

PETITION FOR DETERMINATION OF NON-REGULATED STATUS

***B.t.* Cry1Ac INSECT-RESISTANT COTTON EVENT 3006-210-23**

CBI DELETED COPY

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

Submitted by

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Claim of Data Confidentiality

CONFIDENTIAL BUSINESS INFORMATION DISCLOSURE

The freedom of Information Act (FOIA) specifically exempts federal agencies from releasing information that are “trade secrets and commercial or financial information from a person and privileged or confidential” 5 U.S.C. 552(b)(4). Exemption 4 applies where the disclosure of information would likely cause harm to the competitive position of the person from whom the information was obtained, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to share data with the agency voluntarily.

Dow AgroSciences LLC must keep confidential certain information in this application to maintain its competitive position in a highly competitive market. Disclosure of this information would cause substantial competitive harm to Dow AgroSciences LLC by allowing other companies to unfairly compete with Dow AgroSciences LLC. First, we must keep confidential certain trait(s) which Dow AgroSciences LLC has selected to be of significant agronomic importance that, if imparted to a new seed hybrid, would represent a competitive advantage in the market place. Disclosure of this information would reveal to our competitors our marketing strategy which identifies targets of potential commercial opportunity. Second, we must keep confidential our research: what we are doing, how we are doing it, and how far along we are. Disclosure of this information would enable our competitors to duplicate our research and products without incurring the millions of dollars and many years of research and development expended by Dow AgroSciences LLC. This information would also provide our competitors with commercially valuable knowledge about the particular products Dow AgroSciences LLC is interested in commercializing and the likely time for commercialization. Moreover, we must protect our intellectual property. We must keep research information strictly confidential because in most cases, patent applications have not been filed or patents are pending and have not been published. Third, we must keep confidential commercial development information because such information would reveal our company’s method of operation. Typically, this information would include collaborators, cooperators, and location of the field experiments.

Gene and Regulatory Sequences

Dow AgroSciences LLC’s biotechnology products have a unique combination of genetic components in the vectors transferred to the plants. Each genetic entity in these vectors has the gene form the expression of the trait and regulatory sequences such as promoters, enhancers, and terminators. Disclosure of this information in the vectors will directly provide our competitors with the knowledge of the precise genetic sequence that Dow AgroSciences LLC has found to be most desirable. Disclosure of this information may also reveal the specific modifications we have made in synthesizing the DNA. Disclosure of the genetic modifications made by Dow AgroSciences LLC to enhance the usefulness of the gene would provide our competitors commercially valuable knowledge about the utilization of the gene discovered by Dow AgroSciences LLC. Disclosure of this information may also jeopardize Dow AgroSciences LLC’s intellectual property. Patent applications have not been filed (or patents are pending and have not been published).

Plasmids and Event Designations

Disclosure of this information would allow our competitors to identify the gene and regulatory sequences of interest which has been claimed as CBI. It would also identify this particular plasmid as the most desirable to use. This would be giving away Dow AgroSciences LLC “Know-how”.

Published References

The published references identify donor organisms, genes, and regulatory sequences and other research information which have been claimed as CBI. Although the references appear in the public literature, Dow AgroSciences LLC is not identified or associated with any of the references. Maintaining the confidentiality claimed by Dow AgroSciences LLC protects the use that Dow AgroSciences LLC has made of the information. Dow AgroSciences LLC has used its expertise and resources to discover the value in the public literature, value that would be compromised by disclosure of the references.

Summary

Mycogen Seeds c/o Dow AgroSciences (Dow AgroSciences or DAS) is submitting a Petition for Determination of Non-regulated Status for *Bacillus thuringiensis* (*B.t.*). Cry1Ac (synpro) (Cry1Ac **synthetic protoxin**), hereafter referred to as Cry1Ac, insect-resistant cotton event 3006-210-23. DAS requests a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that Cry1Ac cotton transformation event 3006-210-23 and any cotton lines derived from crosses with event 3006-210-23 (cotton line MXB-7) no longer be considered regulated articles under 7 CFR Part 340.

Cry1Ac is a chimeric, full-length delta endotoxin. A synthetic *cry1Ac* transgene optimized for plant codon usage was transformed into cotton plants, resulting in Cry1Ac expression levels efficacious for control of lepidopteran pests, including tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) in transgenic cotton lines. Safety data generated demonstrate the lack of toxicity to humans and animals, and the absence of adverse effects on non-target organisms and the environment.

In addition to the *cry1Ac* synpro gene, the *pat* gene, which encodes the enzyme phosphinothricin acetyltransferase (PAT), is also present as a selectable marker gene in event 3006-210-23. The *pat* gene is a synthetic version based on the native gene isolated from *Streptomyces viridochromogenes*, a non-pathogenic bacterium, found in soil. The inclusion of the *pat* gene enables plant selection of the *B.t.* lines. The PAT protein does not confer pesticidal activity and there are no known adverse environmental or toxicological effects.

The focus of this petition, transformation event 3006-210-23 was carried forward, through breeding, to produce successful lines. The Cry1Ac event 3006-210-23 will be mainly commercialized as a stack product developed by breeding with the Cry1F event 281-24-236. Introduction of the stack product, along with insect resistance management practices, will reduce selection pressure for resistance to insecticides and help maintain the range of effective control options for Lepidoptera available to cotton growers.

Dow AgroSciences' Cry1Ac event (3006-210-23) was developed by transforming a proprietary cotton cultivar using disarmed *Agrobacterium tumefaciens*, and backcrossing the selected transformants with a commercial variety, to create line designation MXB-7 that will be used in subsequent commercial line development. Cotton event 3006-210-23 has been field tested in 1999, 2000, 2001 and 2002 in the major cotton growing regions of the United States as well as in Puerto Rico. All field tests have occurred under field notifications granted by USDA APHIS (Appendix 1). Data and information regarding the agronomic characteristics, and disease and pest resistance characteristics collected during those trials are presented herein along with laboratory analyses, reports and literature references. This information demonstrates that event 3006-210-23 exhibits no plant pathogenic properties and is unlikely to harm other insects that are beneficial to agriculture. The *B.t.* Cry1Ac protein is unlikely to increase the weediness potential of cotton or any other cultivated plant or wild species. In summary, cotton event 3006-210-23 is not likely to:

- become a weed of agriculture or be invasive of natural habitats
- cross with wild relatives and create hybrid offspring which may become weedy or invasive
- become a plant pest
- have an impact on non-target species, including humans
- have an impact on biodiversity

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 that are unfavorable to the petition.

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Abbreviations and Scientific Terms

APHIS	Animal and Plant Health Inspection Service (USDA)
<i>B.t.</i>	<i>Bacillus thuringiensis</i>
BC3F1	The third backcross of the first filial generation
BC3F4	The third backcross of the fourth filial generation
BAW	beet armyworm, <i>Spodoptera exigua</i>
BW	boll weevil, <i>Athonomus grandis grandis</i>
CA	cotton aphid, <i>Aphis gossypii</i>
CBW	cotton bollworm, <i>Helicoverpa zea</i>
CFSAN	FDA Center for Food Safety and Nutrition
CL	Cabbage looper, <i>Trichoplusia ni</i>
Cry1Ac	Cry1Ac synthetic protoxin (synpro)
<i>cry1Ac</i>	Gene encoding Cry1Ac synthetic protoxin (synpro)
DAS	Mycogen Seeds c/o Dow AgroSciences LLC
DNA	Deoxyribonucleic Acid
EC ₅₀	Estimated concentration to cause a 50%effect
EEC	Estimated environmental concentration
ELISA	Enzyme Linked ImmunoSorbent Assay
EPA	U.S. Environmental Protection Agency
ERS	U.S. Economic Research Service
<i>ery^R</i>	Erythromycin resistance gene
Event 3006-210-23	cotton event expressing Cry1Ac (synpro) ICP and PAT
FAW	fall armyworm, <i>Spodoptera frugiperda</i>
FDA	U.S. Food and Drug Administration
FWS	U.S. Fish and Wildlife Service
GI ₅₀	Concentration estimated to reduce growth by 50%
HEEE	High end exposure estimate
ICP	Insecticidal crystalline protein
Kb	Kilo-base pair
kDa	Kilo Dalton, a measurement of protein molecular weight
LC ₅₀	Lethal concentration estimated to kill 50%
LD ₅₀	Lethal dose estimated to kill 50%
mRNA	Messenger ribonucleic acid
MALDI-TOF	Matrix-assisted laser desorption ionization time-of flight
<i>mas</i>	Probe for the synthetic promoter (4OCS)Δmas 2'
MS	mass spectrometry
MXB-7	Cotton line containing Cry1Ac event 3006-210-23
MXB-9	Cotton line containing Cry1F event 281-24-236
MXB-13	Cotton line containing Cry1F event 281-24-236 and Cry1Ac event 3006-210-23 (Cry1F/Cry1Ac stack cotton line)
NASS	U.S. National Agricultural Statistic Service
OECD	Organization of Cooperative Economic Development
<i>ORF25</i>	Probe for the ORF25PolyA bi-directional terminator
ORF25 polyA	Bi-directional terminator from <i>Agrobacterium tumefaciens</i> pTi15955
pMYC3006	Plasmid carrying the <i>cry1Ac</i> (synpro) and <i>pat</i> genes
<i>pat</i>	synthetic gene from <i>Streptomyces viridochromogenes</i> which encodes the phosphinothricin acetyl transferase protein
PAT	Phosphinothricin acetyl transferase protein; confers tolerance to glufosinate-ammonium herbicides

PBW	pink bollworm, <i>Pectinophora gossypiella</i>
PCR	polymerase chain reaction technique
PIP	Plant-Incorporated-Protectant
RNA	ribonucleic acid
SDI	Strategic Diagnostics Inc.
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBW	Tobacco budworm, <i>Heliothis virescens</i>
T-DNA	Fragment of DNA inserted in the plant genome during <i>Agrobacterium</i> -mediated transformation
TSN	Test Substance Number
<i>ubi</i>	Probe for the Ubi Zm1 promoter
Ubi Zm1	<i>Zea mays</i> L. ubiquitin promoter plus ubiquitin intron and a 5' untranslated region
U.S.	United States of America
USDA	United States Department of Agriculture
(4OCS) Δ mas 2'	Synthetic promoter derived from mannopine synthase promoter from <i>Agrobacterium tumefaciens</i> strain LBA 4404 pTi15955

I. Rationale

The commercial introduction of transgenic cotton expressing the *Bacillus thuringiensis* (*B.t.*) Cry1Ac insecticidal crystal protein (ICP) will provide growers with a simple, inexpensive, highly effective, and environmentally benign means of insect control. Event 3006-210-23 will allow farmers the opportunity to have a new and alternate reduced-risk¹ means to control Lepidoptera pests.

B.t. Cry1Ac cotton lines derived from event 3006-210-23 (designated MXB-7) demonstrate efficacy against the major economic pests in cotton, i.e. the cotton bollworm (*Heliothis zea*), pink bollworm (*Pectinophora gossypiella*), and tobacco budworm (*Heliothis virescens*). In addition, event 3006-210-23 has demonstrated control of sporadic lepidopteran pests. Event 3006-210-23 will also contribute to a decrease in insecticide use in cotton (Gianessi, *et al.*, 2002). Cotton is the most important fiber crop in the United States and lepidopteran insects are the main insect problem. In 2001, 68% of all acres planted to cotton in the United States were treated with insecticides, accounting for over 21 million pounds of applied chemicals (USDA NASS, 2002).

Event 3006-210-23, when deployed in a stack with Cry1F event 281-24-236, will help extend the durability of *B.t.* cotton as well as other control options for lepidopteran pests. Deployment of the two proteins means that insects will need to be resistant to both Cry1Ac and Cry1F in order to survive on the stack product. Such resistant insects are likely to be very rare and spread very slowly. The stack product is also expected to delay the development of resistance to other commercially available *B.t.* cotton products that express Cry1Ac protein.

DAS has submitted a FIFRA Section 3 application for registration of Cry1Ac (as part of the registration package for the end use product Cry 1F (synpro)/Cry 1Ac (synpro) 281/3006) to the US EPA in November 2002. DAS also has submitted a petition for an exemption from the requirement of a tolerance for Cry 1Ac and all genetic material required for its expression, under FFDC Section 408, to the EPA. DAS currently has an experimental use permit (EUP) pending with the US EPA for the 2003 field research season.

In the 1st quarter of 2003, DAS is scheduled to submit a Food and Feed Safety Assessment Summary for Cry1Ac to the FDA, Center for Food Safety and Nutrition (CFSAN). Also in the 1st quarter 2003, DAS plans submissions for novel food and novel feed approval to Canada and Japan. Additional international submissions will follow in the months thereafter.

¹ All Plant-Incorporated-Protectants, or PIPs are defined as reduced risk pesticides by the USEPA and are assigned to review in the Biopesticides and Pollution Prevention Division, BPPD, of the Agency.

II. The Cotton Family

II.A. Cotton as a Crop

Cotton is considered to be one of the most valuable crops known to agriculture because of the diversity of products it provides: fibers for an array of textile products; oil for human consumption; feed for livestock; and base chemicals for a plethora of industrial products (Smith and Cothren, 1999).

Cotton is also the single most important fiber in the world and is grown in over 90 countries, 75 of which are developing nations. The world's four largest producing and consuming countries are China, the United States (U.S.), India, and Pakistan. Together these four countries account for around 60 percent of world cotton production and consumption. The next three largest consuming countries are Turkey, Brazil, and Mexico, all of which produce cotton but are often large importers of cotton as well (Meyer and MacDonald, 2002a).

Trade is particularly important for cotton. Thirty percent of cotton fibers consumed by the world cross international borders before processing. The U.S. and Uzbekistan are the largest exporters of cotton (Meyer and MacDonald, 2002b).

Annual values of the U.S. cotton sold overseas have recently averaged more than \$3 billion. The U.S. commonly supplies 7 million bales or more of the world's cotton exports, accounting for about 25 percent of the total world export market. The largest customers for U.S. raw cotton are in Asia and Mexico. The export of U.S.-manufactured textile products continues to grow, with the equivalent of more than 5 million bales shipped in 2000 (National Cotton Council, 2002).

Cotton is produced in 17 southern U.S. states from Virginia to California, covering more than 12 million acres or about 19,000 square miles. Major areas of concentrated production include:

- ◆ the Texas High and Rolling Plains
- ◆ the Mississippi, Arkansas, and Louisiana Delta
- ◆ California's San Joaquin Valley
- ◆ Central Arizona
- ◆ Southern Georgia.

According to USDA-NASS data, approximately 16.3 million acres of cotton were planted in 2001 which was a 5% increase over 2000. It is estimated that U.S. farmers planted around 14.8 million acres (USDA-NASS, 2002) in 2002.

U.S. cotton farmers annually harvest about 17 million bales or 7.2 billion pounds of cotton. More than half of this crop (64%) goes into apparel, 28% into home furnishings and 8% into industrial products. Business revenue stimulated by the crop in the U.S. economy is estimated at some \$120 billion (National Cotton Council, 2002).

II.A.1. Use of Genetically Modified Cotton

From 1996 to 2001 the acreage planted in genetically engineered crops increased rapidly. Crops carrying insect-resistant traits (plant-incorporated protectants or PIPs) have also been widely adopted. The use of *B.t.* cotton expanded quickly, reaching 15 percent of cotton acreage in 1997, 32 percent in 1999, and 37 percent in 2001. The top two reasons stated by U.S. farmers for adopting *B.t.* cotton were increase of the yield through improved pest control and decrease of pesticide input costs (Fernandez-Cornejo and McBride, 2000).

Adoption of *B.t.* cotton varies by state and sub-state regions is shown by Frisvold *et al.*, (1999) using 1998 data. Regional *B.t.* adoption rates vary widely, and are highest (over 60%) in Alabama, Arizona, Louisiana, and Mississippi. Although the Texas High Plains and the San Joaquin Valley account for a quarter of cotton acreage, there has been virtually no *B.t.* adoption in these areas. Lack of *B.t.* varieties adapted to local growing conditions is a constraint in the Texas High Plains. California's One-Variety Cotton Law has slowed introduction of *B.t.* varieties in that state. The San Joaquin Valley also faces less pressure from pests controlled by *B.t.* varieties, such as the tobacco budworm and pink bollworm.

II.B. Taxonomy of Cotton

Gossypium L. is the cotton genus of the Malvaceae family. The genus includes approximately 50 species that can be found in the tropical and subtropical regions of the world. Four of those are generally cultivated worldwide: *G. hirsutum*, *G. arboreum*, *G. barbadense* and *G. herbaceum* (Fryxell, 1984). The most commonly grown species worldwide is the allotetraploid ($2n=4x=52$) *Gossypium hirsutum*, which owes its current predominance to its relatively high productivity and wide adaptability. Approximately 90% or more of world cotton production is planted to cultivars of *G. hirsutum* types, many of which were derived from American Upland cultivars (Niles and Feaster, 1984). *Gossypium barbadense*, also an allotetraploid, is a distant second to *G. hirsutum* in US and worldwide production. Very small acreages of the diploid ($2n=26$) species *G. herbaceum* and *G. arboreum* are cultivated in Southeast Asia, primarily on dry and unproductive areas of India and Pakistan, not suited for *G. hirsutum* and *G. barbadense* (Niles and Feaster, 1984).

In the United States, four species of *Gossypium* are found. Two are cultivated species: *G. hirsutum*, the primary and important commercial species, also sometimes referred to as Upland cotton, and *G. barbadense*, the secondary species, also sometimes referred to Sea Island, or "Pima" cotton (Niles and Feaster, 1984). The two other *Gossypium* species, *G. thurberi* and *G. tomentosum*, also New World Allotetraploids, are wild plants of Arizona and Hawaii (Percival *et al.*, 1999), respectively.

II.C. Genetics of Cotton

The genus *Gossypium* includes approximately 45 diploid and 5 allotetraploid species (Brubaker *et al.*, 1999). The majority of the wild species are diploid, and they have been divided into cytologically based genome groups based on similarities in chromosome size and structure. They exist in three primary centers of diversity: the Africa-Asian species (A-, B-, E- and F-genomes), the Australian species (C-, G-, and K- genomes), and the New World species (D- genome) (Small and Wendel, 2000).

Genomes typically are similar among close relatives, and this is reflected in the ability of related species to form hybrids that display normal meiotic pairing and high F1 fertility. However, wider crosses are often difficult or impossible to effect, and those that are successful typically are characterized by meiotic abnormalities. Table 1 summarizes the eight diploid genome groups designated A through G, plus K. The collective observations of pairing behavior, chromosome sizes, and relative fertility in interspecific hybrids were used to create these groups (Brubaker *et al.*, 1999).

Table 1. Genomic Types of *Gossypium*, Number of Species and Geographic Distribution (adapted from Brubaker *et al.*, 1999).

Genomes	Number of species	Geographic location
A	2	Africa/Asia
B	4	Africa
E	7	Arabia
F	1	Africa
C	2	Australia
G	3	Australia
K	12	Australia
D	13	New World
AD	5	New World

Many subtypes of the genomic types have been identified. As discussed earlier, two diploid species, *G. arboreum* and *G. herbaceum*, are of regional agronomic importance in Asia. The most important agricultural cottons, *G. hirsutum* and *G. barbadense*, appear to have arisen from the A and D genomes. Other members of this group are *G. tomentosum* (a wild plant native to Hawaii), *G. mustelinum* (Brazil), *G. darwinii* (Galapagos Island) and *G. lanceolatum* (Mexico). New world allotetraploids in their wild forms grow near the ocean as invaders of the strand and its environs. It has been suggested that cultivated species developed from these species (Fryxell, 1979).

II.D. Pollination of Cotton

Cotton is predominantly self-pollinated but cross-pollination may occur via insects. Cotton flowers open in the morning, pollen is shed, and flowers begin to wither at the end of the first day. Pollen is not readily windborne due to its heavy and sticky nature (Poehlman, 1994).

When outcrossing does occur in cultivated cotton, bumble bees (*Bombus spp.*), Melissodes bees, and honeybees (*Apis mellifera*) are the primary pollinators. Cotton pollen is well suited to insect transport but is not equally attractive to all bees. The pollen's spiny shape makes it difficult for honeybees to pack it in their pollen baskets. Thus, honeybees rarely collect pollen deliberately, although they pick up pollen as they visit flowers for nectar (Oosterhuis and Jernstedt, 1999).

A study conducted in the state of Alabama (Ward and Ward, 2000) suggested a positive impact of supplemental honeybees on cotton yield indicators in *B.t.* cotton fields. Concentration of suitable pollinators varies from location to location depending on the nature of crops grown nearby, weed control effectiveness in or near the crop, and insecticide use (McGregor, 1976).

Umbeck *et al.* (1991) studied pollen and gene movement in Mississippi, and showed that pollen movement decreases rapidly after 40 feet (12 meters). A similar study was conducted by Llewellyn and Fitt (1996) in the Namoi Valley, Australia, and the results indicated that 20 meter buffer zones would serve to limit dispersal of transgenic pollen from small-scale field tests.

II.E. Weediness of Cotton

Although cotton allotetraploids of the New World show some tendencies to "weediness" (Fryxell, 1979), the genus has no particularly strong weedy characteristics. Cotton lacks notable characteristics relating to sexual and asexual reproduction that would predispose it to weediness. *Gossypium hirsutum* propagates through the production of seeds. Vegetative dispersal of cultivated cotton does not occur in the field. The seeds are large, covered with thick fibers, and enclosed in a tough boll that retains most of the seeds on the plant. It is generally not regarded as a weedy species due to lack of a good seed dispersal mechanism (via wind, bird, or animal), and there is essentially no volunteerism from seed (Llewellyn and Fitt, 1996). There are no *Gossypium* species considered to be noxious or problematic weeds in the U.S., its possessions or territories (Wendel, 2000).

Cotton is a woody perennial grown as an annual crop. If allowed to develop on its own the cotton plant would lapse into dormancy during winter dry seasons and become vegetative again with the onset of rain the following season. In the continental United States, feral *Gossypium hirsutum* does occur in parts of southern Florida where the period of freezing temperatures is not long enough to kill the cotton plants and seeds. Freezing conditions in other parts of the Cotton Belt prevent cotton from over-wintering (Smith and Coethren, 1999). In recognition of the fact that cotton is a perennial crop and could overwinter in Southern Florida, the USEPA does not allow the cultivation of *B.t.* cotton in Southern Florida (USEPA, 2001b).

II.F. Modes of Gene Escape in Cotton

Genetic material of *Gossypium hirsutum* and *Gossypium barbadense* may potentially escape from a planting site by vegetative material, by seed, or by pollen; however, it is highly unlikely (Thies, 1953 and Llewellyn and Fitt, 1996).

Vegetative propagation is not a common mechanism by which cotton reproduces. The vegetative material of cotton would be unlikely to survive to the freezing temperatures which occurs during the winter in most of the growing regions of the United States (Smith and Coethren, 1999).

Volunteerism is practically nonexistent for cotton, thus gene escape via seed is unlikely to happen. Cotton seeds are contained inside bolls, and due to their characteristics are not dispersed by any of the common mechanisms of seed dispersal such as birds, wind or terrestrial animals (Llewellyn and Fitt, 1996).

There are few wild relatives with which cultivated cotton may be sexually compatible. This is because cross-fertilization is possible only between those plants with similar chromosomal types. In the U.S., this would be only those allotetraploids with AADD genomes. Within the United States, cultivated *Gossypium hirsutum* and *Gossypium barbadense* could hypothetically hybridize with two wild species found in the United States: *Gossypium tomentosum* Nuttall ex Seeman and *Gossypium thurberi* Todaro. *Gossypium thurberi* occurs in the mountains for southern Arizona and northern Mexico at altitudes of 2500 to 5000 feet. It is normally found on rocky slopes and the sides of canyons in late summer and autumn. Any gene exchange between plants of *G. hirsutum* and *G. thurberi*, if it did occur, would result in triploid (3x=39 chromosomes) sterile plants because *G. hirsutum* is an allotetraploid (4x=52 chromosomes) and *G. thurberi* is a diploid (2x=26 chromosomes). Such sterile hybrids have been produced in controlled environments but they would not persist in the wild. No fertile allotetraploids (6x=78 chromosomes) have been reported in the wild.

The second wild *Gossypium* species known in the U.S., *G. tomentosum*, is native to Hawaii, and occurs on the islands of Kahoolawe, Lanai, Maui, Molokai, Nihau and Oahu. *Gossypium hirsutum*, *Gossypium barbadense* and *Gossypium tomentosum* (Hawaiian) are all tetraploids that can crossbreed and produce fertile F1 plants that can survive in the wild (EPA, 2000). Even though fertile offspring can occur, cross pollination is unlikely in natural conditions due to the following natural inhibitors: the stigma of *G. tomentosum* is elongated, and the plant seems incapable of self-pollination until contacted by an insect pollinator. Lepidopterans, presumably moths, are the primary pollinators; the flowers of *G. tomentosum* stay open at night and most *Gossypium* flowers are ephemeral, meaning that they open in the morning and wither at the end of the same day (Fryxell, 1979 and Lackey, 1996). These flowering characteristics make it unlikely that *G. tomentosum* and the cultivated *G. hirsutum* or *G. barbadense* species would cross pollinate in nature.

II.G. Characteristics of the Nontransformed Cultivar

Dow AgroSciences Cry1Ac event 3006-210-23 was developed by transforming the cotton cultivar 'Germain's Acala GC510' (*Gossypium hirsutum* L.) released in 1984 in the USA, by Germain's Agribusiness, Inc. and backcrossing the original transformant (T₀) with PhytoGen Seed Company PSC355 germplasm. The GC510 variety was a proprietary Acala cotton variety adapted for the San Joaquin Valley of California. The GC510 variety was used because of its positive response to the tissue culture system used in the process to produce transgenic plants. Researchers (Trolinder *et al.* - personal communication) have demonstrated that GC510 has a genetic precondition to respond favorably to tissue culture. GC510 variety, although no longer widely grown, is still a commercially acceptable variety.

PhytoGen Seed Co., LLC developed PSC355 from germplasm licensed from Mississippi State University and has applied for variety protection under the 1994 amendments to the U.S. Plant Variety Protection Act of 1970. All testing of the Cry1Ac trait has been conducted with lines introgressed into PSC355 to various degrees. PSC355 was specifically developed for production in the Mississippi Delta region of the U.S., but it has demonstrated broad adaptation for production throughout the South. The Cry1Ac trait will be transferred into other commercial cotton varieties by using traditional breeding techniques.

III. Description of the Transformation System

Event 3006-210-23 was transformed with plasmid pMYC3006, using *Agrobacterium*-mediated transformation (Narva, *et al.*, 2001; Appendix 2). Plasmid pMYC3006 contained the cry1Ac and pat coding sequences along with the regulatory components necessary for their expression in the cotton genome.

Cotyledon segments of cotton germplasm GC510 were isolated from 7-10 day old seedlings germinated *in vitro*. The segments were co-cultivated with disarmed *Agrobacterium tumefaciens* strain LBA4404 that contained the plasmid pMYC3006 encoding the cry1Ac and pat genes, respectively. Following the transformation procedure, treated segments were transferred to callus induction medium that contained glufosinate-ammonium to select only those calli that contained expressed the PAT protein and thus had been successfully transformed. The induction medium also contained the antibiotic carbenicillin to eliminate any remaining *Agrobacterium*.

The transgenic plants were transplanted into soil, maintained in growth chambers for acclimation, and subsequently transferred to the greenhouse. Southern analysis of the event 3006-210-23 confirmed the presence of *cry1Ac* and *pat* genes. The primary transformants were tested for insect resistance against a target pest, cotton bollworm, by conducting bioassays on leaf discs. Event 3006-210-23 was cross-pollinated with the elite genotype PSC355 to obtain seeds for further research and development.

IV. The Donor Genes and Regulatory Sequences

A summary of the genetic elements contained in the T-DNA of plasmid pMYC3006 are given in Table 2 and diagrams of the whole plasmid and linear T-DNA region in Figures 1 and 2, respectively.

IV.A. The *cry1Ac* Insecticidal Crystal Protein Gene

The *cry1Ac* gene was synthesized based on the peptide structure of the Cry1Ac protein (See Section V.E). Cry1Ac synthetic protoxin is an insecticidal crystal protein (ICP; also referred to as a delta-endotoxin) whose core toxin was originally identified in *Bacillus thuringiensis* var. *kurstaki* strain HD73.

The *cry1Ac* gene is driven by the maize Ubiquitin 1 (Ubi Zm1) promoter and terminated by the bi-directional polyadenylation signal, ORF25 polyA (Table 2). The *cry1Ac* gene and regulatory elements were transformed into cotton using T-DNA plasmid pMYC3006.

IV.A.1. Peptide Sequence of Transgenic Cry1Ac

The plant optimized *cry1Ac* transgene encodes a full-length insecticidal crystal protein comprising 1156 amino acids as shown below:

1	MDNPNINIEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
51	VPGAGFVLGL	VDI IWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAI SRL
101	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLFAV
151	QNYQVPLLSV	YVQAANLHLS	VLRDVS VFGQ	RWGFDAATIN	SRYNDLTRLI
201	GNYTDYAVRW	YNTGLERVWG	PDSRDWVRYN	QFRRELTTLV	LDIVALFPNY
251	DSRRYP IRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIERS	IRSPHLM DIL
301	NSIT IYTD AH	RGYYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQORI
351	VAQLGQGVYR	TLSS TLYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
401	YRKS GTVDSL	DEIPPQNNNV	PPRQGF SHRL	SHVSMFRSGF	SNSSVSIIRA
451	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFN GSVISG	PGFTGGDLVR
501	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
551	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
601	DRFEFIPVTA	TLEAESDLER	AQKAVNALFT	SSNQIGLKTD	VTDYHIDRVS
651	NLVECLSDEF	CLDEKKELSE	KVKHAKRLSD	ERNLLQDPNF	RGINRQLDRG
701	WRGSTDITIQ	GGDDVFKENY	VTLLGTFDEC	YPTYLYQKID	ESK LKAYTRY
751	QLRGYIEDSQ	DLEIYLIRYN	AKHETVNVPG	TGSLWPLSAP	SPIGKCAHHS
801	HHFSLDIDVG	CTDLNEDLGV	WVIFKIKTQD	GHARLGNLEF	LEEKPLVGEA
851	LARVKRAEKK	WRDKREKLEW	ETNIVYKEAK	ESVDALFVNS	QYDR LQADTN
901	IAMIHAADKR	VHSIREAYLP	ELSVIPGVNA	AIFEELEGRI	FTAFSLYDAR
951	NVIKNGDFNN	GLSCWNVKGH	VDVEEQNNHR	SVLVVPEWEA	EVSQEV R VCP
1001	GRGYILRVTA	YKEGYGEGCV	TIHEIENNTD	ELKFSNCVEE	EVYPNNTVTC
1051	NDYTATQEEY	EGTYTSRNRG	YDGAYESNSS	VPADYASAYE	EKAYTDGRRD
1101	NPCESNRGYG	DYTPLPAGYV	TKELEYFPET	DKVWIEIGET	EGTFIVDSVE
1151	LLLMEE				

IV.B. The *pat* (Phosphinothricin Acetyltransferase) Gene

The *pat* gene codes for phosphinothricin acetyltransferase from *Streptomyces viridochromogenes*. The *pat* gene was synthesized, based on the amino acid sequence from *S. viridochromogenes*, to optimize plant codon usage for expression in plants (Van Wert, 1994; OECD, 1999).

The *pat* gene is driven by the 4OCSΔMas2' promoter and terminated by the bi-directional polyadenylation signal, ORF25 polyA. The chimeric 4OCSΔMas2' promoter contains the mannopine synthase promoter derived from *Agrobacterium tumefaciens* strain LBA4404 plasmid pTi15955 and four (4) copies of the octopine (OCS) synthase enhancer from *A. tumefaciens* tumor inducing plasmid pTiAch5. The *pat* gene and regulatory elements were transformed into cotton, resulting in PAT expression conferring tolerance to chemically synthesized phosphinothricin products such as the herbicide glufosinate-ammonium.

IV.B.1. Peptide Sequence of Transgenic PAT

The *pat* gene present in event 3006-210-23 is a synthetic version of the native bacterial *pat* gene sequence from *Streptomyces viridochromogenes*. The synthetic version was produced in order to modify the guanine and cytosine codon bias to a level more typical for plant DNA (Van Wert, 1994). The plant-optimized *pat* transgene encodes a protein of 183 amino acids. The amino acid sequence of the PAT protein encoded by the transgene is identical in sequence to the native bacterial PAT protein and is shown below:

```
1  MSPERRPVEI  RPATAADMAA  VCDIVNHYIE  TSTVNFRTPEP  QTPQEWIDDL
51  ERLQDRYPWL  VAEVEGVVAG  IAYAGPWKAR  NAYDWTVEST  VYVSHRHQRL
101 GLGSTLYTHL  LKSMEAQGFK  SVVAVIGLPN  DPSVRLHEAL  GYTARGTLRA
151 AGYKHGGWHD  VGFWQRDFEL  PAPPRPVRPV  TQI*
```

IV.C. The pMYC3006 Transformation Vector

The pMYC3006 transformation vector is a binary T-DNA vector carrying the transgenes for insertion into the plant genome and a bacterial antibiotic resistance marker to facilitate cloning and maintenance of the plasmid in bacterial hosts. The plasmid backbone was derived from plasmid RK2 (Schmidhauser and Helinski, 1985) from which the tetracycline resistance gene was deleted and replaced with a DNA fragment containing the erythromycin resistance coding sequence for bacterial expression. The transgene insert is flanked by T-DNA border sequences from *Agrobacterium tumefaciens* pTi15955 (Barker *et al.* 1983).

Table 2. Genetic Elements of the T-DNA Region of the Plasmid pMYC3006.

Sizes are based on updated plasmid map as in Green *et al.*, 2002.

Genetic element	Size (kbp)	Location (bp)	Details
Ubi Zm1	1.99	6080-8072 (complementary)	<i>Zea mays</i> promoter plus <i>Zea mays</i> exon1 (untranslated enhancer) and intron1 (Christiansen <i>et al.</i> , 1992, Plant Mol Biol 18: 675-689) (US Patent 5614399, GenBank Accession I38571)
<i>cry1Ac</i> (synpro)	3.47	2587-6057 (complementary)	Synthetic, plant-optimized, full length version of Cry1Ac1 from <i>B.t.</i> var. <i>kurstaki</i> . Nucleotides 1-1844 of the coding sequence encode the toxic portion of Cry1Ac1. Nucleotides 1845- 1951 (<i>Xho</i> I to <i>Pvu</i> I) encode a portion of the Cry1C protoxin. Nucleotides 1952-3481 encode a portion of the Cry1Ab1 protoxin.
ORF25 polyA	0.73	1835-2561	Bidirectional terminator from <i>Agrobacterium tumifaciens</i> pTi15955 (Barker <i>et al.</i> , 1983) Plant Mol. Biol. 2, 335-350, GenBank Locus ATACH5, Accession X00493)
<i>pat</i>	0.55	1276-1827	The synthetic plant optimized glufosinate resistance gene, based on a phosphinothricin acetyltransferase gene sequence from <i>Streptomyces viridochromogenes</i> (Eckes <i>et al.</i> , 1989. J. Cell. Biochem. 13D, 334)
(4OCS) Δ mas 2'	0.61	643-1251	Mannopine synthase promoter from pTi15955 (Barker <i>et al.</i> , 1983, Plant Mol. Biol. 2, 335-350, GenBank Locus ATACH5, Accession X00493), including 4 copies of the octopine synthase (OCS) enhancer from pTiAch5 (Ellis <i>et al.</i> , 1987, EMBO Journal 6:3202-3208, GenBank Accession Numbers I05704 to I05712).

