Petition for the Determination of Nonregulated Status

Event COT67B

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

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OECD Unique Identifier: SYN-IR67B-1

No Confidential Business Information Included

Release of Information

Syngenta is submitting the information in this petition for purposes of review by USDA pursuant to the regulations at 7 CFR Part 340.6. By submitting this information, Syngenta does not authorize its release to any third party prior to a determination that this article is not regulated except to the extent that the following three conditions are satisfied: (i) it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., §§ 551 *et seq*.; (ii) USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and (iii) this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Syngenta does not authorize the release, publication or other distribution of this information (including website posting) without Syngenta's prior notice and consent. The information contained in this document may not be used or cited by any third party, including but not limited to any regulatory authority other than USDA, to support the regulatory approval of this product or any other product, or for any other purpose, without Syngenta's prior written consent.

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 all relevant data and information known to the petitioner that are unfavorable to the petition.

_____

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Petition for the Determination of Nonregulated Status for Event COT67B

Summary

Event COT67B

Syngenta Seeds, Inc. has developed a new transgenic cotton event, COT67B (OECD ID No. SYN-IR67B-1) *via* recombinant DNA techniques with broad-spectrum lepidopteran insect resistance. Event COT67B cotton (hereafter referred to as COT67B), produces a full-length Cry1Ab protein originally derived from the bacterium *Bacillus thuringiensis* subsp. *kurstaki* HD-1 (*B.t.k*), which has activity against several important pestiferous lepidopteran species of cotton including, but not limited to, *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm) and *Trichoplusia ni* (cabbage looper).

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167), to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulation 7 CFR Part 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Therefore, to enable unrestricted introduction, Syngenta Seeds, Inc. is submitting this request to APHIS for a determination of nonregulated status for COT67B, any progeny derived from crosses between COT67B and conventional cotton varieties, and any progeny derived from crosses of COT67B with other genetically-enhanced cotton that has also been granted nonregulated status under 7 CFR Part 340.

Overview of Petition

The data and information submitted to support this petition follow the regulations at 7 CFR Part 340.6 and guidance provided by USDA APHIS BRS (Biotechnology and Regulatory Services) (http://www.aphis.usda.gov/brs/). The following are included and briefly summarized below.

1. Cotton biology, production and rationale for developing COT67B
2. Inserted genetic material and molecular analysis of COT67B
3. Description of the FLCry1Ab protein and its quantification in COT67B tissue and seeds
4. Phenotypic, agronomic and ecological assessment of COT67B
5. Compositional and nutritional characteristics of COT67B seed

6. Environmental consequences from the unrestricted introduction of COT67B
7. Adverse consequences including assessment of cumulative effects

1) Cotton biology, production and rationale for developing COT67B

Cotton, genus *Gossypium*, is one of the oldest and most important crops under cultivation. The seed hairs can be spun into fine strong threads and have been valued as a textile fiber for thousands of years in both the old and new worlds. Bits of cotton cloth found by archeologists in caves of Mexico proved to be at least 7,000 years old while the crop is recorded to have been grown, spun and woven into cloth in the Indus River Valley in Pakistan by 3,000 BC. Today, cotton is the single most important textile fiber crop in the world representing over 40% of the total fiber used globally and is cultivated on every major continent or other area between 47 degrees north latitude and 32 degrees south latitude.

In addition to the United States, the major cotton producing countries include Australia, China, Brazil, India, Pakistan, Mexico and Turkey. Over 88 million acres were produced globally in 2004/05 with ca. 12 million acres produced in 17 of the United States from Virginia to California.

Cotton producers in the United States are among the most technically advanced in the world annually harvesting about 17 million bales or 7.2 billion pounds of cotton. By the adoption of new technologies, including agronomic traits delivered through biotechnology, the yields of cotton lint per acre in the U.S. ranks among the highest in the world. Since the introduction of genetically engineered insect resistant and herbicide tolerant cotton, transgenic varieties have been widely adopted by American cotton farmers. These varieties offer excellent protection from the damage incurred by insect pests as well as the ability to utilize environmentally benign herbicides on an as needed basis. It is not surprising that transgenic cotton varieties were planted on 95.5% of United Sates cotton acreage in 2006 (USDA AMS 2006).

Growers are limited in their choice of insect resistant cotton varieties to those containing the *Bacillus thuringiensis* (*Bt*) Cry1Ac, a combination of Cry2Ab2 and Cry1Ac or a combination of the Cry1F and Cry1Ac insecticidal proteins. These “Bt cotton” varieties are registered as plant incorporated protectants with the US Environmental Protection Agency and are sold as Bollgard II (Cry2Ab2 + Cry1Ac), Bollgard (Cry1Ac) and Widestrike (Cry1F + Cry1Ac). Of these, Bollgard or Bollgard II varieties were planted on more than 99% of the cotton acreage planted with transgenic varieties.

Despite the wide adoption of Bollgard or Bollgard II varieties to control lepidopteran insects such as *Heliothis virescens* (tobacco budworm), *Helicoverpa zea* (cotton bollworm) and *Pectinophora gossypiella* (pink bollworm), these same species continue to be the most economically important pests of the crop. In 2005, it was estimated that the Heliothine complex of *H. virescens* and *H. zea* alone reduced yields across the cotton belt by 380,000 bales or 109.1 million dollars ($288 per 480-pound bale).

Syngenta is petitioning the USDA to deregulate Event COT67B. Event COT67B produces a full-length Cry1Ab (FLCry1Ab) protein originally derived from the bacterium *Bacillus thuringiensis* subsp. *kurstaki* HD-1 (*B.t.k*). When expressed in COT67B, this protein provides excellent protection from the feeding damage incurred by several important lepidopteran pests of cotton, which include, but are not limited to, *H. zea* (cotton bollworm), *H. virescens* (tobacco budworm), *P. gossypiella*, (pink bollworm), and *Trichoplusia ni* (cabbage looper). This event, alone or combined by traditional breeding with other genetically-modified insect resistant cotton varieties will be a valuable addition to the pest management options available to U.S. cotton producers.

2) Inserted genetic material and molecular analysis of COT67B

Two binary vectors were introduced through *Agrobacterium tumefaciens*-mediated transformation of cotton (*Gossypium hirsutum* L.) cultivar Coker 312 to develop COT67B. One vector contains a full-length c*ry1Ab* gene under the regulatory control of the actin-2 promoter derived from *Arabidopsis thaliana*, which confers expression of FLCry1Ab protein throughout the plant. Data from Southern analyses and DNA sequencing of the T-DNA insert of COT67B confirmed that a single copy of the *flcry1Ab* gene was present at a single locus in the cotton genome with all expression elements intact and no plasmid bacterial backbone present.

The second binary vector contains the selectable marker gene *aph4*, encoding the enzyme hygromycin-B phosphotransferase, which was originally derived from *Eschericia coli.* The *aph4* gene is under the regulatory control of the ubiquitin-3 promoter from *A. thaliana*. Expression of the *aph4* gene product allows for growth of transformed plant cells on artificial growth medium containing hygromycin B. The *aph4* marker gene segregated away from the *flcry1Ab* gene in the T1 generation and is no longer present in COT67B. Absence of the gene and related regulatory sequences was demonstrated through Southern analyses.

Segregation analysis over three generations confirmed the heritability and stability of the inserted *flcry1Ab* coding sequence. The results of this analysis are consistent with the findings of a single active site of insertion that segregates according to the Mendelian law of genetics.

3) Description of the FLCry1Ab protein and its production in COT67B tissue and seeds

The native or wild-type *cry1Ab* gene is a product of the genetic recombination of the *B. thuringiensis kurstaki* HD-1 genes, *cry1Aa* and *cry1Ac* (Geiser *et al*., 1986). It is well documented that mutations resulting in deletions of stretches of DNA are common during genetic recombination. Such was the case for the native *cry1Ab* gene. This loss of DNA resulted in a diminished capacity of native *cry1Ab* to encode Cry1Ab protein in fermentative cultures of *B. thuringiensis* under the customary fermentation temperatures. It was subsequently discovered that this inefficiency was attributable to the absence of 26 amino acids encoded by the stretch of DNA lost as a result of recombination. Syngenta "repaired" the gene by replacing the deleted coding region with the functional region from

cry1Aa. This "full-length" version of the gene was used in transformation to produce COT67B and is referred to as full-length *cry1Ab* (*flcry1Ab)*.

The *flcry1Ab* gene encodes the same full-length Cry1Ab protein produced by *B. thuringiensis kurstaki* HD-1, except for the additional 26 amino acids in the C-terminal portion of the protein. The 26-amino acid sequence is referred to by Syngenta scientists as the 'Geiser motif' (Geiser[1] and Moser, 1991; Koziel, *et al.* 1997).

