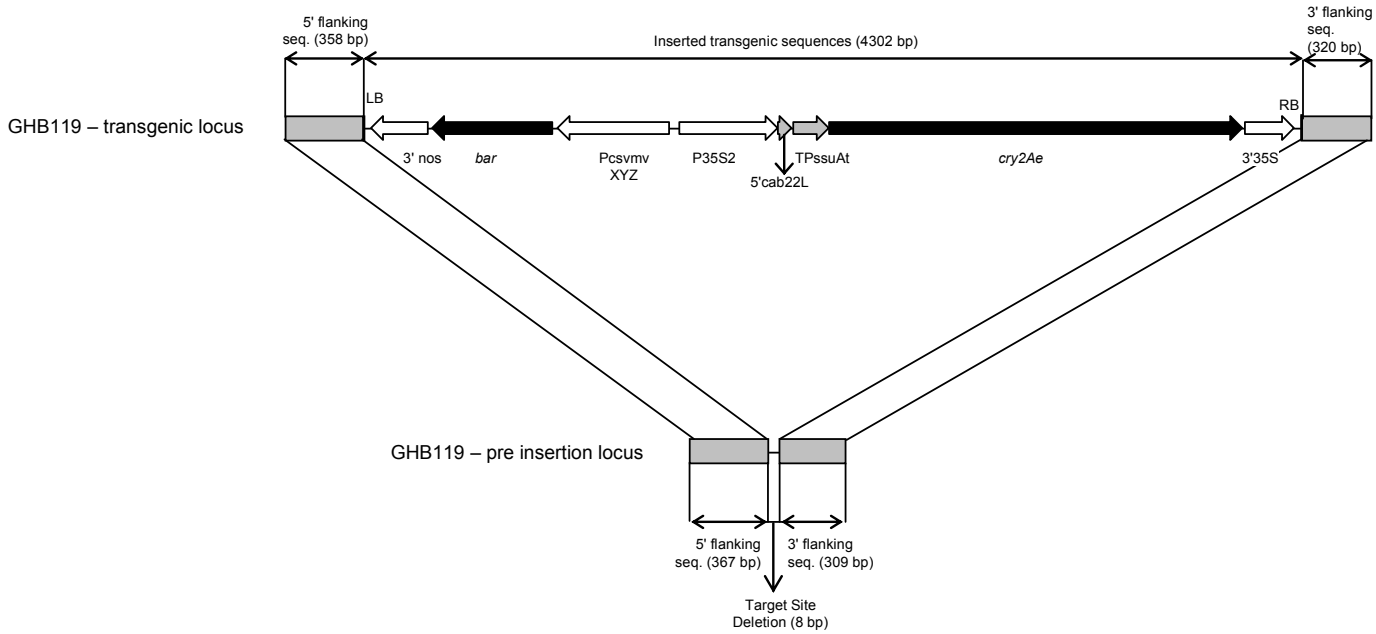


Figure 3.18 Schematic overview of the transgenic and pre-insertion locus of the cotton event GHB119

b. Identification of potentially disrupted genes at the integration site

The DNA of the pre-insertion locus of the cotton event GHB119 was sequenced and subjected to bioinformatic tools in order to identify endogenous cotton genes and/or regulatory elements that may be influenced by the insertion of the transgenic DNA fragment.

Homology was found with cotton genomic DNA but no function could be assigned to these sequences. Also homology was found with repetitive sequences indicating that the insertion of the GHB119 T-DNA took place in a region containing repetitive elements. In order to identify the presence of known functional genes in the pre-insertion locus of event GHB119, a BLASTx similarity search was performed, but no known proteins were found.

Using the prediction tools GetORF and FGENESH, no genes or ORFs were predicted that could be disrupted by the introduction of the transgenic DNA in the genome.

The surrounding sequence of the pre-insertion locus was subjected to a homology analysis comparing it with regulatory elements involved in transcription. Two promoter sequences were predicted (Figure 3.19). Since no known endogenous genes were found or predicted, it is highly unlikely that these predicted promoters are biologically active.