Figure 1. Plasmid Map of pMYC3006

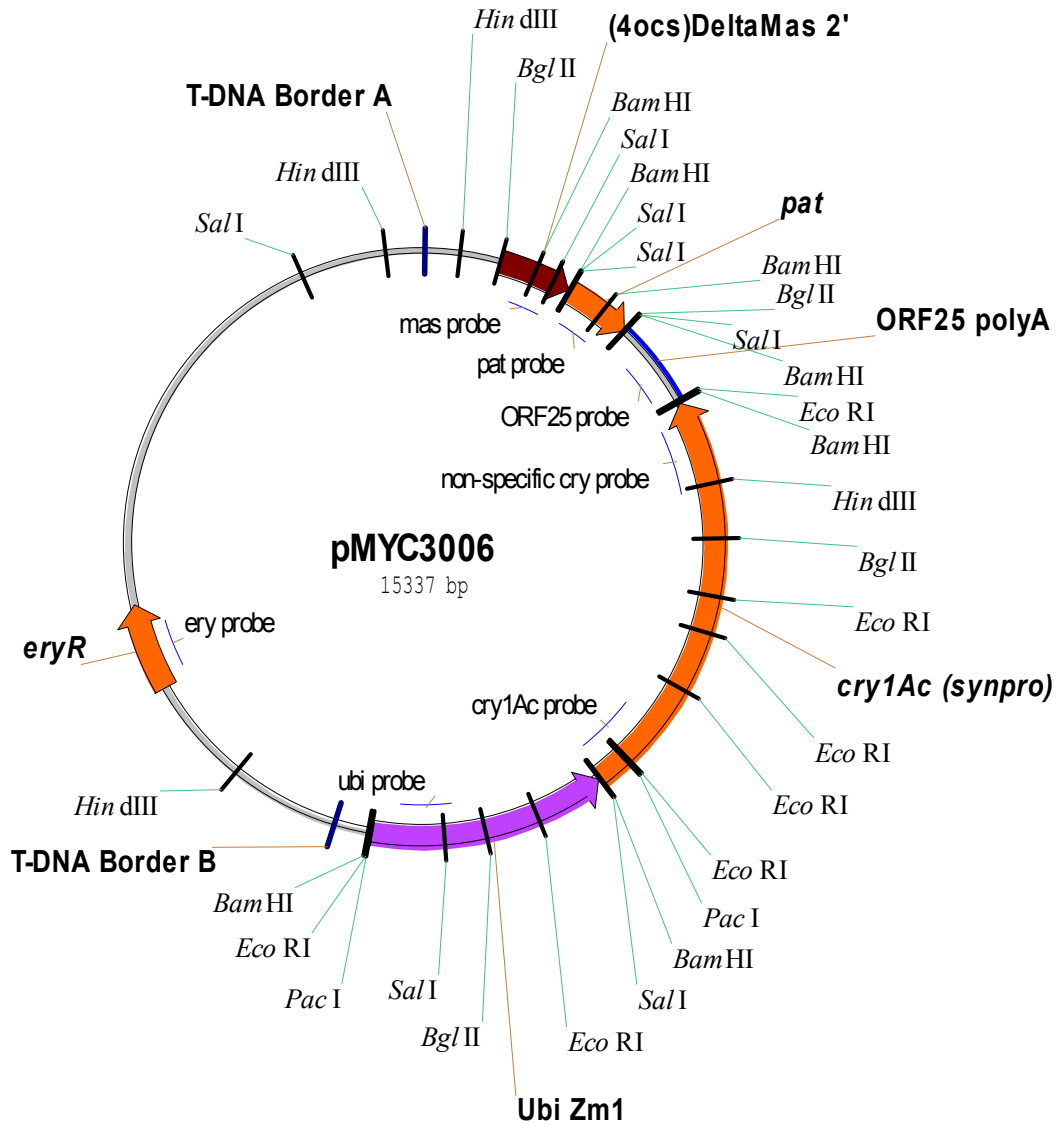
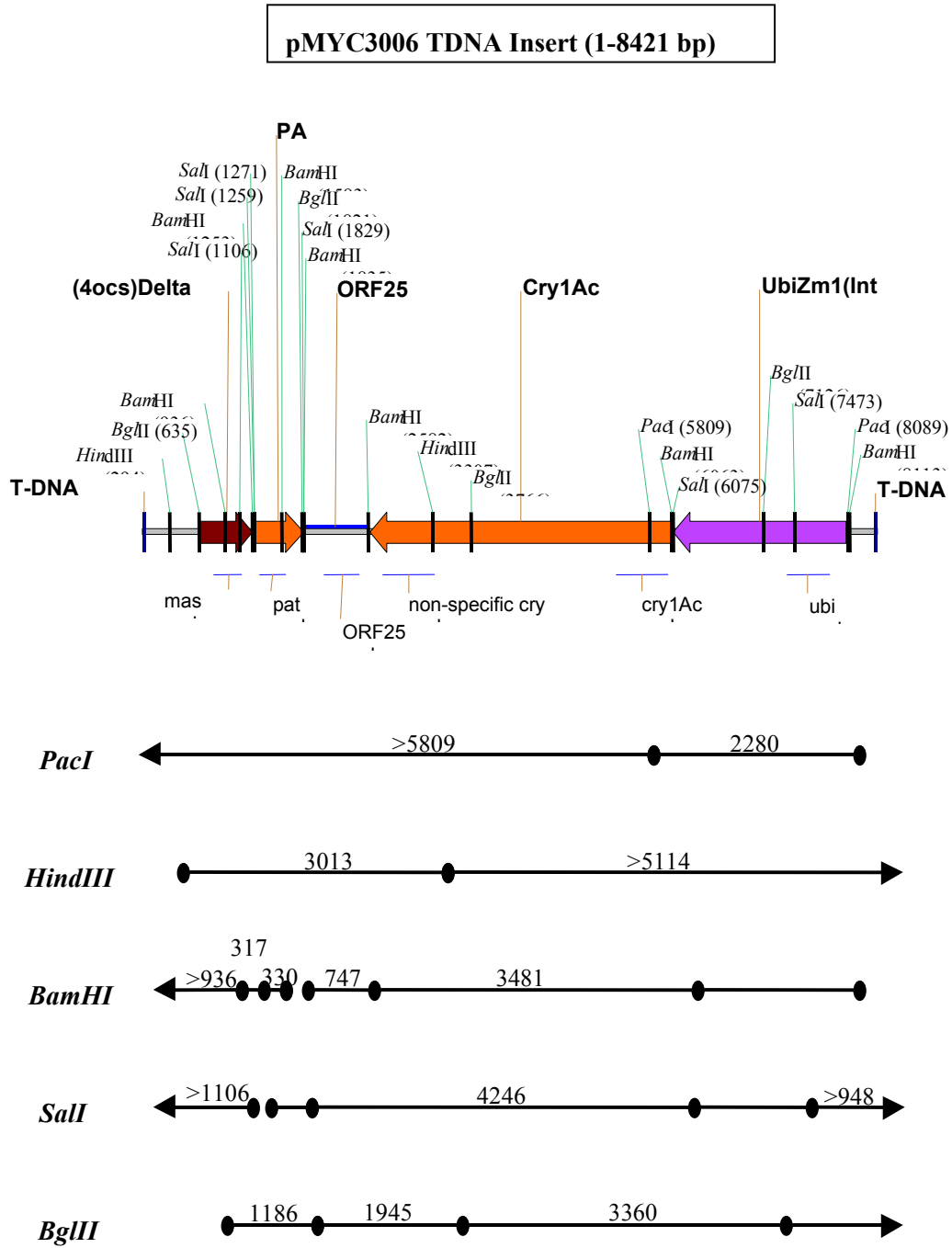


Figure 2. Schematic Diagram of the Insert and Associated Restriction Fragments of Plasmid pMYC3006 T-DNA Region



V. Genetic Analysis and Agronomic Performance of Event 3006-210-23

Southern blot analysis demonstrated that event 3006-210-23 contains a single integration of the transgenic DNA, including one intact copy of the *cry1Ac* gene, one intact copy of the *pat* gene, and single, intact copies of each of the regulatory elements (Green *et al.*, 2002; Appendix 2). In addition, it was also confirmed that the gene encoding for bacterial erythromycin resistance (*ery^R*) was not integrated into event 3006-210-23. The data generated also suggest that inheritance of the *cry1Ac* and *pat* genes is stable across generations and within a segregating generation of event 3006-210-23 (Green, 2003).

Average expression levels of across matrices ranged from not detected (ND) to 1.92 ng/mg sample weight. The expression levels of PAT were rarely detected across matrices (Phillips *et al.*, 2002; Appendix 2).

Agronomic data indicate that event 3006-210-23 shows no differences with respect to the non-transformed parent line or other commercial cotton varieties.

The details of the genetic analyses are summarized in sections V.A. and V.B., along with the data on Mendelian segregation in section V.C. Expression data of the Cry1Ac and PAT proteins on event 3006-210-23 can be found in section V.D. Details on the agronomic characteristics of *B.t.* Cry1Ac cotton event 3006-210-23 are described in section V. F. and V.G.

V.A. Characterization of the DNA insert

The initial transformant of GC510 (described earlier) was self pollinated and backcrossed three times with cotton line PSC355 to produce the BC3F1 generation. The same line was self-pollinated three additional generations to produce the BC3F4 generation (for detailed description see section V.C.). Plants of the BC3F4 generation served as test samples for molecular characterization of event 3006-210-23; the BC3F1 and BC3F4 generations were used to establish across generation stability; and, BC3F2 generation was used to demonstrate stability within a segregating generation. Null cotton, BC3F1 plants that did not express any of the transgenes, was used as the negative control and also used as the positive spiked plasmid control in the integration, copy number and stability across generation determinations. In the stability within generation determinations, PSC355 (parental line) DNA was used as the negative control and positive spiked control. DNA of plasmid pMYC3006 was added either to the null control cotton DNA or the parental line DNA to roughly simulate 3 copies of the *cry1Ac* transgene, for use as the positive control. A 1Kb DNA Ladder was used as the molecular weight marker on agarose gels and Southern blot hybridizations.

DNA probes specific to the *cry1Ac*, *pat* and *ery^R* genes, the (4OCS) Δ mas 2' promoter (*mas*), the Ubi Zm1 promoter (*ubi*) and the ORF25 polyA signal (*ORF25*) were produced by polymerase chain reaction (PCR) amplification. DNA extraction, quantification, digestion and separation, and Southern blot hybridization followed specific methodology. Probe sizes and sequence locations are described in Table 3 and shown in Figure 2. Experimental methodology utilized for molecular characterization of Event 3006-210-23 is described in detail by Green *et al.* (2002; Appendix 2).

Table 3. Description, Location in Sequence and Size of DNA Probes used in Southern Analysis of Cotton Event 3006-210-23

Probe	Genetic Element	Position in pMYC3006(bp)	Length (bp)
<i>cry1Ac</i>	5' portion of the <i>cry1Ac</i> gene	5423-6007	585
	3' portion of the <i>cry1Ac</i> gene	2747-3347	601
<i>pat</i>	<i>pat</i> gene	1327-1630	304
<i>ery^R</i>	erythromycin resistance gene	10360-10796	437
<i>ubi</i>	Ubi Zm1 promoter	7383-7861	479
<i>mas</i>	(4OCS) Δ mas2'promoter	815-1111	297
<i>ORF25</i>	ORF25 polyA signal	2081-2466	386

V.A.1. Analysis of Integration Number of the *cry1Ac* and *pat* Genes

Three DNA samples from the BC3F4 generation of transgenic cotton event 3006-210-23 were cleaved with restriction enzymes *Pac* I and *Hind* III to determine the number of insertions of the *cry1Ac* and *pat* genes in the cotton genome. These enzymes were employed because at least one of their restriction fragments relies on a recognition site in the genomic DNA, flanking the insert. Since the flanking restriction site is unique to each insertion, independent insertions of the transgene can produce unique fragments upon restriction enzyme digestion. The number of bands produced from enzyme digestions relying on a site in the genome is directly related to integration complexity: the greater the number of bands, the more integration sites in the genome. The size of these fragments cannot be predicted in advance since they rely on unknown sequence at the site of integration.

The *cry1Ac* probe consists of two different DNA fragments while the *pat* probe was a single fragment. Because *Pac* I and *Hind* III cut the insert DNA in a position between the binding sites of the two *cry1Ac* probe fragments, the *cry1Ac* probe should hybridize to two restriction fragments, i.e. one band for each *cry1Ac* probe fragment. Alternatively, these enzymes do not cut within the *pat* gene resulting in only one fragment hybridizing to the *pat* probe. The *Hind* III digest provides integration information for only one of the *cry1Ac* fragments, the second *cry1Ac* fragment and the *pat* probe hybridize a 3.0 Kb *Hind* III fragment contained within the insert (Figure 2).

The Southern blots hybridized with the *cry1Ac* and *pat* probes are shown in Figures 3 and 4. The data obtained from these Southern blots are summarized in Table 4. This table contains the figure number of the Southern blot, the probe and enzyme used, the predicted and observed fragment size for the corresponding enzyme/probe combination, and the lane assignment for those samples.

The *cry1Ac* and *pat* probes were found to hybridize to only the predicted number and size of DNA fragments after digestion with *Pac* I and *Hind* III (Table 4; Figure 3, Lanes 3-5, 8-10 and Figure 4, Lanes 3-5, 8-10). These data suggest that there is a single insertion of the *cry1Ac* and *pat* genes in event 3006-210-23 transgenic cotton.

Table 4. Observed and Predicted Hybridizing Fragments in Southern Analysis of Cotton Event 3006-210-23

Probe	Restriction Enzyme	Figure	Event 3006-210-23			PMYC3006		
			Lanes	Fragment Size (bp)		Lane	Fragment Size (bp)	
				Predicted	Observed		Predicted	Observed
<i>cry1Ac</i>	<i>Pac I</i>	3	2-5	2280, >5809	2300, >10,000	6	2280, 13057	2300, >10,000
<i>cry1Ac</i>	<i>Hind III</i>	3	8-10	3013, >5114	3000, 5500	11	3013, 6026	3000, 6000
<i>cry1Ac</i>	<i>Bgl II</i>	3	15-18	1945, 3360	2000, 3500	19	1945, 3360	2000, 3500
<i>cry1Ac</i>	<i>Bam HI</i>	3	21-23	3481	3500	24	3481	3500
<i>cry1Ac</i>	<i>Sal I</i>	3	26-28	4246	4300	29	4246	4300
<i>pat</i>	<i>Pac I</i>	4	2-5	>5809	>10,000	6	13057	>10,000
<i>pat</i>	<i>Hind III</i>	4	8-10	3013	3000	11	3013	3000
<i>pat</i>	<i>Bgl II</i>	4	15-18	1186	1200	19	1186	1200
<i>pat</i>	<i>Bam HI</i>	4	21-23	330	300	24	330	300
<i>mas</i>	<i>Pac I</i>	5	2-5	>5809	>10,000	6	13057	>10,000
<i>mas</i>	<i>Hind III</i>	5	8-10	3013	3000	11	3013	3000
<i>mas</i>	<i>Bgl II</i>	5	15-18	1186	1200	19	1186	1200
<i>ubi</i>	<i>Pac I</i>	6	2-5	2280	2300	6	2280	2300
<i>ubi</i>	<i>Hind III</i>	6	8-10	>5114	5600	11	5699	5700
<i>ubi</i>	<i>Bgl II</i>	6	15-18	>1295	4000	19	8846	8800
<i>ubi</i>	<i>Bam HI</i>	6	21-23	2050	2000	24	2050	2000
<i>ORF25</i>	<i>Pac I</i>	7	2-5	>5809	>10,000	6	13057	>10,000
<i>ORF25</i>	<i>Hind III</i>	7	8-10	3013	3000	11	3013	3000
<i>ORF25</i>	<i>Bgl II</i>	7	15-18	1945	2000	19	1945	2000
<i>ORF25</i>	<i>Bam HI</i>	7	21-23	747	700	24	747	750
<i>ORF25</i>	<i>Sal I</i>	7	26-28	4246	4300	29	4246	4200

Figure 3. Southern Blot Analysis of Cotton Event 3006-210-23 with the *cry1Ac* Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lanes</u>	<u>DNA Sample</u>		<u>Lanes</u>	<u>DNA Sample</u>	
			15	Null Control (102582-4)	<i>Bgl</i> II
1	MW Marker		16	BC3F4 (102577-2)	<i>Bgl</i> II
2	Null control (102582-4)	<i>Pac</i> I	17	BC3F4 (102577-3)	<i>Bgl</i> II
3	BC3F4 (102577-2)	<i>Pac</i> I	18	BC3F4 (102577-5)	<i>Bgl</i> II
4	BC3F4 (102577-3)	<i>Pac</i> I	19	pMYC3006 Plasmid + Null Control	<i>Bgl</i> II
5	BC3F4 (102577-5)	<i>Pac</i> I	20	Empty	
6	pMYC3006 Plasmid + Null Control	<i>Pac</i> I	21	BC3F4 (102577-2)	<i>Bam</i> HI
7	Empty		22	BC3F4 (102577-3)	<i>Bam</i> HI
8	BC3F4 (102577-2)	<i>Hind</i> III	23	BC3F4 (102577-5)	<i>Bam</i> HI
9	BC3F4 (102577-3)	<i>Hind</i> III	24	pMYC3006 Plasmid + Null Control	<i>Bam</i> HI
10	BC3F4 (102577-5)	<i>Hind</i> III	25	Empty	
11	pMYC3006 Plasmid +Null Control	<i>Hind</i> III	26	BC3F4 (102577-2)	<i>Sal</i> I
12	Empty		27	BC3F4 (102577-3)	<i>Sal</i> I
13	MW Marker		28	BC3F4 (102577-5)	<i>Sal</i> I
14	Empty		29	pMYC3006 Plasmid + Null Control	<i>Sal</i> I

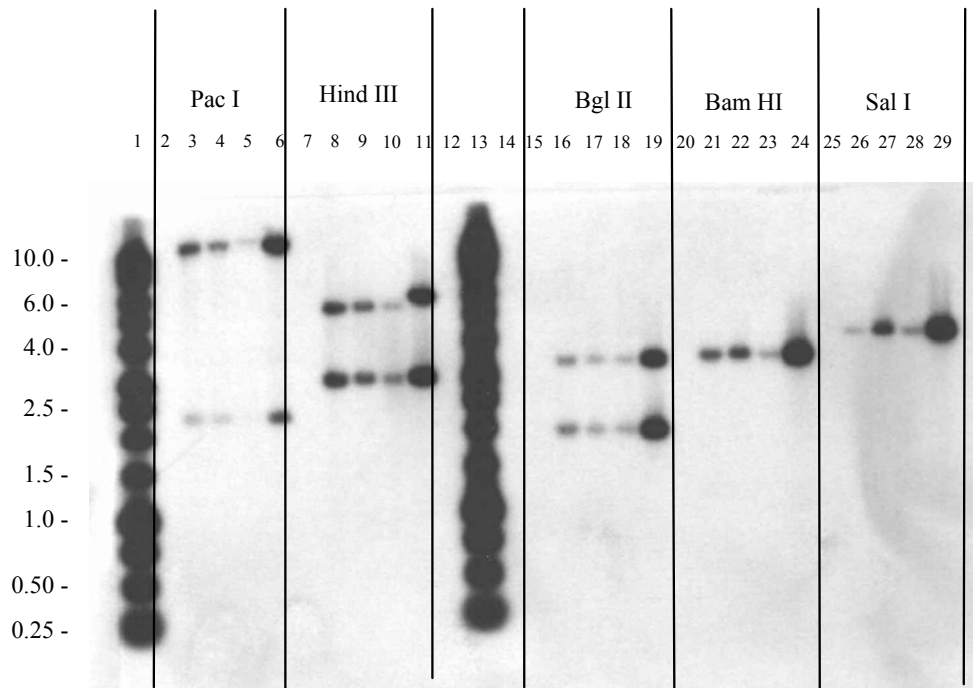
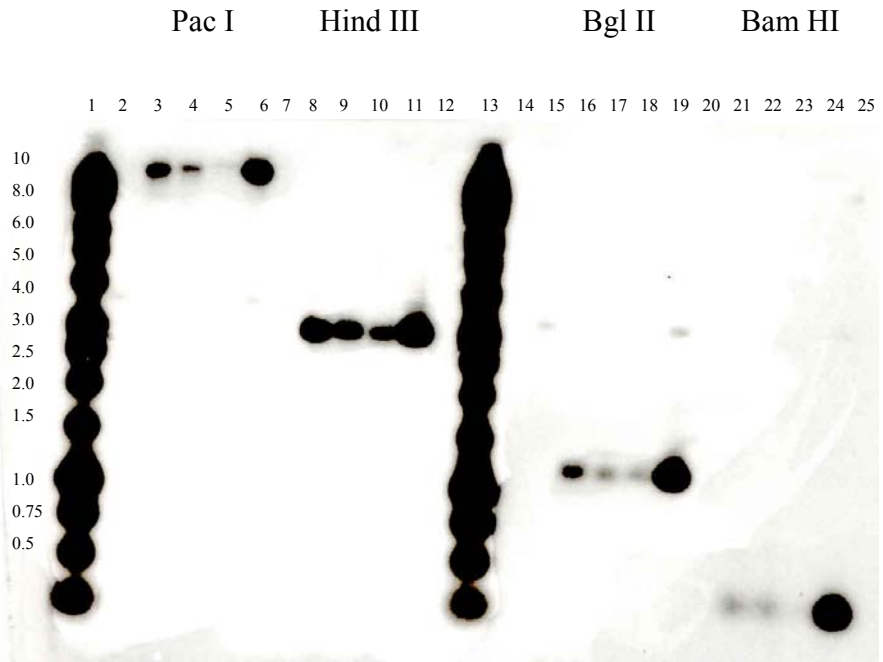


Figure 4. Southern Blot Analysis of Cotton Event 3006-210-23 with the *pat* Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of cry1Ac spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lanes</u>	<u>DNA Sample</u>		<u>Lanes</u>	<u>DNA Sample</u>	
			15	Null Control (102582-4)	<i>Bgl</i> II
1	MW Marker		16	BC3F4 (102577-2)	<i>Bgl</i> II
2	Null control (102582-4)	<i>Pac</i> I	17	BC3F4 (102577-3)	<i>Bgl</i> II
3	BC3F4 (102577-2)	<i>Pac</i> I	18	BC3F4 (102577-5)	<i>Bgl</i> II
4	BC3F4 (102577-3)	<i>Pac</i> I	19	pMYC3006 Plasmid + Null Control	<i>Bgl</i> II
5	BC3F4 (102577-5)	<i>Pac</i> I	20	Empty	
6	pMYC3006 Plasmid + Null Control	<i>Pac</i> I	21	BC3F4 (102577-2)	<i>Bam</i> HI
7	Empty		22	BC3F4 (102577-3)	<i>Bam</i> HI
8	BC3F4 (102577-2)	<i>Hind</i> III	23	BC3F4 (102577-5)	<i>Bam</i> HI
9	BC3F4 (102577-3)	<i>Hind</i> III	24	pMYC3006 Plasmid + Null Control	<i>Bam</i> HI
10	BC3F4 (102577-5)	<i>Hind</i> III	25	Empty	
11	pMYC3006 Plasmid +Null Control	<i>Hind</i> III			
12	Empty				
13	MW Marker				
14	Empty				



V.A.2. Analysis of Gene Copy Number and Integrity

The same DNA samples that were analyzed for integration were cleaved with restriction enzymes *Bam* HI, *Bgl* II, and *Sal* I to determine the copy number and integrity of the *cry1Ac* gene, and were also cleaved with *Bam* HI and *Bgl* II for the *pat* gene in event 3006-210-23. These enzymes were employed because they have more than one recognition site within the transgene insert (Figure 2). This “internal cut” allows for the comparison between observed fragments and those predicted by the sequence. The *cry1Ac* and *pat* probes hybridized with the predicted size fragments in every lane (Table 4; Figure 3, Lanes 16-18, 21-23, 26-28 and Figure 4, Lanes 16-18, 21-23). These data indicate the presence of single, intact copies of the *cry1Ac* and *pat* genes in event 3006-210-23.

V.A.3. Presence of Regulatory Elements

In addition to confirming the presence of the transgenes in cotton event 3006-210-23, the blots were probed with *mas*, *ubi* and *ORF25* probes to detect the presence of the regulatory elements. Restriction enzymes that cleave the insert to provide information on integration number, copy number, and integrity were used. In all cases, the two promoters, (4OCS) Δ mas 2' and Ubi Zm1, and the terminator, ORF25 polyA, were detected by Southern analysis with bands corresponding to the predicted fragment size (Table 4, Figures 5, 6 and 7), suggesting the presence of single, intact copies of each of the regulatory elements in event 3006-210-23.

Figure 5. Southern Blot Analysis of Cotton Event 3006-210-23 with the *mas* Probe.

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lanes</u>	<u>DNA Sample</u>		<u>Lanes</u>	<u>DNA Sample</u>	
1	MW Marker		15	Null Control (102582-4)	<i>Bgl</i> II
2	Null control (102582-4)	<i>Pac</i> I	16	BC3F4 (102577-2)	<i>Bgl</i> II
3	BC3F4 (102577-2)	<i>Pac</i> I	17	BC3F4 (102577-3)	<i>Bgl</i> II
4	BC3F4 (102577-3)	<i>Pac</i> I	18	BC3F4 (102577-5)	<i>Bgl</i> II
5	BC3F4 (102577-5)	<i>Pac</i> I	19	pMYC3006 Plasmid + Null Control	<i>Bgl</i> II
6	pMYC3006 Plasmid + Null Control	<i>Pac</i> I			
7	Empty				
8	BC3F4 (102577-2)	<i>Hind</i> III			
9	BC3F4 (102577-3)	<i>Hind</i> III			
10	BC3F4 (102577-5)	<i>Hind</i> III			
11	pMYC3006 Plasmid +Null Control	<i>Hind</i> III			
12	Empty				
13	MW Marker				
14	Empty				

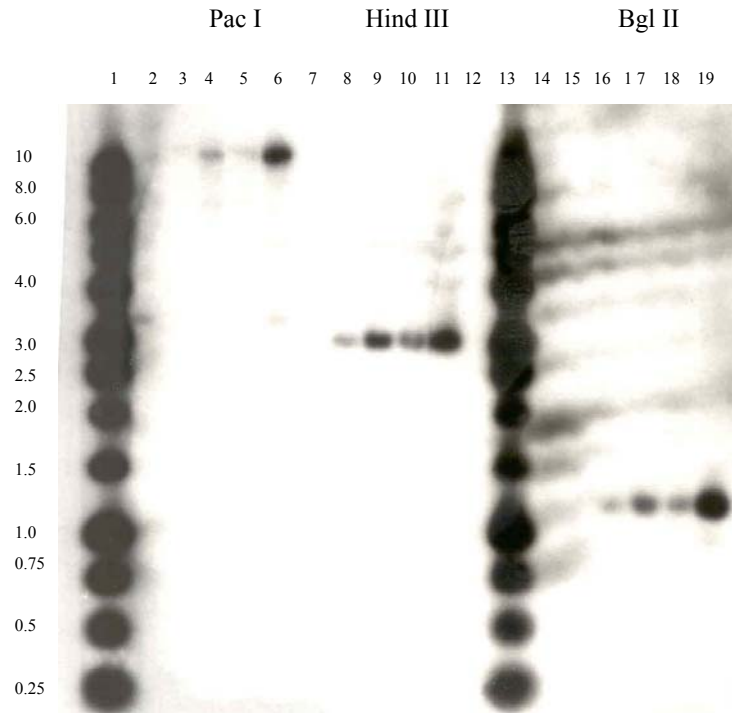


Figure 6. Southern Blot Analysis of Cotton Event 3006-210-23 with the *ubi* Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lanes</u>	<u>DNA Sample</u>		<u>Lanes</u>	<u>DNA Sample</u>	
			15	Null Control (102582-4)	<i>Bgl</i> II
1	MW Marker		16	BC3F4 (102577-2)	<i>Bgl</i> II
2	Null control (102582-4)	<i>Pac</i> I	17	BC3F4 (102577-3)	<i>Bgl</i> II
3	BC3F4 (102577-2)	<i>Pac</i> I	18	BC3F4 (102577-5)	<i>Bgl</i> II
4	BC3F4 (102577-3)	<i>Pac</i> I	19	pMYC3006 Plasmid + Null Control	<i>Bgl</i> II
5	BC3F4 (102577-5)	<i>Pac</i> I	20	Empty	
6	pMYC3006 Plasmid + Null Control	<i>Pac</i> I	21	BC3F4 (102577-2)	<i>Bam</i> HI
7	Empty		22	BC3F4 (102577-3)	<i>Bam</i> HI
8	BC3F4 (102577-2)	<i>Hind</i> III	23	BC3F4 (102577-5)	<i>Bam</i> HI
9	BC3F4 (102577-3)	<i>Hind</i> III	24	pMYC3006 Plasmid + Null Control	<i>Bam</i> HI
10	BC3F4 (102577-5)	<i>Hind</i> III	25	Empty	
11	pMYC3006 Plasmid +Null Control	<i>Hind</i> III	26	BC3F4 (102577-2)	<i>Sal</i> I
12	Empty		27	BC3F4 (102577-3)	<i>Sal</i> I
13	MW Marker		28	BC3F4 (102577-5)	<i>Sal</i> I
14	Empty		29	pMYC3006 Plasmid + Null Control	<i>Sal</i> I

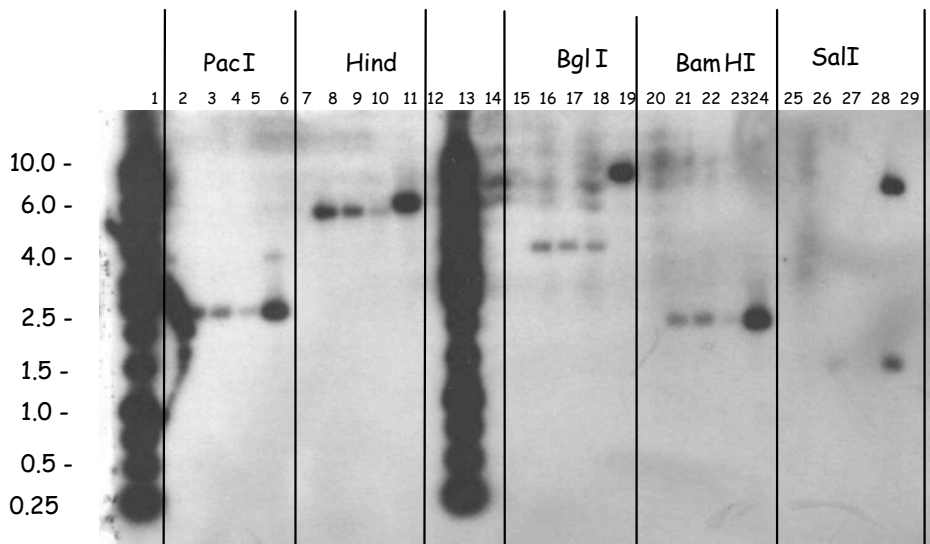
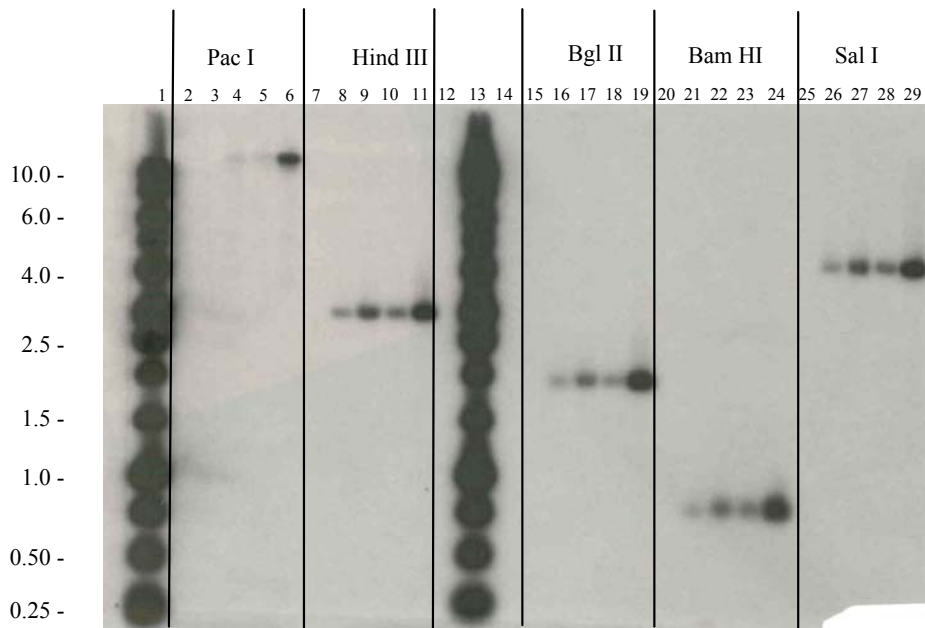


Figure 7. Southern Blot Analysis of Cotton Event 3006-210-23 with the *ORF25* Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lanes</u>	<u>DNA Sample</u>		<u>Lanes</u>	<u>DNA Sample</u>	
			15	Null Control (102582-4)	<i>Bgl</i> II
1	MW Marker		16	BC3F4 (102577-2)	<i>Bgl</i> II
2	Null control (102582-4)	<i>Pac</i> I	17	BC3F4 (102577-3)	<i>Bgl</i> II
3	BC3F4 (102577-2)	<i>Pac</i> I	18	BC3F4 (102577-5)	<i>Bgl</i> II
4	BC3F4 (102577-3)	<i>Pac</i> I	19	pMYC3006 Plasmid + Null Control	<i>Bgl</i> II
5	BC3F4 (102577-5)	<i>Pac</i> I	20	Empty	
6	pMYC3006 Plasmid + Null Control	<i>Pac</i> I	21	BC3F4 (102577-2)	<i>Bam</i> HI
7	Empty		22	BC3F4 (102577-3)	<i>Bam</i> HI
8	BC3F4 (102577-2)	<i>Hind</i> III	23	BC3F4 (102577-5)	<i>Bam</i> HI
9	BC3F4 (102577-3)	<i>Hind</i> III	24	pMYC3006 Plasmid + Null Control	<i>Bam</i> HI
10	BC3F4 (102577-5)	<i>Hind</i> III	25	Empty	
11	pMYC3006 Plasmid +Null Control	<i>Hind</i> III	26	BC3F4 (102577-2)	<i>Sal</i> I
12	Empty		27	BC3F4 (102577-3)	<i>Sal</i> I
13	MW Marker		28	BC3F4 (102577-5)	<i>Sal</i> I
14	Empty		29	pMYC3006 Plasmid + Null Control	<i>Sal</i> I



V.A.4. Stability Across Generations

Three DNA samples from each of two generations (BC3F1 and BC3F4) of event 3006-210-23 transgenic cotton were cleaved with the restriction enzymes *Pac* I and *Bgl* II to determine the stability of inheritance of the *cry1Ac*, and *pat* genes across generations. The *Pac* I restriction digest provides information on integration number and the *Bgl* II digest provides information on copy number and gene integrity (Figure 2).

The Southern blot hybridized with the *cry1Ac* probe is shown in Figure 8 and the one probed with *pat* is shown in Figure 9. The data obtained from these Southern blots are summarized in Table 5.

As observed in Figures 8 and 9 and summarized in Table 5, the banding patterns are the same for samples from both generations and correspond to the predicted size bands. This data indicates that the inheritance of the single copies of the *cry1Ac* and *pat* genes is stable across generations.

Table 5. Generation Stability of the Transgenes in Cotton Event 3006-210-23

Probe	Restriction Enzyme	Sample	Figure	Lanes	Fragment Size (bp)	
					Predicted	Observed
<i>cry1Ac</i>	<i>Pac</i> I	3006-210-23, BC3F4	8	3-5	2280, >5806	2200, >10,000
<i>cry1Ac</i>	<i>Pac</i> I	3006-210-23, BC3F1	8	7-9	2280, >5806	2200, >10,000
<i>cry1Ac</i>	<i>Pac</i> I	pMYC3600 control	8	10	2280, 13057	2200, >10,000
<i>cry1Ac</i>	<i>Bgl</i> II	3006-210-23, BC3F4	8	13-15	1945, 3360	2000, 3300
<i>cry1Ac</i>	<i>Bgl</i> II	3006-210-23, BC3F1	8	17-19	1945, 3360	2000, 3300
<i>cry1Ac</i>	<i>Bgl</i> II	pMYC3600 control	8	20	1945, 3360	2000, 3300
<i>pat</i>	<i>Pac</i> I	3006-210-23, BC3F4	9	3-5	>5806	>10,000
<i>pat</i>	<i>Pac</i> I	3006-210-23, BC3F1	9	7-9	>5806	>10,000
<i>pat</i>	<i>Pac</i> I	PMYC3006 control	9	10	13057	>10,000
<i>pat</i>	<i>Bgl</i> II	3006-210-23, BC3F4	9	13-15	1186	1200
<i>pat</i>	<i>Bgl</i> II	3006-210-23, BC3F1	9	17-19	1186	1200
<i>pat</i>	<i>Bgl</i> II	PMYC3006 control	9	20	1186	1200

Figure 8. Southern Blot Analysis Demonstrating Across Generational Stability of Cotton Event 3006-210-23/ BC3F1 and BC3F4 Generations Digested with *Pac I* and *Bgl II* and Hybridized with the *cry1Ac* Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lane</u>	<u>Sample</u>		<u>Lane</u>	<u>Sample</u>	
1	MW Marker		14	BC3F4 (102577-3)	<i>Bgl II</i>
2	Null Control (102582-1)	<i>Pac I</i>	15	BC3F4 (102577-5)	<i>Bgl II</i>
3	BC3F4 (102577-2)	<i>Pac I</i>	16	Empty	
4	BC3F4 (102577-3)	<i>Pac I</i>	17	BC3F1 (102608-1)	<i>Bgl II</i>
5	BC3F4 (102577-5)	<i>Pac I</i>	18	BC3F1 (102608-4)	<i>Bgl II</i>
6	Empty		19	BC3F1 (102608-5)	<i>Bgl II</i>
7	BC3F1 (102608-1)	<i>Pac I</i>	20	PMYC3006 Plasmid + NullControl	<i>Bgl II</i>
8	BC3F1 (102608-4)	<i>Pac I</i>			
9	BC3F1 (102608-5)	<i>Pac I</i>			
10	pMYC3006 Plasmid + Null Control	<i>Pac I</i>			
11	Empty				
12	Null Control (102582-1)	<i>Bgl II</i>			
13	BC3F4 (102577-2)	<i>Bgl II</i>			

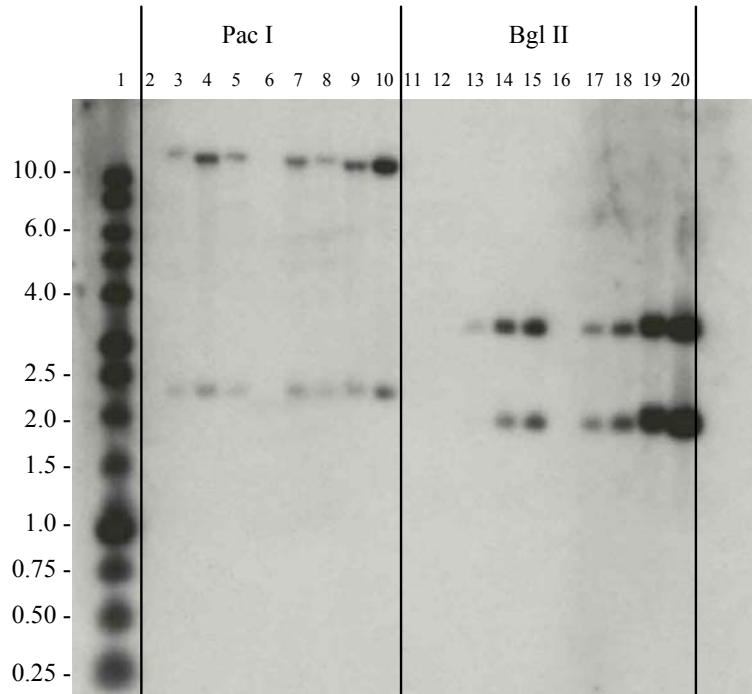
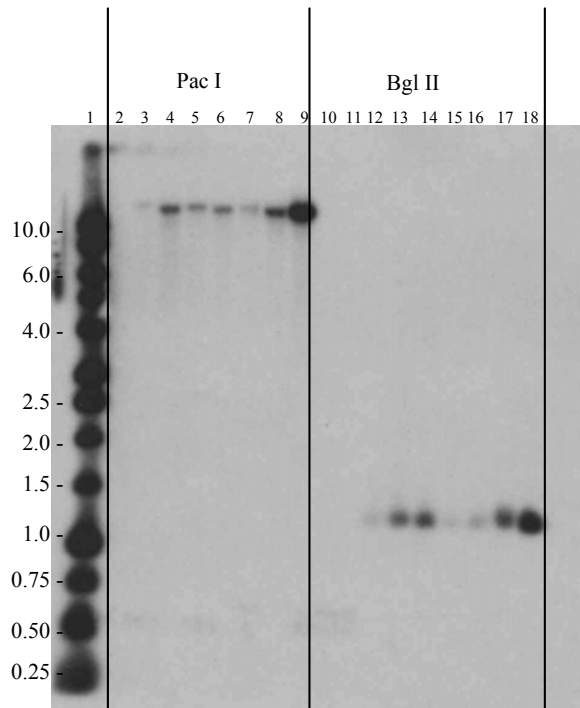


Figure 9. Southern Blot Analysis Demonstrating Across Generational Stability of Cotton Event 3006-210-23/ BC3F1 and BC3F4 Generations Digested with *Pac I* and *Bgl II* and Hybridized with the *pat* Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lane</u>	<u>Sample</u>		<u>Lane</u>	<u>Sample</u>	
1	MW Marker		14	BC3F4 (102577-3)	<i>Bgl II</i>
2	Null Control (102582-1)	<i>Pac I</i>	15	BC3F4 (102577-5)	<i>Bgl II</i>
3	BC3F4 (102577-2)	<i>Pac I</i>	16	Empty	
4	BC3F4 (102577-3)	<i>Pac I</i>	17	BC3F1 (102608-1)	<i>Bgl II</i>
5	BC3F4 (102577-5)	<i>Pac I</i>	18	BC3F1 (102608-4)	<i>Bgl II</i>
6	Empty		19	BC3F1 (102608-5)	<i>Bgl II</i>
7	BC3F1 (102608-1)	<i>Pac I</i>	20	PMYC3006 Plasmid + NullControl	<i>Bgl II</i>
8	BC3F1 (102608-4)	<i>Pac I</i>			
9	BC3F1 (102608-5)	<i>Pac I</i>			
10	pMYC3006 Plasmid + Null Control	<i>Pac I</i>			
11	Empty				
12	Null Control (102582-1)	<i>Bgl II</i>			
13	BC3F4 (102577-2)	<i>Bgl II</i>			



V.A.5. Stability within generations

Southern blot analysis was used to demonstrate the stability of inheritance of the *cry1Ac* and *pat* genes within a segregating generation of cotton event 3006-210-23 (Green, 2003). The generation analyzed was BC3F2, the product of self-pollination of a BC3F1 hemizygous line (*cry1Ac+pat/null*). Therefore, segregation of the transgene insert in the BC3F2 generation would be expected to occur at a ratio of 3 *cry1Ac+pat* : 1 null (1 *cry1Ac+pat/cry1Ac+pat* : 2 *cry1Ac+pat/null* : 1 null/null). Plants were tested for expression of the Cry1Ac protein using a lateral flow test strip immunoassay procedure and expression data was correlated with the results from Southern analysis of the *cry1Ac* transgene. All plants, including those testing negative for Cry1Ac protein expression, were analyzed by Southern blot hybridization with the *cry1Ac* and *pat* probes using 3 restriction enzyme digests, *Bam* HI, *Hind* III, and *Eco* RI.