The Cry1Ab protein is also present in a number of *B. thuringiensis* corn plant incorporated protectants registered by the EPA since 1996 and re-registered in 2001 and 2006. The FDA completed food and feed safety consultations for these products and the EPA, through its statutory authority under the Federal Food Drug and Cosmetic Act, established a permanent exemption from the requirement of a tolerance for the Cry1Ab protein and the genetic material necessary for its production in all plants (40 CFR 180.1173).

The FLCry1Ab protein produced in COT67B was characterized and its biochemical equivalence to microbially produced FLCry1Ab protein used in mammalian and invertebrate safety studies was demonstrated through a number of analytical tests including Western blot, peptide mass mapping, N-terminal amino acid sequence analysis and insect bioassays. The concentration of FLCry1Ab protein was measured in tissues of COT67B plants grown at four U.S. field trial locations in 2004. Concentrations were determined by enzyme-linked immunosorbent assay (ELISA) and where relevant, the concentration of FLCRY1Ab in particular tissues (leaf, root, flowers, bolls, seed) was determined at several growth stages throughout the life of the plant. Results demonstrated that the mean FLCry1Ab protein levels for all tissues across all growth stages and trial sites ranged from 4.97 – 91.78 ug/g fresh weight.

The Cry1Ab in COT67B is referred to as 'FLCry1Ab' throughout this petition. It is recognized, however, that studies in which ELISA was used to measure the concentrations of Cry protein (using a polyclonal anti-Cry1Ab antibody) cannot distinguish between intact, full-length Cry1Ab and smaller immunoreactive derivatives. Therefore, the concentrations of 'FLCry1Ab' measured by ELISA and described herein do not necessarily reflect solely the concentration of FLCry1Ab, but may include smaller Cry1Ab polypeptides.

4) Phenotypic, agronomic and ecological assessment of COT67B

A comprehensive phenotypic, agronomic and ecological assessment was conducted for COT67B during the 2004, 2005 and 2006 growing seasons. Thirty-five characteristics were evaluated at up to 22 locations representative of the U.S. cotton production belt. Among the 35 characteristics evaluated were seed dormancy and germination, plant emergence, vegetative and reproductive growth, seed and fiber production, and disease, insect, and abiotic stressor-plant interactions.

[1] M. Geiser was employed by the former Ciba-Geigy Corp., a legacy company of Syngenta.

Analysis of the data failed to identify a consistent trend of difference between COT67B and control cotton for any of the phenotypic and agronomic characteristics measured. The few differences identified were typically small, site specific and unlikely to be biologically meaningful. The evaluation of ecological interactions at the same locations, based on monitoring of specific insect, disease, and abiotic stressors such as heat and drought again failed to identify trends for differences in susceptibility to pests or environmental stress.

Consequently, these phenotypic, agronomic and ecological data support the conclusion that COT67B does not confer a detectable selective advantage to cotton that would result in increased weed or pest potential compared to control cotton other than the intended trait.

5) Compositional and nutritional characteristics of COT67Bseed

COT67B seed was harvested from replicated field trials conducted in the U.S. in 2004 and was shown to be substantially similar to conventional cottonseed with regard to 41 compositional and nutritional characteristics including proximates, fatty acids, amino acids, minerals, gossypol, cyclopropenoid fatty acids and vitamin E.

Of the 41 analytes measured, there were few instances where the mean values for COT67B were significantly different from those of the control, Coker 312. However, these differences were not consistent across locations. No pattern emerged that would indicate the noted differences were caused by the transgene insertion or expression of the novel proteins. Furthermore, the values measured for both COT67B and Coker 312 fell well within the established range for cottonseed and are considered to be biologically and nutritionally insignificant. Therefore, cottonseed produced from COT67B is compositionally similar to cottonseed produced from control cotton.

6) Environmental consequences from the unrestricted introduction of COT67B

The environmental consequences of pollen flow from genetically enhanced cotton products has been reviewed by EPA in its environmental reassessment of the registered *B. thuringiensis* plant-incorporated protectants and its findings are summarized in the "Biopesticides Registration Action Document" for these products (US EPA, 2001a.). In that reassessment, EPA reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories, and concluded that the possibility for gene transfer exists only in limited geographic locations where wild or feral cotton relatives exist, *i.e.* in Florida, Hawaii, and the Caribbean. Additionally, USDA APHIS BRS has made this same determination under its statutory authority for other deregulated cotton products, which include Bollgard®, Bollgard II® and Widestrike®. These conclusions made with respect to commercial *B. thuringiensis* cotton varieties are also applicable to COT67B and support the conclusion that COT67B is no more likely to become a plant pest than conventional cotton.

7) Adverse consequences including the analysis of CEQ factors to assess significance of deregulation of COT67B

Syngenta is seeking deregulation of COT67B under APHIS regulation 7 CFR part 340.6. The National Environmental Policy Act (NEPA) 42 U.S.C. 4321 *et seq.* requires agencies

undertaking such actions to provide a detailed statement of the environmental impact of the proposed action, any adverse environmental effects, direct, indirect or cumulative, that cannot be avoided, and alternatives to the action (42 U.S.C. 4332). Syngenta knows of no study results or observations associated with COT67B that would be anticipated to result in adverse consequences to endangered species, unique geographic areas, environmental safety, public health and safety, quality of the human environment, genetic diversity of cotton, farmer or consumer choice, insect resistance or the economy, either within or outside the U.S. COT67B cotton plants produce a similar insectidical Cry1Ab protein as a number of other deregulated products and offers additional choice for protection from feeding damage caused by Lepidopteran pests. As such, COT67B is expected to produce the same beneficial effects as previously deregulated Bt cotton products also registered by the EPA as plant-incorporated protectants and which are commercially available. These benefits include additional grower choice, increased competition and extended useful life of Bt cotton technology generally. The information provided in the adverse consequences section addresses potential beneficial and adverse consequences of deregulation of COT67B cotton to show that the introduction of COT67B will not significantly affect the quality of the human environment. The direct, indirect or cumulative impacts of deregulation of COT67B is analyzed within the framework of the factors listed by the Council on Environmental Quality (CEQ) 40CFR 1508.27 (b).

Conclusion

In the past 10 years, transgenic insect resistant crop plants have proven to be an essential tool for agricultural integrated pest management programs. The technology allows the crop plant to deliver its own means of protection against insect attack, which results in an environmentally sound means to preserve yield while reducing chemical, mechanical and physical inputs. Additional environmental and consumer benefits may also be realized, including:

1. reducing risks associated with environmental spills or misapplications of chemical insecticides,
2. eliminating unwanted effects on beneficial insect populations (which are often susceptible to conventional chemical applications),
3. reducing the consumption of fossil fuels required to manufacture and apply chemical inputs, and
4. contributing to the availability of a more reliable, high-quality, and plentiful source of food, feed and fiber.

The data provided in this request demonstrate that COT67B does not represent a plant pest risk. Therefore, Syngenta requests that COT67B, and any progeny derived from crosses of COT67B with conventional cotton varieties, and any progeny derived from crosses of COT67B with transgenic cotton varieties that have also received a determination of nonregulated status, no longer be considered regulated under 7 CFR Part 340.6.

ABBREVIATIONS USED IN THIS PETITION

aadA	Adenylytransferase gene
Act2	Promoter isolated from *Arabidopsis* actin-2 gene
AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride HCl
Ala	Alanine
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service
APN	Aminopeptidase N
APH4	Selecable marker protein hygromycin B phosphotransferase
aph4	Gene encoding the selectable marker protein APH4
Arg	Arganine
Asp	Asparagine
BBMV	Brush border membrane vescicles
BC	Backcross generation; a population resulting from a cross of a hybrid with one of its parents
BCA	Bicinchoninic acid
Bis-Tris	bis[2-hydroxymethyl]imino-tris [hydroxymethyl]methane
Bp	Base pair
BRS	Biotechnology Regulatory Services
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki* strain HD-1
Bwt	Body weight
ca.	*circa* (approximately)
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CBW	Cotton bollworm
CEQ	Council on Environmental Quality
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
ColE1	*E.coli* origin of replication
CPFA	Cyclopropenoid fatty acids
Cry	Crystal protein delta endotoxins
Cys	Cysteine
DDD	Daily dietary dose

DF	Dilution factor
DIG	digoxigenin
DNA	Deoxyribonucleic acid
DT_{50}	Time to dissipation of 50% of the initial bioactivity
DTT	dithiothreitol
dwt	Dry weight
ECB	European corn borer
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EEC	Estimated exposure concentration
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EST	Expressed sequence tag
F1	First filial
FAO	Food and Agriculture Organization
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
flcry1Ab	Full-length *cry1*Ab gene
FLCry1Ab	Full-length Cry1Ab protein
fwt	Fresh weight
G ai	Grams of active ingredient
g, µg	Gram, microgram
gdw	Gram dry weight
gfw	Gram fresh weight
GLP	Good Laboratory Practices
Glu	Glutamine
Gly	Glycine
GSS	Genome survey sequence
HCl	Hydrochloric acid
His	Histidine
HPLC	High Performance Liquid Chromatography
hr	hours(s)
HRP	Horseradish peroxidase
HTGS	High throughput genome sequence
HVI	High Volume Instrumentation