These bioinformatic analyses on the pre-insertion locus of cotton event GHB119, using the current databases and bioinformatic tools, allow us to conclude that the probability that a functional gene was interrupted is highly unlikely.

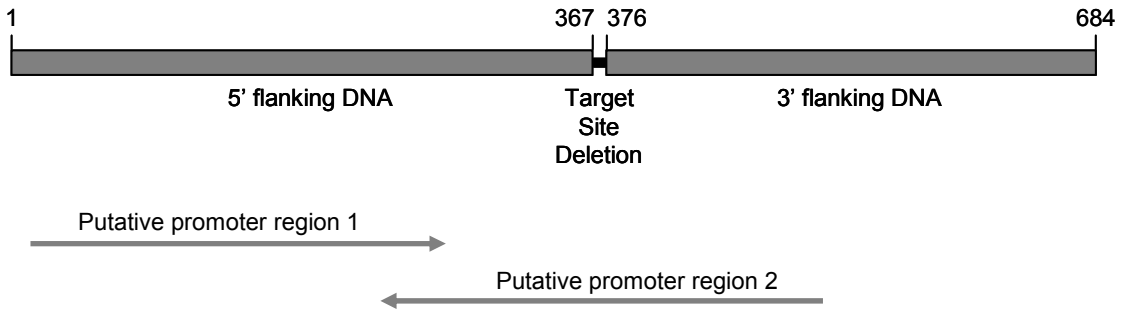


Figure 3.19 Schematic overview of the regulatory element search results on the pre-insertion locus of GHB119

c. Identification of potentially unintended newly-created genes

A bioinformatic analysis was performed to detect the possible presence of cryptic expression due to the formation of newly created open reading frames (ORFs) in the 5' or 3' junction region in event GHB119. These junctions are formed by the insertion of a *cry*2Ae - *bar* gene construct into the cotton genome.

Several *in silico* tools were used to look for ORFs and regulatory elements taking into account the current scientific knowledge of gene expression. Three newly created chimeric ORFs were identified. The surrounding nucleotides of the three ORFs were also subjected to a homology analysis, comparing them with regulatory elements important for transcription and translation. Three newly created chimeric ORFs were identified. The surrounding nucleotides of the three ORFs were also subjected to a homology analysis, comparing them with regulatory elements important for transcription and translation.

- ORF-1 at the 3' junction (sense strand): two promoters were predicted at the 5' end of ORF-1, however more than 200 bp away from the ATG codon. No homology was found with a RBS.

- ORF-2 at the 3' junction (sense strand): two promoters were predicted at the 5' end of ORF-2 however more than 200 bp away from the ATG codon. No homology was found with a RBS.
- ORF-3 at the 5' junction (antisense strand): no promoters were predicted at the 5' end of ORF-3 and no homology was found with a RBS.

ORF-3 is very likely inactive since no regulatory elements that could lead to transcription or translation were predicted. Initiation of transcription of ORF-1 and ORF-2 cannot be excluded, but translation is very unlikely.

One promoter crossing the 5' junction was predicted but it is highly unlikely that this predicted promoter region would lead to a changed expression level of endogenous genes.

As not all regulatory elements were present in the DNA sequence at the 5' or 3' end of the newly created ORFs, the probability of an expression of newly created proteins due to the insertion of DNA containing the *cry*2Ae - *bar* gene construct, is highly unlikely.

Appendix 4. REGIONAL AGRONOMIC DATA

For ease of understanding:

- "GA treated" means "treated with glufosinate ammonium"
- "Sprayed" means "sprayed with conventional insecticides"