The data from this study indicated that cotton event 3006-210-23 contains a single integration of transgenic DNA containing the *cry1Ac* and *pat* genes and that the inheritance of *cry1Ac* and *pat* is stable within a segregating generation (for representative data see Figures 10 and 11). All positive scoring plants were found to be positive for both Cry1Ac protein expression as well as the expected *cry1Ac* and *pat* DNA fragments, while null plants tested negative for the presence of transgenic protein and DNA. The expected number of positive and negative plants for a sample set of 56 was 42 and 14 respectively. Forty-seven (47) positives and 9 negatives were observed. The expected and observed ratios were not significantly different based on a binomial proportions test ($P > 0.05$, SAS version 8).

Additional segregation data demonstrating expected Mendelian segregation of Cry1Ac in segregating populations of Event 3006-210-23 are presented in section V.C.

Figure 10. Southern Blot Analysis Demonstrating Within Generational Stability of Cotton Event 3006-210-23/*Hind* III Digest with the *cry1Ac* probe

DNA isolated from cotton event 3006-210-23 and unmodified cotton was digested with *Hind* III and probed with the *cry1Ac* probe. Ten (10) µg of DNA was digested and loaded per lane. The plasmid control contained 3 gene copy equivalents per cotton genome of pMYC3006 and 10 µg of DNA isolated from PSC355 (neg. control). The numbers in () for each lane represent sample ID numbers. The lanes contained:

<u>Lane</u>	<u>Sample</u>		<u>Lane</u>	<u>Sample</u>	
1	MW Marker		19	BC3F2 (103670-13)	<i>Hind</i> III
2	Negative Control (103656-2)	<i>Hind</i> III	20	BC3F2 (103670-14)	<i>Hind</i> III
3	Negative Control (103656-3)	<i>Hind</i> III	21	BC3F2 (103670-15)	<i>Hind</i> III
4	Negative Control (103656-4)	<i>Hind</i> III	22	BC3F2 (103670-16)	<i>Hind</i> III
5	pMYC3006 Plasmid + Neg. Control	<i>Hind</i> III	23	BC3F2 (103670-17)	<i>Hind</i> III
6	Empty		24	BC3F2 (103670-18)	<i>Hind</i> III
7	BC3F2 (103670-1)	<i>Hind</i> III	25	BC3F2 (103670-19)	<i>Hind</i> III
8	BC3F2 (103670-2)	<i>Hind</i> III	26	BC3F2 (103670-20)	<i>Hind</i> III
9	BC3F2 (103670-3)	<i>Hind</i> III	27	BC3F2 (103670-21)	<i>Hind</i> III
10	BC3F2 (103670-4)	<i>Hind</i> III	28	BC3F2 (103670-22)	<i>Hind</i> III
11	BC3F2 (103670-5)	<i>Hind</i> III	29	BC3F2 (103670-23)	<i>Hind</i> III
12	BC3F2 (103670-6)	<i>Hind</i> III	30	BC3F2 (103670-24)	<i>Hind</i> III
13	BC3F2 (103670-7)	<i>Hind</i> III	31	BC3F2 (103670-25)	<i>Hind</i> III
14	BC3F2 (103670-8)	<i>Hind</i> III	32	BC3F2 (103670-26)	<i>Hind</i> III
15	BC3F2 (103670-9)	<i>Hind</i> III	33	BC3F2 (103670-27)	<i>Hind</i> III
16	BC3F2 (103670-10)	<i>Hind</i> III	34	BC3F2 (103670-29)	<i>Hind</i> III
17	BC3F2 (103670-11)	<i>Hind</i> III	35	BC3F2 (103670-30)	<i>Hind</i> III
18	BC3F2 (103670-12)	<i>Hind</i> III	36	MW Marker	

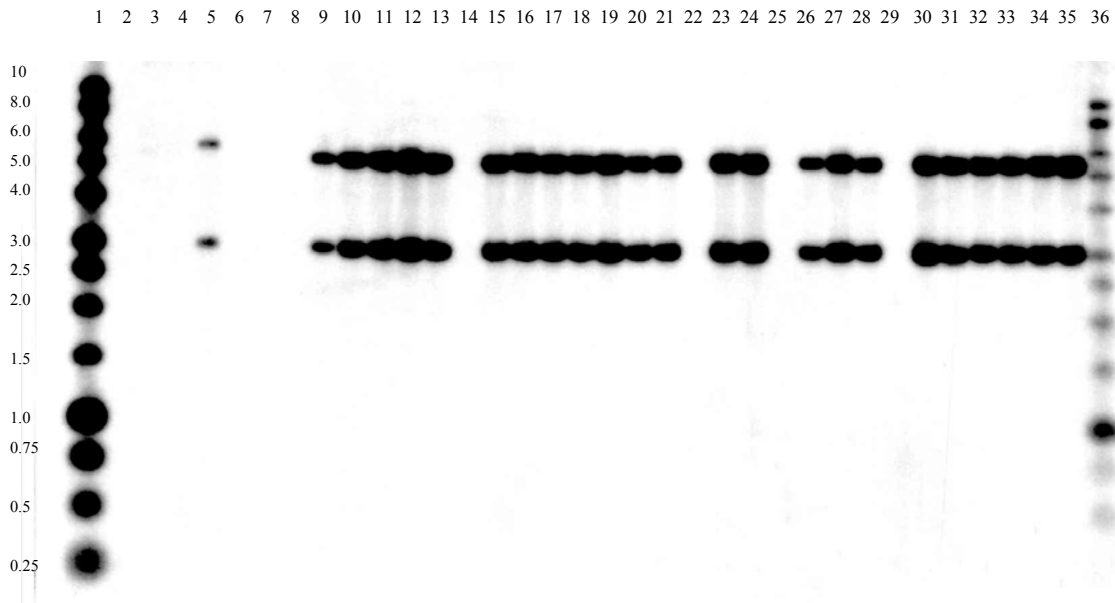
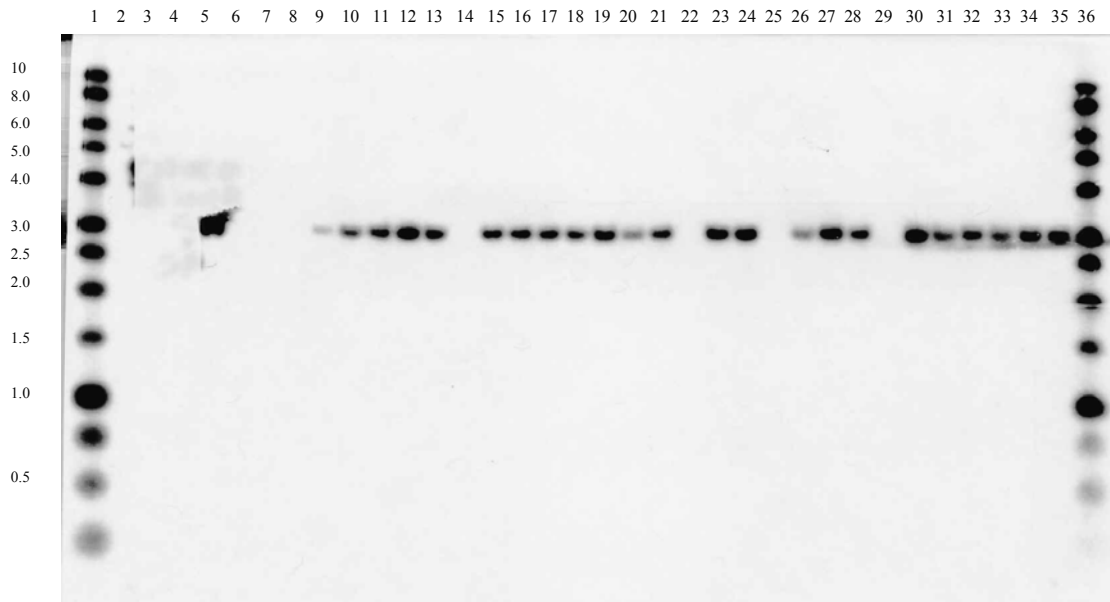


Figure 11. Southern Blot Analysis Demonstrating Within Generational Stability of Cotton Event 3006-210-23/ *Hind* III Digest with the *pat* Probe

DNA isolated from cotton event 3006-210-23 and unmodified cotton was digested with *Hind* III and probed with the *pat* probe. Ten (10) µg of DNA was digested and loaded per lane. The plasmid control contained 3 gene copy equivalents of pMYC3006 and 10 µg of DNA from PSC355 (neg. control). The number in () in each lane designation represents the sample ID. The lanes contained:

Lane	Sample		Lane	Sample
1	MW Marker		19	BC3F2 (103670-13) <i>Hind</i> III
2	Negative Control (103656-2)	<i>Hind</i> III	20	BC3F2 (103670-14) <i>Hind</i> III
3	Negative Control (103656-3)	<i>Hind</i> III	21	BC3F2 (103670-15) <i>Hind</i> III
4	Negative Control (103656-4)	<i>Hind</i> III	22	BC3F2 (103670-16) <i>Hind</i> III
5	pMYC3006 Plasmid + Neg. Control	<i>Hind</i> III	23	BC3F2 (103670-17) <i>Hind</i> III
6	Empty		24	BC3F2 (103670-18) <i>Hind</i> III
7	BC3F2 (103670-1)	<i>Hind</i> III	25	BC3F2 (103670-19) <i>Hind</i> III
8	BC3F2 (103670-2)	<i>Hind</i> III	26	BC3F2 (103670-20) <i>Hind</i> III
9	BC3F2 (103670-3)	<i>Hind</i> III	27	BC3F2 (103670-21) <i>Hind</i> III
10	BC3F2 (103670-4)	<i>Hind</i> III	28	BC3F2 (103670-22) <i>Hind</i> III
11	BC3F2 (103670-5)	<i>Hind</i> III	29	BC3F2 (103670-23) <i>Hind</i> III
12	BC3F2 (103670-6)	<i>Hind</i> III	30	BC3F2 (103670-24) <i>Hind</i> III
13	BC3F2 (103670-7)	<i>Hind</i> III	31	BC3F2 (103670-25) <i>Hind</i> III
14	BC3F2 (103670-8)	<i>Hind</i> III	32	BC3F2 (103670-26) <i>Hind</i> III
15	BC3F2 (103670-9)	<i>Hind</i> III	33	BC3F2 (103670-27) <i>Hind</i> III
16	BC3F2 (103670-10)	<i>Hind</i> III	34	BC3F2 (103670-29) <i>Hind</i> III
17	BC3F2 (103670-11)	<i>Hind</i> III	35	BC3F2 (103670-30) <i>Hind</i> III
18	BC3F2 (103670-12)	<i>Hind</i> III	36	MW Marker



V.B. Absence of Genes Outside the Insert Borders

V.B.1. Absence of Erythromycin Resistance Gene

Green *et al.* (2002; Appendix 2) confirmed that the gene encoding for erythromycin resistance on plasmid pMYC3006 has not been integrated into transgenic cotton event 3006-210-23. Three BC3F4 DNA samples from event 3006-210-23 were cleaved with restriction enzymes *Pac I*, *Hind III*, *Bgl II*, *Bam HI* and *Sal I* to confirm the absence of the *ery^R* gene. As seen in Figure 12 and summarized in Table 6 the *ery^R* probe does not bind to any fragments of the experimental samples or negative controls. The probe does bind to the expected fragments in each of the plasmid controls.

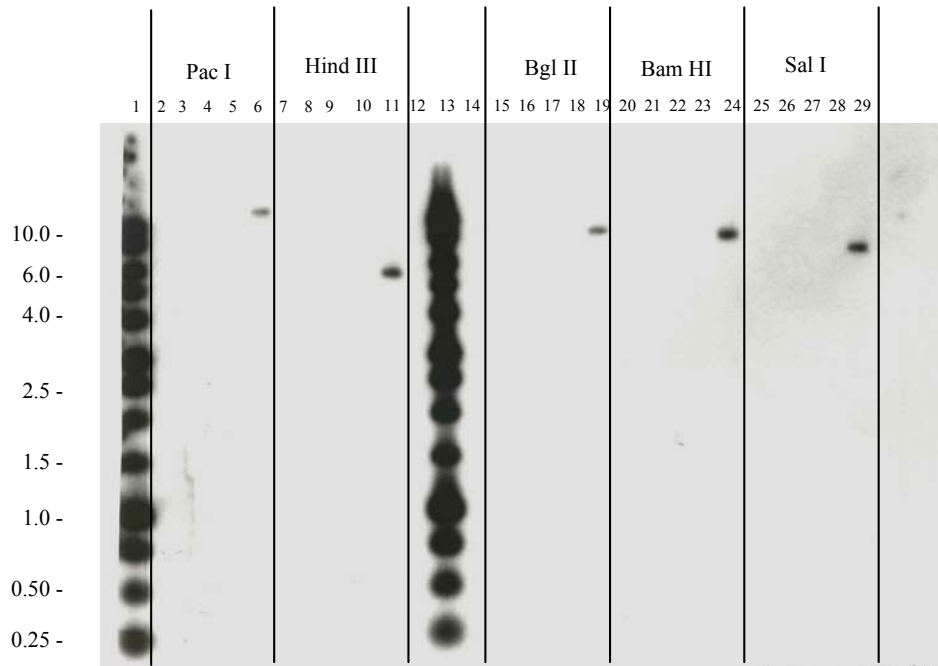
Table 6. Predicted and Observed Hybridizing Fragments in Southern Analysis for the Presence of the Erythromycin Resistance Gene (*ery^R*) in Cotton Event 3006-210-23

Probe	Restriction Enzyme	Figure	Event 3006-210-23			PMYC3006			
			Lanes	Fragment Size (bp)		Lane	Fragment Size (bp)		
				Predicted	Observed		Predicted	Observed	
<i>ery^R</i>	<i>Pac I</i>		12	2-5	NA	None	6	13057	>10,000
<i>ery^R</i>	<i>Hind III</i>		12	8-10	NA	None	11	5699	5700
<i>ery^R</i>	<i>Bgl II</i>		12	15-18	NA	None	19	8846	8800
<i>ery^R</i>	<i>Bam HI</i>		12	21-23	NA	None	24	8160	8200
<i>ery^R</i>	<i>Sal I</i>		12	26-28	NA	None	29	6842	6800

Figure 12. Southern Blot Analysis of Cotton Event 3006-210-23 with the *ery*^R Probe

All digested young leaf tissue DNA samples contained 8 µg DNA. The pMYC3006 plasmid DNA, ~3 gene copies of *cry1Ac* spiked into null cotton DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples were from greenhouse material. The lanes contained:

Lanes	DNA Sample		Lanes	DNA Sample	
			15	Null Control (102582-4)	<i>Bgl</i> II
1	MW Marker		16	BC3F4 (102577-2)	<i>Bgl</i> II
2	Null control (102582-4)	<i>Pac</i> I	17	BC3F4 (102577-3)	<i>Bgl</i> II
3	BC3F4 (102577-2)	<i>Pac</i> I	18	BC3F4 (102577-5)	<i>Bgl</i> II
4	BC3F4 (102577-3)	<i>Pac</i> I	19	pMYC3006 Plasmid + Null Control	<i>Bgl</i> II
5	BC3F4 (102577-5)	<i>Pac</i> I	20	Empty	
6	pMYC3006 Plasmid + Null Control	<i>Pac</i> I	21	BC3F4 (102577-2)	<i>Bam</i> HI
7	Empty		22	BC3F4 (102577-3)	<i>Bam</i> HI
8	BC3F4 (102577-2)	<i>Hind</i> III	23	BC3F4 (102577-5)	<i>Bam</i> HI
9	BC3F4 (102577-3)	<i>Hind</i> III	24	pMYC3006 Plasmid + Null Control	<i>Bam</i> HI
10	BC3F4 (102577-5)	<i>Hind</i> III	25	Empty	
11	pMYC3006 Plasmid +Null Control	<i>Hind</i> III	26	BC3F4 (102577-2)	<i>Sal</i> I
12	Empty		27	BC3F4 (102577-3)	<i>Sal</i> I
13	MW Marker		28	BC3F4 (102577-5)	<i>Sal</i> I
14	Empty		29	pMYC3006 Plasmid + Null Control	<i>Sal</i> I



V.C. Mendelian inheritance of Cotton Event 3006-210-23

Segregation studies were conducted on plants carrying the *cry1Ac* gene from event 3006-210-23. Trials were conducted at the Dow AgroSciences field station in Woodland, CA. Data on the Mendelian segregation of transgenes provide evidence for the stable inheritance of newly introduced genetic material.

Plants were tested for the presence or absence of the Cry1Ac protein by the use of qualitative ELISA strips that detect the protein product of the genes. These data are presented as Ac+ (positive for Cry1Ac protein) and Ac- (negative for Cry1Ac protein). Plants were tested for gene segregation under a variety of genetic conditions to confirm proper Mendelian segregation patterns. Ratios were determined at both the F1 and F2 generations. Plants from three different variety backgrounds were used that were also at various backcross generations.

The cotton lines carrying event 3006-210-23 were developed through a series of backcrosses and self-pollinations (Figure 13). The variety GC510 was used in the transformation and the original transformed lines (T0) were crossed to the variety PSC355. The F1 of this cross was both self-pollinated and backcrossed to PSC355. Successive backcrosses produced later backcross F1 generations (BC2, BC3, BC4) that would be expected to segregate in a 1:1 phenotypic ratio. Self-pollinations of the F1 hemizygotes produced the F2 generation that should segregate in a 3:1 phenotypic ratio for Cry1Ac. Outcrosses were made between F1 plants of PSC355 and other elite varieties (Phy78 and Phy72). The resulting F1 plants were then further crossed to non-transgenic recurrent parent plants to produce later backcross F1 generations.

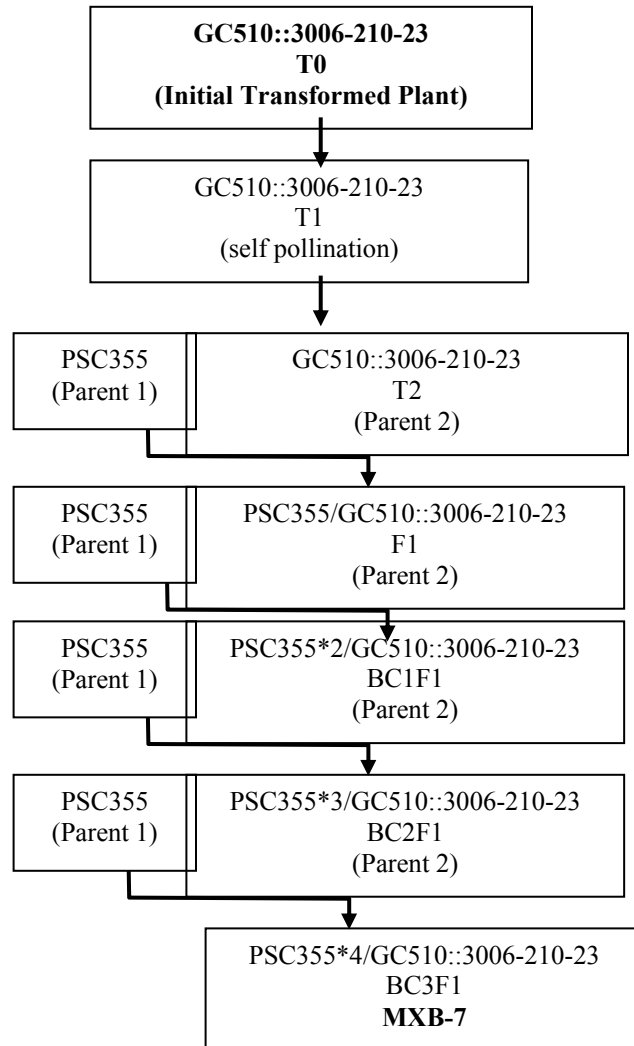
Two sets of approximately 100 F1 plants segregating for the *cry1Ac* gene were tested (Table 7). Chi square analysis for fit to the expected 1:1 ratio showed no significant difference. Two sets of approximately 100 F2 plants segregating for the *cry1Ac* gene were also tested (Table 7). Chi square analysis for fit to the expected 3:1 ratio also showed no significant difference.

Segregation analysis of the *cry1Ac* gene in the 3006-210-23 event was tested under multiple genetic conditions. Conditions included F1 and F2 generation segregation patterns, multiple backcross generations, and three variety backgrounds. Under all conditions tested, the transgene segregated properly according to standard Mendelian genetics.

Table 7. Gene Segregation Analysis of Cry1Ac Event 3006-210-23

Trait	Variety	Generation	Plants Tested	Observed		Expected		Chi Square
				Ac+	Ac-	Ac+	Ac-	
Cry1Ac	Phy72	BC3F1	100	47	53	50.0	50.0	0.36
Cry1Ac	Phy78	BC2F1	108	54	54	54.0	54.0	0.00
Cry1Ac	PSC355	BC1F2	102	77	25	76.5	25.5	0.01
Cry1Ac	PSC355	BC3F2	104	76	28	78.0	26.0	0.21

Figure 13. Breeding Schematic for Cry1Ac Event 3006-210-23 in Cotton Variety PSC355



*Arrows indicate progeny

V.D. Expression of Cry1Ac and PAT Protein in the Plant

Field expression trials of the event 3006-210-23 and control non-transgenic cotton lines were conducted in 2001 at six field sites under USDA 01-093-17n: Maricopa Arizona, Fresno California, Wayside Mississippi, Sunbury North Carolina, East Bernard Texas, and Claude Texas (Appendix 1). These sites represent regions of diverse agronomic practices and environmental conditions for cotton. Samples were collected at various times during development and were analyzed for Cry1Ac and PAT protein levels using quantitative enzyme-linked immunosorbent assay (ELISA) methods. Additional analyses were conducted for cottonseed processed products. The details of the filed expression study are given in Phillips *et al.* (2002; Appendix 2).

For event 3006-210-23, all matrices except nectar, hulls, meal, and oil expressed the Cry1Ac protein at measurable levels. Average expression levels across all matrices ranged from not detected (ND) to 1.92 ng/mg sample weight. PAT protein was rarely detected in Cry1Ac event 3006-210-23 samples.

V.D.1. Determination of Cry1Ac Protein in Cotton Samples

Samples of cotton were analyzed for the amount of Cry1Ac protein in event 3006-210-23 using a validated method (Phillips *et al.*, 2002; Appendix 2). The analytical method has a validated limit of quantitation of 0.001 to 0.375 ng/mg Cry1Ac protein/sample weight (dependent upon the matrix). In this method, the soluble extractable Cry1Ac protein is quantified using an ELISA kit purchased from Strategic Diagnostics, Inc. (SDI).

In the analytical method, the Cry1Ac protein was buffer extracted from cotton tissues. Following centrifugation, the aqueous supernatant was collected and analyzed using the Cry1Ac ELISA kit that employs a double sandwich format using Cry1Ac specific monoclonal and polyclonal antibodies. A calibration curve was generated and the Cry1Ac concentration in unknown samples was calculated from the polynomial regression equation. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported. The results of the Cry1Ac protein expression in various cotton tissues and cottonseed processed products are summarized in Tables 8 and 10, respectively.

Table 8. Summary of the Expression of Cry1Ac Protein in 3006-210-23 Cotton Tissues as Determined by ELISA

Cotton Tissue	Cry1Ac ng/mg Tissue Dry Weight ^a		
	Mean ^b	Std. Dev.	Min/Max Range
Young Leaf (3-6 week)	1.92	0.7	0.46 - 3.5
Terminal Leaf	1.44	0.5	0.24 - 2.4
Flower	1.92	0.3	1.3 - 2.4
Square	1.84	0.5	1.0 - 3.1
Boll (Early)	0.77	0.2	0.46 - 1.1
Whole plant (seedling)	1.59	0.4	0.8 - 2.2
Whole plant (pollination)	1.15	0.5	0.57 - 2.1
Whole plant (defoliation)	0.81	0.3	0.31 - 1.3
Root (seedling)	0.20	0.1	(0.09) ^c - 0.44
Root (pollination)	0.10	0.07	ND ^d - 0.23
Root (defoliation)	(0.05)	0.04	ND - 0.11
Pollen	1.44	0.5	0.9 - 2.4
Nectar		NA ^e	
Seed	0.57	0.09	(0.33) - 0.78

^a Results based on fresh tissue weight for pollen, nectar and seed.
^b Means are calculated from samples taken across all six locations. Samples were analyzed in duplicates.
^c Values in () = Calculated concentration is less than the LOQ of the method.
^d ND = Absorbance value of sample was less than the lowest standard absorbance.
^e NA = Not analyzed.

V.D.2. Determination of PAT Protein in Cotton Samples

All cotton samples were analyzed to determine the expression levels of the PAT protein. Samples were analyzed using a validated ELISA method. The procedure has a validated limit of quantitation of 0.002 to 0.4 ng/mg PAT protein/dry sample weight.

The PAT protein was buffer extracted from cotton tissues. Following centrifugation, the aqueous supernatant was collected, and analyzed using a PAT ELISA kit from Envirologix, Inc. The kit employs a double sandwich format ELISA and PAT-specific antibodies. A calibration curve was generated and the PAT concentration in unknown samples was calculated. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported. The results of the PAT protein expression in various cotton tissues and cottonseed processed products are summarized in Tables 9 and 10, respectively.

Table 9. Summary of the Expression of PAT Protein in Event 3006-210-23 Cotton Tissues as Determined by ELISA

Cotton Tissue	PAT ng/mg Tissue Dry Weight ^a		
	Mean ^b	Std. Dev.	Min/Max Range
Young Leaf (3-6 week)	ND ^c	NA ^d	ND – 0.20
Terminal Leaf	ND	NA	ND – 0.12
Flower	ND	NA	ND - ND
Square	ND	NA	ND – 0.08
Boll (Early)	ND	NA	ND – 0.08
Whole plant (seedling)	ND	NA	ND – 0.09
Whole plant (pollination)	ND	NA	ND – 0.14
Whole plant (defoliation)	0.11	0.05	ND – 0.20
Root (seedling)	ND	NA	ND – 0.07
Root (pollination)	ND	NA	ND – ND
Root (defoliation)	ND	NA	ND – ND
Pollen	ND	NA	ND – ND
Nectar	ND	NA	ND
Seed	(0.06) ^e	0.06	ND – (0.23)

^a Results based on fresh tissue weight for pollen, nectar and seed.
^b Means are calculated from samples taken across all six locations. Samples were analyzed in duplicates.
^c ND = Absorbance value of sample was less than the lowest standard absorbance.
^d NA = Not applicable.
^e Values in () = Calculated concentration is less than the LOQ of the method.

Table 10. Summary of the Expression of Cry1Ac and PAT Proteins in Cotton Processed Fractions of Event 3006-210-23

Cotton Processed Fraction	ng Protein/mg Tissue Fresh Weight	
	Cry1Ac	PAT
Cottonseed	0.62	(0.09) ^a
Kernel	0.41	(0.23)
Hulls	ND ^b	ND
Toasted Meal	ND	ND
Refined Oil	ND	ND

^a Values in () = Calculated concentration is less than the LOQ of the method.

^b ND = Absorbance value of sample was less than the lowest standard absorbance.

V.E. Characterization of the Plant-Expressed and Microbe-Derived Cry1Ac and PAT Proteins

Large quantities of protein are required to perform toxicology, eco-toxicology, biochemical, and insecticidal activity studies. Because it is very difficult, or impossible, to extract and purify sufficient quantities of the subject protein from the transgenic cotton plants for the aforementioned studies, Cry1Ac protein was produced in *Pseudomonas fluorescens* through recombinant DNA technology. The recombinant full length Cry1Ac protein was produced in *P. fluorescens* strain MR1620. The molecular identity and biochemical characteristics of the Cry1Ac expressed *in planta* in the event 3006-210-23 and in the microbial-expression system were examined by Gao *et al.* (2001 and 2002; Appendix 2). The purified cotton Cry1Ac protein was characterized, including detection of glycosylation, N-terminal sequencing, and peptide mapping. The *in planta* and microbe-expressed Cry1Ac proteins were found to be biochemically equivalent.

Similarly, microbe-derived PAT was compared to event 3006-210-23 PAT and they were found to be equivalent. The data are presented in Section V.E.2.

V.E.1. Cry1Ac Protein Characterization

Molecular Weight, Immunoreactivity and Glycosylation

The Cry1Ac protoxin expressed in event 3006-210-23 is a chimeric, full-length δ -endotoxin comprised of the core toxin of Cry1Ac1 from *Bacillus thuringiensis* strain HD73 and the nontoxic portions of Cry1Ca3 and Cry1Ab1 proteins. Together, the portions of Cry1Ca3 and Cry1Ab1 that comprise the chimeric C-terminal domain are approximately those removed by alkaline proteases during the formation of the active Cry1Ac core toxin.

Many *B.t.* endotoxins are known to have protease recognition sites on the exposed surfaces of the molecules, which are vulnerable to protease cleavage. The Cry1Ac protoxin has a molecular weight of 131 kDa, and is highly vulnerable to alkaline protease cleavage. The recombinant *P. fluorescens*-produced Cry1Ac preparation containing the protoxin was readily cleaved into various intermediate partially-truncated forms during production and preparation, and a stable truncated form (approximately 65 kDa) was obtained following trypsin treatment of the protoxin (Figure 14A, Lanes 2 and 3, respectively). Immunodetection of the full-length and truncated microbe-derived Cry1Ac protein is shown in Figure 14B, Lanes 2 and 3, respectively (Gao, *et al.*, 2001; Appendix 2).

Due to the expression level of Cry1Ac in cotton being relatively low (Phillips *et al.* 2002; Appendix 2), full-length Cry1Ac could not be detected from plant tissue using western blot analysis. However, the full-length Cry1Ac protein is sensitive to protease cleavage *in planta* resulting in the truncated Cry1Ac core toxin which was readily purified (Figures 15, lanes 4-11) and detected by western blot analysis (Figure 16, lanes 4-11) (Gao *et al.*, 2002; Appendix 2).

No detectable carbohydrates were identified, using a commercial glycoprotein staining kit, in either the *in planta* protein or microbe-derived Cry1Ac protein (Figure 17, lane 4 and Figure 18, lane 4).

Figure 14. SDS-PAGE and Western Blot of *Pseudomonas fluorescens* Produced Cry1Ac Protein

Panel A: 4-15% SDS-PAGE stained with CBB.

Panel B: Western blot of SDS-PAGE gel; probed with rabbit anti-Cry1Ac polyclonal antibody.

The lanes contained:

Lane 1: Pre-stained molecular weight markers (Bio-Rad, broad range), 7 μ L

Lane 2: *P. fluorescens* derived Cry1Ac toxicology lot loaded 1.4 μ g for SDS-PAGE, 0.9 μ g for Western blot

Lane 3: *P. fluorescens* derived truncated Cry1Ac loaded 1.3 μ g for SDS-PAGE, and 0.4 μ g for Western blot

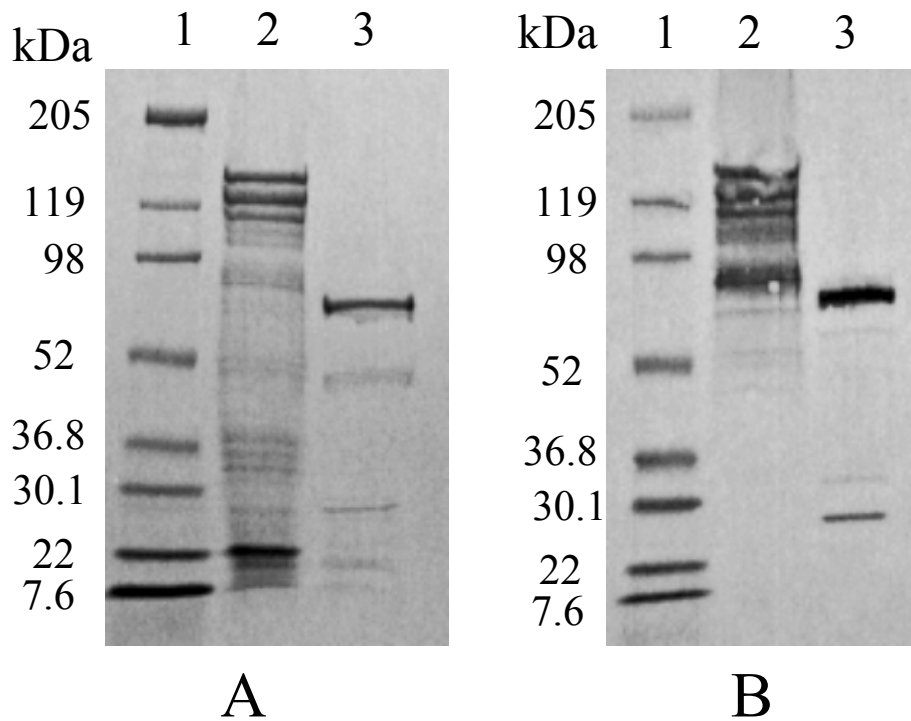


Figure 15. SDS-PAGE Profile of the Immunoaffinity Chromatography Fractions of Plant-Produced Cry1Ac

Each lane was loaded 10 μ L of the concentrated fractions. The arrow points to the truncated Cry1Ac protein. The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β -mercaptoethanol (β -ME), and boiled for 5 min at 100 $^{\circ}$ C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. After electrophoresis the proteins were stained with Coomassie Brilliant Blue (CBB). The lanes contained:

Lane M: BenchMark protein ladder, Invitrogen, 7 μ L

Lane S: *P. fluorescens*-derived truncated Cry1Ac protein standard, 0.3 μ g.

Lanes 4-11: The numbers represent the fraction numbers of the immunoaffinity chromatography of transgenic cotton.

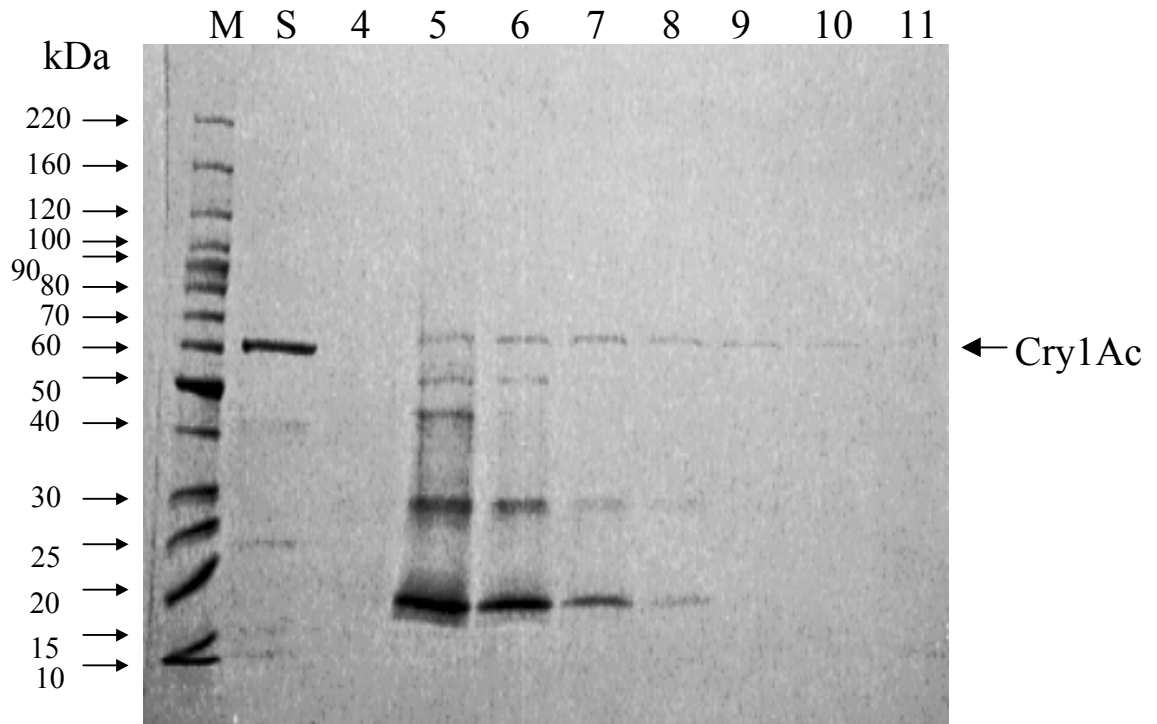


Figure 16. Western Blot of the Immunoaffinity Chromatography Fractions of Plant-Produced Cry1Ac

The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β -mercaptoethanol, and boiled for 5 min at 100 °C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. After separation, the gel was electro-blotted to a nitrocellulose membrane with a Mini Trans-Blot electrophoretic transfer cell for 60 min under a constant voltage of 100 V. For immunodetection of the blot, a specific rabbit polyclonal antibody (PAb) against the truncated Cry1Ac was used as the primary antibody. A conjugate of goat anti-rabbit IgG (H+L)-alkaline phosphatase was used as the secondary antibody. A substrate solution containing 100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, 0.025% 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 0.05% p-nitroblue tetrazolium (NBT) was used for colorimetric development of the immunoreactive protein bands on the membrane. Each lane was loaded 10 μ L of the concentrated fractions. The arrow points to the truncated Cry1Ac protein. The lanes contained:

- Lane M: Pre-stained molecular weight standards from Bio-Rad, 7 μ L
- Lane S: *P. fluorescens*-derived truncated Cry1Ac protein standard, 0.3 μ g.
- Lanes 4-11: The numbers represent the fraction numbers of the immunoaffinity chromatography of transgenic cotton.