H. virescens	*Heliothis virescens* (Hv)
H. zea	*Helicoverpa zea* (Hz)
IgG	Immunoglobulin G
ILSI	International Life Sciences Institute
IRM	Insect resistance management
Ile	Isoleucine
Kb	Kilobase
kDa	Kilo Dalton
LB	Left border
LC_{50}	50% lethal concentration
LC_{90}	90% lethal concentration
LD_{50}	50% lethal dose
Leu	Leucine
LOD	Limit of detection
LOQ	Level of quantitation
LSD	Least Significant Difference
Lys	Lysine
MAFF	Ministry of Agriculture, Forestry and Fisheries (Japan)
MES	Morpholinoethanesulfonoic acid
Met	Methionine
Mg ai	Milligrams of active ingredient
MHLW	Ministry of Health, Labour and Welfare (Japan)
MiliQ®	Millipore system Ultrapure Organex Cartridge
min	minute(s)
MOE	Margins of exposure
MOE	Ministry of Environment (Japan)
MW	Molecular weight
NEPA	National Environmental Policy Act
nm	namometer
NOAEC	No observable adverse effect concentration
NOEC	No observable effect concentration
NOEL	No observable effect level
nos	Nopaline synthase terminator
nptII	Gene encoding the neomycin phosphotransferase enzyme
NS	Not significant

NTO	Nontarget organism
OECD	Organization for Economic Cooperation and Development
OD	Optical density
ORFs	Open reading frames
PBS	Phosphate buffered saline
PBW	Pink bollworm
PCR	Polymerase chain reaction
pH	-log of hydrogen ion concentration
Phe	Phenylalanine
PIP	Plant-incorporated protectant
PM	Plant mapping
PPQ	Plant Protection and Quarantine
Pro	Proline
psi	Pounds per square inch
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
Q-TOF	Quadrupole time-of-flight mass spectrometer
RB	Right border
RepA	Bacterial replication protein
SBI	Syngenta Biotechnology, Inc.
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SG	Seed germination
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOP	Standard operating procedure
SP	Seed production
spec	Spectinomycin resistance gene
STS	Sequence tagged site
T0	First generation transgenic
T1	Second generation transgenic
TBST	Tris buffered saline with Tween 20
TBW	Tobacco budworm
T-DNA	Transfer DNA

Thr	Threonine
Ti-plasmid	Tumor inducing plasmid
TMB	3,3',5,5'-tetramethylbenzidine
TRIS	Tris(hydroxymethyl)aminomethane
Tween® 20	Polyoxyethylene sorbitanmonolaurate
Tyr	Tyrosine
Ubq3	Ubiquitin-3 gene isolated from Arabidopsis.
Ubq3int	Promoter plus the first intron isolated from *Arabidopsis* ubiquitin-3 gene.
USDA	United States Department of Agriculture
US EPA	United States Environmental Protection Agency
Val	Valine
VIPs	Vegetative insecticidal proteins
VS1	*Agrobacterium* origin of replication
w/w	weight/weight
WHO	World Health Organization
WLA	Winnsboro, Louisiana
XAD-4	A non-ionic polymeric adsorbent resin
x *g*	Times gravity

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CHAPTER 1. PETITION FOR A DETERMINATION OF NONREGULATED STATUS UNDER 7 CFR PART 340.6 FOR EVENT COT67B

A. Basis of the Request

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167), to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulation 7 CFR Part 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

B. Rationale for the Deregulation of COT67B

Cotton plant incorporated protectants based on *Bacillus thuringiensis* (Bt) endotoxin proteins have been widely adopted and were planted either alone or in conjunction with plant incorporated herbicide tolerance on 95.5% of cotton acres planted in the U.S. in 2006 (USDA AMS 2006). Three Bt Cry1 endotoxin protein-based cotton plant incorporated protectants are currently deregulated by the USDA and registered by the EPA under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA): Bollgard[2], Bollgard II[3] and WideStrike[4]. Bollgard produces only the Cry1Ac protein. Bollgard II and WideStrike are combinations of two Bt Cry proteins, Cry1Ac plus Cry2Ab and Cry1Ac plus Cry1F, respectively. These three products, of which Bollgard and Bollgard II represent more than 99% of the insect resistant cotton varieties sold in the US in 1996, provide essentially complete protection from the damage incurred by tobacco budworm. However, they may require supplemental insecticide applications for the control of cotton bollworm. Additional benefits of these transgenic varieties include: (1) reduced insecticide use; (2) reduced yield loss due to inadequate or poorly timed pest control; (3) the potential for fewer environmental spills or misapplications of chemical insecticides; (4) greater preservation of beneficial insect populations (which are often susceptible to conventional chemical applications); (5) less consumption of fossil fuels required to manufacture and apply chemical inputs; and (6) an overall contribution to the production of a more reliable and plentiful source of quality fiber and cottonseed products.

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[2] http://www.aphis.usda.gov/brs/aphisdocs2/94_30801p_com.pdf

[3] http://www.aphis.usda.gov/brs/aphisdocs2/00_34201p_com.pdf

[4] http://www.aphis.usda.gov/brs/aphisdocs2/03_03601p_com.pdf and http://www.aphis.usda.gov/brs/aphisdocs2/03_03602p_com.pdf

Despite such extensive adoption of transgenic cotton, the principal insect targets for these products, the cotton bollworm and tobacco budworm complex, remained the top pests in the U.S. In 2004 alone, these pests, of which 94% are cotton bollworm, infested 11 million acres and reduced production by *ca*. 380,000 bales or 109.1 million dollars ($288 per 480 pound bale) (Williams 2005a, b).

Event COT67B cotton (hereafter COT67B) produces a full-length Cry1Ab protein originally derived from the bacterium *Bacillus thuringiensis* subsp. *kurstaki* HD-1 (*B.t.k*). When expressed in COT67B, this protein provides excellent protection from the feeding damage incurred by several important lepidopteran pests of cotton, which include, but are not limited to, *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm) and *Trichoplusia ni* (cabbage looper). This event, alone or in combination through traditional breeding with other genetically-modified insect resistant cotton varieties will be a valuable addition to the panoply of pest management options available to U.S. cotton producers.

C. Submissions to Other Regulatory Agencies

A permanent tolerance exemption for Cry1Ab proteins in all crops was established by the EPA on August 2, 1996 and is published in the Code of Federal Regulations (40 CFR 180.1173). Thus, COT67B does not require further interaction with the Agency regarding tolerances. Syngenta Seeds, Inc. submitted a FIFRA Section 3 application for registration of a breeding stack of COT102 (Vip3A) x COT67B on December 15, 2006. Concurrent with the Section 3 application, Syngenta also petitioned the Agency for a permanent exemption from the requirement of a tolerance for Vip3A and the genetic material required for its production, under Federal Food, Drug and Cosmetic Act (FFDCA) Section 408.

Event COT67B falls within the scope of the Food and Drug Administration's (FDA) policy statement concerning regulation of products derived from new plant varieties, including those produced through genetic engineering (FDA, 1992). Syngenta voluntarily initiated and intends to complete a consultation process with FDA following their policy. A safety and nutritional assessment of food and feed derived from COT67B will be submitted to the FDA in June of 2007. Regulatory submissions for import and production clearances will be made to countries that import U.S. cottonseed and cottonseed products and have regulatory approval processes in place. These will include submissions to a number of foreign government regulatory agencies, including Japan's Ministries of Agriculture, Forestry and Fisheries (MAFF), Health, Labor and Welfare (MHLW), and Environment (MOE), as well as the Canadian Food Inspection Agency (CFIA) and Health Canada.

D. Regulatory Permit Status with USDA APHIS

Field trials of COT67B have been conducted in the US since 2003. The notifications were issued under the research event name of CE43-67. The protocols for these trials include field performance, agronomics, and generation of field materials and data necessary for this petition. In addition to the phenotypic assessment data provided for

COT67B, observational data on pest and disease stressors were collected from these product development trials. The majority of these final reports have been submitted to the USDA. A list of trials conducted under USDA notification and the status of the final reports for these trials are provided in Table 1-1.