Table 4.1 Agronomic data across regions from BCS Regulatory trials in 2007

Growth Habit & Phenotype					
Agronomic Parameter (locations)*	Coker	TwinLink GA treated	TwinLink Non treated	Significance	
				LSD	CV
Plant Stand (7)	2.11b	2.73a	2.79a	0.15	1.84
Days to bloom (7)	54.19a	51.81b	51.86b	2.11	1.24
% open bolls (7)	47.86b	59.86a	58.95b	9.73	5.40
Plant height in cm (6)	101.69a	96.87b	96.59b	3.56	1.02
Total # of plant nodes (7)	17.89a	16.97b	16.95b	0.76	1.36
Height to Node Ratio (6)	5.65a	5.63a	5.63a	0.21	1.04
# of 1st position bolls (7)	4.40a	4.70a	4.61a	1.10	7.40
% retention 1st position (2)	100.00b	167.11a	171.28a	9.57	0.98
# of total bolls (7)	9.51a	9.05a	8.89a	1.81	6.10
Strain Uniformity (7)	1.43a	1.43a	1.43a	0	0
Boll Type (7)	4.29a	4.29a	4.29a	0	0
Lodging (7)	1.14a	1.14a	1.14a	0	0

* Numbers in parentheses indicate the number of sites used to generate data for that parameter

Table 4.2 Yield and Fiber Quality data across regions from BCS Regulatory trials in 2007

Yield and Fiber Quality					
Agronomic Parameter (locations)*	Coker	TwinLink GA treated	TwinLink Not treated	Significance	
				LSD	CV
Yield (7)	608.28a	619.12a	608.00a	78.59	3.96
Fiber Length (7)	1.21a	1.20b	1.19b	0.02	0.46
Fiber Strength (7)	31.73a	31.74a	31.82a	0.57	0.55
Fiber Uniformity % (7)	84.93a	84.31a	84.37a	0.69	0.25
Micronaire (7)	4.26a	4.50a	4.55a	0.31	2.13
% lint (7)	38.65a	39.02a	38.71a	0.64	0.51

* Numbers in parentheses indicate the number of sites used to generate data for that parameter

Table 4.3 Agronomic data from BCS Development Efficacy trials in 2007

Growth Habit & Phenotype						
Agronomic parameter (locations)*	Coker Not sprayed	Coker Sprayed	TwinLink Not sprayed	TwinLink Sprayed	Significance	
					LSD	CV
Plant Stand (1)	1.31cd	1.26d	1.49bc	1.36cd	0.21	10.0
days to bloom (1)	60.00ab	61.00ab	61.33ab	62.00a	2.69	2.52
# open bolls (1)	88.75 ab	70.00 e	82.50 cd	90.00ab	5.40	4.33
Days to 1st open bolls (1)	123.33a	123.00a	123.33a	123.00a	1.33	0.62
Lodging (1)	8.00a	7.50a	5.00b	3.75bc	1.58	22.13

* Note: All data from this table from a single location, Sellers, South Carolina

Table 4.4 Yield and Fiber Quality data across regions from BCS Development Efficacy trials in 2007

Yield and Fiber Quality						
*Agronomic Parameter (locations)**	*Coker Not sprayed*	*Coker Sprayed*	*TwinLink Not sprayed*	*TwinLink Sprayed*	*Significance*	
					LSD	*CV*
Yield (5)	815.72e	1009.30c	860.14de	968.66cd	118.60	2.95
Fiber Length (1)		1.23a		1.23a	0.04	1.85
Fiber strength (1)		31.50b		32.30b	2.23	4.21
Fiber uniformity % (1)		87.05b		86.98b	0.98	0.7
Micronaire (1)		4.65b		4.75b	0.37	4.78
% lint (3)	38.94cd	38.76d	39.51bc	39.45bc	0.61	0.31

* Numbers in parentheses indicate the number of sites used to generate data for that parameter
Non-sprayed plots were analyzed from only one location