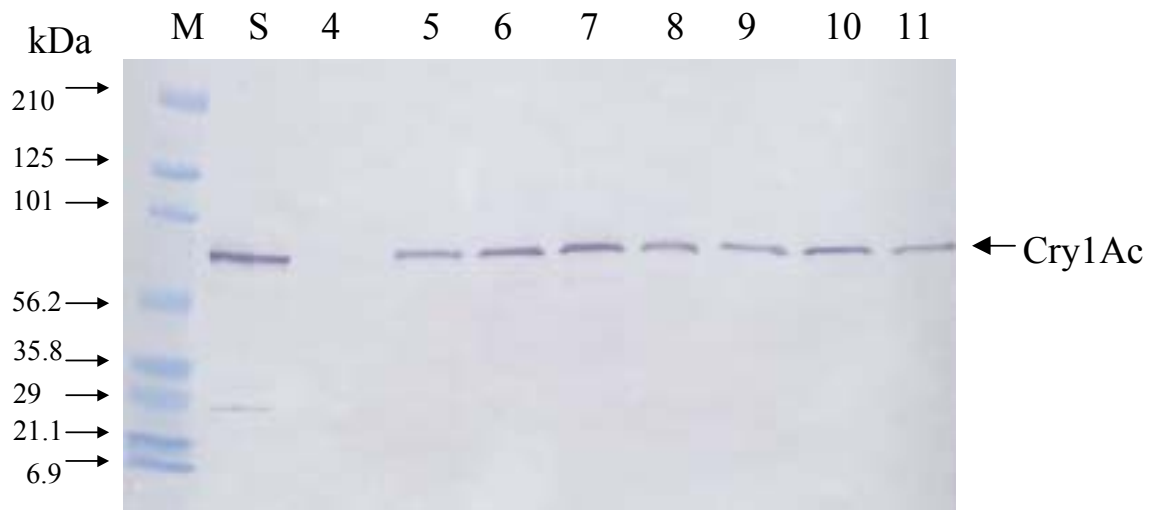


Figure 17. Analysis of Glycoprotein on Cry1Ac Plant-Produced Protein

The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β -mercaptoethanol, and boiled for 5 min at 100 °C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. After electrophoresis, the gels were stained either with Coomassie Brilliant Blue (CBB) to visualize all protein bands (**Panel A**), or with GelCode Glycoprotein Staining Kit (**Panel B**) to visualize glycoproteins only. For detection of glycosylation, the kit supplier's instruction manual was followed with slight modifications. The lanes contained:

- Lane 1: Pre-stained molecular weight markers (Bio-Rad), 7 μ L
- Lane 2: Horseradish peroxidase, 2 μ g
- Lane 3: Soybean trypsin inhibitor, 2 μ g
- Lane 4: 3006-210-23 cotton -derived truncated Cry1Ac protein (immunoaffinity purified fractions #6-8), loaded 22 μ L

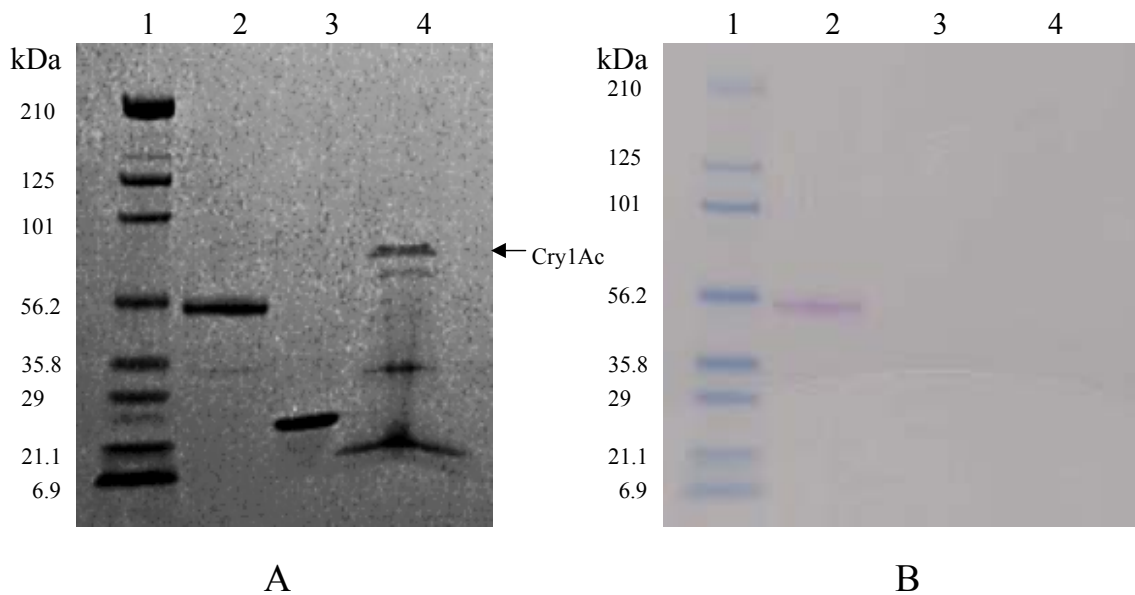
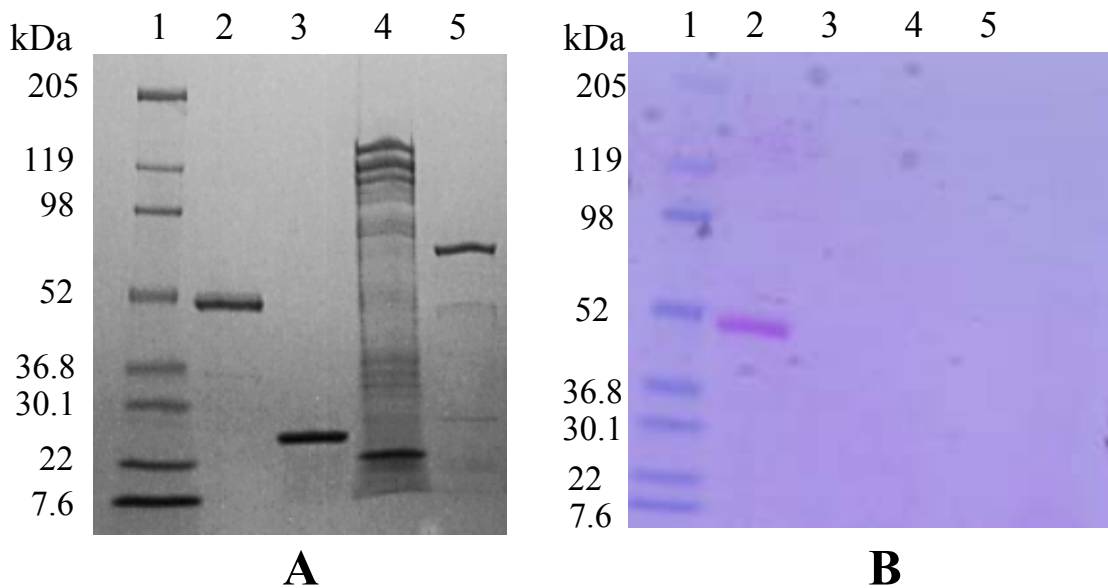


Figure 18. Analysis of Glycoproteins on Microbe-Derived Cry1Ac Protein

The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β mercaptoethanol, and boiled for 5 min at 100 °C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. After electrophoresis, the gels were stained either with Coomassie Brilliant Blue (CBB) to visualize all protein bands (**Panel A**), or with GelCode Glycoprotein Staining Kit (**Panel B**) to visualize glycoproteins only. For detection of glycosylation, the kit supplier's instruction manual was followed with slight modifications. The lanes contained:

- Lane 1: Pre-stained molecular weight markers (Bio-Rad), 7 μ L
- Lane 2: Horseradish peroxidase, a glycoprotein, 2 μ g
- Lane 3: Soybean trypsin inhibitor, a non-glycoprotein, 2 μ g
- Lane 4: *Pseudomonas fluorescens* derived Cry1Ac toxicology lot, 1.4 μ g
- Lane 5: *Pseudomonas fluorescens* derived truncated Cry1Ac, 0.9 μ g



N-terminal Sequencing and Mass Peptide Fingerprinting

The amino acid residues at the amino terminus of the microbe-derived and plant-produced Cry1Ac protein samples were sequenced using the Edman degradation reaction. The 17 N-terminal amino acid residues determined from the *in planta* truncated Cry1Ac protein and the microbe-derived Cry1Ac were found to correspond to the #29 to #45 residues of the full-length Cry1Ac (Table 11). It suggests that during exposure of full length Cry1Ac to alkaline proteases such as trypsin, truncation occurs not only at the C-terminus, removing the chimeric nontoxic C-terminal domain; but also at the N-terminus, removing the first 28 amino acid residues (between residue 28 and 29 is a trypsin cleavage site).

The microbe-derived and plant-derived truncated Cry1Ac samples were further separated from impurities on SDS-PAGE, and the Cry1Ac band was excised and subjected to in-gel digestion by trypsin. The resulting peptide mixture was analyzed by matrix-assisted laser desorption ionization time-of flight mass spectrometry (MALDI-TOF MS) to determine the peptide mass

fingerprint coverage. The masses of the detected peptides were compared to those deduced based on potential trypsin cleavage sites in the sequence of Cry1Ac.

Twenty-nine peptides were identified from the plant-derived sample matching the theoretical deduced peptide masses of Cry1Ac (Table 12) and 27 peptides were identified from the microbe-derived sample matching the theoretical deduced peptide masses of Cry1Ac (Table 13).

Table 11. N-Terminal Sequence of Microbe-Derived and Plant-Derived Cry1Ac Protein

I.	M ¹ DNNPNINEC ¹⁰ IPYNC ¹⁵ LSNPEVEVLGGER ²⁸ ↓IETGYTPIDISLSLT ⁴³
II.	XDNNPNINEX ¹⁰ IPYNX ¹⁵
III.	XETGYTPIDISLSLT
IV.	XETGYTPIDXSLXL T

- I: Expected N-terminal sequence of the first 43 amino acid residues of Cry1Ac(synpro);
II: Detected N-terminal sequence of *P. fluorescens* produced full length Cry1Ac
III: Detected N-terminal sequence of *P. fluorescens* produced truncated Cry1Ac
IV: Detected N-terminal sequence of 3006-210-23 cotton-derived truncated Cry1Ac
↓ indicates a trypsin cleavage site.

Table 12. Tryptic Peptide Mass Data (m/z [M+H]⁺) of the Truncated Cry1Ac Protein Derived from 3006-210-23 Cotton

Cry1Ac residue #	Theoretical mass (m/z)	Cotton Cry1Ac^a
88-93	764.39	764.38
116-127	1398.67	1398.64
116-131	1943.89	1943.86
174-181	907.46	907.45
182-192	1237.60	1237.58
193-198	781.38	781.37
199-209	1284.66	1284.65
210-217	1038.50	1038.49
218-224	816.40	816.39
218-228	1372.67	1372.65
229-233	727.35	727.34
229-234	883.45	883.44
259-265	804.46	804.45
266-281	1900.91	1900.89
293-311	2197.11	2197.09
350-360	1203.68	1203.66
361-368	940.51	940.50
403-423	2277.15	2277.11
404-423	2149.05	2149.02
404-429	2861.39	2861.38
424-437	1688.84	1688.82
430-437	976.50	976.49
438-449	1253.65	1253.64
450-458	1144.57	1144.55
459-478	2118.11	2118.07
479-500	2211.12	2211.08
512-526	1703.88	1703.86
666-671	733.40	733.47
696-702	930.48	930.50

^a A peptide was considered a match if the standard deviation is within 500 ppm.

Table 13. Tryptic Peptide Mass Data (m/z [M+H]⁺) of Truncated Cry1Ac Protein Obtained by MALDI-TOF Mass Spectroscopy Derived from recombinant *P. fluorescens*

Cry1Ac residue #	Theoretical mass (m/z)	<i>P. f.</i> truncated Cry1Ac^a
100-115	1902.96	1902.72
116-127	1398.67	1398.55
116-131	1943.89	1943.67
174-181	907.46	907.42
182-192	1237.60	1237.51
199-209	1284.66	1284.56
210-217	1038.50	1038.44
218-224	816.40	816.38
229-233	727.35	727.33
235-253	2179.17	2178.90
254-265	1489.86	1490.59
266-281	1900.91	1900.69
293-311	2197.11	2196.84
350-360	1203.68	1203.61
369-402	3731.12	3729.37
403-423	2277.15	2276.85
404-423	2149.05	2148.79
404-429	2861.39	2860.09
430-437	976.50	976.45
430-449	2211.13	2210.84
438-449	1253.65	1253.54
450-458	1144.57	1144.49
459-478	2118.11	2117.85
479-500	2211.12	2210.84
512-526	1703.88	1703.66
591-602	1249.65	1249.56
649-665	1942.88	1943.67

V.E.2. PAT Protein Characterization

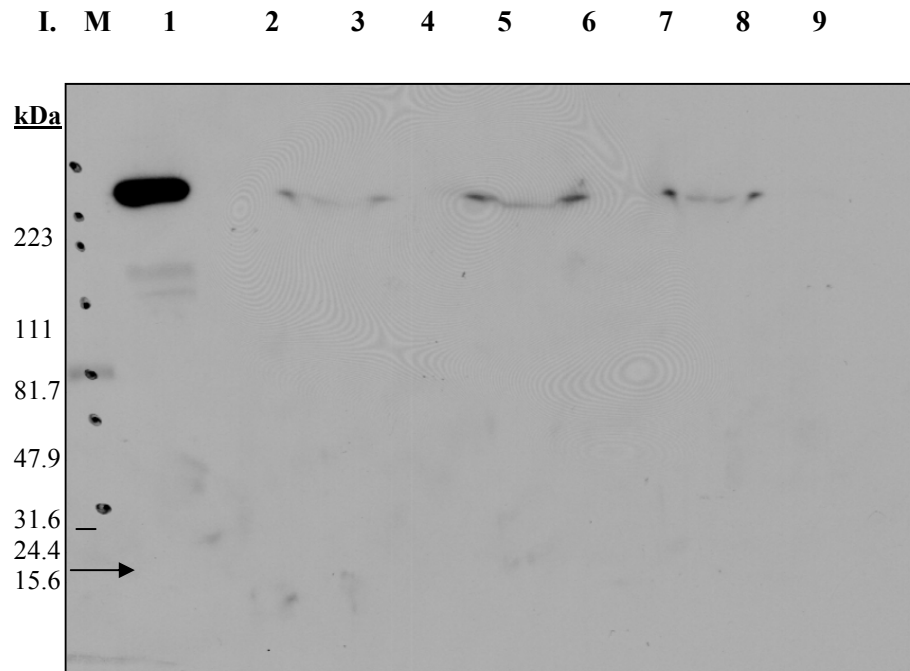
The PAT protein is known to be a homodimer of approximately 43 kD in its native form, comprised of two components of approximately 22 to 23 kD. PAT protein extracts were prepared from microbe-expressed PAT protein and lyophilized flower buds from event 3006-210-23. Flower buds were used due to the high expression in this tissue. The membrane was incubated with the rabbit specific anti-PAT polyclonal antibody. A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary/detection antibody. The microbe-derived PAT protein and the plant-derived PAT protein showed a positive signal of expected size by western Blot analysis (Figure 19, Lanes 1 and 3, respectively).

Further biochemical analyses were conducted and the results confirmed that there was no difference between the microbe-derived PAT and the PAT expressed in event 3006-210-23 (Schafer and Schwedler, 2002; Appendix 2).

Figure 19. Western Blot Analysis of Microbe -Derived PAT and Cotton Flower Bud Extracts.

The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β mercaptoethanol, and boiled for 5 min at 100 °C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. After electrophoresis, the gel was blotted on to a nylon membrane for western analysis. The western blot was probed with rabbit anti-PAT polyclonal antibody and antibody binding was detected with a chemiluminescent substrate. The arrow denotes *E. coli*-derived PAT protein standard. Due to the large amount of protein extract added to each lane, noticeable distortion occurred in the SDS-PAGE gel lanes 2-9. The molecular weight markers were manually transcribed onto the western blot film after development. The lanes contained:

- Lane M: TriChromRanger pre-stained molecular weight markers (Pierce Chemical)
- Lane 1: *E. coli* -derived PAT (MW: 23.3 kDa), 1.6 ng/lane
- Lane 2: Blank
- Lane 3: Transgenic Cotton Extract (Event 3006-210-23), 40 μ L/lane
- Lane 4: Blank
- Lane 5: NA – not applicable to this petition
- Lane 6: Blank
- Lane 7: NA – not applicable to this petition
- Lane 8: Blank
- Lane 9: Non-transgenic Cotton Extract, 40 μ L/lane



V.F. Disease and Pest Resistance Characteristics

B.t. Cry1Ac cotton event 3006-210-23 has been field tested since 1999 in the major cotton growing regions of the United States as well as in Puerto Rico, under USDA notifications 02-302-12n, 02-249-02n, 02-066-09n, 02-066-07n, 02-066-06n, 01-093-17n, 01-093-14n, 01-052-10n, 01-052-08n, 01-052-06n, 00-049-15n, 99-067-09n (Appendix 1). The breeders conducting each field test visually monitored disease and pest resistance characteristics of event 3006-210-23 and non-modified (parental) cotton lines. There were no differences reported in severity of disease symptoms or insect damage (other than the targeted organisms susceptible to the Cry1Ac) between the plants from event 3006-210-23 and non-modified cotton lines.

V.F.1. Efficacy of Cotton Event 3006-210-23 Against Lepidopteran Pests of Cotton

Cry1Ac event 3006-210-23 has been tested for efficacy against lepidopteran pests of cotton in field trials during 2001 and 2002 where comparisons were made to the non-transgenic recurrent parent PSC355. The results of these studies establish that cotton event 3006-210-23 has very good efficacy against tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) and moderate levels of efficacy against the beet armyworm (*Spodoptera exigua*) and soybean looper (*Psuedoplusia includens*). When commercialized, this event will be stacked with Cry1F event 281-24-236, which provides additional efficacy against most of these pests as well as efficacy against additional pests.

V.G. Agronomic Characteristics of Cotton Event 3006-210-23

Agronomic trials were conducted at diverse locations across the major regions of the US cotton-belt to characterize growth habit, field emergence, vegetative vigor, flowering period and reproductive potential of the Cry1Ac line 3006-210-23 and the MXB-13 line (the stack expressing both Cry1Ac and Cry1F). The agronomic characteristics of these lines were compared to those of PSC355, the non-transgenic recurrent parent for the transgenic lines, since true isolines of these events were not available.

The agronomic characteristics of MXB-7, the line which contains event 3006-210-23, and MXB-13 were comparable to the PSC355 parent (Table 14). Since the BC3 transgenic lines were compared to the PSC355 recurrent parent and not to a true isoline, it is reasonable to find some differences in agronomic characteristics that would result from residual genes of the original transformed variety (GC510 Acala). Occasional small differences, well within the range of typical variability within cotton varieties, were seen, but are not biologically or commercially significant. Improvements in various fiber properties were noted which reflect the Acala background in the transgenic events, represent added value of lines containing event 3006-210-23.

Yield, in terms of pounds of lint per acre, is not different among any of the transgenic lines and the PSC355. The total germination for the generation of seed harvested from the agronomic trials shows no difference among cotton lines and provides a more representative comparison of seed quality and viability than does field emergence of the prior generation of seed where winter nursery seed of uneven quality were used. When viewing the overall range of values for the measured parameters, they are all within the range obtained for traditional cotton lines. Therefore, the Cry1Ac line containing event 3006-210-23 and the derivative event MXB-13 exhibit no phenotypic characteristics diminishing agronomic utility and value, or that would increase weediness potential.

Breeding trials conducted at locations across the southern US support the conclusion that transgene insertion has not adversely affected the agronomic characteristics of event 3006-210-23 and derivative lines.

Table 14. Agronomic Characteristics of Event 3006-210-23 and the Stack Product (MXB-13) Cotton Lines Expressing Cry1Ac Protein in Comparison to Parent Variety PSC355

Variable	Units	3006-210-23 (Cry1Ac)	MXB-13 (Cry1Ac/Cry1F)	PSC355 (null)	Number of Locations
Growth Habit					
Plant height	inches	39.9	40.2	41.5	17
Total nodes	number per plant	17.4	17.4	17.6	16
Height to node ratio	inches per plant	2.29	2.32	2.35	17
Node of the 1st fruiting branch	node	6.7	6.8	6.6	17
Fruting branches	number per plant	11.7	11.6	12.1	16
Total fruiting positions	number per plant	25.6	24.7	26.6	17
Vegetative bolts	number per plant	2.3	1.7	1.6	16
Germination and Emergence					
Field emergence	%	63.6	78.9	82.3	19
Cool vigor	%	32	36	38	20
4 day warm	%	64	64	65	20
7 day warm	%	80	80	82	20
Total germination	%	85	84	87	20
Dormant seed	%	0.6	0.5	0.3	20
Vegetative Vigor					
Vegetative branches	number per plant	2.9	2.8	2.6	16
Flowering Period					
Days to first flower	days	61.9	61.4	60.6	18
Node of white flower - 15 days	node	12.9	12.9	12.9	17
Node of white flower - 30 days	node	17.0	16.9	16.8	15
Reproductive Potential					
Percent retention - total	%	49.0	45.8	44.4	16
Percent retention - 1st position	%	58.5	62.4	54.3	16
Percent open bolls	% per plant	73.5	76.5	75.4	17
Seed cotton weight per boll	grams per boll	5.5	5.3	5.1	19
Lint percent	%	37.9	37.1	37.3	19
Seed index (fuzzy)	grams per 100 seeds	11.0	11.3	10.7	17
Lint per acre	pounds per acre	1005	1000	993	17
Fiber Quality					
Length	inches	1.160	1.177	1.147	19
Strength	grams per tex	31.9	33.0	32.6	19
Micronaire	micronaire units	4.51	4.51	4.96	19
Length uniformity	%	85.8	85.8	85.7	19
Reflectance	%	75.7	76.0	74.6	19
Yellowness	Hunter's +b scale	8.4	8.3	8.4	19

V.H. Compositional and Nutritional Equivalence of Cotton Event 3006-210-23 as Compared to Non-Transgenic Control

Event 3006-210-23 and the non-transgenic control lines were grown in six locations in the major cotton-growing regions of the US under USDA ID 01-093-17n (Appendix 1). The non-transgenic control line was selected in the F1 segregating generation after intercrossing Cry1Ac and Cry1F BC3F1 lines both derived from backcrosses with the cotton variety PSC355. The null plants and the Cry1F event BC3F1 3006-210-23 line were self-pollinated to the F4 generation to provide the seed for use in the following nutritional composition studies. The event 3006-210-23 and the non-transgenic cottonseed were analyzed for proximates in order to establish compositional and nutritional equivalence (Phillips *et al.*, 2002; Appendix 2). In addition, cottonseed harvested from these locations was processed and the proximate composition was determined for kernel, toasted meal, refined oil and hulls.

The compositional analysis of cottonseed indicated no significant differences between event 3006-210-23 and non-transgenic control lines (Table 15). Cottonseed moisture and carbohydrates were marginally lower for both event 3006-210-23 and non-transgenic in comparison to typically reported ranges for cotton; this is likely due to sample preparation and is not of consequence relative to cottonseed quality. There were no differences observed for components of processed products (kernels, toasted meal, refined oil and hulls; Table 16) and results for both event 3006-210-23 and non-transgenic control were consistent with literature reported values. The results from the compositional analyses demonstrate equivalence between the control line and transgenic cotton event line. Thus, there is no evidence from these analyses that the insertion of the *cry1Ac* gene and its expression of Cry1Ac protein in event 3006-210-23 cotton plants has altered the levels of proximates with respect to the non-transgenic parent plant.

Table 15. Summary of the Proximate Analysis of Event 3006-210-23 and Non-Transgenic Cottonseed from All Sites

Seed was analyzed from sites throughout the cotton growing regions of the US: AZ, NC, MS, CA, TX (2)

Matrix Treatment	Literature Values	Seed Control	Seed Cry1Ac
Proximate (%)			
Ash	3.76-4.85 ^a	3.9	4.0
Total Fat	15.4-23.8 ^a	21.9	23.3
Moisture	3.97-8.47 ^a	3.2	2.8
Protein	21.8-28.2 ^a	26.7	27.3
Carbohydrates	45.6 – 53.6 ^a	44.3	42.8
Calories (Kcalories/100g)	NA ^c	481	490
Crude Fiber (%)	15.4-20.9 ^a	17.0	15.7
Acid Detergent Fiber (%)	26, 37.5 ^{b,a}	24.4	22.6
Neutral Detergent Fiber (%)	37, 52.6 ^{b,a}	34.7	34.1

^a OECD Draft Consensus Document, 2002.
^b NCPA, Cottonseed Feed Products Guide.
^c Literature values not available.

Table 16. Proximate Analysis of Cottonseed Processed Fractions from Event 3006-210-23 and Non-Transgenic Control

Seed was analyzed from sites throughout the cotton growing regions of the US: AZ, NC, MS, CA, TX (2)

Matrix Treatment	Literature Values	Kernel Control	Kernel Cry1Ac
<u>Proximate (%)</u>			
Moisture	NA ^a	7.6	7.8

Matrix Treatment	Literature Values	Hulls Control	Hulls Cry1Ac
<u>Proximate (%)</u>			
Ash	2.4 - 4.0 ^b	2.7	2.6
Total Fat	1.0 - 3.3 ^b	2.7	1.6
Moisture	NA ^a	10.3	11.4
Protein	4.0 - 6.9 ^b	6.4	5.7
Carbohydrates	NA ^a	77.9	78.7
Calories (Kcalories/100g)	NA ^a	362	352

Matrix Treatment	Literature Values	Toasted Meal Control	Toasted Meal Cry1Ac
<u>Proximate (%)</u>			
Ash	4.6 - 9.8 ^b	5.9	6.1
Total Fat	0.6 - 4.7 ^b	4.5	2.4
Moisture	NA ^a	2.2	9.1
Protein	43.0 - 52.4 ^b	46.2	47.0
Carbohydrates	NA ^a	41.2	35.4
Calories (Kcalories/100g)	NA ^a	390	351
Crude Fiber (%)	8.4 - 15.3 ^b	12.1	8.3
Acid Detergent Fiber (%)	12.2 - 23.9 ^b	18.1	12.9
Neutral Detergent Fiber (%)	15.8 - 32.4 ^b	23.7	17.1

Matrix Treatment	Literature Values	Refined Oil Control	Refined Oil Cry1Ac
<u>Proximate (%)</u>			
Total Fat	NA ^a	100.2	100.1
Moisture	NA ^a	< 0.1	< 0.1
Protein	NA ^a	< 0.1	< 0.1

^a Literature value not available.

^b Feedstuffs, 1995.

V.H.1. Toxicants and Allergens Present in Event 3006-210-23

In order to assess the potential for an introduced allergen in event 3006-210-23, a sequence evaluation scheme was used to assess the similarity of the Cry1Ac and PAT proteins to known protein allergen sequences contained in several widely accepted databases. An immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids. In studies conducted on the Cry1Ac and PAT proteins, no immunologically significant sequence identity was detected, indicating that no homology to known allergens based on amino acid sequence occurs in Cry1Ac or PAT (Stelman, 2001; Appendix 2). *In vitro* simulated gastric fluid (SGF) digestibility studies were also conducted on the proteins. Within one minute of exposure to SGF, both Cry1Ac and PAT were rapidly digested and no longer detectable by SDS-PAGE or western blot analysis (Korjagin, 2001 and 2002; Appendix 2). Thermolability results for Cry1Ac also indicated that the protein was not biologically active following exposure to elevated temperatures (>75°C) (Herman and Gao, 2001; Appendix 2). The results of these studies indicate that the Cry1Ac and PAT proteins do not exhibit characteristics commonly attributed to an allergenic protein.

Several acknowledged toxicants occur naturally in cotton: gossypol, tocopherols, and phytic acid, and the cyclopropenoid fatty acids, malvalic, sterculic and dihydrosterculic. Appropriate cottonseed matrices were measured for content of these anti-nutrients. The levels of all of these anti-nutrients were comparable to those of the control non-transgenic control and were within the acceptable literature ranges for cottonseed and its processed products (Tables 17 to 20, Phillips *et al.*, 2002; Appendix 2).

Table 17. Tocopherol Analysis of Event 3006-210-23 and Non-Transgenic Control Cottonseed Oil

Matrix		Refined Oil	Refined Oil
Treatment		Control	Cry1Ac
Sample ID	Literature	375444601	37544602
Covance LIMS	Values ^a	20300932	20300933
Total Tocopherols (mg/kg)			
Alpha Tocopherol	320	549	502
Beta Tocopherol	ND ^b	< 60.0	< 60.0
Gamma Tocopherol	313	344	365
Delta Tocopherol	ND	< 60.0	< 60.0

^a Cottonseed Oil, 1990.

^b ND = not detected.

Table 18. Anti-Nutrient Analysis of Event 3006-210-23 and Non-Transgenic Control Cotton Leaves and Squares

Site	NC	NC	TX1	TX1	NC	NC	TX1	TX1
Matrix	Terminal leaf	Terminal leaf	Terminal leaf	Terminal leaf	Square	Square	Square	Square
Treatment	Control	Cry1Ac	Control	Cry1Ac	Control	Cry1Ac	Control	Cry1Ac
Sample ID	35008701	35009501	35106701	35107501	35014101	35016801	35112101	35114801
Covance LIMS	20101731	20101732	20101735	20101736	20101739	20101740	20101743	20101744
Total Polyphenols (%)	1.53	1.60	0.56	0.73	0.74	0.70	0.68	0.73
Total Gossypol (%)	0.031	0.056	< 0.020	0.087	0.081	0.081	0.094	0.109

Table 19. Summary of Anti-Nutrient Analysis of Event 3006-210-23 and Non-Transgenic Control Cottonseed

Matrix Treatment	Literature Values ^a	Seed Control	Seed Cry1Ac
Cyclopropanoid Fatty Acids (%)			
Sterculic	0.13 - 0.66	0.311	0.289
Malvalic	0.17 - 0.61 1.9	0.384	0.340
Dihydrosterculic	0.11 - 0.22	0.213	0.212
Aflatoxins (ppb)			
AHB1	NA ^b	< 1.00	< 1.00
AHB2	NA	< 1.00	< 1.00
AHG1	NA	< 1.00	< 1.00
AHG2	NA	< 1.00	< 1.00
Total Gossypol (%)	0.71 - 1.24	0.841	0.852

^a OECD Draft Consensus Document, 2002.

^b Literature value not available.

Table 20. Anti-Nutrient Analysis of Cottonseed Processed Products of Event 3006-210-23 and Non-Transgenic Control Line

Matrix		Kernel	Kernel
Treatment		Control	Cry1Ac
Sample ID	Literature	37540301	37540304
Covance LIMS	Values ^a	20300920	20300923
Free Gossypol (%)	NA	0.839	1.00
Total Gossypol (%)	NA	0.976	1.15

^a Literature data not available.

Matrix		Toasted Meal	Toasted Meal
Treatment		Control	Cry1Ac
Sample ID	Literature	37543801	37543804
Covance LIMS	Values ^a	20300928	20300931
Free Gossypol (%)	0.02 – 0.07	0.044	0.045
Total Gossypol (%)	0.93 – 1.43	0.907	1.09

^a OECD Draft Consensus Document, 2002.

Matrix		Refined Oil	Refined Oil
Treatment		Control	Cry1Ac
Sample ID	Literature	375444601	37544604
Covance LIMS	Values ^a	20300932	20300935
Free Gossypol (%)	ND	< 0.002	< 0.002
Total Gossypol (%)	0.09	< 0.002	< 0.002
Cyclopropenoid Fatty Acids (%)			
Sterculic	0.58	0.217	0.223
Malvalic	0.41	0.272	0.255
Dihydrosterculic	0.22	0.212	0.202

^a OECD Draft Consensus Document, 2002.

VI. Human Health and Environmental Consequences of Introduction of the Transformed Cultivar

The safety of the Cry1 class of proteins is supported by decades of safe use in microbial sprays. The microbial *B.t.* products have never caused any significant adverse human health or environmental effects in more than 40 years of widespread use. In EPA’s 1998 Registration Eligibility Decision (USEPA, 1998), the Agency concluded that microbial *B.t.* products pose no unreasonable adverse effects to humans or the environment and that all uses of those products are eligible for re-registration. Specifically, foliar-applied biopesticides containing Cry1Ac protein have been registered and used safely for over 30 years.

The recent history of transgenic crops expressing Cry1 proteins further supports the safety of Cry1Ac protein. The Cry1Ac protein has been expressed in corn (*B.t.* Xtra) and in cotton (Bollgard I®²). EPA evaluated each of these products, and the expressed protein, for safety prior to their registration, and USDA has deregulated them prior to their commercial use within the US. These products have demonstrated no adverse human health or environmental effects.

VI.A. Cry1Ac Protein Bio-equivalency

The toxicity of Cry1Ac protein to non-target organisms was examined in several studies using a microbial source of Cry1Ac protein test material. In section V.E the biochemical equivalency of Cry1Ac protein produced by recombinant *P. fluorescens* to that produced in transgenic cotton event 3006-210-23 is described. Additional studies were performed to further substantiate protein equivalence in terms of biological activity.

Bioassays were conducted on sensitive and insensitive insects to compare the biological activity of cotton event 3006-210-23 expressed Cry1Ac protein and microbe-derived Cry1Ac protein (Herman and Collins, 2002; Appendix 2). Three insect pests were chosen for inclusion in this study based on their varied susceptibilities to the Cry1Ac delta-endotoxin. Tobacco budworm (TBW), *Heliothis virescens*, was chosen as a highly susceptible pest, cotton bollworm (CBW), *Helicoverpa zea*, as a susceptible pest, and beet armyworm (BAW), *Spodoptera exigua*, as a relatively less susceptible pest. Potency results summarized in the Table 21 show the biological equivalency of the microbial and plant-produced Cry1Ac protein.

Table 21. Potency Estimates with Plant-Produced and Microbe-Produced Cry1Ac (in ng ai/cm²)

Insect	Microbe-produced Cry1Ac		Plant-produced Cry1Ac	
	GI ₅₀ in ng ai/cm ²	(95% CL)	GI ₅₀ in ng ai/cm ²	(95% CL)
beet armyworm	0.80	(0.42-1.5)	2.2*	(0.24-21.4)
cotton bollworm	0.15	(0.11-0.20)	0.12	(0.050-0.30)
tobacco budworm	0.028	(0.018-0.043)	0.0040	(0.0020-0.0080)
	LC ₅₀ in ng ai/cm ²	(95% CL)	LC ₅₀ in ng ai/cm ²	(95% CL)
tobacco budworm	1.2	(0.93-1.7)	1.3	(0.54-8.1)

CL = confidence limits.

* GI₅₀ is above the highest concentration tested.

² Bollgard® is a registered trademark of the Monsanto Company

Based on the results from the insect bioassays, the Cry1Ac proteins from both sources appear to have similar potencies against the three insect species compared in this study. Both materials are most active against tobacco budworm, followed by cotton bollworm and beet armyworm. The GI₅₀s for the two protein sources are statistically indistinguishable for beet armyworm and cotton bollworm. Although the tobacco budworm GI₅₀ for the plant powder appeared significantly lower than for the microbe-derived Cry1Ac, the LC₅₀ values from both sources were virtually identical for this pest indicating equivalent potency.

VI.B. Effects on Non Target Organisms

The activity of Cry proteins is restricted to specific insect species within a given order (Lepidoptera, Coleoptera, Diptera, and Orthoptera) (Glare and O'Callaghan, 2000); therefore, large margins of safety are shown for non-target terrestrial and aquatic species. There is no evidence for Cry1 proteins originating from *Bacillus thuringiensis* to have harmful effects on the health of humans or animals (McClintock *et al.*, 1995). Nor is there evidence that Cry proteins have activity against hemimetabolic insects (insects of the class Exopterygota which encompass crop beneficial insects) (Glare and O'Callaghan, 2000). There is no demonstrated toxicity at environmentally relevant concentrations of Cry1Ac protein to vertebrate or invertebrate species other than lepidopteran insects.

VI.B.1. Selectivity of Cry1Ac Protein

Bacillus thuringiensis subspecies are differentiated from one another based on their insecticidal activity. Generally, only insect species within a given order (Lepidoptera, Coleoptera, Diptera, and Orthoptera) are susceptible to a given Cry protein. Therefore, insect susceptibility results provide general information about the δ -endotoxin(s) expressed by particular *B. thuringiensis* strains. In the case of the Cry1 proteins, the greatest activity is shown for the order Lepidoptera. This has been confirmed in selectivity studies for microbe-derived Cry1Ac protein (Herman, 2001; Appendix 2).