Table 1-1. USDA Notifications Approved for COT67B and Status of Trials Conducted Under These Notifications

USDA No.	Effective Dates	Release Site (State)	Trial Status
2003 Field Trials			
03-098-08n	5/8/2003	MS	Report Submitted to USDA July 28, 2004
03-268-04n	10/25/2003	PR	Report Submitted to USDA September 21, 2004
2004 Field Trials			
04-041-01n	3/26/2004	AR, FL, GA, LA, MS, NC, TN	Report Submitted to USDA August 24, 2005
04-064-05n	4/8/2004	AZ	Report Submitted to USDA October 12, 2005
04-079-01n	4/18/2004	TX	Report Submitted to USDA August 9, 2005
2005 Field Trials			
05-034-02n	3/28/2005	AL, AR, AZ, GA, LA, MO, MS, NC, SC, TX	Report Submitted to USDA October 30, 2006
05-102-01n	5/17/2005	CA	Report Submitted to USDA October 30, 2006
05-266-01n	11/7/2005	HI	In Progress
2006 Field Trials			
05-339-04n	1/4/2006	MS, PR	Report Submitted to USDA February 20, 2007
06-039-16n	3/14/2006	AL, AR, AZ, CA, FL, GA, LA, MO, MS, NC, SC, TN, TX	In Progress
06-060-04n	5/2/2006	NC, TX	In Progress
06-223-109n	10/26/2006	HI	In Progress
06-223-110n	10/26/2006	HI	In Progress

CHAPTER 2. THE COTTON FAMILY

The following was excerpted from the USDA Agricultural Biotechnology website (http://www.aphis.usda.gov/brs/cotton.html) This website provided the basis of information and was supplemented by Syngenta.

A. Cotton as a Crop

Four species of the genus *Gossypium* are known as cotton, which is grown primarily for the seed hairs that are made into textiles. Cotton is predominant as a textile fiber because the mature dry hairs twist in such a way that they can be spun into fine, strong threads. Other products, such as cottonseed oil, meal, and cotton linters are by-products of fiber production. Cotton, a perennial plant cultivated as an annual, is grown in the United States mostly in areas from Virginia southward and westward to California in an area often referred to as the Cotton Belt (McGregor 1976). Cotton varieties are classified as either New World or Old World depending on the derivation of the genomes. New World cotton production is the most agriculturally significant of the two types (see part C. below).

B. Taxonomy of Cotton

The genus *Gossypium*, a member of the Malvaceae, consists of 39 species, 4 of which are generally cultivated (Fryxell 1984). The most commonly cultivated species is *G. hirsutum* L. Other cultivated species are *G. arboreum L.*, *G. barbadense* L., and *G. herbaceum* L. Four species of *Gossypium* occur in the United States (Fryxell 1979, Kartesz and Kartesz 1980). *G. hirsutum* is the primary cultivated cotton. *G. barbadense* is also cultivated. The other two species, *G. thurberi Todaro and G. tomentosum* Nuttall ex Seemann, are wild plants of Arizona and Hawaii, respectively. *G. tomentosum* is known from a few isolated locations very close to the ocean.

C. Genetics of Cotton

At least seven complete sets of genes, designated A, B, C, D, E, F, and G, are found in the genus (Endrizzi 1984). Diploid species (2n=26) are found on all continents, and a few are of some agricultural importance. The A genome is restricted in diploids to two species (*G. arboreum* and *G. herbaceum*) of the Old World. The D genome is restricted in diploids to some species of the New World, such as *G. thurberi*. By far the most important agricultural cottons are *G. hirsutum* and *G. barbadense*. These are both allotetraploids of New World origin and presumably resulted from an ancient cross between Old World A genomes and New World D genomes. How and when the original crosses occurred is speculative. Euploids of these plants have 52 somatic chromosomes and are frequently designated as AADD. Four additional New World allotetraploids occur in the genus, including *G. tomentosum*, the native of Hawaii. *G. tomentosum* has been crossed with *G. hirsutum* in breeding programs. The New World allotetraploids are peculiar in the genus because the species, at least in their wild forms, grow near the ocean as invaders in the constantly disturbed habitats of

strand and associated environs. It is from these "weedy" or invader species that the cultivated cottons developed (Fryxell 1979).

D. Pollination of Cotton

Gossypium hirsutum is generally self-pollinating but, in the presence of suitable insect pollinators, it can cross-pollinate. Bumble bees (*Bombus spp*.), Black bees, (*Melissodes spp*.), and honey bees (*Apis mellifera*) are the primary pollinators (McGregor 1976). Concentration of suitable pollinators varies from location to location and by season, and is considerably suppressed by herbicide use. If suitable bee pollinators are present, distribution of pollen decreases considerably with increasing distance. McGregor (1976) reported results from an experiment in which a cotton field was surrounded by a large number of honeybee colonies, and movement of pollen was traced by means of fluorescent particles. At 150 to 200 feet from the source plants, 1.6 percent of the flowers showed the presence of the particles. The isolation distance for Foundation, Registered, and Certified seeds in 7 CFR Part 201 are 1,320, 1,320, and 660 feet, respectively. Unlike *G. hirsutum*, *G. tomentosum* seems to be pollinated by lepidopterans, presumably moths (Fryxell 1979). The stigma in *G. tomentosum* is elongated so that the plant seems incapable of self-pollination until acted upon by an insect pollinator. The flowers are unusual, too, because they stay open at night; most *Gossypium* flowers are ephemeral they open in the morning and wither at the end of the same day.

E. Weediness of Cotton

Although the New World allotetraploids show some tendencies to "weediness" (Fryxell 1979), the genus shows no aggressive, weedy tendencies in the South. Cotton is a poor competitor in most of the southern U.S. cotton-growing regions and is not allowed to overwinter. In more northerly areas, where freezing conditions occur, the cotton plant cannot overwinter, and there is essentially no volunteerism from seed.

EPA recently concluded its environmental reassessment of the registered Bt plant-incorporated protectants and summarized its findings in the "Biopesticides Registration Action Document" for these products (US EPA, 2001). In its reassessment of Bt cotton, EPA reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories, and concluded that the possibility for gene transfer exists only in limited geographic locations where wild or feral cotton relatives exist, *i.e.* in Florida, Hawaii, and the Caribbean.

The USDA/APHIS has made this same determination under its statutory authority in deregulating Bt insect resistant cotton varities such as Bollgard, Bollgard II, WideStrike and COT102[5]. The USDA has stated that "APHIS notes that there have

[5] http://www.aphis.usda.gov/brs/aphisdocs2/03_15501p_com.pdf

been no reports of increased weediness associated with other lepidotperan insect resistant cotton lines that have been granted nonregulated status" (USDA, 2005). In fact, a comparison of environmental impacts of biotechnology derived and traditional cotton crops has not identified weediness associated with insect resistant cotton lines being grown in the U.S. (Carpenter et al., 2002). Further, weediness is a multigenic trait with attributes mostly pertaining to sexual or asexual reproductive advantage within natural or agricultural ecosystems (Baker, 1965). Lepidopteran resistant FLCry1Ab cotton is expected to be cultivated in a managed agroecosystem; the likelihood of sufficient selection pressure for this single gene trait to result in expression of weediness is remote. These conclusions, made with respect to previously deregulated cotton varieties, are also applicable to COT67B. Accordingly, the same geographical restrictions imposed by EPA (*e.g.,* no commercial plantings in Hawaii and south Florida) and currently in effect for Bollgard, Bollgard II or WideStrike cotton varieties are expected to apply to COT67B varieties.

F. Modes of Gene Escape in Cotton

Genetic material of *G. hirsutum* may escape from a planting site by vegetative material, by seed, or by pollen. Vegetative propagation is not a common mechanism by which cotton reproduces. Movement of genetic material by pollen is possible only to those plants with the proper chromosomal type, in this instance only to those allotetraploids with AADD genomes. In the United States, this group would include only the cultivated species *G. hirsutum*, *G. barbadense*, and the wild species *G. tomentosum*. *G. thurberi*, the native diploid from Arizona with a DD genome, is not a suitable recipient. Movement to *G. hirsutum* and *G. barbadense* is possible if suitable insect pollinators are present and if there is a short distance from transgenic plants to recipient plants. Physical barriers, intermediate pollinator-attractive plants, and other temporal or biological impediments would reduce the potential for pollen movement. Movement of genetic material to *G. tomentosum* is less well documented. The plants are chromosomally compatible with *G. hirsutum*, but there is some doubt as to the possibility for pollination. The flowers of *G. tomentosum* seem to be pollinated by moths, not bees, and the flowers are receptive at night, not in the day. Both these factors would seem to minimize the possibility of cross-pollination. However, Fryxell (1979) reports that *G. tomentosum* may be losing its genetic identity from introgression hybridization of cultivated cottons by unknown means.