Table 4.5 Agronomic data across regions from BCS Regulatory trials in 2008

Growth Habit & Phenotype					
*Agronomic Parameter (locations)**	*Coker*	*TwinLink GA treated*	*TwinLink Not treated*	*Significance*	
				LSD	*CV*
Plant stand (plants/ft) (2)	1.20b	1.55a	1.53a	0.02	0.25
Days to bloom (2)	45.0a	45.0a	45.0a	0	0
Days to 1st open boll (2)	98.0a	98.0a	98.0a	0	0
% open bolls (2)	60.0a	60.0a	60.0a	0	0
Plant height in cm (2)	32.17a	32.33a	30.33b	1.02	0.48
Total # of plant nodes (2)	18.83a	18.00b	17.17c	0.62	0.52
Height to Node Ratio (2)	2.12a	1.71b	1.62b	0.35	2.89
# of 1st position bolls (2)	7.50c	8.83a	8.33b	0.41	0.74
# of total bolls (2)	15.33b	20.17a	18.50a	2.85	2.37
Strain Uniformity (2)	5.00a	5.00a	5.00a	0	0
Leaf Uniformity (2)	1.00a	1.00a	1.00a	0	0
Boll Type (2)	5.00a	5.00a	5.00a	0	0
Disease reaction (2)	1.00a	1.00a	1.00a	0	0
Lodging (2)	3.00a	3.00a	3.00a	0	0

* Numbers in parentheses indicate the number of sites used to generate data for that parameter

Table 4.6 Yield and Fiber Quality data across regions from BCS (Regulatory) trials in 2008

Yield and Fiber Quality					
*Agronomic Parameter (locations)**	*Coker*	*TwinLink GA treated*	*TwinLink Not treated*	*Significance*	
				LSD	*CV*
Yield (2)	689.83a	805.33a	812.67a	188.00	3.66
Fiber Length (2)	1.29a	1.30a	1.28a	0.02	0.22
Fiber strength (2)	35.58	34.23a	34.77a	1.87	0.80
Fiber uniformity % (2)	87.32b	87.87a	86.78c	0.14	0.02
Fiber elongation (2)	5.30a	5.47a	5.65a	0.41	1.11
Micronaire (2)	3.92a	4.12a	3.77a	0.45	1.70
% lint (2)	36.78a	35.97b	35.97b	0.58	0.24

* Numbers in parentheses indicate the number of sites used to generate data for that parameter

Table 4.7 Agronomic data across regions from University and USDA Efficacy trials in 2008

Growth Habit and Phenotype						
*Agronomic Parameter (locations)**	*Coker Not sprayed*	*Coker Sprayed*	*TwinLink Not sprayed*	*TwinLink Sprayed*	*Significance*	
					LSD	*CV*
Plant Stand (3)	2.98b	3.21ab	3.23ab	3.57a	0.36	1.65
Plot uniformity (2)	1.00b	1.50ab	2.00a	1.25ab	0.76	40.51
Plant Height in cm (2)	92.04bc	94.68ab	89.57c	94.42ab	3.79	0.50
Total nodes (2)	22.73a	23.23a	20.70c	23.00a	0.60	0.39
Height-Node ratio (2)	2.27ab	2.25ab	2.45a	1.98c	0.25	1.38
1st position bolls (2)	6.55bc	6.75ab	6.65b	6.25bc	0.65	1.23
2nd position bolls (2)	3.45ab	3.08abc	3.53a	3.23abc	0.41	1.65
3rd position bolls (1)	3.50a	3.35a	3.05a	4.00a	1.96	41.74
Total Bolls (2)	13.98a	12.95ab	13.08ab	12.38b	1.25	1.21
% retention 1st position (2)	58.93d	67.13b	62.84c	59.89d	2.40	0.47
% retention 2st position (2)	29.65a	25.05ab	26.49ab	22.87ab	7.68	4.01
% retention 3rd position (1)	29.35a	24.44a	24.49a	24.45a	18.81	48.81
Plant Uniformity (1)	1.00b	1.50ab	2.00a	1.5b	0.50	29.18
Disease Reaction (1)	1.00a	1.00a	1.00a	1.00a	0	0
Lodging (1)	1.00a	1.00a	1.00a	1.00a	0	0
Leaf morphology (1)	1.00a	1.00a	1.00a	1.00a	0	0
Plant morphology (1)	1.00a	1.00a	1.00a	1.00a	0	0
Boll morphology (1)	1.00a	1.00a	1.00a	1.00a	0	0

* Numbers in parentheses indicate the number of sites used to generate data for that parameter
NOTE: Data received from only one location was analyzed individually.