Selectivity of the Cry1Ac protein was determined for eight insect species exposed to microbially-expressed Cry1Ac protein in artificial diet studies (Herman, 2001; Appendix 2). The insects considered represented taxonomically diverse cotton pests from three orders (Lepidoptera, Homoptera and Coleoptera) and four families (Aphididae, Curculionidae, Noctuidae and Gelchiidae). The insects evaluated in this study were cotton aphid (CA) (*Aphis gossypii*), tobacco budworm (TBW) (*Heliothis virescens*), cotton bollworm (CBW) (*Helicoverpa zea*), beet armyworm (BAW) (*Spodoptera exigua*), boll weevil (BW) (*Athonomus grandis grandis*), fall armyworm (FAW) (*Spodoptera frugiperda*), cabbage looper (CL) (*Trichoplusia ni*) and pink bollworm (PBW) (*Pectinophora gossypiella*). The activity of the Cry1Ac protein was restricted to lepidopteran insects in this study.

VI.B.2. Results of Ecotoxicity Studies

Mammals

A microbial protein preparation containing 14% Cry1Ac protein was evaluated for acute oral toxicity from gavage administration to male and female CD1 mice (Brooks and Yano, 2001; Appendix 2). All mice survived and there were no adverse effects in terms of body weights, detailed clinical observations, and gross pathological lesions during the two-week observation period. Under the conditions of this study, the LD₅₀ of the Cry1Ac microbial protein in male and female CD-1 mice was greater than 700 mg a.i./kg.

Birds

An 8-day acute avian dietary study with bobwhite quail investigated the effect of a 10% cotton meal diet containing 0.012 µg per g Cry1Ac protein in combination with Cry1F protein (Gallagher and Beavers, 2002; Appendix 2). There were no adverse effects of treatment; therefore the LC₅₀ was >100,000 µg meal/g diet (>1200 ng Cry1Ac per g diet).

Soil invertebrates

Earthworm. Microbe-derived Cry1Ac protein, alone or in combination with Cry1F protein, showed no toxicity to earthworms (*Eisenia foetida*). The LC₅₀ was >107 mg Cry1Ac protein per kg (soil dry weight basis) (Sindermann *et al.*, 2001; Appendix 2). This represents concentrations that are 3066-fold higher than the EEC expected with incorporation of event 3006-210-23 cotton plants into the top 15 cm of soil (see Expected Environmental Concentrations, section VI.C).

Collembola. Collembola plays a major role in soil ecosystems due to its feeding on decaying plant materials. Therefore, a laboratory study to determine the chronic effects of Cry1Ac protein on survival and reproduction of the soil dwelling invertebrate collembola (*Folsomia candida*) was conducted using microbe-derived Cry1Ac added to Brewer's yeast (standard food for collembola) (Teixeira, 2002; Appendix 2). The fortification concentration tested was 22.6 mg Cry1Ac protein per kg diet, either alone or in combination with Cry1F protein. The study also evaluated the chronic effects of diets containing lyophilized event 3006-210-23 cotton leaves at concentrations of 5% and 50% of diet. Cry1Ac protein caused no significant effects on adult survival and reproduction. There was, however, an anomalous effect of Cry1Ac microbial protein alone resulting in reduced numbers of off-spring when administered in diet with Brewer's yeast at a nominal concentration of 22.6 mg Cry1Ac/kg diet (a level 647-fold above the EEC for Cry1Ac expressed by event 3006-210-23 in soil – see section VI.C). This effect was not shown with lyophilized leaf tissue from event 3006-210-23 cotton plants at levels of exposure representing 5% and 50% lyophilized tissue of in a diet with Brewer's yeast (representing approximately 16.45 and 164.5 µg Cry1Ac protein per kg diet on the basis of high-end leaf expression of Cry1Ac; see Estimated Environmental Concentrations, section VI.C). The study results *in toto* show no anticipated effect of Cry1Ac protein at approximately 5× the soil EEC for event 3006-210-23 (34.9 µg/kg; section VI.C). The EC₅₀ > 164.5 µg Cry1Ac protein per kg diet.

Effects on aquatic organisms

Daphnia magna. There are no known adverse effects of Cry proteins on the aquatic invertebrate *Daphnia magna*. A 48-hour static limit test with *Daphnia* was conducted with 2,500 µg/L Cry1Ac protein in combination with Cry1F protein (Marino and Yaroch, 2002a; Appendix 2). This rate of fortification represents >13,000× the anticipated EEC for Cry1Ac protein from event 3006-210-23 in surface water (see Estimated Environmental Concentrations in section VI.C). There were no observed adverse effects of treatment in terms of immobility or sublethal effects; therefore, the 24- and 48-hour EC₅₀ is > 2,500 µg Cry1Ac/L.

Fish. The acute dietary toxicity of Cry1Ac protein to the rainbow trout (*Oncorhynchus mykiss*) was determined for fish exposed for eight days to a commercial-grade, pelleted trout diet containing 10% cotton meal prepared from cotton seed expressing Cry1Ac and Cry1F proteins (Marino and Yaroch, 2002; Appendix 2). This produced a diet containing an initial dosing of 0.118 µg Cry1Ac per g-food in combination with Cry1F protein. The control diet consisted of the same commercial fish diet prepared with non-transgenic cotton meal. No fish mortality or sublethal effects were observed for either the control or treatment diet. Therefore, based on the biological observations, the 8-day LC₅₀ value with

rainbow trout is greater than 0.118 mg/kg-diet, representing 618× the anticipated EEC for Cry1Ac protein from event 3006-210-23 in surface water (see Estimated Environmental Concentrations in section VI.C.).

Effects on non-target arthropods

Honeybee. There was no effect on mean survival to emergence for honeybee exposed to either 2 mg pollen from a Cry1Ac-expressing event or to 11.94 µg per mL of Cry1Ac protein in combination with Cry1F protein (Maggi, 2001; Appendix 2). The LC₅₀ for exposure to Cry1Ac protein is >11.94 µg per mL (11.94 µg per g) and represents 5× the high-end expression in event 3006-210-23 pollen.

Green Lacewing. The dietary LC₅₀ for green lacewing (*Chrysoperia carnea*) larvae exposed to Cry1Ac protein, alone or in combination with Cry1F protein, has been investigated in a series of studies with microbial protein administered in a diet of moth eggs (Sindermann *et al.*, 2002; Appendix 2). There was no effect of Cry1Ac protein alone at a concentration of 46.8 µg per g diet; whereas, a significant effect of 46.8 µg per g Cry1Ac in combination with 5.2 µg per g Cry1F was shown in one study but not in a second. There was no effect of 4.68 µg per g Cry1Ac in combination with 0.52 µg per g Cry1F. Previous studies have shown that Cry1Ac is practically nontoxic to green lacewing (USEPA, 1995b). The conservatively projected LC₅₀ for green lacewing exposed to Cry1Ac protein is greater than 4.68 µg per g. The exposure level to Cry1Ac protein represents a concentration 94× higher than the high-end expression in event 3006-210-23 nectar and 2× higher than that in pollen. Safety factors based on a relevant food source (aphids consuming transgenic plant tissue) would be significantly higher (as much as 100- to > 4,000-fold higher; see *Phytophagus Insects* under Estimated Environmental Concentrations, section VI.C).

Toxicity to green lacewing larvae is not considered ecologically relevant to the risk assessment for event 3006-210-23 cotton, since exposure, if it occurs, will be indirect and field census results show no impact of Cry1Ac as expressed in the stacked product MXB-13 on green lacewing abundance (Mahill and Storer, 2002; Appendix 2).

Parasitic wasp. Parasitic hymenoptera (*Nasonia vitripennis*) were exposed to a single limit concentration of Cry1Ac protein, alone and in combination with Cry1F protein, in sugar water for up to 10 days. There were no significant differences in mortality between treatment groups and a sugar water control. The LC₅₀ was greater than 46.8 µg a.i. per mL of microbially-expressed Cry1Ac protein (Sindermann *et al.*, 2002; Appendix 2). The exposure level represents concentrations greater than 900× higher than the high-end expression of Cry1Ac protein event 3006-210-23 nectar and greater than 19× higher than that in pollen. Safety factors based on a relevant food source (lepidopteran larvae consuming transgenic plant tissue) would be significantly higher (from 9- to 286-fold higher; see *Phytophagus Insects* under Estimated Environmental Concentrations).

Ladybird beetle. Adult ladybird beetles (*Hippodamia convergens*) were unaffected when exposed to microbially-expressed Cry1Ac protein, alone or in combination with Cry1F protein (Porch and Krueger, 2001; Appendix 2). Ladybirds fed *ad libitum* over 15-days on a diets containing 22.5 µg Cry1Ac protein per mL of food, alone or in combination, were monitored for mortality and clinical signs of toxicity. The LC₅₀ for exposure to Cry1Ac protein is >22.5 µg Cry1Ac protein per mL, equivalent > 9× the high-end exposure in event 3006-210-23 pollen.

Monarch butterfly. Incidental exposure of a sensitive larval stage of a non-target butterfly or moth to Cry1Ac protein may occur if event 3006-210-23 pollen is present on host plants and it is consumed. Monarch larvae feeding on milkweed containing transgenic pollen is a surrogate for indirect exposure of a

hypothetical sensitive non-target lepidopteran larvae to cotton pollen. The response of first instars of monarch butterfly (*Danaus plexippus* L.) exposed to Cry1Ac in artificial diet for 7 days is reported in studies by Hellmich *et al.* (2001). The dietary concentration resulting in 50% growth reduction relative to controls (EC₅₀) was 0.9 ng a.i./mL for Cry1Ac. Hellmich *et al.* (2001) present bridging calculations for translation of artificial diet results to upper bound estimates of effect levels expressed in terms of pollen consumption on leaves of a host plant for monarch, common milkweed (*Asclepias curassavica* L.). On this basis, the equivalent effects levels in terms of pollen density on leaves of the host plant is 11 grains event 3006-210-23 pollen per cm² leaf consumed for Cry1Ac protein. Thus, the EC₅₀ for Cry1Ac protein expressed in cotton pollen is > 10× higher than the estimated environmental concentration in pollen from event 3006-210-23 (see, section VI.C).

Hazard summary

Table 22. Summary of guideline hazard tests for effect of Cry1Ac protein.

Guideline	Study Title	Results
OECD 401	Acute Toxicity – Mouse	LD ₅₀ > 700 mg Cry1Ac /kg
OPP B, 71-2	Acute Dietary Toxicity – Northern Bobwhite Quail	8-day LC ₅₀ > 100,000 µg meal /kg diet (> 1200 ng Cry1Ac /kg diet)
OECD 207	Acute Toxicity – Earthworm	14-day LC ₅₀ > 107 mg a.i.Cry1Ac /kg soil 3,066× EEC in soil
OECD proposed	Chronic Toxicity – Collembola	EC ₅₀ > 164.5 µg Cry1Ac /kg 5× EEC in soil
OECD 202	Acute Dietary Toxicity – <i>Daphnia magna</i>	48-hour EC ₅₀ > 2500 µg Cry1Ac/L 13,000× EEC in water
OECD 203	Acute Dietary Toxicity – Rainbow Trout	8-day LC ₅₀ > 0.118 mg /kg diet 618× EEC in water
OPPTS 885.4380	Acute Dietary Toxicity LD ₅₀ – Honeybees	LC ₅₀ > 11.94 µg Cry1Ac/ g diet 5× high-end expression in pollen
OPPTS 885.4340	Non-target Insect – Green Lacewing	LC ₅₀ > 4.68 µg Cry1Ac/g of diet 2× high-end expression in pollen 94× high-end expression in nectar
OPPTS 885.4340	Non-target Insect – Parasitic Hymenoptera	LC ₅₀ > 46.8 µg a.i. Cry1Ac /mL 19× high-end expression in pollen 900× high-end expression in nectar
OPPTS 885.4340	Non-target Insect – Ladybird Beetle	LC ₅₀ > 22.5 µg a.i. Cry1Ac /mL 9× high-end expression in pollen

VI.C. Environmental Exposure

Non-target organisms may be exposed to Cry1Ac protein expressed in event 3006-210-23 through either direct or indirect routes. Exposure estimates for organisms directly feeding on cotton tissues expressing Cry1Ac protein are based on the high-end expression for the relevant plant tissue to which a non-target organism of concern may be exposed through direct ingestion. High-end exposure estimates (HEEE) represent the 90% upper bound of the reported expression (USEPA, 1997b). Indirect exposures represent inadvertent exposures to Cry1Ac protein through soil, water, pollen on host plant tissues, or multitrophic interactions. These exposures are expressed as Estimated Environmental Concentrations (EEC) and are conservatively calculated using high-end estimates for input parameters.

VI.C.1. Exposure Routes

Direct feeding on plants or plant parts constitutes the primary route of exposure of organisms to Cry1Ac protein expressed in event 3006-210-23. Plant parts subject to feeding are predominantly leaves, roots, stems and pollen, and perhaps nectar as well. Organisms directly feeding on cotton as a primary food source within agroecosystems would be characterized as plant pests and are not germane to this assessment. Organisms incidentally exposed to plant residues or organisms consuming cotton plants or plant parts as an occasional or supplementary food source are considered non-target organisms of concern in this exposure assessment. Secondary exposure to protein residues by tritrophic interactions may occur for predators or parasites of plant-feeding organisms. Residues occurring in soil or water matrices may constitute an additional secondary route of exposure to Cry1Ac protein. The no-effect levels for non-target ecotoxicity tests show large margins of safety relative to conservatively projected environmental exposure concentrations (see section VI.C.), and these observations are supported in field monitoring for species abundance (Mahill and Storer, 2002; Appendix 2). Thus, the exposure routes postulated here are relevant only to exposure and risk characterization for potentially sensitive taxa of Lepidoptera.

VI.C.2. Environmental Fate of Cry1Ac Protein Incorporated into Soil

The conditions supporting degradation of Cry1Ac protein in soil were described in studies where either plant- or microbe-derived Cry1Ac protein were mixed with soil, incubated under standard laboratory conditions, and then sampled for bioassay at various intervals (Herman *et al.*, 2001; Herman and Collins, 2001; Appendix 2). Insect bioassays were conducted to measure degradation as loss in biological activity by applying aqueous-agar mixtures of soil samples to the top of artificial diet and allowing neonate tobacco budworms (*Heliothis virescens*) to feed on the treated media. Cry1Ac microbial protein did not decay when applied to a microbially non-vital soil (Herman *et al.*, 2001; Appendix 2), but rapid decay was found when lyophilized cotton leaf tissue from MXB-13 cotton, containing Cry1F and Cry1Ac protein was applied to viable soil (Herman and Collins, 2001, Appendix 2). These results are consistent with microbially-mediated soil degradation of Cry1Ac protein. Based on the bioassay results (GI_{50}) for soil amended with lyophilized cotton tissue, the half-life of the Cry1F/Cry1Ac insecticidal crystal proteins was 1.3 days under laboratory conditions, indicating a rapid decay rate in soil. The GI_{50} for the microbially-produced Cry1Ac insecticidal crystal protein was previously determined to be 0.028 ng ai/cm² (Herman and Collins, 2002; Appendix 2); therefore, the highest concentration of plant powder used in this study (167 ug/cm²) contained over 17-times as much Cry1Ac as required to inhibit growth of a highly sensitive organism by 50%.

Previous studies have shown microbially-expressed Cry1Ac ICP to exhibit soil half-lives of 9 to 20 days as compared to a 41-day half-life for *B.t.* cotton tissue expressing Cry1Ac ICP (cited in Head *et al.*,

2001). Cry1Ac residues were not detectable in soils continuously cropped to Bollgard®cotton for up to six years as determined by either chemical immunoassay or insect bioassay (Head *et al.*, 2001).

VI.C.3. Estimated Environmental Concentrations

Evaluation of protein expression levels and routes of exposure allows for development of estimated levels of exposure conservatively projected to occur in the environment.

High End Exposure Estimates

High-end exposure estimates (HEEE, calculated as Mean + 1.96 × Standard Deviation of expression values reported in Table 8) and are shown in Table 23 for those tissues relevant to conservatively estimating exposure concentrations by an identified route of exposure.

Table 23. High End Exposure Estimates for Expression of Cry1Ac Protein

Cotton Tissue	High End Exposure Estimate, ng/mg tissue
Leaf (young)	3.29
Whole plant (seedling)	2.18
Root (seedling)	0.40
Pollen	2.42
Nectar	< 0.05 ng/ul
Seed	0.75

Soil and water. Estimated environmental concentrations (EEC) in soil and water matrices were calculated to conservatively represent exposure by indirect routes for comparison against ecotoxicity endpoints. The basis for EEC computations is expression data (Table 23) for event 3006-210-23 that describes HEEE for Cry1Ac in relevant plant tissues (leaves, stems, and roots) at the time of maximal expression, and conservatively based models that predict concentration in soil and water.

The basis for the calculation of EEC reported here for Cry1Ac occurring by environmentally relevant exposure routes in the environment is predicted biomass production and partitioning as determined for average cotton yield in the US, 1999-2001 (1.35 bales per acre; NASS, 2002). From this, and literature estimates of biomass production and dry matter partitioning in cotton, the HEEE for expression are converted into EEC in soil and water (Appendix 3). The EEC in soil for Cry1Ac is 0.0349 mg a.i./kg soil and conservatively represents the worst case upper bound soil concentration of Cry1Ac protein which could occur through cropping of event 3006-210-23 cotton.

The EEC for Cry1Ac occurrence in surface water was estimated using the GENEEC farm pond scenario (USEPA, 2000). The Cry1Ac returned to the soil through incorporation to a 6-inch depth was assumed available for runoff/erosion to an edge of field pond in the two days immediately post-incorporation. Additional input assumptions were soil degradation rate based on bioavailable DT₅₀ (1.3 days, see Environmental Fate), no degradation in other environmental compartments, and K_{OC} and solubility for Cry1Ac protein of 100 L kg⁻¹ and 1000 g L⁻¹, respectively (conservative estimates). The EEC in water is 191 ng per L for Cry1Ac protein (Appendix 3) and conservatively represents the worst case upper bound aquatic concentration of Cry1Ac protein which could occur through cropping of event 3006-210-23 cotton.

Pollen. Cotton is predominately a self-pollinated crop with some amount of cross-pollination facilitated by bumblebees, Melissodes bees, and honeybees; lepidopteran insects are not pollinators of cultivated

cotton (McGregor, 1976). As a consequence, environmental exposure of sensitive lepidopterans to cotton pollen will be indirect through contamination of food sources. Indirect exposure to cotton pollen is negligible. Transgenic cotton pollen dissemination into the 23 border rows adjacent to a transgenic cotton field resulted in an average of 0.76% out-crossing (Umbeck *et al.*, 1991). Zhang *et al.* (1997) found out-crossing frequency from transgenic cotton declined from 0.61% at 5-m off-source to 0.03% at 50 m, and was not detectable at 100 m. Because of limited out-crossing (and, thus, limited environmental dissemination of cotton pollen), the occurrence of cotton pollen grains as contaminants on a host plant, will be negligible. A cotton pollen density of 1 grain per cm² leaf, of a host plant for a hypothesized non-target organism of concern is a conservative environmental exposure estimate for off-source occurrence.

Phytophagous insects. Both target and non-target insect herbivores serve as food sources for beneficial insect predators and prey and, therefore, constitute a relevant exposure route within a multitrophic context (Groot and Dicke, 2002). The levels of transgenic protein occurring in these food chain intermediates and their by-products are more relevant to exposure and risk to beneficial insects than are the levels of plant tissue expression on which hazard doses are based. The concentration of transgenic protein found in foliar feeding herbivores clearly shows the reduction in protein that can be expected within a multitrophic context (Head *et al.*, 2001; Raps *et al.*, 2001). The concentrations of transgenic protein found in aphid were a minimum of 100-fold lower upwards to several thousand-fold lower than in food sources containing the protein; the protein concentration was further lowered in excreted honeydew. Similarly, Lepidoptera showed reduction in transgenic protein concentration in comparison to their food source, but the level of reduction was somewhat less dramatic than for aphid, ranging from 9- to 286-fold; excreted feces showed that transgenic protein largely passed through the insect (Raps *et al.*, 2001).

Exposure Estimates for the Stacked Product MXB-13 Cotton

Cotton line MXB-13 is the end-use product containing Cry1Ac protein originating from event 3006-210-23. MXB-13 cotton is a cross between Cry1Ac event 3006-210-23 and Cry1F event 281-24-236. Phillips *et al.* (2002; Appendix 2) have reported expression levels of Cry1Ac protein in MXB-13 cotton, and Wolt (2002; Appendix 2) has considered the exposure concentrations relevant to non-target organisms. As shown in table 24, the relevant exposure concentrations for Cry1Ac protein expressed in event 3006-210-23 are comparable with those for MXB-13 cotton.

Table 24. Comparison of Estimated Exposure Concentrations for Cry1Ac Protein from Event 3006-210-23 and the Stacked Product (MXB-13)

Matrix	Exposure Concentration, ng/mg	
	Event 3006-210-23	MXB-13
	<u>Cry1Ac</u>	
Leaves	3.29	2.09
Whole plants	2.18	0.99
Roots	0.4	0.19
Pollen	2.42	2.43
Nectar	< 0.05	< 0.05
Seed	0.75	0.6872
Soil	0.0349	0.0196
Water, ng/L	191	107

VI.D. Potential Ecological Effects

VI.D.1. Likelihood of Exposure Exceeding Effects Thresholds and Taxa at Risk

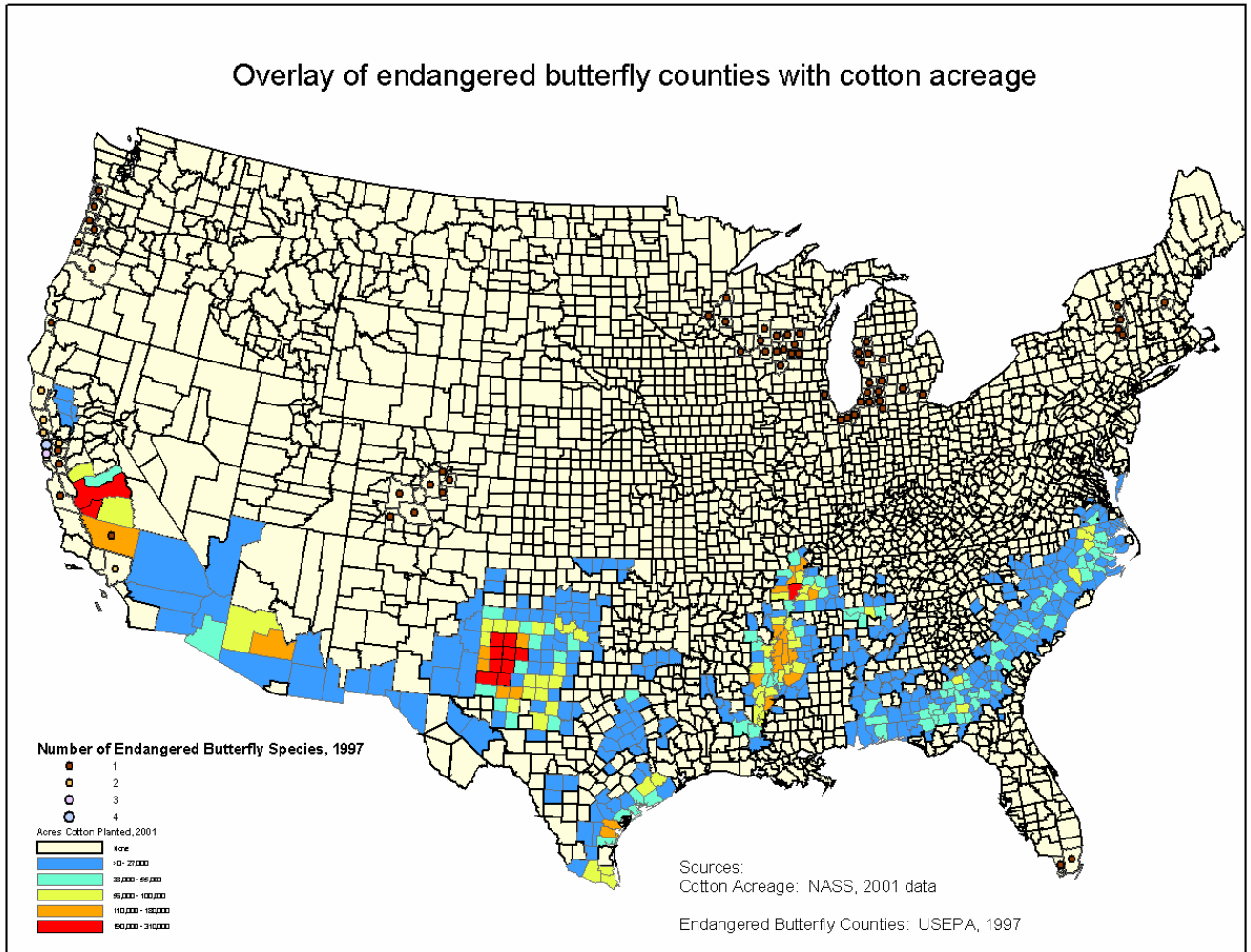
Sensitive non-target Lepidoptera. Incidental exposure of a sensitive larval stage of a non-target butterfly or moth to Cry1Ac may occur if event 3006-210-23 pollen is present on host plants and it is consumed. Indirect exposure of a hypothetical sensitive non-target lepidopteran larvae to cotton pollen is negligible, as exemplified by the case of monarch feeding on milkweed as a surrogate for a non-target lepidopteran species with host plants occurring in or near cotton fields. The likelihood of exposure is remote due to the insignificant outflow of pollen from cotton; thus, there is negligible risk to non-target butterflies or moths from cropping of event 3006-210-23 cotton. Monarch butterfly itself, is only a surrogate species for the purposes of this assessment, as it will be minimally exposed to pollen of event 3006-210-23 pollen, since spring migration through cotton growing regions will occur well in advance of cotton flowering.

Beneficial insect considerations. The consequences of cultivation of cotton expressing Cry1Ac protein on beneficial insects of cotton has been described by Wolt (2002; Appendix 2). Well in excess of 300 different species of beneficial insects are known to inhabit cotton fields. Common arthropod predators and parasites of cotton fields represent orders that are insensitive to the Cry1 proteins. Additionally, these beneficial organisms are predominantly predators and parasites and only in relatively few instances are plant products (pollen and nectar) consumed, and in these instances consumption is by a non-sensitive life stage (adults). Therefore, direct risks of beneficials from exposure to Cry1Ac protein expressed in event 3006-210-23 cotton are negligible. Risk from indirect exposure to Cry1Ac protein through tritrophic feeding on insect host/prey is also negligible due to the low levels of exposure anticipated in comparison to effect levels shown in testing of surrogates. (Safety margins shown in hazard studies are quite conservative, since they are based on plant expression levels, when actual secondary exposure would be much reduced from tritrophic feeding on prey/hosts of beneficials; see section VI.C).

Endangered species considerations. The overlay map shown in Figure 20 describes the county level distribution of endangered lepidopteran species as listed by the U.S. Fish and Wildlife Service (FWS, 1997) relative to cotton producing counties in the US (NASS, 2002). These endangered lepidopteran species have very restricted host-range specificity. All but one of these endangered lepidopteran taxa is not known to occur in counties where cotton is grown. The Kern primrose sphinx moth (*Euproserpinus*

euterpe) is known to occur in Kern County, CA but cotton is not a host plant for this species, nor do host-range considerations place habitat in or near cotton fields (FWS, 1984).

Figure 20. Cotton Producing Counties in the United States in Relation to Those Counties Where Endangered Lepidopteran Taxa are Known to Occur.



A recent risk assessment by EPA relating to the renewal for registration for Bollgard® cotton expressing Cry1Ac protein (USEPA, 2001b) considered a Biological Opinion from the Department of the Interior Fish and Wildlife Service (18 December 1986) concerning the possible effect of foliar spray of *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) on beneficial and endangered species. Based on the difference in exposure scenarios between *B.t.* foliar spray and expression of *B.t.* in cotton plants, EPA concluded that the Biological Opinion specific to foliar sprays was inapplicable to Cry protein expressed in cotton, and that re-initiation of consultation was not required.

Specifically, with respect to Cry1Ac protein expressed in Bollgard® cotton, EPA concluded pollen drift out of fields was of negligible concern in recognition that Cry1Ac “was not toxic to the test species

representative of organisms likely to be exposed to such pollen when cotton plants containing the Cry1Ac gene are grown.” EPA further concluded,

The amount of pollen that would drift from these cotton plants onto plants fed upon by endangered/beneficial species, would be very small compared to the levels fed to the test species. Therefore, EPA does not expect that any endangered/beneficial species will be adversely affected by pollen containing the Cry1Ac delta-endotoxin. (USEPA, 2001b)

In view of the toxicological and expression data presented here, these conclusions also follow for Cry1Ac protein as expressed in event 3006-210-23 cotton.

Ecological Relevance. Based on the analysis presented herein, there are no ecologically relevant concerns arising from cropping of event 3006-210-23 cotton expressing Cry1Ac protein. Selectivity, exposure routes, and exposure concentrations restrict potential risks to lepidopteran insects directly exposed to event 3006-210-23 residues. For these taxa, the likelihood of an adverse environmental consequence is negligible as shown by toxicity testing of non-target organisms, which indicated no-effect concentrations at doses well above conservatively projected environmental exposure concentrations. Field abundance supports lack of risk to taxa characteristic of cotton agroecosystems.

Field Census Study. The lack of adverse non-target effects of Cry1Ac at environmentally relevant exposure concentrations is substantiated in field monitoring studies for MXB-13, the stacked product containing Cry1Ac protein derived from event 3006-210-23. The beneficial arthropods present in field plots of MXB-13 cotton were compared to those in field plots of non-transgenic cotton with comparable genetics, with and without insecticide application, at locations in Louisiana and Arizona (Mahill and Storer, 2002; Appendix 2). Preliminary results show no adverse effect of MXB-13 on the numbers of insects from over 50 taxa monitored using scouting, whole plant sampling, and sweeps. Synthetic insecticide treatment, however, reduced the population of some taxa of non-target arthropods for certain times of sampling. The field plots were sufficiently large (1,000 m²) to minimize plot to plot movement of most species, although the most mobile insects are likely to have moved among plots.

VI.E. Potential Adverse Effects on Human and Animal Health

Plant compositional analysis (section V.D.) and toxicity tests (section VI.B.) show no adverse consequences of event 3006-210-23 or the Cry1Ac protein it expresses on human or animal health.

VI.E.1. Human Health Risk

Proteins as a class are generally not highly toxic to humans, nor are they likely to bioaccumulate in fatty tissue or to persist in the environment. The Cry1 proteins used in crop production, either as formulated microbial sprays or as PIPs, show no mammalian toxicity, do not correspond to known allergens, and are rapidly digestible. The Cry1Ac protein conforms to properties of proteins that are not toxic to humans.

Human consumption of cotton products is limited. Typically, cotton by-products occur as blended items and comprise a minor component of daily dietary intake. The genetic modification of event 3006-210-23 cotton to express Cry1Ac protein will not alter consumption patterns for cotton products. The introduced gene product is not toxic, and exposure will be negligible in foods. Dietary exposure is significantly restricted by the rapid digestibility of Cry1Ac protein.

Cotton products comprise a minor component of the human diet and because of market share, food products containing Cry1Ac cotton-derived food ingredients will represent only a small fraction of all

cotton ingredients in foods. In addition, Cry1Ac protein is rapidly digested (non-detectable in <1 min in simulated gastric fluid) and the Cry1Ac protein is not toxic to mammals. Therefore, the dietary exposure to Cry1Ac protein from event 3006-210-23 is negligible.

Because Cry1Ac protein is contained within the plant there is minimal potential for human exposure via dermal, eye, or inhalation exposure routes.

VI.E.2. Animal Health Risk

Animals may consume cottonseed, cottonseed meal, cottonseed hulls, or cotton gin by-products (gin trash) as a portion of their diet. On the basis of various scenarios for cotton product consumption by livestock, the worst case exposure from consumption of Cry1Ac residues in cotton products is that of dairy cattle consuming 100% of their total protein as cottonseed meal. This high-end estimate of cottonseed meal consumption by a high producing mature dairy cow indicates negligible exposure to Cry1Ac protein in animal diets.

VI.F. Weediness

Introduction of the gene coding for Cry1Ac, which confers lepidopteran-resistance to cotton, is unlikely to increase weediness of cotton. In order that domesticated cotton have the potential to become a weed, the transgenic characteristic would have to confer to cotton a weedy characteristic otherwise absent in the parental, non-transgenic cotton line. Weediness is a multigenic trait with attributes mostly pertaining to sexual or asexual reproductive advantage within natural or agricultural ecosystems (Baker, 1965). Lepidopteran-resistant Cry1Ac cotton is expected to be cultivated in a managed agroecosystem; the likelihood is remote that sufficient selective pressure for this single gene trait would result in expression of weediness. Nor is their evidence that cotton lines expressing Cry1Ac protein have other characteristics that are indicative of increased weediness. Agronomic characteristics for Cry1Ac cotton (other than those related to lepidopteran insect resistance) do not pose biologically relevant differences to comparable non-transgenic cotton (see section V.G.). Agronomic reports from field studies indicate that no increase in volunteers from seed, regrowth from stubble, or increase in seed dormancy, were observed in event 3006-210-23, versus the parent plant PSC355.

In the Environmental Assessment for Bollgard® (Cry1Ac, lepidopteran-resistant) cotton event 531, USDA concluded that “*G. hirsutum* does not show any appreciable weedy characteristics or tendencies, and that the genus seems to be devoid of any such characteristics.” USDA also concluded that the introduction of the lepidopteran resistance trait into cotton is unlikely to increase weediness of this cotton, and that any increase in the weediness of this lepidopteran resistant cotton would have to result from the transgenic plant having a competitive advantage over the parental, non-transgenic line. Because lepidopteran resistant cotton is expected to be cultivated like any other cotton in a managed agricultural environment, the likelihood that sufficient selective pressure would be present for the lepidopteran-resistant cotton to become a weed is low. These same conclusions can be made regarding the lepidopteran-resistant Cry1Ac event 3006-210-23. It can thus be concluded that event 3006-210-23 will not pose a weed hazard.

VI.G. Product Durability Plan for Cry1Ac in *B.t.* Cotton Stacked Product MXB-13

The availability of novel lepidopteran-resistance traits in cotton lessens the selection pressure for pest adaptation to existing pest control methods. By providing an additional option to cotton producers for managing lepidopteran pests, each new product adds to the sustainability of other products. The cost of discovering and developing commercial cotton traits conferring high levels of resistance to pests is very high. To bring the advantage of such technology to the cotton industry in an economically sustainable manner, Dow AgroSciences intends to implement measures that protect the durability of the traits by reducing the rate at which pests adapt. The product durability plan is a set of scientifically based practical measures that are intended to achieve this aim (Storer, 2002; Appendix 2).

Resistance management for *B.t.* cotton has become well established since the first *B.t.* cotton was commercially grown in 1996. Cotton growers, consultants, and entomologists, as well as regulatory authorities, all agree on the need to preserve the benefit of the technology, and that the best tool is the planting and managing of refugia consisting of non-*B.t.* cotton. Several refuge options are available to growers, and have proven to be effective at maintaining pest susceptibility in the face of extensive use of *B.t.* cotton.

Cry1Ac, as expressed in event 3006-210-23 cotton, is similar in efficacy against key target pests to Cry1Ac in existing commercial *B.t.* cotton (Bollgard®). Thus the refuge options for Bollgard® will also be effective for commercial cotton derived from event 3006-210-23. In addition, Cry1Ac protein expressed in event 3006-210-23 will be deployed in a stack (MXB-13) with Cry1F event 281-24-236 to extend the spectrum of activity of the cotton in the commercial product. An additional benefit of the stack is that it increases the number of target sites in the midgut of several key sensitive pest species. Multiple resistance mechanisms are required in an individual insect for it to experience enhanced survival. It has long been accepted that a combination of insecticidal compounds or proteins requiring multiple resistance mechanisms in an individual insect is far more durable than a single compound or protein requiring a single resistance mechanism. Computer models developed by Dow AgroSciences indicate that this is likely to be true for the stack of Cry1F and Cry1Ac in insect species that are sensitive to both proteins. Therefore, it is likely that deployment of Cry1Ac stacked with Cry1F will slow the development of resistance to Cry1Ac and thus preserve the durability of MXB-13 cotton and the durability of other *B.t.* cottons. This in turn will help preserve the durability of other control tools by expanding the range of tools available and reducing the dependence on any single one.

VI.H. Interactions with Sexually Compatible Species

Cross-pollination of cultivated cotton varieties does and will occur between event 3006-210-23 and other commercial varieties. This transfer, however, is not considered to be of consequence to cotton farmers in the U.S. because seed produced from event 3006-210-23 will have to meet existing cotton certification standards in order to be sold commercially.

Successful sexual transmission of genetic material via pollen is possible only to certain cotton relatives and requires sexual compatibility, overlap in flowering and pollen receptivity (nick), geographic proximity, and a vector for pollen transmission. In the United States and possessions, the sexually compatible species with cultivated cotton are *G. hirsutum* (wild or under cultivation), *G. barbadense* (wild or cultivated Pima cotton), and *G. tomentosum*. Any potential effects of lepidopteran insect resistance conferred by Cry1Ac protein are not expected to alter the potential for gene transmission to wild cotton populations and transmission, should it occur, is not expected to alter the weediness attributes of wild cotton. Concerns relative to pollen and gene flow can be addressed through geographical

isolation, since sexually compatible feral and wild cotton populations are remote from areas of cultivated cotton production.

Cultivated cotton, including cotton expressing Cry1Ac protein, is chromosomally compatible with wild *G. hirsutum* and *G. barbadense*. Movement of genetic materials between *G. hirsutum* and *G. barbadense* is widespread in cultivated stocks but it is low or absent in natural populations where *G. hirsutum* and *G. barbadense* co-occur. The absence of natural introgression may be caused by isolating mechanisms such as pollination, fertilization, ecology, gene incompatibility, or chromosome incompatibility (Percy and Wendel, 1990). Any movement of genetic material *G. hirsutum* or *G. barbadense* expressing Cry1Ac protein into non-transgenic *G. hirsutum* or *G. barbadense* is likely to be the result of intentional breeding practice rather than accidental crossing. Unintended movement of gene material between cultivated *G. hirsutum* or *G. barbadense* expressing Cry1Ac ICP and cultivated would be uncommon. Even if such movement did occur, it would not offer the progeny any clear selective advantage over the parents.