G. Characteristics of the Non-transformed Cultivar

COT67B was produced via disarmed non-pathogenic *Agrobacterium tumefasciens* transformation of the parental cotton cultivar Coker 312 (*G. hirsutum*). This cotton line was released by the Coker Pedigreed Seed Company in 1974, and the variety is currently owned by the SeedCo Corporation of Lubbock, TX. Coker 312 is quite

amenable to modern tissue culture techniques and is considered desirable for molecular transformation purposes. Coker 312 is not widely planted and it is still considered a viable commercial cultivar and, therefore, an acceptable genetic background for the purposes of agronomic performance evaluations.

H. Cotton as a Test System in this Petition

Appropriate test and control materials were evaluated in developing the data to support this petition.

In general, the genetic background of the test material was matched with that of the control material so the effect of the genetic insert could be assessed in an unbiased manner. Where feasible, positive (COT67B) and negative (COT67B(-)) isolines were used as test and control materials, respectively. The nontransgenic cotton cultivar Coker 312, which was transformed to develop COT67B, is also included as a comparator in many evaluations. Descriptions of the test, control, and reference materials are provided in the methods and materials sections for each experiment.

CHAPTER 3. IDENTIFICATION AND CHARACTERIZATION OF THE INTRODUCED GENETIC MATERIAL IN COT67B

A. Introduction

This chapter is comprised of data and information describing the molecular analyses of the introduced genetic material in COT67B. The chapter begins with a description of the transformation method followed by a detailed molecular and genetic assessment. Southern analyses with probes for transgenes, promoters and backbone sequences of the transformation vectors, and sequencing of the T-DNA insert and flanking 5' and 3' regions were used to characterize COT67B. Stability of the transgenes was assessed by Mendelian inheritance analysis. The genetic and regulatory elements are expected to function as intended to encode only those proteins necessary to confer insect resistance to COT67B. The production of other proteins which might harm cotton, other plants or the environment is highly unlikely. The genetic analyses together with the results from protein characterization studies (Chapter 4) provide a basis to assess possible direct or indirect consequences of the genetic modification which resulted in COT67B.

In summary, data from Southern analyses demonstrated that:

1. COT67B contains a single intact T-DNA insert,
2. A single copy of the full-length *cry1Ab* gene (*flcry1Ab*) and a single copy of the ACT2 promoter are present in the T-DNA insert in COT67B,
3. COT67B does not contain the selectable marker gene, hygromycin B phosphotransferase (*aph4*), or the Ubq3 promoter sequence from the transformation plasmid pNOV1914,
4. COT67B does not contain any of the backbone sequences from the transformation plasmids pNOV4641 or pNOV1914, and
5. The insert is stable over several generations of COT67B.

Sequence analysis of the entire T-DNA insert present in COT67B:

1. Confirmed the overall integrity of the insert and that contiguousness of the functional elements has been maintained, and
2. Revealed that some truncation occurred at the right and left border ends of the T-DNA during the transformation process; these deletions are commonly associated with *Agrobacterium* transformation and do not affect the functionality of the T-DNA itself.

Sequence analysis of the cotton genome sequences flanking the T-DNA insert:

1. Demonstrated that the COT67B T-DNA insert does not disrupt any known endogenous cotton gene, and

2. Revealed no novel open reading frames spanning either the 5' or 3' junctions between the COT67B T-DNA insert and the cotton genomic sequence.

Lastly, statistical analysis of segregation patterns over several generations of COT67B confirmed the expected Mendelian inheritance ratio for *flcry1Ab* demonstrating that the transgenic locus in COT67B is inherited across generations in an expected manner as a single locus dominant trait.

B. Event COT67B

Syngenta's COT67B was produced via disarmed non-pathogenic *Agrobacterium tumefasciens* transformation of the parental cotton cultivar Coker 312 (*Gossypium hirsutum*). COT67B contains a full-length c*ry1Ab* gene (*flcry1Ab*, 3546bp) which encodes a full-length crystal protein comprising 1181 amino acids (Appendix 1-Figure 1). Full-length c*ry1Ab* in COT67B was originally derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (*Btk*). The native or wild-type *cry1Ab* gene is a product of the genetic recombination of the *B. thuringiensis kurstaki* HD-1 genes, *cry1Aa* and *cry1Ac*. It is well documented that mutations resulting in deletions of stretches of DNA are common during genetic recombination. Such was the case for the native *cry1Ab* gene (Geiser *et al*., 1986). This loss of DNA resulted in a diminished capacity of native *cry1Ab* to encode Cry1Ab protein in fermentative cultures of *B. thuringiensis* under the customary fermentation temperatures. It was subsequently discovered that this inefficiency was attributable to the absence of 26 amino acids encoded by the stretch of DNA lost as a result of recombination. Syngenta "repaired" the gene by replacing the deleted coding region with the functional region from *cry1Aa*, thus restoring the original fermentative properties of *cry1Ab*. The additional 26 amino acids in the C-terminal portion of the FLCry1Ab protein are referred to as the 'Geiser motif' (Geiser, 1986, 1991; Koziel, *et al.* 1997) and are encoded by the "full-length" cry1Ab (*flcry1Ab)* gene. This "full-length" version of the gene was used in transformation to produce COT67B and is referred to as full-length *cry1Ab*.

Full-length *cry1Ab* in COT67B produces the same full-length Cry1Ab protein as that produced by *Btk*, except for the 26 amino acids in the C-terminal portion of the protein responsible for the efficient production of Cry1Ab protein in fermentative cultures of Bt. The additional amino acid sequence has no apparent functionality in plants, and is not contained in the region of the protein responsible for insecticidal activity. The nucleotide sequence of *flcry1Ab* has been codon optimized for enhanced expression in plants. The expression of the *flcry1Ab* gene is driven by the constitutive promoter Act2 from *Arabidopsis thaliana.* COT67B does not contain a selectable marker gene nor any backbone sequences of the selectable marker plasmid as only those plants identified as having lost the *aph4* marker gene through segregation were used for commercial development. A complete description of the FLCry1Ab protein is found in Chapter 4.

C. Description of the Transformation System and Method

COT67B was produced through the introduction of DNA into petioles of *Gossypium hirsutum* L. cv. Coker 312 *via Agrobacterium tumefaciens*-mediated transformation technology. Using this method, genetic elements contained between the left and right border regions of the transformation plasmid are efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions are generally not transferred. The insecticide gene (*flcry1Ab*) and the selectable marker gene (*aph4*) were delivered *via* separate transfer DNA (T-DNA) inserts as separate constructs, pNOV4641 (*flcry1Ab*) and pNOV1914 (*aph4*), respectively (Figures 3-1 and 3-2). Both T-DNA's were derived from disarmed strains of *A. tumifaciens* and lack the phytohormone genes that cause crown gall disease. Descriptions of the genetic elements comprising both plasmids are outlined in detail in Section D of this chapter. Donor Genes and Regulatory Sequences. After self-pollination of the initial transformed plant (the T0 plant) and during production of T1 seed (first generation of transgenic seed), the insect-resistant and selectable marker traits segregated according to Mendelian genetics. TaqMan® PCR (Ingham *et al.*, 2001) was used to identify those T1 plants containing the *flcry1Ab* gene but lacking the *aph4* gene. COT67B was identified in this manner and produces only the FLCry1Ab protein; it no longer contains the selectable marker gene. A detailed description of the transformation methodology follows:

Surface-sterilized petioles from three to five week old plants were pre-cultured for several days prior to transformation *via A. tumefaciens* strain GV3101 harboring plasmids pNOV4641 and pNOV1914. The *Agrobaterium* inoculation solution was applied to the petioles for five to ten minutes, after which they were incubated at 24ºC for 48 hours on co-culture plates. The petioles were then transferred to culture medium supplemented with cefotaxime (500 mg/L) to clear the *A. tumefaciens* from the plant tissue. Hygromycin (10 mg/L) was added to the culture medium as a seletion agent to facilitate the growth and identification of plant tissue carrying only the introduced genes. The explants were transferred to fresh culture medium on a regular basis until callus tissue formed. When calli were of a size deemed capable of continued growth, they were dissected away from the original explant and transferred to fresh growth medium supplemented with cefotaxime and hygromycin. After several more rounds of subculture to fresh growth medium the surviving callus tissue was broken into small pieces and placed into a liquid culture system until small white slightly round cell clusters were visible. These clusters indicated the tissue was embryogenic. The embryogenic suspension culture cells were plated onto solid growth medium until embryoids large enough to germinate into plantlets had formed. The embryoids were placed onto semi-solid regeneration medium and cultured until true leaves had formed. The plantlets were transferred to flasks containing hydrated peat plugs until they grew large enough to be transplanted into the glasshouse.

Plants carrying the intended genes were identified by TaqMan® PCR as noted above. They were screened for insect resistance and field performance. Of the many transformation events screened, COT67B was selected as the leading commercial candidate. Regulatory studies on COT67B were initiated to establish that it was

substantially similar to conventional cotton and to demonstrate the human health and environmental safety of the FLCry1Ab protein. A flow chart of the major steps involved in the transformation, selection, and development of COT67B is shown in Figure 3-3.