Table 4.8 Yield and Fiber Quality data across regions from University and USDA Efficacy trials in 2008

Yield and Fiber Quality						
*Agronomic Parameter (locations)**	*Coker Not sprayed*	*Coker Sprayed*	*TwinLink Not sprayed*	*TwinLink Sprayed*	*Significance*	
					LSD	*CV*
Yield (2)	1078.66bc	1070.26bc	1030.46c	1018.46c	58.57	0.69
Fiber Length (1)	1.11a	1.11a	1.11a	1.11a	0.04	2.3
Fiber strength (1)	30.90bc	33.60ab	30.05c	30.53bc	3.53	7.46
Fiber uniformity % (1)	82.48ab	83.15a	82.40ab	81.75b	1.07	0.88
Micronaire (1)	5.45a	5.10abc	5.25ab	5.20abc	0.36	4.73
% lint (1)	44.08bcd	43.84cd	45.40b	44.83bcd	1.46	2.19

* Numbers in parentheses indicate the number of sites used to generate data for that parameter

Table 4.9 Agronomic data across regions from BCS (Development) Efficacy trials in 2008

Growth Habit & Phenotype						
*Agronomic Parameter (locations)**	*Coker Not sprayed*	*Coker Sprayed*	*TwinLink Not sprayed*	*TwinLink Sprayed*	*Significance*	
					LSD	*CV*
Stand uniformity (3)	1.00a	1.00a	1.00a	1.00a	0	0
Plant uniformity (3)	1.00a	1.00a	1.00a	1.00a	0	0
Disease rating (3)	1.00a	1.00a	1.00a	1.00a	0	0
Leaf morphology (3)	1.00a	1.00a	1.00a	1.00a	0	0
Boll morphology (3)	1.00a	1.00a	1.00a	1.00a	0	0
Plant morphology (3)	1.00a	1.00a	1.00a	1.00a	0	0
Plant Height in cm (2)	91.18bc	88.15cd	90.71bc	92.32b	3.57	0.49
Total nodes (2)	17.73b	17.53b	17.63b	18.15ab	0.83	0.58
Bolls P1 (2)	4.30d	4.58cd	4.80bc	4.60cd	0.46	1.21
Bolls P2 (2)	2.40bc	2.53abc	2.65ab	2.30cd	0.32	1.65
Total bolls (2)	10.45ab	10.55ab	9.48bc	9.58bc	1.40	1.77
Height Node Ratio (2)	3.62ab	3.75ab	3.74ab	3.51bc	0.28	0.98
Boll Retention P1 (2)	46.12c	50.18bc	52.80ab	50.86bc	6.07	1.47
Boll Retention P2 (2)	36.48b	38.14ab	40.78a	39.53ab	4.14	1.33

* Numbers in parentheses indicate the number of sites used to generate data for that parameter

Table 4.10 Yield and Fiber Quality data from BCS Development Efficacy trials in 2008

*Agronomic Parameter (locations)**	*Coker Not sprayed*	*Coker Sprayed*	*TwinLink Not sprayed*	*TwinLink Sprayed*	*Significance*	
					LSD	*CV*
Yield (1)	1227.1c	1326.9bc	1334.6bc	1434.0ab	162.60	7.82
Fiber Length (1)	1.27a	1.25ab	1.22bc	1.22c	0.03	1.57
Fiber strength (1)	36.00b	35.80b	33.80c	34.20c	1.41	2.65
Fiber uniformity % (1)	86.10a	85.30ab	84.80b	84.80b	1.07	0.84
Micronaire (1)	4.90ab	4.90a	4.70b	4.70b	0.22	3.07
Lint % (1)	40.80cd	41.70bc	41.90bc	42.4b	1.29	2.04

* Numbers in parentheses indicate the number of sites used to generate data for that parameter
Note: All data from this table from a single location in Sellers, South Carolina