Wild *G. hirsutum* and *G. barbadense* populations are geographically isolated from cultivated cotton and, therefore, do not cross with cultivated cotton species. Within the United States, wild *G. hirsutum* is found only in southern Florida (in the Florida Keys). Additionally, within U.S. territories and possessions in tropical and subtropical regions, there is the potential for wild *G. barbadense* or *G. hirsutum* to occur as feral populations. While some evidence suggests wild populations may occur in Puerto Rico and the U.S. Virgin Islands, there is no current commercial cotton production in these locations.

The potential for movement of genetic material from cultivated cotton expressing the Cry1Ac ICP to *G. tomentosum* is uncertain. The wild species of *G. tomentosum* occur throughout the Hawaii and this species is chromosomally compatible with *G. hirsutum* and *G. barbadense*. There is, however, uncertainty about the vector for pollination. The flowers of *G. tomentosum* seem to be pollinated by moths, not bees, and they are reportedly receptive at night, not in the day; thus the potential for gene transmission appears remote. The USEPA restricts the commercial sale of transgenic cotton seed within Hawaii and further restricts the size and location of cotton breeding nurseries in order to mitigate against the potential for gene transmission from transgenic cotton to *G. tomentosum*. Regardless, neither the weediness nor the survival of *G. tomentosum* will be affected by the cultivation of cotton expressing Cry1Ac protein. Event 3006-210-23 cotton itself poses no potential for increased weediness (see section VI.F.) and, in the unlikely event the two species were to successfully cross in nature, the added trait would confer no selective advantage in the wild species habitat.

G. thurberi Todaro (*Thurberia thespiodes* Gray) is a wild cotton species occurring commonly on rocky slopes and sides of canyons in the mountains of Southern Arizona and northern Mexico. Gene transmission from cultivated cotton is not possible because of sexually incompatibility (*G. thurberi* is a diploid species and crosses with cotton tetraploids are unsuccessful), lack of nick with cultivated cotton, and geographic isolation from cultivated cotton. Therefore, a concern for gene transfer from cultivated event 3006-210-23 cotton to *G. thurberi* is not warranted.

VII. Adverse Consequences of New Cultivar Introduction

The evidence provided in this petition supports the conclusion that cotton lines derived from event 3006-210-23 present negligible risk to human health and the environment and do not present a plant pest risk. Based on exposure estimates and the results of toxicological studies, there is negligible risk to non-target organisms and beneficial insects from expression of the Cry1Ac protein in cotton lines derived from event 3006-210-23. There are no identified concerns for endangered species as a consequence of cultivation of transgenic cotton expressing Cry1Ac protein. Cotton lines derived from event 3006-210-23 exhibit typical

agronomic characteristics and composition, and normal Mendelian inheritance of the introduced genetic material. Transformation of cotton to express Cry1Ac protein imparts no biologically relevant alterations in the phenotype of cotton other than resistance to lepidopteran pests. There has been no evidence of increased susceptibility to insect pests or disease in cotton lines derived from event 3006-210-23 when compared to conventional cotton lines.

The *pat* gene and PAT protein have been the subject of a previous determination of non-regulated status by the USDA (USDA, 1995). USDA determined that events T14 and T25 developed by AgrEvo: (1) exhibit no plant pathogenic properties; (2) are no more likely to become weeds than other cotton developed by traditional breeding techniques; (3) are unlikely to increase the weediness potential for any other cultivated or wild species with which they can interbreed; (4) will not harm other organisms that are beneficial to agriculture; and (5) show no adverse consequence to processed agricultural commodities. Similarly, Cry1Ac cotton lines derived from event 3006-210-23 expressing the identical PAT protein are not expected to present any significant plant pest or environmental risk. The PAT protein has been shown to present no significant human health environmental risk based on acute oral toxicity studies and in vitro digestibility studies (USEPA, 1997a; USEPA, 1995a).

VII.A. Statement of Grounds Unfavorable

The results of all field release data and laboratory studies presented herein establish that there are no unfavorable grounds associated with Cry1Ac cotton event 3006-210-23.

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Appendix 1. USDA Field Trial Approvals for Event 3006-210-23

MS #	USDA #	Counties of Release	Notification Issue Date
MS266	02-302-12n	Not yet planted	11/28/2002
MS259	02-249-02n	Not yet planted	9/16/02
MS234	02-066-09n	MS: Washington, SC: Darlington, TX: Haskell	4/6/02
MS232	02-066-07n	AL: Bladwin, AR: Drew, Jackson, Woodruff, Lonoke, Phillips, AZ: Pinal, Yuma, Maricopa, CA: Fresno, Kings, GA: Decatur, Decatur, LA: ST. Landry, Rapides, Franklin, MS: Oktibbeha, Washington, Webster, NC: Washington, Martin, NM: Dona Ana, SC: Barnwell, TN: Haywood, Shelby, TX: San Patricio, Waller, Burleson	04/06/2002
MS231	02-066-06n	AZ: Pinal, GA: Mithcell, MS: Washington, TN: Shelby, TX: Lubbock	04/06/2002
MS198	01-093-17n	AZ: Pinal, CA: Fresno, MS: Washington, TX: Armstrong, Wharton, VA: Gates	5/3/01
MS196	01-093-14n	AZ: Pinal, MS: Bolivar, SC: Darlington,	4/23/01
MS185	01-052-10n	AZ: Pinal, GA: Mitchell, MS: Washington, TN: Shelby, TX: Lubbock	3/23/01
MS183	01-052-08n	MS: Washington	3/23/01
MS181	01-052-06n	AL: Baldwin, AZ: Pinal, CA: Kings, Fresno, LA: Franklin, MS: Washington (3), Oktibbeha, NC: Martin, PR: Santa Isabel	3/21/01
MS105	00-049-15n	MS: Washington, Oktibbeha, CA: Kings, NC: Martin	3/20/00
MS077	99-067-09n	MS: Oktibbeha, Washington	4/1/99

Appendix 2. Studies Submitted for Section 3 EPA Submission

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Appendix Table 2.1. List of Studies Submitted for Section 3 EPA Submission

Volume No.	Title	Author/Year	No. of Pages	MRID #
Volume 1	Product Characterization Data for <i>Bacillus thuringiensis</i> var. <i>kurstaki</i> Cry1Ac(synpro) Insect Control Protein as Expressed in Cotton	Narva, K.E., Palta, A., and Pellow, J.W./2001	338	45607901
Volume 2	Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Event 3006-210-23	Green, S.B, Ernest A.D., Bevan, S.A./2002	57	45808402
Volume 3	Cloning and Characterization of DNA Sequences in the Insert and Flanking Border Regions of B.t. Cry1Ac Cotton 3006-210-23	Song, P./2002	28	45818602
Volume 4	Comparison of the Amino Acid Sequence of the <i>Bacillus thuringiensis</i> var. <i>kurstaki</i> Cry1Ac(synpro) Insect Control Protein as Expressed in Cotton to Known Protein Allergens	Stelman, S.J./2001	114	45542310
Volume 5	Comparison of the Amino Acid Sequence of the phosphinothricin acetyltransferase (PAT) Protein as Expressed in Cotton to Known Protein Allergens	Stelman, S.J./2001	113	45542311
Volume 6	Characterization of Cry1Ac(synpro) Delta-endotoxin Derived from <i>Pseudomonas Fluorescens</i> and Transgenic Cotton	Y. Gao, Y., Gilbert, J.R., Ni, W. and Xu, X./2001	52	45542306
Volume 7	Purification and Characterization of Cry1Ac Delta-Endotoxin from Transgenic Cotton Event 3006-210-23	Gao, Y, Ni, W. and Xu, X./2002	38	45808404
Volume 8	Characterization of Phosphinothricin Acetyltransferase (PAT) from Recombinant <i>Escherichia coli</i> and Transgenic Cotton	Schafer, B.W. and Schwedler, D.A./2002	44	45808405
Volume 9	Biological Equivalency of Cotton (Event 3006)- and <i>Pseudomonas</i> -expressed Cry1AC B.t. Delta-Endotoxin	Herman, R.A./2001	27	45542304
Volume 10	Microbial B.t. Cry1Ac(synpro) Delta-Endotoxin: Cotton-Insect-Pest Susceptibility Study	Herman, R.A./2001	23	45542308
Volume 11	Efficacy of Cry1F/Cry1Ac Cotton Against a Wide Range of Lepidopteran Pests	Pellow, J.W./2002	100	45808407

Volume 12	Field Expression of Cry1F (synpro), Cry1Ac (synpro), and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products; and Compositional Analysis of Cottonseed and Cottonseed Processed Products	Phillips, A.M., Embrey, S.K., Shan, G. and Korjagin, V.A./2002	408	45808408
Volume 13	Cry1Ac-(Synpro) Microbial Protein: Acute Oral Toxicity Study in CD-1 Mice	Brooks, K.J. and Yano, B.L./2001	41	45542313
Volume 14	Cry1F(synpro) Delta Endotoxin and Cry1Ac(synpro) Delta Endotoxin: A Dietary Toxicity Study with the Ladybird Beetle	Porch, J.R. and Krueger, H.O./2001	31	45542315
Volume 15	Microbial Cry1F Delta-Endotoxin, Microbial CryAC Delta-Endotoxin Pollen Expressing Cry1F Delta-Endotoxin, and Pollen Expressing Cry1AC Delta-Endotoxin: Evaluation of Dietary Exposure on Honey Bee Development	Maggi, V.L./2001	61	45542316
Volume 16	Assesment of Chronic Toxicity of Diets Containing Cry1F and Cry1Ac Microbial Protein, Lyophilized Cry1Ac Cotton Leaf Tissue or PSC355 Control Cotton Leaf Tissue to Collembola (<i>Folsomia candida</i>)	Teixeira, D./2002	54	45808409
Volume 17	Cry1F(synpro) ICP and Cry1Ac(synpro) ICP: Dietary Toxicity to Green Lacewing Larvae (<i>Chrysoperla carnea</i>)	Sindermann, A.B., Porch, J.R., and Krueger, H.O./2002	36	45808410
Volume 18	Cry1F(synpro) ICP and Cry1Ac(synpro) ICP: Dietary Toxicity to Parasitic Hymenoptera (<i>Nasonia vitripennis</i>)	Sindermann, A.B., Porch, J.R., and Krueger, H.O./2002	25	45808411
Volume 19	Cry1F (synpro) and Cry1Ac(synpro) Insecticidal Crystal Proteins: An Acute Toxicity Study With The Daphnid, <i>Daphnia magna</i> Straus	Marino, T.A. and Yaroach, A. M./2002	32	45808412
Volume 20	Fish Food Containing 10% Cotton Meal Prepared from Cotton Seed Expressing B.t. Cry1F and Cry1Ac Proteins: An 8-Day Dietary Toxicity Study with the Rainbow Trout, <i>Oncorhynchus mykiss</i> Walbaum	Marino, T.A. and Yaroach, A.M./2002	33	45808413
Volume 21	Cotton Meal Prepared from Seeds Expressing Cry1F(synpro) and Cry1Ac(synpro) Insecticidal Crystal Proteins: Avian Acute Dietary Test with the Northern Bobwhite	Gallagher, S.P. and Beavers, J.B./2002	43	45808414

Volume 22	Cry1F (synpro) Delta Endotoxin and Cry1Ac (synpro) Delta Endotoxin: Acute Toxicity to the Earthworm in an Artificial Soil Substance	Sindermann, A.B, Porch, J.R., and Krueger, H.O./2001	23	45580701
Volume 23	Degradation of Cotton-Produced B.t. Cry1Ac(synpro) and Cry1F(synpro) in a Representative Cotton Soil	Herman, R.A. and Collins, R.A./2001	22	45556801
Volume 24	In Vitro Simulated Gastric Fluid Digestibility Study of Microbially Derived Cry1Ac(synpro)	Korjagan, V.A./2001	27	45542319
Volume 25	In Vitro Simulated Gastric Fluid Digestibility Study of Recombinant Phosphinothricin Acetyltransferase (PAT)	Korjagin, V.A. and Herman, R.A./2002	47	45808416
Volume 26	Product Durability Plan for Cotton Expressing Cry1F and Cry1Ac Insecticidal Crystal Proteins from <i>Bacillus thuringiensis</i>	Storer, N. P./2002	273	45808415
Volume 27	Investigations into High-Dose Expression of Cry1F and Cry1Ac Proteins Against the Tobacco Budworm in Bt Cotton Line MXB-13	Blanco, C., Herman, R.H. and Storer, N.P./2002	58	45808417
Volume 28	Investigations into the Dose of Cry1Ac and Cry1F Proteins in Bt Cotton Line MXB-13 Against Bollworm	Storer, N.P. and Blanco, C./2002	33	45808418
Volume 29	2002 Field Survey to Evaluate Effects on Non-target Beneficial Arthropods of Cry1F/Cry1Ac Bt Cotton MXB-13	Mahill, J.F. and Storer, N.P./2002	58	45808419
Volume 30	Ecological Risk of Cotton Expressing Cry1F and Cry1Ac Insecticidal Crystalline Proteins to Non-target, Beneficial, and Endangered Insects	Wolt, J.D./2002	57	45808420
Volume 31	Independent Laboratory Validation of Method GRM 02.11, "Determination of Cry1Ac Insecticidal Crystal Protein in Cotton Tissues by Enzyme Linked Immunosorbent Assay"	Shan, G./2002	54	45808422
Volume 32	Development and Characterization of Enzyme Linked-Immunesorbent Assay (ELISA) for the Detection of Cry1Ac Protein	Shan, G./2002	44	45808424

Full text documents of the above listed study reports are provided under separate cover

Appendix 3. Estimated Environmental Concentration Calculations

Appendix Table 3.1. Calculation of Estimated Environmental Concentrations of Cry1Ac and PAT Proteins as Expressed in Event 3006-210-23 Cotton.

	B	C	D	E	F	G	H	I	J	K	L	M
2	Cotton EEC Calculation											
3			yield	delivered to gin			in-field					
4	protein		bales/A	lint	seed	trash	leaves	stems	roots	biomass		information source
5			1.35									US average, 1999-2001;NASS, 2002
6		dry matter production, lb/A		648	1152	188	14549	9363	3796	27707		calculated; Bange and Milroy, 2000
7		lb/bale		480	853	139						Princ. Field Crop Prod., 3rd Ed ^a
8	Cry1Ac	tissue (fresh weight):	ng B.t. toxin/mg fw		7.5	0.96562	0.96562	0.63983	0.12512			calculated for sample moisture as received
9		tissue (dry matter basis):	ng B.t. toxin/mg dm			3.29	3.29	2.18	0.4			measured/calculated as HEE
10			lb B.t. toxin/A		0.0086	0.0006	0.0479	0.0204	0.0015	0.0698		calculated
11		soil:	mg B.t. toxin/ kg				0.0239	0.0102	0.0008	0.0349		calculated, 6-in depth of incorporation
12		water:	ng B.t. toxin/ L							1,290		GENEEC estimate
18												
19	^a J. H. Martin, W. H. Leonard, and D. L. Stamp. 1976. Principles of Field Crop Production. 3rd Ed. Macmillan.											
20	A bale of cotton at the gin contains 480 lbs lint plus 20 lbs bagging.											
21	At the gin an equivalent bale of machine harvested cotton contains 480 lint, 853 lb cotton seed, plus trash (139 lbs if machine picked, 836 lbs if machine stripped).											
22	^b M. P. Bange and S. P. Milroy. 2000. Timing of crop maturity in cotton: Impact of dry matter production and partitioning. Field Crops Res. 68:143-155.											
23	Figure 7b shows the relationship of fruit dry matter (FDM, squares, green bolls, open bolls) to total dry matter (TDM, cotton, leaves, stems, squares, fruit), which can be expressed roughly as:											
24	$\ln \text{FDMg} = 0.36 \ln \text{TDMg} - 7.33$											
25	These values are on gram equivalent basis with glucose adjustment (a factor used to determine dry matter at maturity).											

Cotton EEC Calculation (spreadsheet formulae):

Cotton Yield, Bales/Acre D5=1.35

dry matter production, lb/A

cotton delivered to gin

lint E6=\$D\$5*E7

seed F6=\$D\$5*F7

trash G6=\$D\$5*G7

standard bale composition, lb/bale

lint E7=480

seed F7=853

trash G7=139

in-field biomass

leaves I6=(((EXP((LN((E6+F6)*0.0893))+7.12/2.67))/0.0893)-(E6+F6+G6))*(2.16/(2.16+1.39))

stems J6=I6*(1.39/2.16)

roots K6=(I6+J6)/6.3

total biomass L6=SUM(I6:K6)

Cry1Ac protein produced

tissue (fresh weight): ng B.t. toxin/mg fw

cotton delivered to gin

seed F8=7.5

trash G8=18

in-field biomass

leaves I8=I9*0.2935

stems J8=J9*0.2935

roots K8=0.3128 *K9

tissue (dry matter basis): ng B.t. toxin/mg dm

cotton delivered to gin

trash G9=19

in-field biomass

leaves I9=18.1

stems J9=40.5

roots K9=1.6

lb B.t. toxin/A

cotton delivered to gin

seed F10=F6*F8*10⁽⁻⁶⁾

trash G10=G6*G8*10⁽⁻⁶⁾

in-field biomass

leaves I10=I6*I9*10⁽⁻⁶⁾

stems J10=J6*J9*10⁽⁻⁶⁾

roots K10=K6*K9*10⁽⁻⁶⁾

total biomass L10=SUM(I10:K10)

EEC, soil: mg B.t. toxin/ kg

in-field biomass

leaves I11=I10/2

stems J11=J10/2

roots K11=K10/2

total biomass L11=L10/2

PAT protein produced

Appendix Table 3.2. Runoff of Cry1Ac Protein Through Incorporation of Event 3006-210-23 Cotton Residues at Harvest as Predicted by GENEEC2 (USEPA, 2000)

RUN No.	1 FOR Cry1Ac		ON Cotton			* INPUT VALUES *		
RATE (#/AC) ONE (MULT)	No. APPS & INTERVAL		SOIL Koc	SOLUBIL (PPM)	APPL TYPE (%DRIFT)	NO-SPRAY (FT)	INCRP (IN)	
.070 (.070)	1 1	100.0	1000.0	GRANUL (.0)	.0	6.0

FIELD AND STANDARD POND HALFLIFE VALUES (DAYS)

METABOLIC (FIELD)	DAYS UNTIL RAIN/RUNOFF	HYDROLYSIS (POND)	PHOTOLYSIS (POND-EFF)	METABOLIC (POND)	COMBINED (POND)
1.30	2	N/A	.00-	.00	.00

GENERIC EECs (IN NANOGRAMS/LITER (PPTr))

PEAK GEEC	MAX 4 DAY AVG GEEC	MAX 21 DAY AVG GEEC	MAX 60 DAY AVG GEEC	MAX 90 DAY AVG GEEC
191.35	190.58	189.82	189.05	188.29

CBI - DELETED REVIEW LETTER TO DOW AGROSCIENCES

July 14, 2003

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9330 Zionsville Road
Indianapolis, IN 46268-1054

Subject: Review for Technical Completeness of your petitions numbered 03-036-01p and 03-036-02p for a Determination of Non-regulated Status for the Insect Resistant Cotton Events 281-24-236 and 3006-210-23.

Dear Dr. Gatti:

This letter is in reference to both the Petition for Determination of Non-Regulated Status *B.t.* Cry 1F Insect-Resistant Cotton Event 281-24-36 and the Petition for Determination of Non-Regulated Status *B.t.* Cry 1Ac Insect-Resistant Cotton Event 3006-210-23 submitted to the USDA, Animal and Plant Health Inspection Service/Biotechnology Regulatory Services (APHIS/BRS) on 24 February 2003. APHIS/BRS has assigned these petitions the numbers 03-036-01p and 03-036-02p, respectively. After reviewing these petitions APHIS/BRS has determined that there is a need for additional information and clarification before we can declare these petitions technically complete. Please respond to each of the 30 points listed below, which are relevant to each petition, in the form of an addendum that will then be considered part of each of the petitions, respectively. These points are listed under the relevant heading as they appear in the petitions.

Section IV. The Donor Genes and Regulatory Sequences

1. The *cry* 1Ac and the *cry* 1F insecticidal crystal protein genes are described as synthetic genes based on sequences of either the Cry 1Ac or Cry 1F (page 16) proteins. However, Table 2, Genetic Elements of the T-DNA Region of the Plasmids describes a chimeric gene construct that includes portions of both Cry 1Ca3 and Cry 1Ab1 that are to be synthesized with both the *cry* 1F and *cry* 1Ac genes. This table does not provide a reference for the DNA sequence of this chimeric sequence design. Please provide a literature reference in this table or a complete description of these chimeras.
2. Figures 1 and 2 illustrate "non-specific cry probe"; however Table 3 lists one probe as *cry* 1Ac--3' portion of *cry* 1Ac gene. We assume that these two probes are one and the same. Please explain.

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Section V.A. Characterization of the DNA Insert

3. The description of the null cotton, BC3F1, states that the selected plants did not express any transgenes. Please provide us with a description of the methodology you used to determine presence/absence of any transgenes.
4. All leaf tissue samples analyzed by Southern Blots are listed in each figure legend as samples from the BC3F generations. It is unclear to us if the various numbered samples were collected from different plants. Please provide a more complete description of sampling methodology for the Southern Blot samples and analyses.
5. Figure 4. Southern Blot Analysis of Cotton Event 281-24-236 with *pat* probe, page 26. There are unexplained or miscalculated bands in the *PacI* digest. There are two bands of approximately 8,000 and 9,000 base pairs, not just 8,000 bp as indicated in Table 4, page 23. Are these two bands consistent with there being a complete and a partial *pat* sequence in the tissue sample?
6. Figure 6. Southern Blot Analysis of Cotton Event 281-24-236 with *ubi* probe, page 28. Molecular weight markers are overloaded; therefore, we cannot distinguish the control lanes on either side of molecular weight markers in lane 18. This blot should be repeated or another exposure provided, especially for the *XhoI* and *BamHI* digests.
7. Figure 6. Southern Blot Analysis of Cotton Event 3006-210-23 with the *ubi* Probe, page 28. Digest with *Bgl I*, lane 19, positive control does not give band of expected size. Please discuss the significance of this result.
8. Figure 7. Southern Blot Analysis of Cotton Event 281-24-236 with *ORF25* probe, page 29. There are unexplained high molecular weight bands in the *BamHI* and maybe in the *EcoRI* lanes that do not appear in the negative control lanes. Some of the transgenic plant *EcoRI* digests appear to have doublets where there should be only one band, if in fact there is only one copy. Since there may be two copies of the *pat* gene and *ubi* promoter sequences, are there also two copies of the Poly A sequence? Please provide an explanation of these observations, or another blot that is clearer for the *BamHI* and *EcoRI* digests.
9. Figures 10 and 11. Southern Blot Analysis Demonstrating Within Generational Stability Cotton Event 281-24-236 BC3F2 Plants using *EcoRI* digest with the *cry 1F* probe, page 34 and *pat* probe, page 35. Lanes 17 and 18 of both gels are labeled the same, BC3F2 (1036336-28); however, lane 17 contains one or two bands and lane 18 appears to be empty. Please provide an explanation of these results.

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Section V.D.1 and V.D.2. Determination of Proteins in Cotton Samples

10. Summary of the Expression of Cry 1Ac or Cry 1F and PAT Proteins in Cotton Tissues as Determined by ELISA. Tables 8 and 9 do not indicate the sample size. Please provide us with a more complete description of the samples including the sample size, number of samples per plot and number of plots per location.

Section V.E.1. Cry 1Ac/Cry 1F Protein Characterization

11. Chimeric protein intended to be expressed is described on page 43. Please provide a literature reference describing this construct or indicate if this construct is of your design and include the rationale for this design with relevant references.

12. Figure 14. Alignment of the Polypeptide Sequences of the Plant and Microbial Cry 1F Sequences, page 44. It is unclear whether the sequence information was determined by actual DNA or amino acid sequence analysis of the proteins as they are expressed in the cotton and bacteria or whether they are deduced from the constructs that were introduced. Also, there is no explanation as to why the C-terminal domain from the other Cry proteins was introduced with the Cry1F core toxin. According to Drs. Tagliani and Shan, it was strictly for convenience. The same fragment is included in the Cry1Ac cotton construct. Their rationale needs to be explained and there needs to be a discussion about whether this affects specificity or activity of the Cry1F and Cry1Ac protein. Please provide on figure 14 which sequences were derived from Cry 1F, Cry1Ca3 and Cry 1Ab.

13. SDS-PAGE and Western Blot Analyses of Plant-Produced and Microbe-Derived Cry 1F Protein, Figure 15, page 47. Legend for lanes 4 and 5 do not indicate the amount of protein loaded. Please provide these amounts.

14. Table 12. Tryptic Peptide Mass Data of Cry1F Proteins Obtained by MALDI/TOF MS, page 50. Provide an explanation why some of the residues were not detected.

Section V.G. Agronomic characteristics of Cotton Events 281-24-36 and 3006-210-23

15. Tables 13 and 14, respectively. Agronomic Characteristics of Event(s) and the Stack Product in Comparison to Parent Variety PSC355. Tables do not provide us with enough information about the agronomic characteristics of the parent, transformed and stacked plants. Please include the following information: What is the sample size from each location? What are the ranges of the units that were sampled and reported? What are the standard deviations and statistical methodology? Is seed cotton weight per boll equal to seed weight per boll? Do you have data on number of seeds per boll and number of bolls per plant?

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Section V.H.1. Toxicants and Allergens Present in Events 281-24-36 and 3006-210-23

16. Table 17. Tocopherol Analyses of Events and Non-transgenic Control Cottonseed Oil refers to a publication titled "Cottonseed Oil, 1990". Citation was not found in Section VIII References. Please provide us with this citation.

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Section VI.C.2. Environmental Fate of Cry 1F Protein Incorporated into Soil

25. The petition should state how many soil types were assayed and provide a justification for this number. Please justify the validity of the aqueous agar over-lay technique for tobacco budworm feeding assays.

Section VI.C.3. Estimated Environmental Concentrations

26. At the top (or bottom) of page 68 cotton pollen density is given to be 1 grain per cm² leaf. Please provide us with a reference or explanation of this value.

27. Table 23 High End Exposure Estimates for Expression of Cry 1Ac Protein, page 68. The exposure estimate for nectar is listed in Table 23; however Table 8, page 41 indicates that nectar was not analyzed. Since the nectar was not analyzed, provide us with a description of how you arrived at values for high end exposure estimate.

VI.D.1. Likelihood of Exposure Exceeding Effects Thresholds and Taxa at Risk

28. Figure 20, page 70 or page 71. “Endangered Lepidopterans”. The figure presented refers to 1997 USEPA data. Have any other endangered Lepidopterans been added to this EPA list since 1997?

Field Census Study

29. Preliminary results from the field study comparing the stacked event with non-lepidopteran insecticide treatment to the non-transgenic parent with lepidopteran or non-

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lepidopteran insecticide treatments revealed no significant consistent decline in beneficial arthropods using several sampling methods (Mahill and Storer, 2002, Appendix 2, Volume 32 (Cry 1F) or 29 (Cry 1Ac). Please submit the FINAL results from this study. This data will be required before APHIS' environmental assessment is finalized for comment.

VI.E.1. Human Health Risk

30. Text states that Cry1Ac is rapidly digested in <1 minute in simulated gastric fluid, page 73 and similarly for Cry 1F on page 71. Please provide the titles of the references that demonstrate these results.

Please contact us if you need to discuss further the status of these petition submissions or any questions you may have about the content of this letter.

Sincerely,

/s/

Susan Koehler
Biotechnologist Reviewing ~~03-036-01p~~
Acting Director, APHIS, BRS Regulatory Division

/s/

Lena Carmen Soileau
Biotechnologist Reviewing 03-036-02p

APHIS:BRS:SKoehler:tah:734-4886:7/14/03:stackbtcottondeflet.wpd

RESPONSE TO REVIEW FOR TECHNICAL COMPLETENESS OF PETITION 03-036-01p FOR A DETERMINATION OF NON-REGULATED STATUS FOR THE B.T. CRY1F INSECT RESISTANT COTTON EVENT 281-24-236

In a memo dated July 14, 2003, USDA-APHIS asked for clarification of technical issues pertaining to Dow AgroSciences (DAS) petitions for determination of deregulated status for cotton events 3006-210-23 and 281-24-236. In this response we address those questions relating to cotton event 281-24-236 expressing Cry1F protein. Responses to questions pertaining to cotton event 3006-210-23 expressing Cry1Ac protein are addressed in a separate communication.

Section IV. The Donor Genes and Regulatory Sequences

- 1. The ... cry1F insecticidal crystal protein gene ... [is] described as synthetic ... based on sequences of either the ... [Cry1F protein]. However, Table 2, Genetic Elements of the T-DNA Region of the Plasmids describes a chimeric gene construct that includes portions of both Cry1Ca3 and Cry1Ab1 that are to be synthesized with .. the cry1F ... [gene]. This table does not provide a reference for the DNA sequence of this chimeric sequence design. Please provide a literature reference in this table or a complete description of these chimeras.*

The chimeric cry1F(synpro) gene is of Mycogen/Dow AgroSciences design and the specific gene sequence has not been published. The method used to design the plant optimized synthetic coding sequence was essentially that of Adang et al., US Patent 5,380,831.

The amino acid sequence of Cry1F(synpro) is comprised of the first 604 amino acids of the insecticidal protein of Cry1Fa2 and 544 amino acids from the nontoxic portions of Cry1Ca3 (residues 605-640) and Cry1Ab1 (residues 641-1148). Together, the portions of Cry1Ca3 and Cry1Ab1 that comprise the chimeric C-terminal domain are approximately those removed by alkaline proteases in the insect gut during formation of the active Cry1F core insecticidal protein. The sequence of Cry1F is found in SwissProt Accession Q03746 (Payne and Sick, US Patent 5,188,960), Cry1Ab1 is found in SwissProt Accession P06578 (Geiser et al. 1986), and the sequence of Cry1Ca3 is in SwissProt Accession P05518 (Honee et al. 1988).

- 2. Figures 1 and 2 illustrate “non-specific cry probe”; however Table 3 lists one probe as ...[cry1F]—3’ portion of ...[cry1F] gene. We assume that these two probes are one and the same.*

Yes they are the same. The cry1F probe described in Table 3 is a mixture of two different DNA fragments. One fragment is specific to the 5’ portion of the gene and is designated “cry1F probe” in Figure 2. The “non-specific cry” probe is a fragment from the 3’ portion of the gene. The “non-specific” designation is used because the probe sequence is homologous to the C-terminal sequence that is common to the 3’ portions of both cry1Ac and cry1F.

Section V.A. Characterization of the DNA Insert

3. *The description of the null cotton, BC3F1, states that the selected plants did not express any transgenes. Please provide us with a description of the methodology you used to determine presence/absence of any transgenes.*

Prior to harvesting leaf tissue for the Southern Blot analysis studies, all plants were tested for the presence or absence of Cry1F and Cry1Ac proteins by Lateral Flow Immunodiagnostic Test Strips (Strategic Diagnostics Inc., Newark, DE). The test strips employ a double antibody sandwich format, with antibodies specific to the protein of interest coupled to a color reagent and incorporated into the lateral flow strip. Leaf punches were collected in small tubes, buffer extracted, and lateral flow strips were incubated in the leaf tissue homogenate for approximately 10 min. Following incubation, the strips were scored as positive or negative for the presence of either Cry1F or Cry1Ac. Based on results of the test strips, plants were labeled as null control (negative for Cry1F or Cry1Ac), or Cry1F or Cry1Ac positive. The Cry1Ac and Cry1F specific test strips will be commercially available from Strategic Diagnostics, Inc upon regulatory approval and commercialization of Cry1F/Cry1Ac cotton.

4. *All leaf tissue samples analyzed by Southern Blots are listed in each figure legend as samples from the BC3F generations. It is unclear to us if the various numbered samples were collected from different plants. Please provide a more complete description of sampling methodology for the Southern Blot samples and analyses.*

Each unique number represents an individual plant. Seeds were planted in a Dow AgroSciences Indianapolis greenhouse, the pots uniquely identified by labeled stakes. Approximately 1.5 grams of leaf tissue were harvested from each plant. During sample collection, each leaf tissue sample was assigned a unique sample number that was used for tracking during the study, through sample movement, storage and analysis. DNA was extracted from all harvested tissue. The identity of each DNA sample was carefully maintained.

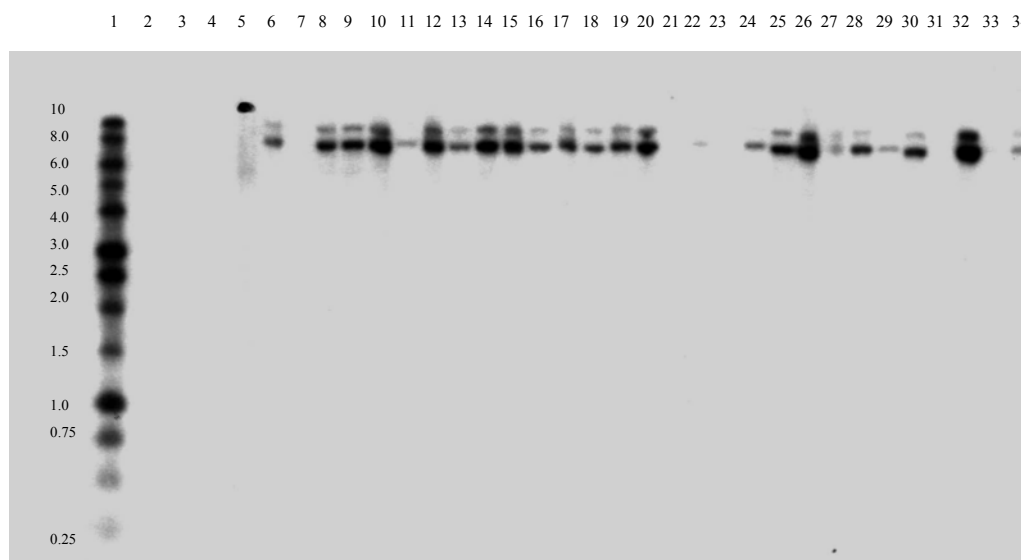
5. *Figure 4. Southern Blot Analysis of Cotton Event 281-24-236 with pat probe, page 26. There are unexplained or miscalculated bands in the PacI digest. There are two bands of approximately 8,000 and 9,000 base pairs, not just 8,000 bp as indicated in Table 4, page 23. Are these two bands consistent with there being a complete and a partial pat sequence in the tissue sample?*

One band was indicated in Table 4 for the *Pac* I digest and *pat* probe because, due to the signal intensity on this blot, it was unclear as to whether there was a second band or partial digestion causing the appearance of a second band. Two bands would be consistent with the presence of a complete and a partial copy of *pat* in event 281-24-236. The data showing two visible distinct bands obtained with the *Hind* III and *Bam*H I digests suggests the likelihood that there is a second band in the *Pac* I digest. A subsequent determination (see attached Figure 17 from Green, 2003) verified this result, indicating the presence of two bands in *Pac* I digests with the *pat* probe.

Figure 17. Southern Blot Analysis of Cotton Event 281-24-236/ *Pac* I Digest with the *pat* Probe (Green 2003)

DNA isolated from cotton event 281-24-236 and unmodified cotton DNA were digested with *Pac* I and probed with the *pat* probe. Ten (10) µg of DNA was digested and loaded per lane. The plasmid control contained 3 gene copy equivalents per cotton genome of pAGM281 and 10 µg of DNA isolated from a neg. control. The BC3F2 generation was expected to be segregating 3:1 (3 Cry1F event 281-24-236 : 1 null).

Lane	Sample	Lane	Sample
1	MW Marker	18	281-24-236 BC3F2 (103636-29) <i>Pac</i> I
2	Negative Control (102632-5) <i>Pac</i> I	19	281-24-236 BC3F2 (103636-30) <i>Pac</i> I
3	Negative Control (102632-5) <i>Pac</i> I	20	281-24-236 BC3F2 (103636-36) <i>Pac</i> I
4	Space	21	281-24-236 BC3F2 (103636-42) <i>Pac</i> I
5	pAGM281 Plasmid + Neg. Control <i>Pac</i> I	22	281-24-236 BC3F2 (103636-43) <i>Pac</i> I
6	281-24-236 BC3F2 (103636-7) <i>Pac</i> I	23	281-24-236 BC3F2 (103636-45) <i>Pac</i> I
7	Space	24	281-24-236 BC3F2 (103636-48) <i>Pac</i> I
8	281-24-236 BC3F2 (103636-10) <i>Pac</i> I	25	281-24-236 BC3F2 (103636-53) <i>Pac</i> I
9	281-24-236 BC3F2 (103636-12) <i>Pac</i> I	26	281-24-236 BC3F2 (103636-60) <i>Pac</i> I
10	281-24-236 BC3F2 (103636-13) <i>Pac</i> I	27	281-24-236 BC3F2 (103671-69) <i>Pac</i> I
11	281-24-236 BC3F2 (103636-15) <i>Pac</i> I	28	281-24-236 BC3F2 (103671-80) <i>Pac</i> I
12	281-24-236 BC3F2 (103636-18) <i>Pac</i> I	29	281-24-236 BC3F2 (103671-82) <i>Pac</i> I
13	281-24-236 BC3F2 (103636-19) <i>Pac</i> I	30	281-24-236 BC3F2 (103671-84) <i>Pac</i> I
14	281-24-236 BC3F2 (103636-20) <i>Pac</i> I	31	281-24-236 BC3F2 (103671-85) <i>Pac</i> I
15	281-24-236 BC3F2 (103636-21) <i>Pac</i> I	32	281-24-236 BC3F2 (103671-88) <i>Pac</i> I
16	281-24-236 BC3F2 (103636-24) <i>Pac</i> I	33	281-24-236 BC3F2 (103671-95) <i>Pac</i> I
17	281-24-236 BC3F2 (103636-27) <i>Pac</i> I	34	281-24-236 BC3F2 (103671-96) <i>Pac</i> I



6. *Figure 6. Southern Blot Analysis of Cotton Event 281-24-236 with ubi probe, page 28. Molecular weight markers are overloaded; therefore, we cannot distinguish the control lanes on either side of molecular weight markers in lane 18. This blot should be repeated or another exposure provided, especially for the XhoI and BamHI digests.*

Attached find a lighter exposure of the blot in question, with improved visibility of the lanes adjacent to the marker in lane 18 (lane 17 is a pAGM281 plasmid control for *Xho* I and lane 19 is the negative control for *Bam*H I).