Figure 3-1. Plasmid map of pNOV4641 indicating the restriction enzyme sites used for Southern analyses

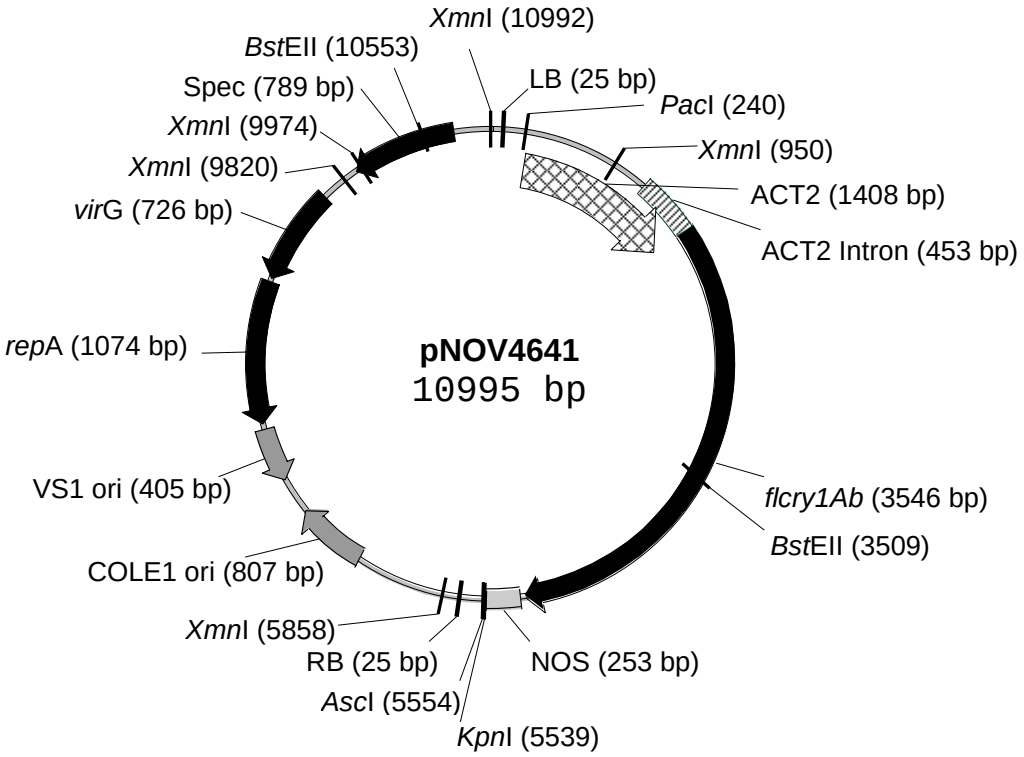


Figure 3-2. Plasmid map of pNOV1914 indicating the restriction enzyme sites used for Southern analyses

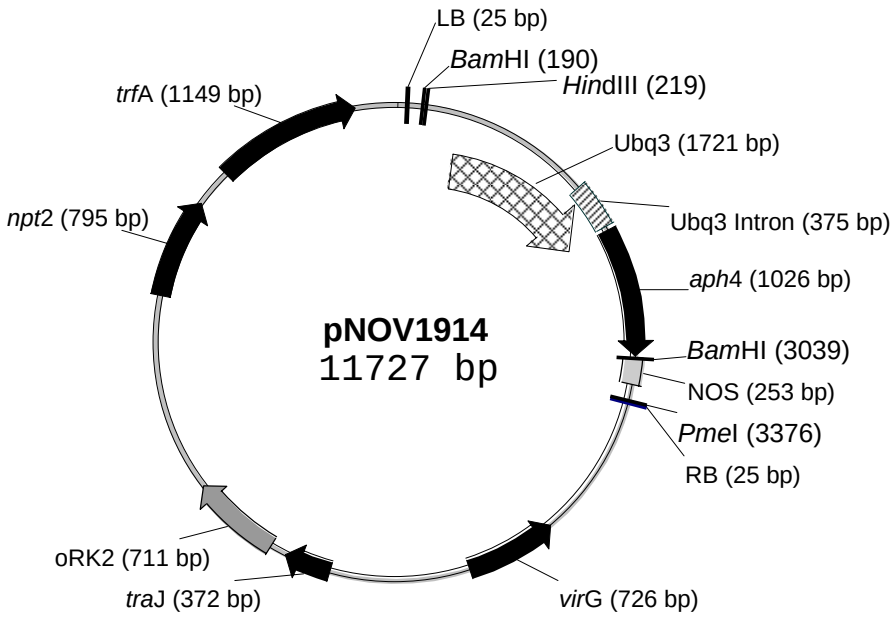
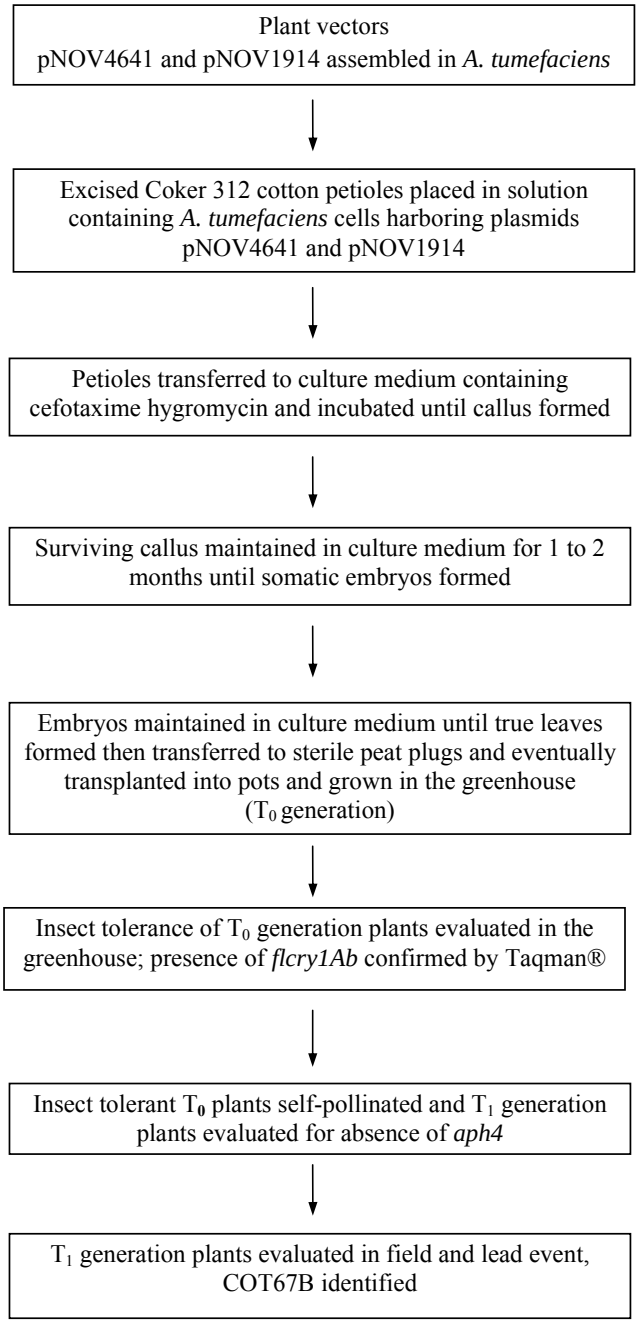


Figure 3-3. Development and selection of Event COT67B



D. The Donor Genes and Regulatory Sequences

This section describes the donor genes and regulatory sequences in constructs pNOV4641 and pNOV1914. Construct pNOV4641 (Table 3-1) carries the *flcry1Ab* insecticide gene driven by the Act2 constitutive promoter. Construct pNOV1914 (Table 3-2) carries the selectable marker gene, *aph4*, which encodes a protein conferring hygromycin B phosphotransferase resistance and is driven by the Ubq3 constitutive promoter.

Table 3-1. Active Ingredient Cassette and Plasmid Backbone Components of Plasmid pNOV4641

Genetic Element	Location in pNOV4641 (bp)	Size (bp)	Function
ACTIVE INGREDIENT CASSETTE			
Act2	267 - 1674	1408	Promoter region from the actin-2 gene of *Arabidopsis thaliana* and its intron (An *et al.,* 1996). It promotes expression of the *flcry1Ab* gene.
flcry1Ab	1684 - 5230	3546	The full-length cry1Ab gene encodes a FLCry1Ab protein identical to Cry1Ab protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, except for an additional 26 amino acids (described as the 'Geiser motif') in the C-terminal portion of the protein (Geiser et al., 1986) and its nucleotide sequence has been codon optimized (Koziel *et al*., 1997). Cry1Ab is insecticidal against certain lepidopteran species.
NOS	5273 - 5525	253	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez Accession Number V00087 (NCBI, 2007)). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).
PLASMID BACKBONE COMPONENTS			
LB (left border)	71 - 95	25	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez Accession Number J01825 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).
spec	9928 - 10716	789	Streptomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (Entrez Accession Number X03043 (NCBI, 2007)). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker (Fling *et al*., 1985).