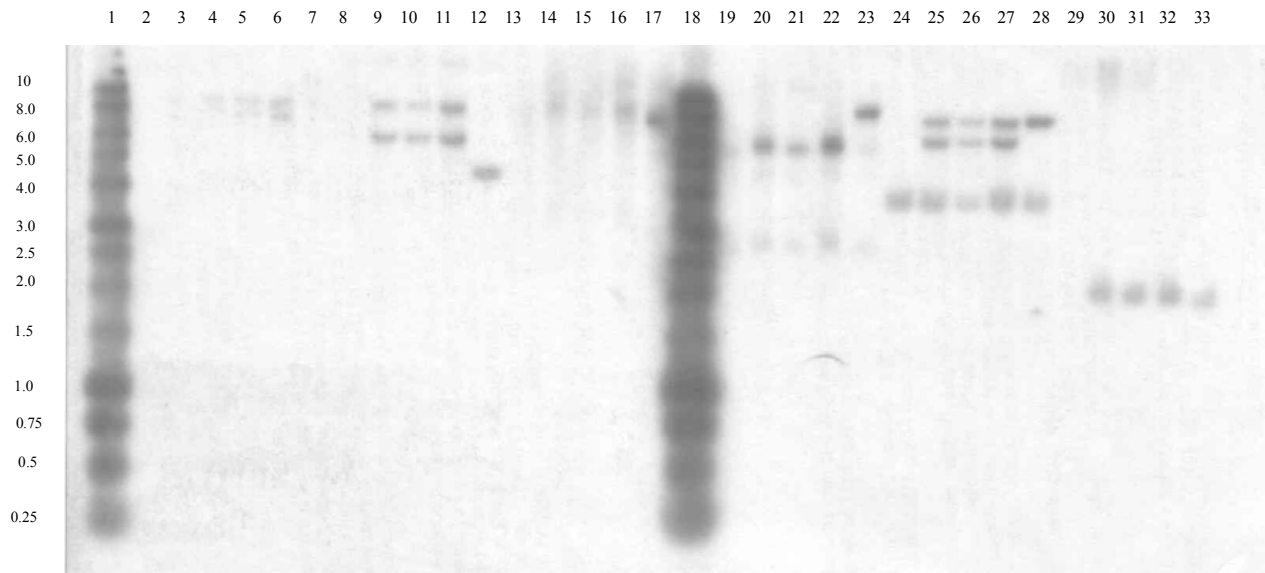
The *Xho* I and *Bam*H I digests show hybridizing bands with the *ubi* probe at approximately 9 kb and 6 kb, respectively. The *Hind* III, *Eco*R I and *Pac* I digests show two bands each, suggesting that 281-24-236 has two integrations of the UbiZm1 promoter or a fragment thereof. The *Bam*H I and *Eco*R I banding patterns also indicate hybridization with cotton genomic sequences as well as the transgene. It is not uncommon to see background hybridization of genomic sequences with some probes. Background signal is identified by the presence of similar bands across both positive test samples and negative controls. For the *ubi* probe, a background signal at approximately 4 kb can be seen in all *Eco*R I digests, and 2 weakly hybridizing bands, one at 3 kb and a second at 6 kb, are present in all *Bam*H I samples. The 6 kb *Bam*H I background signal is approximately the same size as what is assumed to be a transgene band, as determined by increased hybridization signal intensity.

Figure 6. Southern Blot Analysis of Cotton Event 281-24-236 with *ubi* Probe

All digested young leaf tissue DNA samples contained 10 µg DNA. The pAGM281 plasmid DNA, ~3 gene copies spiked into null cotton leaf DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

<u>Lanes</u>	<u>DNA Sample</u>		<u>Lanes</u>	<u>DNA Sample</u>	
1	MW Marker		18	MW Marker	
2	Empty		19	Negative Control (102632-6)	<i>Bam</i> HI
3	Negative Control (102632-6)	<i>Pac</i> I	20	BC3F4 (102578-2)	<i>Bam</i> HI
4	BC3F4 (102578-2)	<i>Pac</i> I	21	BC3F4 (102578-6)	<i>Bam</i> HI
5	BC3F4 (102578-6)	<i>Pac</i> I	22	BC3F4 (102578-7)	<i>Bam</i> HI
6	BC3F4 (102578-7)	<i>Pac</i> I	23	pAGM281 Plasmid + Neg. Control	<i>Bam</i> HI
7	pAGM281 Plasmid + Neg. Control	<i>Pac</i> I	24	Negative Control (102632-6)	<i>Eco</i> RI
8	Negative Control (102632-6)	<i>Hind</i> III	25	BC3F4 (102578-2)	<i>Eco</i> RI
9	BC3F4 (102578-2)	<i>Hind</i> III	26	BC3F4 (102578-6)	<i>Eco</i> RI
10	BC3F4 (102578-6)	<i>Hind</i> III	27	BC3F4 (102578-7)	<i>Eco</i> RI
11	BC3F4 (102578-7)	<i>Hind</i> III	28	pAGM281 Plasmid + Neg. Control	<i>Eco</i> RI
12	pAGM281 Plasmid + Neg. Control	<i>Hind</i> III	29	Negative Control (102632-6)	<i>Pst</i> I
13	Negative Control (102632-6)	<i>Xho</i> I	30	BC3F4 (102578-2)	<i>Pst</i> I
14	BC3F4 (102578-2)	<i>Xho</i> I	31	BC3F4 (102578-6)	<i>Pst</i> I
15	BC3F4 (102578-6)	<i>Xho</i> I	32	BC3F4 (102578-7)	<i>Pst</i> I
16	BC3F4 (102578-7)	<i>Xho</i> I	33	pAGM281 Plasmid + Neg. Control	<i>Pst</i> I
17	pAGM281 Plasmid + Neg. Control	<i>Xho</i> I			

Pac I Hind III Xho I Bam HI Eco RI Pst I



7. This question pertains to Petition 03-036-02 p. See responses to that petition.
8. *Figure 7. Southern Blot Analysis of Cotton Event 281-24-236 with ORF25 probe, page 29. There are unexplained high molecular weight bands in the BamHI and EcoRI lanes that do not appear in the negative control lanes. Some of the transgenic plant EcoRI digests appear to have doublets where there should be only one band, if in fact there is only one copy. Since there may be two copies of the pat gene and ubi promoter sequences, are there also two copies of the PolyA sequence? Please provide an explanation of these observations, or another blot that is clearer for the BamHI and EcoRI digests.*

Attached is another scan of the blot for Figure 7. The conclusion that there is only one copy of ORF25 is based on the following reasons. The ORF25 probe hybridized with the expected sized fragments in the *Pst* I, *Xho* I, *Bam*H I and *Eco*R I lanes. The other two enzymes used here, *Pac* I and *Hind* III, produced integration fragments (i.e., one site in the T-DNA insert and the second site in the flanking border sequence), resulting in a fragment of unpredicted size. Integration fragments are particularly useful in determining integration number, because each integration would be expected to produce a fragment of unique size. Only one band was seen in the *Pac* I and *Hind* III lanes with the ORF25 probe, indicating a single integration of the element. The data for all six enzymes indicate that a single intact copy of the ORF25 element integrated into the genome of event 281-24-236.

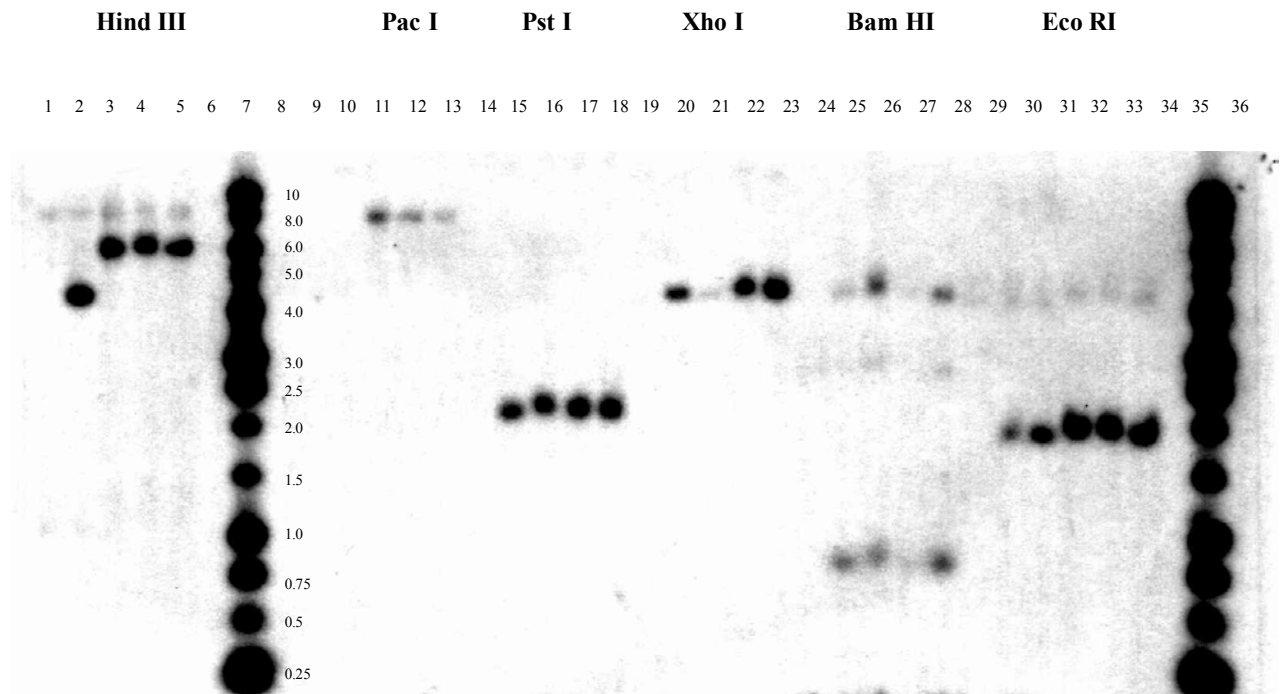
The *Bam*H I digest showed higher molecular weight hybridizing bands at approximately 4 kb and a weaker band at 3 kb, in addition to the expected 750 bp band (Lanes 25-28). Although not clearly visible in the negative control, these bands are visible in the plasmid control, which is plasmid pAGM281 DNA spiked into the negative control cotton DNA (Lane 25). Since the extra bands were seen in both the plasmid control lane and in the sample lanes, they appear to be background hybridization with cotton genomic sequence. In addition, very faint bands of approximately 4.5 kb are observed with the *Eco*R I digest. They appear in the negative control (visible on film) and plasmid control lanes in addition to the sample lanes and are also likely to be background hybridization to cotton sequence.

We do not think there are doublet bands in the *Eco*R I digests. The slight tailing of the *Eco*R I digests is likely a transfer phenomenon. The variations in signal intensity internal to the digests would be due to differences in sample loading and/or the efficiency of transfer of individual DNA samples to the membrane. Also, there are no additional, unpredicted bands with any of the other digests.

Figure 7. Southern Blot Analysis of Cotton Event 281-24-236 with *ORF25* Probe.

All digested young leaf tissue DNA samples contained 10 µg DNA. The pAGM281 plasmid DNA, ~3 gene copies spiked into null cotton leaf DNA, served as the positive control. Null cotton leaf DNA served as the negative control. All plant samples are from greenhouse material. The lanes contained:

Lane	DNA Sample	Enzyme	Lane	DNA Sample	Enzyme
1	Negative control (102582-5)	<i>Hind III</i>	19	Negative control (102582-5)	<i>Xho I</i>
2	pAGM281 plasmid + neg. control	<i>Hind III</i>	20	pAGM281 plasmid + neg. control	<i>Xho I</i>
3	BC3F4 (102578-1)	<i>Hind III</i>	21	BC3F4 (102578-1)	<i>Xho I</i>
4	BC3F4 (102578-2)	<i>Hind III</i>	22	BC3F4 (102578-2)	<i>Xho I</i>
5	BC3F4 (102578-4)	<i>Hind III</i>	23	BC3F4 (102578-4)	<i>Xho I</i>
6	Empty		24	Negative control (102582-5)	<i>Bam HI</i>
7	MW Marker		25	pAGM281 plasmid + neg. control	<i>Bam HI</i>
8	Empty		26	BC3F4 (102578-1)	<i>Bam HI</i>
9	Negative control (102582-5)	<i>Pac I</i>	27	BC3F4 (102578-2)	<i>Bam HI</i>
10	pAGM281 plasmid + neg. control	<i>Pac I</i>	28	BC3F4 (102578-4)	<i>Bam HI</i>
11	BC3F4 (102578-1)	<i>Pac I</i>	29	Negative control (102582-5)	
12	BC3F4 (102578-2)	<i>Pac I</i>	30	pAGM281 plasmid (1 copy)+ neg. control	<i>Eco RI</i>
13	BC3F4 (102578-4)	<i>Pac I</i>	31	pAGM281 plasmid (3 copy)+ neg. control	<i>Eco RI</i>
14	Negative control (102582-5)	<i>Pst I</i>	32	BC3F4 (102578-1)	<i>Eco RI</i>
15	pAGM281 plasmid + neg. control	<i>Pst I</i>	33	BC3F4 (102578-2)	<i>Eco RI</i>
16	BC3F4 (102578-1)	<i>Pst I</i>	34	BC3F4 (102578-4)	<i>Eco RI</i>
17	BC3F4 (102578-2)	<i>Pst I</i>	35	Empty	
18	BC3F4 (102578-4)	<i>Pst I</i>	36	MW Marker	



9. *Figures 10 and 11. Southern Blot Analysis Demonstrating Within Generational Stability Cotton Event 281-24-236 BC3F2 Plants using EcoRI digest with the cry1F probe, page 34 and pat probe, page 35. Lanes 17 and 18 of both gels are labeled the same, BC3F2 (1036336-28); however, lane 17 contains one or two bands and lane 18 appears to be empty. Please provide an explanation of these results.*

Lanes 17 and 18 represent individual extractions from the same plant. The tissue was extracted twice due to the poor quality of DNA obtained in the first extraction, most probably a result of polysaccharide contamination making quantification, sampling and loading of the DNA difficult. Consequently, there was little or no DNA loaded in the lane 18 of figures 10 and 11.

Section V.D.1 and V.D.2. Determination of Proteins in Cotton Samples

10. Summary of the Expression of Cry1Ac or Cry1F and PAT proteins in Cotton Tissues as Determined by ELISA. Tables 8 and 9 do not indicate the sample size. Please provide us with a more complete description of the samples including the sample size, number of samples per plot and number of plots per location.

See supplementary Table A below for details.

Table A. Sample Description for Cotton Expression/Composition Study. Samples were collected from 6 sites; Arizona, California, Mississippi, North Carolina, and 2 sites in Texas.

Cotton Tissue	Plots per site	Sample descriptions	Samples per Plot	Samples collected per site
Young Leaf (3-6 week)	1 Control, 3 transgenic	3 leaves from one plant	3	3 control, 9 transgenic
Terminal Leaf	1 Control, 3 transgenic	3 leaves from one plant	3	3 control, 9 transgenic
Flower	1 Control, 3 transgenic	3 white flowers (one flower per plant)	3	3 control, 9 transgenic
Square	1 Control, 3 transgenic	3 squares (one square per plant)	3	3 control, 9 transgenic
Boll (Early)	1 Control, 3 transgenic	3 bolls (one boll per plant)	3	3 control, 9 transgenic
Whole plant	1 Control, 3 transgenic	1 plant	1	1 control, 3 transgenic
Root	1 Control, 3 transgenic	1 plant	1	1 control, 3 transgenic
Pollen	1 Control, 3 transgenic	Pollen from 20-25 open white flowers	1	1 control, 3 transgenic
Nectar ^a	1 Control, 3 transgenic	0.01 – 0.5 mL	1	1 control, 3 transgenic
Seed	1 Control, 3 transgenic	Seed from 3 mature Bolls (from 1 plant)	3	3 control, 9 transgenic

^a Nectar samples were collected at the Arizona and California sites only.

Section V.E.1. Cry1F Protein Characterization

11. *Chimeric protein intended to be expressed is described on page 43. Please provide a literature reference describing this construct or indicate if this construct is of your design and include the rationale for this design with relevant references.*

Literature references describing the sequences comprising Cry1F(synpro) are provided in the response to question 1.

The full-length chimeric protoxin of Cry1F was developed to achieve improved expression and solubility of the *Bt* toxin. The Cry1Ab C-terminal coding sequence was chosen as the C-terminal module for creating full length Cry1 toxins primarily based on: (1) Improved protein expression demonstrated by Thompson and Schwab (US 5,527,883) for expression of a Cry1F/Cry1Ab chimera in *Pseudomonas*. (2) Increased solubility of *Bt* toxin inclusion bodies containing the Cry1Ab protoxin (Aronson 1995). *Bt* cells produce inclusion bodies that are made up of one or more disulfide cross-linked Cry1 protoxins. Aronson (1995) demonstrated that both insect toxicity and inclusion solubility increased with the presence of Cry1Ab in the inclusion body. The C-terminus of the Cry1Ab protein demonstrating this property is unique in that it contains deletions of several cysteine residues that are present in most other Cry1 protoxins. Since soluble protoxin is the target of insect proteases in the generation of the activated core toxin, solubility is clearly linked to insect toxicity. These data indicate that the Cry1Ab C-terminus may play a role in solubility and therefore it was selected for use in the development of full-length chimeric Cry1 protoxins.

Based on the strategy to improve expression and solubility of *Bt* toxins by addition of the Cry1Ab C-terminus, a modular coding sequence was designed to facilitate construction of full length Cry1 transgenes. The Cry1Ab C-terminal fragment was used with a small portion (35 residues) of the Cry1Ca3 sequence in the development of a chimeric C-terminal tail. The Cry1Ca3 sequence was used in the construction for ease of sequence assembly; the region used was highly homologous (>90%) to Cry1Ab as well. The sequence was synthesized with plant optimized codons and constructed with a cloning site for joining the Cry1Ab tail to any core toxin sequence, such as Cry1Ac or Cry1F. The cloning junction for the Cry1Ab C-terminal fragment was engineered based on the unique *Xho* I site near the end of the core toxin in Cry1Ac. Cry1F was modified to include an *Xho* I site at the analogous position (residue 604, F to L substitution) to enable attachment of the modular Cry1Ab C-terminal coding sequence.

12. *Figure 14. Alignment of the Polypeptide Sequences of the Plant and Microbial Cry1F Sequences, page 44. It is unclear whether the sequence information was determined by actual DNA or amino acid sequence analysis of the proteins as they are expressed in the cotton and bacteria or whether they are deduced from the constructs that were introduced. Also, there is not explanation as to why the C-terminal domain from the other Cry proteins was introduced with the Cry1F core toxin. According to Drs. Tagliani and Shan, it was strictly for convenience. The same fragment is included in the Cry1Ac cotton construct. Their rationale needs to be explained and there needs to be a discussion about whether this affects specificity or activity of the Cry1F and Cry1Ac protein. Please provide on figure 14 which sequences were derived from Cry1F, Cry1Ca3 and Cry1Ab.*

The sequence information in Figure 14 was obtained by direct DNA sequencing of the *cry1F* gene sequences in (1) the microbial transformation construct; (2) the plant transformation construct; and, (3) the event 281-24-236 plant genomic DNA. DNA from each of the

transforming plasmids (microbial and plant) and plant genomic DNA which was cloned from event 281-24-236, was subjected to overlapping sequencing of both DNA strands of the *cry1F* gene sequence. The work was performed at Dow AgroSciences or at external laboratories contracted by Dow AgroSciences to perform the sequencing. The DNA sequences were confirmed to be identical to the expected sequences and were used to deduce the amino acid sequences presented in Figure 14. In addition, Cry1F protein isolated from *P. fluorescens* and from cotton event 281-24-236 was characterized by N-terminal sequence and MALDI-TOF MS fingerprinting analysis, to support confirmation of the protein sequences. All sources of Cry1F gene and protein sequence data confirmed the expected results.

Substitution of the Cry1Ab C-terminus for that found in native Cry1Ac or Cry1F protoxins does not affect specificity or activity of the activated Cry1Ac or Cry1F core toxins. It is well documented (for review see Schnepf et al., 1998) that after ingestion by susceptible insects, Cry1 protoxins are solubilized in the alkaline midgut of the insect and are subsequently processed by midgut proteases which promote hydrolytic cleavage of the C-terminal domain. Thus, the activated, protease resistant core toxin comprises approximately the N-terminal half of the protein representing native Cry1Ac or Cry1F. As mentioned above in response to question 11, the rationale for using the Cry1Ab C-terminal sequence is the effect on the biochemical behavior, i.e., solubility and expression, of the chimeric protoxins.

Attached find Figure 14, with the origin of the Cry1F(synpro) sequence fragments identified by highlighting. Amino acids 1-604 are derived from Cry1Fa2, 605-640 from Cry1Ca3, and 641-1148 from Cry1Ab1. The 4 amino acid differences between the Cry1F sequence expressed in *P. fluorescens* and the Cry1F sequence expressed in cotton represent existing natural variation in the Cry1 sequences. The F to L substitution at residue 604 is due to the introduction of a cloning site to enable attachment of the C-terminal tail. This substitution was made based on a naturally occurring leucine residue at the analogous position in the protein sequence of Cry1Ac. The three remaining residue differences at amino acid positions 608, 624 and 628 reflect natural amino acid variations in the Cry1C portion of the C-terminal domain.

Figure 14. Alignment of the Polypeptide Sequences of the Plant and Microbial Cry1F Sequences

The consensus displays identical amino acid residues between the two versions of Cry1F proteins, Cry1FMR872 is the sequence expressed in *Psuedomonas fluorescens* and Cry1F(synpro) is the sequence expressed in cotton event 281-24-236. The positions of putative protease cleavage sites at the start (about residue R28 or R31) and end (about residue R612 or K615) of the active core protein are marked with a ↓. Note that only four amino acid differences are present in the plant Cry1F polypeptide with respect to Cry1F from the microbial *Psuedomonas fluorescens* MR872 source.

The original source of the sequences comprising Cry1FMR872 and Cry1F(synpro) are noted by highlighting. Amino acids 1-604 are from Cry1Fa2 (light gray), 605-640 from Cry1Ca3 (black), and 641-1148 from Cry1Ab1 (dark gray).

		↓	↓	
	1			50
cry1f.msf{Cry1FMR872}	MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP			
cry1f.msf{Cry1F(synpro)}	MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP			
Consensus	MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP			
	51			100
cry1f.msf{Cry1FMR872}	GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG			
cry1f.msf{Cry1F(synpro)}	GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG			
Consensus	GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG			
	101			150

crylf.msf{CrylFMR872}	LADSYEIIYIE	ALREWEANPN	NAQLREDVRI	RFANTDDALI	TAINNF TLTS
crylf.msf{CrylF(synpro)}	LADSYEIIYIE	ALREWEANPN	NAQLREDVRI	RFANTDDALI	TAINNF TLTS
Consensus	LADSYEIIYIE	ALREWEANPN	NAQLREDVRI	RFANTDDALI	TAINNF TLTS
	151				200
crylf.msf{CrylFMR872}	FEIPLLSVYV	QAANLHLSLL	RDAVSFGQGW	GLDIATVNNH	YNRLINLIHR
crylf.msf{CrylF(synpro)}	FEIPLLSVYV	QAANLHLSLL	RDAVSFGQGW	GLDIATVNNH	YNRLINLIHR
Consensus	FEIPLLSVYV	QAANLHLSLL	RDAVSFGQGW	GLDIATVNNH	YNRLINLIHR
	201				250
crylf.msf{CrylFMR872}	YTKHCLDTYN	QGLLENLRGTN	TRQWARFNQF	RRDLTLTVLD	IVALFPNYDV
crylf.msf{CrylF(synpro)}	YTKHCLDTYN	QGLLENLRGTN	TRQWARFNQF	RRDLTLTVLD	IVALFPNYDV
Consensus	YTKHCLDTYN	QGLLENLRGTN	TRQWARFNQF	RRDLTLTVLD	IVALFPNYDV
	251				300
crylf.msf{CrylFMR872}	RTYPIQTSSQ	LTREIYTSSV	IEDSPVSANI	PNGFNRAEFG	VRPPHLMDFM
crylf.msf{CrylF(synpro)}	RTYPIQTSSQ	LTREIYTSSV	IEDSPVSANI	PNGFNRAEFG	VRPPHLMDFM
Consensus	RTYPIQTSSQ	LTREIYTSSV	IEDSPVSANI	PNGFNRAEFG	VRPPHLMDFM
	301				350
crylf.msf{CrylFMR872}	NSLFVTAETV	RSQTVWGGHL	VSSRNTAGNR	INFPSYGVFN	PGGAIWIAD E
crylf.msf{CrylF(synpro)}	NSLFVTAETV	RSQTVWGGHL	VSSRNTAGNR	INFPSYGVFN	PGGAIWIAD E
Consensus	NSLFVTAETV	RSQTVWGGHL	VSSRNTAGNR	INFPSYGVFN	PGGAIWIAD E
	351				400
crylf.msf{CrylFMR872}	DPRPFYRTLS	DPVFVRGGFG	NPHYVLGLRG	VAFQQTGTNH	TRTRFRNSGTI
crylf.msf{CrylF(synpro)}	DPRPFYRTLS	DPVFVRGGFG	NPHYVLGLRG	VAFQQTGTNH	TRTRFRNSGTI
Consensus	DPRPFYRTLS	DPVFVRGGFG	NPHYVLGLRG	VAFQQTGTNH	TRTRFRNSGTI
	401				450
crylf.msf{CrylFMR872}	DSLDEIPPQD	NSGAPWNDYS	HVLNHVTFVR	WPGEISGSDS	WRAPMFSWTH
crylf.msf{CrylF(synpro)}	DSLDEIPPQD	NSGAPWNDYS	HVLNHVTFVR	WPGEISGSDS	WRAPMFSWTH
Consensus	DSLDEIPPQD	NSGAPWNDYS	HVLNHVTFVR	WPGEISGSDS	WRAPMFSWTH
	451				500
crylf.msf{CrylFMR872}	RSATPTNTID	PERITQIPLV	KAHTLQSGTT	VVRGPGFTGG	DILRRTSGGP
crylf.msf{CrylF(synpro)}	RSATPTNTID	PERITQIPLV	KAHTLQSGTT	VVRGPGFTGG	DILRRTSGGP
Consensus	RSATPTNTID	PERITQIPLV	KAHTLQSGTT	VVRGPGFTGG	DILRRTSGGP
	501				550
crylf.msf{CrylFMR872}	FAYTIVNING	QLPQRYRARI	RYASTTNLRI	YVTVAGERIF	AGQFNK MDT
crylf.msf{CrylF(synpro)}	FAYTIVNING	QLPQRYRARI	RYASTTNLRI	YVTVAGERIF	AGQFNK MDT
Consensus	FAYTIVNING	QLPQRYRARI	RYASTTNLRI	YVTVAGERIF	AGQFNK MDT
	551				600
crylf.msf{CrylFMR872}	GDPLTFQSFS	YATINTAFTF	PMSQSSFTVG	ADTFSSGNEV	YIDRFELIPV
crylf.msf{CrylF(synpro)}	GDPLTFQSFS	YATINTAFTF	PMSQSSFTVG	ADTFSSGNEV	YIDRFELIPV
Consensus	GDPLTFQSFS	YATINTAFTF	PMSQSSFTVG	ADTFSSGNEV	YIDRFELIPV
			↓ ↓		
	601				650
crylf.msf{CrylFMR872}	TATF EAEYDL	ERAQKAVNAL	FTSINQIGIK	TDVTDYHIDR	VSNLVECLSD
crylf.msf{CrylF(synpro)}	TATL EAESDL	ERAQKAVNAL	FTSSNQIGLK	TDVTDYHIDR	VSNLVECLSD
Consensus	TAT-EAE-DL	ERAQKAVNAL	FTS-NQIG-K	TDVTDYHIDR	VSNLVECLSD
	651				700
crylf.msf{CrylFMR872}	EFCLDEKKEL	SEKVKHAKRL	SDERNLLQDP	NFRGINRQLD	RGWRGSTDIT
crylf.msf{CrylF(synpro)}	EFCLDEKKEL	SEKVKHAKRL	SDERNLLQDP	NFRGINRQLD	RGWRGSTDIT
Consensus	EFCLDEKKEL	SEKVKHAKRL	SDERNLLQDP	NFRGINRQLD	RGWRGSTDIT

	701		750
crylf.msf{CrylFMR872}	IQGGDDVFKE	NYVTLLGTFD	ECYPTYLYQK IDESKLKAYT RYQLRGYIED
crylf.msf{CrylF(synpro)}	IQGGDDVFKE	NYVTLLGTFD	ECYPTYLYQK IDESKLKAYT RYQLRGYIED
Consensus	IQGGDDVFKE	NYVTLLGTFD	ECYPTYLYQK IDESKLKAYT RYQLRGYIED
	751		800
crylf.msf{CrylFMR872}	SQDLEIYLIR	YNAKHETVNV	PGTGSLWPLS APSPIGKCAH HSHHFSLDID
crylf.msf{CrylF(synpro)}	SQDLEIYLIR	YNAKHETVNV	PGTGSLWPLS APSPIGKCAH HSHHFSLDID
Consensus	SQDLEIYLIR	YNAKHETVNV	PGTGSLWPLS APSPIGKCAH HSHHFSLDID
	801		850
crylf.msf{CrylFMR872}	VGCTDLNEDL	GVWVIFKIKT	QDGHARLGNL EFLEEKPLVG EALARVKRAE
crylf.msf{CrylF(synpro)}	VGCTDLNEDL	GVWVIFKIKT	QDGHARLGNL EFLEEKPLVG EALARVKRAE
Consensus	VGCTDLNEDL	GVWVIFKIKT	QDGHARLGNL EFLEEKPLVG EALARVKRAE
	851		900
crylf.msf{CrylFMR872}	KKWRDKREKL	EWETNIVYKE	AKESVDALFV NSQYDRLQAD TNIAMIHAAD
crylf.msf{CrylF(synpro)}	KKWRDKREKL	EWETNIVYKE	AKESVDALFV NSQYDRLQAD TNIAMIHAAD
Consensus	KKWRDKREKL	EWETNIVYKE	AKESVDALFV NSQYDRLQAD TNIAMIHAAD
	901		950
crylf.msf{CrylFMR872}	KRVHSIREAY	LPELSVIPGV	NAAIFEELEG RIFTAFSLYD ARNVIKNGDF
crylf.msf{CrylF(synpro)}	KRVHSIREAY	LPELSVIPGV	NAAIFEELEG RIFTAFSLYD ARNVIKNGDF
Consensus	KRVHSIREAY	LPELSVIPGV	NAAIFEELEG RIFTAFSLYD ARNVIKNGDF
	951		1000
crylf.msf{CrylFMR872}	NNGLSCWNVK	GHVDVEEQNN	HRSVLVPEW EAEVSQEV RV CPGRGYILRV
crylf.msf{CrylF(synpro)}	NNGLSCWNVK	GHVDVEEQNN	HRSVLVPEW EAEVSQEV RV CPGRGYILRV
Consensus	NNGLSCWNVK	GHVDVEEQNN	HRSVLVPEW EAEVSQEV RV CPGRGYILRV
	1001		1050
crylf.msf{CrylFMR872}	TAYKEGYGEG	CVTIHEIENN	TDELKFSNCV EEEVYPNNTV TCNDYTATQE
crylf.msf{CrylF(synpro)}	TAYKEGYGEG	CVTIHEIENN	TDELKFSNCV EEEVYPNNTV TCNDYTATQE
Consensus	TAYKEGYGEG	CVTIHEIENN	TDELKFSNCV EEEVYPNNTV TCNDYTATQE
	1051		1100
crylf.msf{CrylFMR872}	EYEGTYTSRN	RGYDGAYESN	SSVPADYASA YEEKAYTDGR RDNPCE SNRG
crylf.msf{CrylF(synpro)}	EYEGTYTSRN	RGYDGAYESN	SSVPADYASA YEEKAYTDGR RDNPCE SNRG
Consensus	EYEGTYTSRN	RGYDGAYESN	SSVPADYASA YEEKAYTDGR RDNPCE SNRG
	1101		1149
crylf.msf{CrylFMR872}	YGDYTPLPAG	YVTKELEYFP	ETDKVWIEIG ETEGTFIVDS VELLLEE*
crylf.msf{CrylF(synpro)}	YGDYTPLPAG	YVTKELEYFP	ETDKVWIEIG ETEGTFIVDS VELLLEE*
Consensus	YGDYTPLPAG	YVTKELEYFP	ETDKVWIEIG ETEGTFIVDS VELLLEE

13. *SDS-PAGE and Western Blot Analyses of Plant-Produced and Microbe-Derived Cry1F Protein, Figure 15, page 47. Legend for lanes 4 and 5 do not indicate the amount of protein loaded. Please provide these amounts.*

There is a typographical error in the units reported for the amount of protein loaded in lanes 2 and 3 of Figure 15. The correct units should be micrograms (μg). The protein amounts loaded in the four lanes of Figure 15 are as follows:

Lane 2: *P. fluorescens* derived full length Cry1F, 1 μg /lane

Lane 3: *P. fluorescens* truncated Cry1F, 0.7 μg /lane

Lane 4: Lyophilized powder of transgenic cotton 281-24-236 leaf extract, 160 μg /lane (containing ~ 85 ng Cry1F)

Lane 5: Lyophilized powder of nontransgenic control cotton leaf extract, 160 μg /lane

14. *Table 12. Tryptic Peptide Mass Data of Cry1F Proteins Obtained by MALDI/TOF MS, page 50. Provide an explanation why some of the residues were not detected.*

A mass spectrum of the peptide mixture resulting from the digestion of a denatured protein by an enzyme provides a fingerprint of such great specificity that it is often possible to identify the protein from this information alone. However, peptide mass fingerprinting is limited to the identification of proteins for which amino acid sequences are already known. The ability to ionize many peptides in a mixture simultaneously makes MALDI-TOF MS a popular choice for rapid analysis of protein digests. Due to various factors, 100% sequence coverage, in most experiments, is infeasible and unnecessary. In general, a protein identification made by peptide fingerprinting is considered to be reliable if the measured coverage of the sequence is 20% or greater, and major peaks in the mass spectrum have been assigned to the protein identified, or to known contaminants.

Peptide mass fingerprinting by MALDI-TOF MS is a complex experimental procedure including such major steps as gel electrophoresis, enzyme digestion (e.g., using trypsin), peptide extraction and purification (e.g., using ZipTip C18), crystallization of peptides with matrix compounds (e.g., α -cyano-4-hydroxycinnamic acid), and laser desorption and ionization. Many events happen during the process, which could affect the generation and detectability of each individual peptide. It is common that for the same protein preparation, each measurement of the peptide mass fingerprint could result in different results of sequence coverage, which means some peptides are detected in one analysis but not in another. Possible reasons include the following: (1) Partial and missed cleavage at protease sites could happen due to variations in digestion conditions such as enzyme to protein ratio and digestion time. Therefore not all theoretical fragments are generated in each digestion. (2) Some peptides are lost during the ZipTip C18 purification. The ZipTip C18 works in the same way as a reverse phase column. Its binding capacity for each peptide varies depending on the hydrophobicity of the peptides. Each purification run is a competitive binding process, and depending on peptide composition and amount, certain peptides may not be sufficiently enriched after ZipTip purification to be detectable by MALDI-TOF MS. (3) Differential ionization and subsequent detection of fragments is experiment specific. During matrix-assisted laser desorption/ionization, the matrix molecules of α -cyano-4-hydroxycinnamic acid absorb the energy from the laser light and transfer it into excitation energy and pass it on to the analyte peptides, which causes the peptides to become ionized. Only ionized peptides can travel through the TOF tube and be

detected by the MS detector. Not all the peptides can be ionized equally. Typically hydrophilic peptides are ionized more efficiently than hydrophobic ones. Also, signals for less efficiently ionized peptides can be suppressed by other dominant and readily ionized peptides. Therefore, for one specific peptide, if its amount in the mixture is not dominant or it is difficult to be ionized, it might be ionized and detected in one experiment but suppressed in another. In Table 12, among the listed 28 peptides of Cry1F, 3 were not detected in the microbial Cry1F sample, 5 were not detected in the cotton Cry1F sample. Based on the spectra among these 8 peptides undetected in either of the two samples, 7 of them are minor peaks. These peptides are either in low quantities in the digests or are difficult to ionize. Therefore, these peptides could sometimes be detected, and sometimes not. Overall, the coverage obtained was 38% for the plant-derived Cry1F protein and 40% for the microbial derived Cry1F, demonstrative of clearly acceptable levels of coverage for protein identification.

15. Table 13. Agronomic Characteristics of Event(s) and the Stack Product in Comparison to Parent Variety PSC355. Tables do not provide us with enough information about the agronomic characteristics of the parent, transformed and stacked plants. Please include the following information: What is the sample size from each location? What are the ranges of the units that were sampled and reported? What are the standard deviations and statistical methodology? Is seed cotton weight per boll equal to seed weight per boll? Do you have data on number of seeds per boll and number of bolls per plant?

The revised Table13 following includes over-location treatment means and indicates statistical significance of observations based on a means comparison according to a Dunnett-Hsu test, $p = 0.05$). Additional Tables labeled as A, B and C are provided below which include the maximum and minimum treatment values and standard deviations, respectively. The number of locations used in any given analysis is indicated in each table.

Revised Table 13. Agronomic Characteristics of Event 281-24-236 and MXB-13 Cotton Lines Expressing Cry1F Protein in Comparison to Parent Variety PSC355.