Table 3-1 Continued

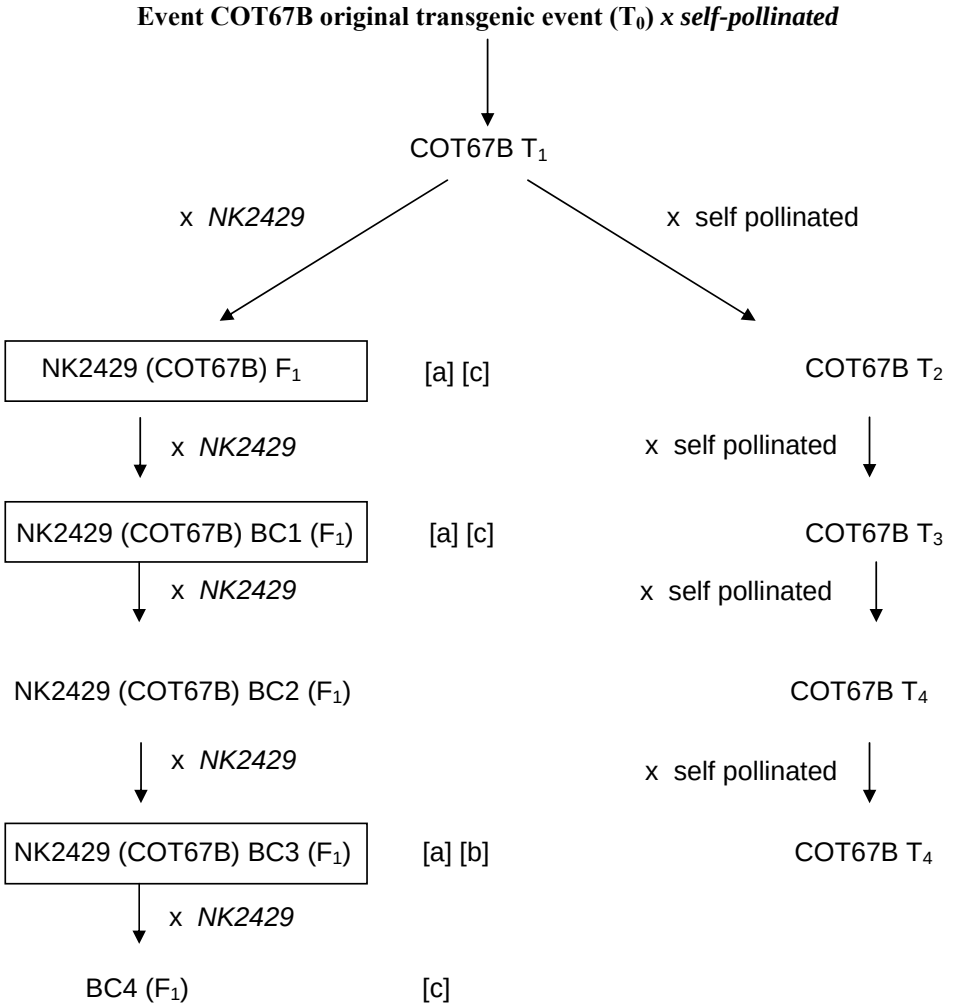
Genetic Element	Location in pNOV4641 (bp)	Size (bp)	Function
virG	8903 - 9628	726	VirGN54D from pAD1289 (similar to Entrez Accession Number AF242881 (NCBI, 2007)). The N54D substitution results in a constitutive virG phenotype. virG is part of the two-component regulatory system for the vir regulon in *Agrobacterium* (Hansen *et al.*, 1994).
repA	7800 - 8873	1074	pVS1 replication protein from Pseudomonas, which is a part of the minimal pVS1 replicon that is functional in gram-negative plant associated bacteria (Entrez Accession Number AF133831 NCBI, 2007) (Heeb et al., 2000).
VS1ori	7353 - 7757	405	Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of Pseudomonas (similar to Entrez Accession Number U10487 (NCBI, 2007)). Serves as origin of replication in Agrobacterium tumefaciens host (Itoh et al., 1984).
ColE1ori	5869 - 6675	807	Origin of replication that permits replication of plasmid in E. coli. (similar to Entrez Accession Number V00268 (NCBI, 2007)) (Itoh and Tomizawa, 1978).
RB (right border)	5732 - 5756	25	Right border region of T-DNA from Agrobacterium tumefaciens nopaline Ti-plasmid (Entrez Accession number J01826 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang et al., 1984).

Table 3-2. Selectable marker cassette and plasmid backbone components of plasmid pNOV1914

Genetic Element	Location in pNOV1914 (bp)	Size (bp)	Function
SELECTABLE MARKER CASSETTE			
Ubq3	245 - 1965	1721	Promoter region plus the first intron from the ubiquitin 3 (ubi3) of *Arabidopsis thaliana* (Norris *et al.*, 1993).
aph4	1997 - 3022	1026	The *aph4* gene encodes a phosphotransferase enzyme (hygromycin B phosphotransferase; an aminocyclitol phosphotransferase) that catalyzes the phosphorylation of hygromycin and some related aminoglycosides (Waldron, 1997). The *aph4* gene, when transformed into some plant cells, enables the transformed cells to grow in the presence of the selection agent hygromycin.
NOS	3056 - 3308	253	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Enterz Accession Number V00087 (NCBI, 2007)). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).
PLASMID BACKBONE COMPONENTS			
LB (left border)	71 - 95	25	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01825, (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).
trfA	10262 - 11410	1149	Encodes the replication initiation protein essential for plasmid replication (Smith and Thomas, 1984).
npt2	9169 - 9963	795	5' region from the *Streptococcus faecalis* gene encoding the 3'5'-aminoglycoside phosphotransferase type III conferring kanamycin resistance (Trieu-Cuot and Courvalin, 1983).
oRK2	6880 – 7590	711	Region covering the origin of replication oriV of plasmid RK2 (Stalker *et al*., 1981)
traJ	6378 – 6749	372	Encodes the relaxosome protein for plasmid replication (Guiney and Yakobsen, 1983).
virG	4543 - 5268	726	VirGN54D from pAD1289 (similar to Entrez Accession Number AF242881 (NCBI, 2007)). The N54D substitution results in a constitutive virG phenotype. virG is part of the two-component regulatory system for the vir regulon in *Agrobacterium* (Hansen *et al*., 1994).
RB (right border)	3390 - 3414	25	Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01826 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*., 1984).

Figure 3-4: Breeding pedigree indicating the generations tested in the molecular and protein analysis of COT67B

Plant material from the F_1, BC1(F_1)and BC3(F_1) generations were used for the Mendelian inheritance study and the generational stability Southern analysis. BC3(F_1) material was used for T-DNA insert sequencing and Southern analysis for copy number of functional elements. The negative control consisted of ten pooled negative segregant plants from the BC3(F_1) generation.



[a] Materials used for Mendelian inheritance and generational stability Southern analyses
[b] Materials used for all Southern analysis and sequencing
[c] Materials used for protein generational stability analysis

E. Genetic Analysis of Event COT67B

E.1. Introduction

Molecular studies were conducted to demonstrate that the transgenes in COT67B had integrated into the cotton genome in the predicted manner. The T-DNA insert and the surrounding integration site were characterized to confirm that no changes to the cotton genome other than those intended had occurred as a result of gene delivery and integration. The behavior of the genes over successive generations was analyzed to verify stability of the transgenic insert. The genetic elements of pNOV4641 and pNOV1914 were analyzed by sequence and Southern analyses and Mendelian inheritance studies. Detailed materials and methods are found in Appendix 1.

Sequence analysis of DNA from COT67B plants was used to assess the following:

1. intactness of the T-DNA insert,
2. contiguousness of the functional elements within the insert,
3. the presence of any rearrangements, deletions and/or basepair changes within the insert,
4. whether the T-DNA insertion occurred in a known functional gene of cotton, and
5. whether novel open reading frames (ORFs) occurred at the junctions of the T-DNA insert and the genome of *G. hirsutum*.

Southern analyses using DNA from COT67B plants were used to assess the following:

1. number of T-DNA insertions (number of integration sites within the cotton genome),
2. copy number of functional elements (number of each element integrated within one insertion site),
3. the presence or absence of plasmid backbone sequences,
4. the presence or absence of the Ubq3 promoter and *aph4* gene sequences, and
5. stability of the inserted T-DNA during conventional breeding.