Variable	Units	281-24-236 (Cry1F)	MXB-13 (Cry1Ac/Cry1F)	PSC355 (null)	Number of Locations
Growth Habit					
Plant height	inches	40.8	40.2 *	41.5	17
Total nodes	number per plant	17.7	17.4	17.6	16
Height to node ratio	inches per plant	2.31	2.32	2.35	17
Node of the 1st fruiting branch	node	6.6	6.8	6.6	17
Fruiting branches	number per plant	12.1	11.6 *	12.1	16
Total fruiting positions	number per plant	26.9	24.7 *	26.6	17
Vegetative bolts	number per plant	2.6 *	1.7	1.6	16
Germination and Emergence					
Field emergence	%	60.9 *	78.9	82.3	19
Cool vigor	%	35	36	38	20
4 day warm	%	62	64	65	20
7 day warm	%	79	80	82	20
Total germination	%	84	84	87	20
Dormant seed	%	0.6	0.5	0.3	20
Vegetative Vigor					
Vegetative branches	number per plant	2.9 *	2.8	2.6	16
Flowering Period					
Days to first flower	days	61.5 *	61.4	60.6	18
Node of white flower - 15 days	node	13.0	12.9	12.9	17
Node of white flower - 30 days	node	17.0	16.9	16.8	15
Reproductive Potential					
Percent retention - total	%	47.6	45.8	44.4	16
Percent retention - 1st position	%	54.8	62.4 *	54.3	16
Percent open bolls	% per plant	75.8	76.5	75.4	17
Seed cotton weight per boll	grams per boll	5.4 *	5.3 *	5.1	19
Lint percent	%	38.1 *	37.1	37.3	19
Seed index (fuzzy)	grams per 100 seeds	10.9	11.3 *	10.7	17
Lint per acre	pounds per acre	1007	1000	993	17
Fiber Quality					
Length	inches	1.147	1.177 *	1.147	19
Strength	grams per tex	31.4 *	33.0	32.6	19
Micronaire	micronaire units	4.67 *	4.51 *	4.96	19
Length uniformity	%	85.4 *	85.8	85.7	19
Reflectance	%	75.6 *	76 *	74.6	19
Yellowness	Hunter's +b scale	8.5	8.3	8.4	19

* = significantly different from the non-transgenic recurrent parent (PSC355) using means comparison according to Dunnett-Hsu at $p = 0.05$.

Table 13A. Maximum Values for Agronomic Characteristics of Event 281-24-236 and MXB-13 Cotton Lines Expressing Cry1F Protein in Comparison to Parent Variety PSC355

Variable	Units	281-24-236 (Cry1F)	MXB-13 (Cry1Ac/Cry1F)	PSC355 (null)	Number of Locations
Growth Habit					
Plant height	inches	59.8	55.2	56.7	17
Total nodes	number per plant	21.0	20.3	20.5	16
Height to node ratio	inches per plant	3.25	3.33	3.14	17
Node of the 1st fruiting branch	node	10.0	11.0	9.5	17
Fruiting branches	number per plant	15.2	15.7	15.5	16
Total fruiting positions	number per plant	38.0	36.7	37.0	17
Vegetative bolts	number per plant	8.7	7.8	4.0	16
Germination and Emergence					
Field emergence	%	96.3	112.5	116.0	19
Cool vigor	%	84	86	82	20
4 day warm	%	98	96	96	20
7 day warm	%	100	100	100	20
Total germination	%	100	100	100	20
Dormant seed	%	6.0	3.0	2.0	20
Vegetative Vigor					
Vegetative branches	number per plant	6.5	6.4	5.8	16
Flowering Period					
Days to first flower	days	80.0	80.0	79.0	18
Node of white flower - 15 days	node	16.5	17.2	18.0	17
Node of white flower - 30 days	node	21.8	20.8	22.2	15
Reproductive Potential					
Percent retention - total	%	74.5	74.8	74.8	16
Percent retention - 1st position	%	75.4	81.1	78.4	16
Percent open bolls	% per plant	100	100	100	17
Seed cotton weight per boll	grams per boll	6.5	6.7	6.0	19
Lint percent	%	43.0	43.5	42.5	19
Seed index (fuzzy)	grams per 100 seeds	12.6	13.4	13.4	17
Lint per acre	pounds per acre	2412	2493	2533	17
Fiber Quality					
Length	inches	1.23	1.27	1.24	19
Strength	grams per tex	35.0	37.4	36.5	19
Micronaire	micronaire units	5.4	5.4	5.9	19
Length uniformity	%	87.3	88.2	88.3	19
Reflectance	%	79.3	78.7	79.0	19
Yellowness	Hunter's +b scale	9.9	9.6	10.2	19

Table 13B. Minimum Values for Agronomic Characteristics of Event 281-24-236 and MXB-13 Cotton Lines Expressing Cry1F Protein in Comparison to Parent Variety PSC355

Variable	Units	281-24-236 (Cry1F)	MXB-13 (Cry1Ac/Cry1F)	PSC355 (null)	Number of Locations
Growth Habit					
Plant height	inches	21.1	22.3	25.7	17
Total nodes	number per plant	13.7	13.8	13.3	16
Height to node ratio	inches per plant	1.54	1.56	1.77	17
Node of the 1st fruiting branch	node	5.2	4.7	4.5	17
Fruiting branches	number per plant	8.3	6.7	8.0	16
Total fruiting positions	number per plant	14.7	9.2	13.5	17
Vegetative bolts	number per plant	0.0	0.0	0.0	16
Germination and Emergence					
Field emergence	%	15.0	40.0	36.0	19
Cool vigor	%	0	2	2	20
4 day warm	%	14	18	12	20
7 day warm	%	16	22	36	20
Total germination	%	23	31	45	20
Dormant seed	%	0.0	0.0	0.0	20
Vegetative Vigor					
Vegetative branches	number per plant	1.0	0.5	0.8	16
Flowering Period					
Days to first flower	days	52.0	52.0	52.0	18
Node of white flower - 15 days	node	7.2	7.2	7.5	17
Node of white flower - 30 days	node	13.8	13.5	13.3	15
Reproductive Potential					
Percent retention - total	%	20.1	23.2	15.5	16
Percent retention - 1st position	%	38.8	43.6	18.8	16
Percent open bolls	% per plant	21.2	34.0	20.0	17
Seed cotton weight per boll	grams per boll	4.2	3.9	4.3	19
Lint percent	%	11.2	12.0	11.2	19
Seed index (fuzzy)	grams per 100 seeds	8.4	9.2	9.0	17
Lint per acre	pounds per acre	331	339	238	17
Fiber Quality					
Length	inches	1.05	1.05	1.04	19
Strength	grams per tex	28.3	29.5	29.1	19
Micronaire	micronaire units	3.8	3.2	4.0	19
Length uniformity	%	83.0	83.5	83.8	19
Reflectance	%	68.0	70.2	69.2	19
Yellowness	Hunter's +b scale	7.0	6.9	6.8	19

Table 13C. Standard Deviations for Agronomic Characteristics of Event 281-24-236 and MXB-13 Cotton Lines Expressing Cry1F Protein in Comparison to Parent Variety PSC355

Variable	Units	281-24-236 (Cry1F)	MXB-13 (Cry1Ac/Cry1F)	PSC355 (null)	Number of Locations
Growth Habit					
Plant height	inches	7.1	6.9	7.0	17
Total nodes	number per plant	1.6	1.7	1.8	16
Height to node ratio	inches per plant	0.37	0.36	0.33	17
Node of the 1st fruiting branch	node	1.0	1.1	1.0	17
Fruiting branches	number per plant	1.8	2.1	1.9	16
Total fruiting positions	number per plant	6.6	6.5	6.0	17
Vegetative bolts	number per plant	1.8	1.4	1.0	16
Germination and Emergence					
Field emergence	%	15.1	14.3	17.0	19
Cool vigor	%	23	23	22	20
4 day warm	%	20	20	18	20
7 day warm	%	19	18	14	20
Total germination	%	30	27	25	20
Dormant seed	%	1.4	0.8	0.6	20
Vegetative Vigor					
Vegetative branches	number per plant	1.4	1.5	1.4	16
Flowering Period					
Days to first flower	days	7.0	6.8	7.1	18
Node of white flower - 15 days	node	2.3	2.2	2.2	17
Node of white flower - 30 days	node	2.0	1.9	2.7	15
Reproductive Potential					
Percent retention - total	%	11.1	12.0	12.7	16
Percent retention - 1st position	%	8.3	7.7	11.4	16
Percent open bolls	% per plant	21.7	19.7	20.6	17
Seed cotton weight per boll	grams per boll	0.5	0.5	0.4	19
Lint percent	%	6.6	6.4	6.7	19
Seed index (fuzzy)	grams per 100 seeds	0.9	1.0	0.8	17
Lint per acre	pounds per acre	473	487	560	17
Fiber Quality					
Length	inches	0.05	0.05	0.05	19
Strength	grams per tex	1.6	1.8	1.6	19
Micronaire	micronaire units	0.4	0.4	0.4	19
Length uniformity	%	0.9	1.0	0.9	19
Reflectance	%	2.2	1.9	2.1	19
Yellowness	Hunter's +b scale	0.6	0.6	0.7	19

Sample size: The sample sizes were the same for each location, and the sample size was dependent on the characteristic being measured. Samples sizes are described as follows:

Field Emergence - # of seedlings emerged per 20' of row. The value is expressed as a percentage of the number of seeds planted in 20' as determined by the number of seeds packaged per row-foot. This evaluation was done in each plot for each of four replicates.

Progeny Seed Germination – A 25-boll sample was picked per plot from each of 4 replicates. The seed was bulked within a treatment (trait) across the four replicates within a location. This resulted in one seed sample for each treatment for each location. Cool vigor tests were conducted with 4 samples of 50 seeds each for each treatment within a location. All other germination tests were conducted with 8 samples of 50 seeds each for each treatment within a location.

Seed cotton weight per boll, Lint percent, Seed index and Lint per acre – These characteristics were determined with the use of one 25-boll sample of seedcotton harvested from each plot in each of 4 replicates. Therefore, there would be one value per plot or 4 values for each treatment (trait) per location.

Fiber Quality – The lint from the 25 boll samples was used for fiber quality measurements. One 25-boll sample was harvested from each plot in each of 4 replications. Therefore, there would be one value per plot or 4 values for each treatment (trait) per location. The number of determinations conducted on the fiber by the High Volume Instrumentation (HVI) at the International Textile Center varied depending on the fiber property. The standard analysis of 2 micronaire, 4 length, 4 strength, 1 reflectance and 1 +b was used for all samples.

All other plant measurements – 6 plants were observed from each plot for each of the 4 replicates within a location.

Seedcotton: The agronomic performance data report seedcotton weight per boll. Seedcotton is the raw material harvested out of the field. Seedcotton is a combination of lint, seed, moisture and plant trash. For hand-picked samples, as was used here, the amount of trash and moisture is typically very low and uniform across varieties/treatments. Therefore, seedcotton weight of hand-picked samples is commonly used as a measure of lint plus seed. To determine seedcotton weight per boll, a 25-boll sample of seedcotton (excluding the burr) was harvested, weighed and divided by 25 to get the average seedcotton weight per boll. Cotton breeders commonly refer to this as “boll size.”

Seeds per boll: Since all values relative to seedcotton, lint, and seed were determined based on a bulk sample of 25 bolls, values on a per boll basis can only be determined as an average of 25. The number of seeds per boll can be calculated given the values presented in Table 13. The explanation and calculation is as follows – Seedcotton weight per boll is the combination of lint and seed weight. Lint percent is the percentage of seedcotton that is lint. Seed percent can be calculated as $[100 - \text{Lint Percent} = \text{Seed Percent}]$. Seed weight per boll can then be calculated as $[\text{Seedcotton weight per boll} \times \text{Seed Percent} / 100 = \text{Seed Weight per Boll}]$. Using the Seed Index (grams/100 seeds) the Number of Seeds per Boll can be calculated by $[\text{Seed Weight per Boll} / (\text{Seed Index} / 100) = \text{Seeds per boll}]$. Using the over-location treatment means in Table 13, the number of seed per boll is calculated below.

Seedcotton weight per boll of Event 281-24-236 (MBX-9) and the Stack Product (MBX-13) Cotton Lines Expressing Cry1Ac and/or Cry1F Protein in Comparison to Parent Variety PSC355

Characteristic	MXB-9	MXB-13	PSC355
Seedcotton weight per boll	5.4	5.3	5.1
Lint percent	38.1	37.1	37.3
Seed percent	61.9	62.9	62.7
Seed weight per boll	3.3	3.3	3.2
Seed index	10.9	11.3	10.7
Number of seed per boll	30.7	29.5	29.9

Bolls per plant: The number of bolls per plant developing on fruiting branches and vegetative branches is shown in the table below. Counts were taken when plants were mature. Bolls were counted if they were open or green and at least the size of a dime (18 mm). As with most other plant measurements, 6 plants were evaluated per plot with 4 plots (reps) per treatment (trait) per location. The data in the table below are from 16 locations.

Number of Bolls per Plant on Fruiting and Vegetative Branches of Event 281-24-236 (MBX-9) and the Stack Product (MBX-13) Cotton Lines Expressing Cry1Ac and/or Cry1F Protein in Comparison to Parent Variety PSC355

Characteristic	MXB-9	MXB-13	PSC355
Fruiting branch bolls per plant			
Mean	12.4	10.9	11.4
Std. Dev.	3.2	2.7	3.2
Max	19.1	16.2	17.2
Min.	8.0	7.1	5.9
Vegetative branch bolls per plant			
Mean	2.6	1.7	1.6
Std. Dev.	1.5	1.3	0.7
Max	5.6	5.1	2.9
Min	0.5	0.4	0.3

Statistical methodology: In the over location summary, Dunnett's test was used to compare all lines to the non-transgenic control, PSC-355, using least square means. In individual experiments, Duncan's New Multiple Range Test was used to evaluate differences among treatment means. The analyses were run in SAS version 8.12 and used linear mixed model ANOVA techniques where the treatments (four lines) were treated as a fixed factor and trial locations, replications within trial locations and trial*line were treated as random factors. In all cases where linear combinations of error terms were combined to construct test statistics, Satterthwaite's approximation was used to adjust degree of freedom values in the tests.

Section V.H.1. Toxicants and Allergens Present in Events 281-24-236

16. Table 16. *Tocopherol Analyses of Events and Non-transgenic Control Cottonseed Oil* refers to a publication titled "Cottonseed Oil, 1990". Citation was not found in Section VIII. References. Please provide us with this citation.

Cottonseed Oil, National Cottonseed Products Association: Memphis, TN, 1990.

Dow AgroSciences is enclosing here a revised section VI.B.2 of the petition for deregulated status for cotton event 281-24-236. This revision addresses minor errors in response to questions 17-24 of the USDA-APHIS review dated July 14, 2003, and additionally incorporates minor points of clarification.

VI.B.2. Results of Ecotoxicity Studies

Cry1F protein will occur in cotton as a stack with Cry1Ac protein, therefore, many of the ecotoxicity studies were conducted with Cry1F protein alone as well as in combination with Cry1Ac protein. Table 21 presents the hazard result for the lowest observed effect level observed in ecotoxicity tests. This result may represent Cry1F protein alone or in combination with Cry1Ac protein, depending on the test material used, the dosing strategy, and the observed result. The results of ecotoxicity studies are described below. A detailed summary of the ecotoxicity results for the studies conducted with combined Cry1Ac and Cry1F proteins can additionally be found in Wolt (2002).

Comment on Positive Controls: For studies of plant incorporated protectants appropriate positive control substances have been difficult to identify, and interpretations using candidate positive controls substances have been controversial. A FIFRA Scientific Advisory Panel called December 8, 1999 to make recommendations concerning "Characterization and Non-Target Organism Data Requirements for Protein Plant-Pesticides" concluded that positive controls generally are not required for NTO testing.

Mammals

A microbial protein preparation containing 30% Cry1F protein (full length) was evaluated for acute oral toxicity from gavage administration to male and female CD1 mice (Brooks and Andrus, 1999; Appendix 2). All mice survived and there were no adverse effects in terms of body weights, detailed clinical observations, and gross pathological lesions during the two-week observation period. Under the conditions of this study, the LD₅₀ of the Cry1F microbial protein in male and female CD-1 mice was greater than 600 mg a.i./kg.

Birds

An 8-day acute avian dietary study with bobwhite quail investigated the effect of a 10% cotton meal diet using meal prepared from cottonseed expressing Cry1F and Cry1Ac proteins. This produced a diet containing 0.021 µg Cry1F protein per g in combination with Cry1Ac protein. The LC₅₀ was >100,000 µg meal/g diet (>2100 ng Cry1F per g diet) (Gallagher and Beavers, 2002; Appendix 2).

Cotton meal prepared from 281-24-236/3006-210-23 cottonseed was used as 10% of the diet. This level of feeding approximates 400 seeds/kg body weight per bird per day (Payne, 1995), and represents the maximum amount of cotton meal that can be tolerated in the quail's diet.

Feral birds would not consume cottonseed or otherwise be exposed to Cry1F protein in their diet. Based on average measured Cry1F in feed, the diet contained Cry1F protein at less than the high-end exposure in cotton seed since Cry1F protein was degraded when cottonseed was processed into meal. This is consistent with the anticipated fate of the protein when processed into food or feed.

In addition to the subject study, an acute oral gavage study has been conducted for bobwhite quail where Cry1F protein was administered in combination with Cry1Ac protein as a single oral dose. No mortality occurred and the no-observed-effect level was 113.6 mg /kg (Gallagher and Beavers, 2002).

Soil invertebrates

Earthworm. Microbially-derived Cry1F protein, alone or in combination with Cry1Ac protein, showed no toxicity to earthworms (*Eisenia foetida*). The LC₅₀ was >247 mg Cry1F protein per kg (soil dry weight basis) (Sindermann *et al.*, 2001; Appendix 2). This represents concentrations that are 762-fold higher than the EEC expected with incorporation of event 281-24-236 cotton plants into the top 15 cm of soil (see Expected Environmental Concentrations, section VI.C).

Collembola. Collembola plays a major role in soil ecosystems due to its feeding on decaying plant materials. Therefore, a laboratory study to determine the chronic effects of Cry1F protein on survival and reproduction of the soil dwelling invertebrate collembola (*Folsomia candida*) was conducted using microbe-derived Cry1F added to Brewer's yeast (standard food for collembola) (Teixeira, 2002; Appendix 2). The fortification concentration tested was 709 mg Cry1F protein per kg diet or 702 mg Cry1F protein per kg diet in combination with Cry1Ac protein. There was no effect shown from Cry1F protein exposure in the diet. The EC₅₀ was > 702 mg Cry1F protein per kg diet representing an exposure of 2167× the soil EEC for event 281-24-236 (0.324 mg/kg; section VI.C).

Effects on aquatic organisms

Daphnia magna. There are no known adverse effects of Cry proteins on the aquatic invertebrate *Daphnia magna*. A 48-hour static limit test with *Daphnia* was conducted with 510 µg/L Cry1F protein in combination with Cry1Ac protein (Marino and Yaroch, 2002a; Appendix 2). This rate of fortification represents 395× the anticipated EEC for Cry1F protein from event 281-24-236 in surface water (1.21 µg/L; see Estimated Environmental Concentrations in section VI.C). Protein solubility at these concentrations is not complete. The study was therefore conducted at the maximum practical concentration although this level was not measured. No effect was seen using a saturated solution. There were no observed adverse effects of treatment in terms of immobility or sublethal effects; therefore, the 24- and 48-hour EC₅₀ is > 510 µg Cry1F/L.

Fish. The acute dietary toxicity of Cry1F protein to the rainbow trout (*Onchorynchus mykiss*) was determined for fish exposed for eight days to a commercial-grade, pelleted trout diet containing 10% cotton meal prepared from cotton seed expressing Cry1F and Cry1Ac proteins (Marino and Yaroch, 2002; Appendix 2). This produced a diet containing an initial dosing of 0.209 mg Cry1F per g-food in combination with Cry1Ac protein. The control diet consisted of the same commercial fish diet prepared with non-transgenic cotton meal. No fish mortality or sublethal effects were observed for either the control or treatment diet. Therefore, based on the biological observations, the 8-day LC₅₀ value with rainbow trout is greater than 0.209 mg/kg-diet, representing 162× the anticipated EEC for Cry1F protein from event 281-24-236 in surface water (see Estimated Environmental Concentrations in section VI.C.).

Effects on non-target arthropods

Honeybee. There was no effect on mean survival to emergence for honeybee exposed to either 2 mg pollen from a Cry1F-expressing event or to 1.98 µg per mL of Cry1F protein in combination with Cry1Ac protein (Maggi, 2001; Appendix 2). The LC₅₀ for exposure to Cry1F protein is >1.98 µg per mL (1.98 µg per g) and represents approximately 3× the high-end expression in event 281-24-236 pollen. This dose represents 19.8 ng/larvae, an amount equivalent to that which would be present in 28.2 mg of pollen based on the HEE of 0.7ng/mg of pollen.

Green Lacewing. The dietary LC₅₀ for green lacewing (*Chrysoperia carnea*) larvae exposed to Cry1F protein, alone or in combination with Cry1Ac protein, has been investigated in a series of studies with microbial protein administered in a diet of moth eggs (Sindermann *et al.*, 2002a; Appendix 2). There was an effect of Cry1F at 5.2 micrograms/g in combination with Cry1Ac in 1 test, but not in the second test. The test with Cry1F alone showed no effect. The dietary LC₅₀ for green lacewing is ≤ 5.2 µg per g on this basis and represents an exposure level to Cry1F protein 104× higher than the high-end expression in event 281-24-236 nectar and > 7× higher than that in pollen. Safety factors based on a relevant food source (aphids consuming transgenic plant tissue) would be significantly higher (as much as 100- to > 4,000-fold higher; see *Phytophagus Insects* under Estimated Environmental Concentrations, section VI.C).

Toxicity to green lacewing larvae is not considered ecologically relevant to the risk assessment for event 281-24-236 cotton, since exposure, if it occurs, will be indirect and field census results show no impact of Cry1F as expressed in the stacked product MXB-13 on green lacewing abundance (Mahill and Storer, 2002; Appendix 2).

Parasitic wasp. Parasitic hymenoptera (*Nasonia vitripennis*) were exposed to a single limit concentration of Cry1F protein, alone and in combination with Cry1Ac protein, in sugar water for up to 10 days. There were no significant differences in mortality between treatment groups and a sugar water negative control on day 9, which was the last observation point before mortality increased beyond the criterion set in the protocol for the negative control (20%). Beyond this level of mortality in the wasp assay, the test results are considered compromised. The LC₅₀ was greater than 5.2 µg a.i. per mL of microbe-expressed Cry1F protein (Sindermann *et al.*, 2002b; Appendix 2). The exposure level represents concentrations greater than 104× higher than the high-end expression of Cry1F protein event 281-24-236 nectar and greater than 7× higher than that in pollen. Safety factors based on a relevant food source (lepidopteran larvae consuming transgenic plant tissue) would be significantly higher (from 9- to 286-fold higher; see *Phytophagus Insects* under Estimated Environmental Concentrations).

Ladybird beetle. Adult ladybird beetles (*Hippodamia convergens*) were unaffected when exposed to microbe-expressed Cry1F protein, alone or in combination with Cry1Ac protein (Porch and Krueger, 2001; Appendix 2). Ladybirds fed *ad libitum* over 15-days on a diets containing 300 µg Cry1F protein per mL of food, alone or in combination, were monitored for mortality and clinical signs of toxicity. The LC₅₀ for exposure to Cry1F protein is >300 µg per mL, equivalent > 428× the high-end exposure in event 281-24-236 pollen.

Monarch butterfly. Incidental exposure of a sensitive larval stage of a non-target butterfly or moth to Cry1F protein may occur if event 281-24-236 pollen is present on host plants and it is consumed. Monarch larvae feeding on milkweed containing transgenic pollen is a surrogate for indirect exposure of a hypothetical sensitive non-target lepidopteran larvae to cotton pollen.

The response of first instars of monarch butterfly (*Danaus plexippus* L.) exposed to Cry1F in artificial diet for 7 days is reported in studies by Hellmich *et al.* (2001). The dietary concentration resulting in 50% growth reduction relative to controls (EC₅₀) was 5,220 ng a.i./mL for Cry1F. Hellmich *et al.* (2001) present bridging calculations for translation of artificial diet results to upper bound estimates of effect levels expressed in terms of pollen consumption on leaves of a host plant for monarch, common milkweed (*Asclepias curassavica* L.). On this basis, the equivalent effects levels in terms of pollen density on leaves of the host plant is are $> 4.5 \times 10^5$ grains event 281-24-236 pollen per cm² leaf consumed for Cry1F protein. Thus, the EC₅₀ for Cry1F protein expressed in cotton pollen is $> 450,000\times$ higher than the estimated environmental concentration in pollen from event 281-24-236 (see, section VI.C).

Hazard summary

Table 21. Summary of Guideline Hazard Tests for Effect of Cry1F Protein.

Guideline	Study Title	Protein Source	Results
OECD 401	Acute Toxicity – Mouse	Microbe-derived Cry1F protein	LD ₅₀ > 600 mg Cry1F /kg
OPP B, 71-2	Acute Dietary Toxicity – Northern Bobwhite Quail	Cotton meal prepared from 3006-210/281-24-236 cottonseed	8-day LC ₅₀ > 100,000 µg meal /kg diet
OECD 207	Acute Toxicity – Earthworm	Microbe-derived Cry1F, alone or in combination with microbe-derived Cry1Ac protein	14-day LC ₅₀ > 247 mg Cry1F /kg soil 762× EEC in soil
OECD proposed	Chronic Toxicity – Collembola	Microbe-derived Cry1F, alone or in combination with microbe-derived Cry1Ac protein	LC ₅₀ > 702 µg Cry1F /kg 2167× EEC in soil
OECD 202	Acute Dietary Toxicity – <i>Daphnia magna</i>	Combination of microbe-derived Cry1F and Cry1Ac proteins	48-hour EC ₅₀ > 510 µg Cry1F/L 395× EEC in water
OECD 203	Acute Dietary Toxicity – Rainbow Trout	Cotton meal prepared from 3006-210/281-24-236 cottonseed	8-day LC ₅₀ > 0.209 mg /kg diet 162× EEC in water
OPPTS 885.4380	Acute Dietary Toxicity LD ₅₀ – Honeybees	Combination of microbe-derived Cry1F and Cry1Ac proteins	LC ₅₀ > 1.98 µg Cry1F/ g diet 2.8× high-end expression in pollen
OPPTS 885.4340	Non-target Insect – Green Lacewing	Combination of microbe-derived Cry1F and Cry1Ac proteins	LC ₅₀ ≤ 5.2 µg Cry1F/g of diet 7× high-end expression in pollen 104× high-end expression in nectar
OPPTS 885.4340	Non-target Insect – Parasitic Hymenoptera	Microbe-derived Cry1F, alone or in combination with microbe-derived Cry1Ac protein	LC ₅₀ > 5.2 µg Cry1F /mL 7× high-end expression in pollen 104× high-end expression in nectar
OPPTS 885.4340	Non-target Insect – Ladybird Beetle	Microbe-derived Cry1F, alone or in combination with microbe-derived Cry1Ac protein	LC ₅₀ > 300 µg Cry1F /mL 428× high-end expression in pollen

Section VI.C.2. Environmental Fate of Cry1F Protein Incorporated into Soil

25. The petition should state how many soil types were assayed and provide a justification for this number. Please justify the validity of the aqueous agar over-lay technique for tobacco budworm feeding assays.

One soil representative of the cotton agro-ecosystem was run in the laboratory study. Decay of the protein was very rapid and was consistent with what we have seen for other Bt proteins in a variety of soils in our laboratory (Herman et al. 2001; 2002; Herman and Scherer 2002). The submitted study demonstrates the ability of soil microbes to rapidly effect the degradation of the subject Bt proteins. Additionally, field studies encompassing multiple sites and varied soils are planned to address potential accumulation of Cry1F and Cry1Ac proteins as expressed in cotton. A published report on one such multiple field study has reported no such accumulation (Head et. al. 2002. Environ. Entomol. 31: 30-36) and will serve as the model for the study we conduct for 3006-210-23/281-24-236 cotton.

The aqueous-agar overlay technique was validated as part of the study. Reproducible concentration-response curves were generated that accurately indexed the insecticidal activity of the soil (see spiked controls vs. zero time point). The progressively lower activity of the soil over time in this study (as well as the aforementioned published studies) further validates this method as having sufficient precision to index the decay of Bt proteins in soil. This method has consistently provided smooth decay curves as illustrated in the published studies. We use overlay techniques in our laboratory to avoid potential decay of the proteins at the temperatures typically employed in diet-incorporation studies. We have found that such temperatures are capable of denaturing some Bt proteins. Furthermore, our experience is that diet overlay is almost always a more sensitive technique when compared to diet incorporation. Diet overlay is a standard bioassay technique (for example, Sims 2000; Marcon et. al. 1999; Herman et. al. 2002).

Section VI.C.3. Estimated Environmental Concentrations

26.[On] page 68 cotton pollen density is given to be 1 grain per cm² leaf. Please provide us with a reference or explanation of this value.

The rationale for this estimate comes from a bridging comparison of cotton versus corn out-crossing percentages and knowledge of the quantity of corn pollen that occurs incidentally on non-target food sources adjacent to fields. The average out-crossing for cotton (0.76% in the 23 rows of a receptor field adjacent to a source field) was compared to the average for corn (32.87% over approximately the same distance off-source). This was considered in conjunction with the reported mean corn pollen density on milkweed adjacent to a corn field (8.1 grains/cm² leaf at 4-5 m from the source field). Thus, as stated in the petition, "A cotton pollen density of 1 grain per cm² leaf, of a host plant for a hypothesized non-target organism of concern is a conservative environmental exposure estimate for off-source occurrence," when one considers (1) the roughly 40-fold lower out-crossing to adjacent receptor plants for cotton versus corn, (2) the fact that the out-crossing for cotton will be dominantly from insects versus pollen flow, and (3) that for corn, a plant where pollen flow account for the majority of out-crossing, the off-source pollen occurrence on receptors is low.

27. *The exposure estimate for nectar is listed ...; however ... nectar was not analyzed. Since the nectar was not analyzed, provide us with a description of how you arrived at values for high end exposure estimate.*

Attempts were made to detect protein in nectar in samples collected for the field expression study, and all results were ND (not detected). Due to the lack of nectar matrix that was available, we were not able to fully validate the ELISA methods. On the basis of our knowledge concerning overall analytical sensitivity of the ELISA procedure, we determined that the ND was likely 0.05 ng/ul; thus, we have used < 0.05 ng/uL for the high end estimate based on our attempts to analyze the nectar. Thus, the result is a reasonable estimate based on our attempts to detect protein in nectar and our knowledge of the assay sensitivity.

VI.D.1. Likelihood of Exposure Exceeding Effects Thresholds and Taxa at Risk

28. *Figure 20, page 70 “Endangered Lepidopterans”. The figure presented refers to 1997 USEPA data. Have any other endangered Lepidopterans been added to this EPA list since 1997?*

We used the 1997 FWS listing as this was the most current, publicly available list (of which we were aware) that described occurrence of endangered and threatened species to the county level. The FWS Endangered Species List (<http://endangered.fws.gov/wildlife.html#Species>) shows one additional listing for Fender’s Blue Butterfly (*Icaricia icarioides fenderi*) which was listed as endangered on 25 January 2000. This species is known to occur only in Oregon and, therefore, is not in the range of cotton production. The conclusions of the endangered species assessment for cotton event 281-24-236 are unchanged.

Field Census Study

29. *Preliminary results from the field study comparing the stacked event with non-lepidopteran insecticide treatment to the non-transgenic parent with lepidopteran or non-lepidopteran insecticide treatments revealed no significant consistent decline in beneficial arthropods using several sampling methods (Mahill and Storer, 2002, Appendix 2, V 30 (Cry1F)...). Please submit the final results from this study. This data will be required before APHIS’ environmental assessment is finalized for comment.*

A decline in beneficial arthropods, which act as specialist predators on insects targeted for control, is consistent with the efficacy of plant-expressed Cry1F protein in protecting against key lepidopteran pests of cotton, since if the food source is absent so will be the predator. Field census data for 2003 (year 2) are pending and will be reported to USDA-APHIS as soon as results can be compiled and analyzed. On the basis of 2002 field census results, knowledge of Cry1F protein activity (in combination with that of Cry1Ac protein), and the biology of beneficial arthropods of cotton, there are no unanticipated adverse effects to beneficial arthropods identified to date in the evaluation of cotton event 281-24-236.

VI.E.1. Human Health Risk

30. *Text states that [Cry1F] is rapidly digested in <1 minute in simulated gastric fluid, ... on page 71. Please provide the titles of the references that demonstrate these results.*

V. A. Korjagin. *In Vitro* Simulated Gastric Fluid Digestibility Study of Microbially Derived Cry1F(synpro). Dow AgroSciences Study ID. 010081.

References

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November 25, 2003

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B.t. CRY1F COTTON EVENT 281-24-236 / CRY1AC COTTON EVENT 3006-210-23
PETITION NUMBERS 03-036-01p and 03-036-02p
RESPONSE TO USDA TECHNICAL REVIEW LETTER DATED JULY 14, 2003

On January 31, 2003, Mycogen Seeds c/o Dow AgroSciences submitted petitions for determination on the regulatory status of *B.t.* Cry 1F cotton event 281-24-236 and any cotton lines derived from crosses of *B.t.* cotton line MXB-9 and *B.t.* Cry 1Ac Cotton event 3006-210-23 and any cotton lines derived from crosses of *B.t.* cotton line MXB-7. On July 14, 2003, we received your review for technical completeness of our petitions numbered 03-036-01p and 03-036-02p. In response to Question #29 of the USDA technical review letter and our reply to you dated September 15, 2003, we are submitting 2 copies of the field survey results (1 CBI and 1 CBI Deleted) for the Cry1F and Cry1Ac cotton event.

Contents of Submission:

- **Transmittal document** (this letter)
- **Study Title:** Field Surveys to Evaluate Effects on Non-Target Beneficial Arthropods of Cry1F/Cry1Ac *Bt* Cotton (CBI)

Authors: N.P. Storer
Study ID: GH-C 5692
Pages: 1-127
Study Date: 11/19/03
(2 copies)

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If you have questions or need further information, please contact me or Paula McKinnies, Registration Assistant (317/337-4679 / pmckinnies@dow.com).

Sincerely,

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Enclosures

/pkm

SUMMARY

(In accordance with 40 CFR part 152, this summary is available
for public release after registration)

STUDY TITLE

Field Surveys to Evaluate Effects on Non-target Beneficial Arthropods of Cry1F/Cry1Ac *Bt*
Cotton

DATA REQUIREMENTS

None

AUTHOR(S)

N. P. Storer

STUDY COMPLETED ON

November 19, 2003

PERFORMING LABORATORY

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LABORATORY STUDY ID

GH-C 5692

Field Surveys to Evaluate Effects on Non-target Beneficial Arthropods of Cry1F/Cry1Ac *Bt* Cotton

SUMMARY

Dow AgroSciences has developed a new line of *Bt* cotton expressing Cry1F and Cry1Ac insecticidal crystal proteins. The stack of the two events will be commercialized as WideStrike* *Bt* cotton. Studies have been conducted to evaluate the non-target effects of WideStrike *Bt* cotton in the field compared with the non-target effects of conventional cotton pest management. The studies were conducted at two locations (Arizona and Louisiana) in two years (2002 and 2003). At Maricopa, Arizona, conventional cotton pest management was targeted at pink bollworm (*Pectinophora gossypiella*) control; at Winnsboro, Louisiana, the target was tobacco budworm (*Heliothis virescens*).

Treatments consisted of non-Bt cotton without insecticides to control Lepidoptera, WideStrike Bt cotton without insecticides to control Lepidoptera, and non-Bt cotton with insecticides to control Lepidoptera as needed. All treatments allowed control of non-lepidopteran pests. There were two or three replicates of each treatment at each location each year. The non-Bt cotton variety used (PSC355) is the recurrent parent of the WideStrike cotton line.

In 2002, plot size was 2,667 sq. ft at Winnsboro and 6,400 sq. ft at Maricopa. In 2003, the plot sizes were expanded to 1 acre and 0.5 acres respectively. Plots were separated either by bare ground or by plantings of alternate hosts. Sampling methods consisted of visual plant inspections, sweep nets, shake sheets, sticky traps, and pitfall traps. Sampling for arthropods was conducted in the center of each plot to minimize edge effects and the effects of insect dispersal. Sampling intensity varied across method, location, and year. There were totals of 29, 42, 26, and 48 data points per plot for Arizona 2002, Louisiana 2002, Arizona 2003, and Louisiana 2003, respectively. Each data point was composed of multiple samples.

Non-target arthropods sampled in these studies included insects from multiple orders filling diverse ecological roles, including phytophagous, predatory, parasitic and saprophagous modes of feeding. The abundance and diversity of non-target arthropods, including natural enemies, tended to be lower in non-*Bt* cotton that is treated with conventional insecticides to control Lepidoptera than in non-*Bt* cotton that does not receive insecticides to control Lepidoptera. By contrast, the non-target arthropod abundance and diversity in WideStrike *Bt* cotton was similar to non-*Bt* cotton that is similarly managed for other pests but receives no sprays for Lepidoptera. Some minor but statistically significant differences were detected (for example, greater numbers of predatory Hemiptera, greater numbers of lacewings, lower numbers of phorid, and flies greater numbers of phytophagous Hemiptera) but these were not consistent across years, across locations or across sampling methods. Principal response curve analysis of the Maricopa 2003 data revealed no community-level effects of WideStrike *Bt* cotton, but a decline in community abundance at the end of the season in the plots sprayed for Lepidoptera. The only major or consistent differences in arthropod abundance between WideStrike *Bt* cotton and unsprayed-for-Lepidoptera non-*Bt* cotton were in Lepidoptera larvae, which are the target of the WideStrike *Bt* cotton.

This set of studies, at two locations over two years, provides strong support for the hypothesis that WideStrike *Bt* cotton, like other *Bt* crops, has minimal effects on non-target arthropods in field settings.

* Trademark of Dow AgroSciences LLC

STUDY TITLE

Field Surveys to Evaluate Effects on Non-target Beneficial Arthropods of Cry1F/Cry1Ac *Bt*
Cotton

DATA REQUIREMENTS

None

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STUDY COMPLETED ON

November 19, 2003

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LABORATORY STUDY ID

GH-C 5692

CBI Begins [Pages 2 – 15]

1.] End CBI

Appendix A-Field Survey to evaluate Effects on Non-Target Beneficial Arthropods of
WideStrike Cry1F/Cry1Ac Bt Cotton in Louisiana, 2003

Begin CBI [Pages 16 – 103]

] End CBI

Appendix B-2003 DAS NTO Assessment, Maricopa, Arizona

Begin CBI [

] End CBI

Appendix C-Copies of References

Begin CBI [Pages 104 – 127]

] End CBI