Taqman® PCR genotyping followed by Chi square analysis was used to assess the mode of inheritance of the insert.

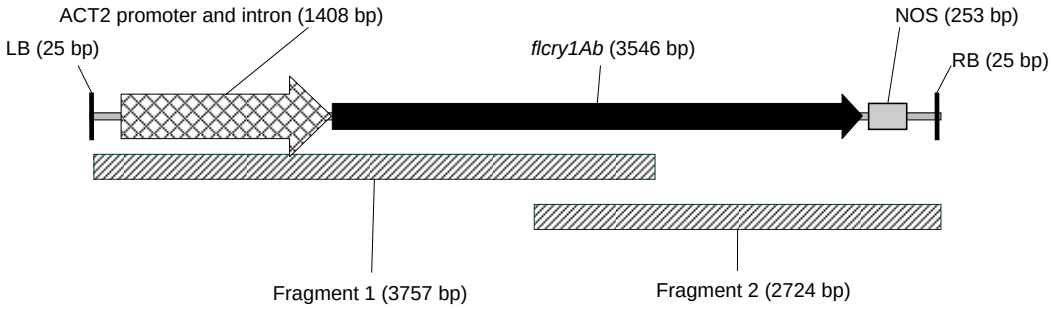
E.2. T-DNA Insert Sequencing

The COT67B insert was amplified from DNA derived from the BC3(F_1) generation (Figure 3-4) as two individual overlapping fragments (Figure 3-5). PCR-amplified products were cloned into a TOPO plasmid and three separate clones for each PCR product were identified and sequenced. Sequence analysis was done using the Phred,

Phrap, and Consed package from the University of Washington and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998). The final consensus sequence was determined by combining the sequence data from the six individual clones (three for each PCR product) to generate one consensus sequence of the COT67B insert. Sequence alignment was performed using the ClustalW program described in Thompson *et al.*, 1994.

The consensus sequence data for the entire COT67B T-DNA insert demonstrates the overall integrity of the insert and that contiguousness of the functional elements within the insert as intended in pNOV4641 has been maintained (Appendix 1-Figure 4). The left border (LB) and the adjacent 13 bp of the insert along with 24 bp of the right border (RB) were deleted during insertion of the T-DNA. However, such deletions are common in *A. tumefaciens*-mediated transformation and do not affect the functionality of the T-DNA itself (Tinland and Hohn, 1995).

Figure 3-5. Locations of PCR-amplified fragments from COT67B to determine insert sequence



E.3. Flanking Sequence

Genomic *G. hirsutum* sequences flanking the 5' (Appendix 1-Figure 2) and 3' (Appendix 1-Figure 3) regions of the COT67B T-DNA insert were screened for homology with sequences found in public databases. BLAST analysis was performed using the BLASTN program (Altschul *et al.*, 1997) (version 2.2.6, April 9, 2003) to compare the genomic sequences with sequences in the latest version of the National Center for Biotechnology Information (NCBI) nr (non-redundant) database (NCBI, 2007). The nr database contains all sequences from the National Institutes of Health genetic sequence database (GenBank®), RefSeq Nucleotides, the European Molecular Biology Laboratory (EMBL), the DNA Database of Japan (DDBJ) and sequences derived from the three-dimensional structure (PDB) database. Whereas GenBank is an archival repository of all sequences, the RefSeq database is a non-redundant set of reference standards that includes chromosomes, complete genomic molecules (organelle genomes, viruses, and plasmids), intermediate assembled genomic contigs, curated genomic regions, mRNAs, RNAs, and proteins. The nr database does not contain any EST, STS, GSS, or phase 0, 1, or 2 HTGS sequences. At the time of this analysis, the nr database contained 4,746,158

unique sequences and was last updated on January 12, 2007. The query sequence was not filtered for low complexity. Parameters for the BLASTN analysis were as follows:

- Expect = 10. The Expect value (E-value) is the significance threshold for reporting matches against database sequences; the default value is 10, meaning that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). Those results for which the Expect value is close to 0 will be unlikely to have occurred at random and, therefore, will be statistically significant hits. Those hits with E-values close to 10 could have happened randomly and are, therefore, not statistically significant.
- The scoring scheme (bits) used is the default for nucleotides: +1 for a match and –3 for a mismatch. Gap penalties: Existence = 5, Extension = 2. A gap is a space introduced into an alignment to compensate for insertions and deletions in one sequence relative to another. Gap existence and extension penalties are chosen empirically. To prevent the accumulation of excessive gaps in an alignment, the introduction of a gap causes the deduction of a fixed amount from the alignment score. Extension of the gap to encompass additional nucleotides is also penalized in determining the score of an alignment. The resultant score is derived from the number of identical matches between the query sequence and the database entry, with higher scores indicating more homology between the two sequences.

To ascertain if any putative novel open reading frames (ORFs) were generated at the point of insertion in the *G. hirsutum* genome, the junctions between genomic sequences and the COT67B T-DNA insert were examined for the presence of ORFs using Vector NTI® (version 9.0) software from InforMax. An ORF was defined in the bioinformatic program as a region corresponding to at least fifty amino acids in length initiating with an ATG codon and ending with any of the three stop codons: TAA, TAG or TGA. All six possible reading frames at both the 5' and 3' regions were examined using the above criteria.

E.3.a. Results

BLASTN analysis of the cotton genomic sequence flanking the 5' region (Appendix 1-Figure 2) of the COT67B T-DNA insert shows homology (79% over 86 bp) to sequence defined as a genomic microsatellite locus from *G. hirsutum* [Entrez Accession Number DQ908605 (NCBI, 2007)]. BLASTN analysis of the cotton genomic sequence flanking the 3' region (Appendix 1-Figure 3) of the COT67B T-DNA insert also shows homology to two sequences defined as genomic microsatellite loci from *G. hirsutum* [Entrez Accession Numbers DQ908555 and AF351368 (NCBI, 2007)]. Both DQ908555 and AF351368 have homology in two areas of the 3' region (81% over 292 bp and 84% over 160 bp; and 80% over 169 bp and 84% over 166 bp, respectively). The 3' region also shows some homology to an unknown chloroplast sequence [Entrez Accession Number AF497429 (NCBI, 2007)] (80% over 160 bp) that is located 190 bp from the COT67B T-DNA insert sequence. The presence of organelle sequences in the nuclear genome is not

without precedent as this observation has been made previously in several conventional (non-GM) plant species (Figueroa *et al*., 1999a and 1999b; Fukuchi *et al*., 1991; Goff *et al*., 2002; Kemble *et al*., 1983) and is unlikely to adversely impact plant growth and development. Thus, the results confirm that introduction of the T-DNA insert into COT67B was as intended, and no disruption of endogenous cotton genes occurred.

Open reading frame analysis of all six potential reading frames at both the 5' and 3' T-DNA to cotton genome junctions did not detect the presence of any putative novel ORFs of 50 amino acids or greater. Consequently, there is no reason to expect novel proteins to be produced as a result of T-DNA integration.

E.4. Mendelian Inheritance of Transgene Insert

The inheritance pattern of the T-DNA insert derived from pNOV4641 in COT67B was investigated. The initial COT67B plant (T_0 generation) was self pollinated to create the T_1 generation. Homozygous plants from this generation were then crossed with the recurrent parent, inbred cotton line NK2429, creating the F_1 generation. NK2429 (COT67B) plants from this F_1 generation were then crossed into NK2429 again creating the BC1(F_1) (backcross 1) generation. This process was carried out twice more yielding the BC2(F1) and BC3(F_1) generations. Material from the F_1, BC1(F_1) and BC3(F_1) generations (Figure 3-4) was used in the Mendelian inheritance study.

Individual plants from each generation were assayed by TaqMan® PCR which confirmed the presence or absence of the *flcry1Ab* gene. The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait in BC1(F_1) and BC3(F_1) populations is 1:1. All progeny of the F_1 generation are expected to be positive for the trait. Genotypic data were used to assess the goodness-of-fit of the observed genotypic ratio to the expected genotypic ratio using Chi Square (X^2) analysis with Yates correction factor (Strickberger, 1976).

$$\chi^2 = \sum (O - E - 0.5)^2 / E$$

The critical value to reject a hypothesis that the *flcry1Ab* gene present in the COT67B T-DNA insert is segregating in a Mendelian fashion at the 5% level of probability using a Chi-square analysis is 3.84 (Strickberger, 1976). Since the Chi-squared value is less than 3.84 (Table 3-3), the hypothesis that the genetic trait is behaving in a Mendelian fashion is accepted for all COT67B generations examined. As expected all plants in the F_1 generation were positive for the genetic trait while in the BC1(F_1) and BC3(F_1) generations, the trait segregated in a 1:1 ratio.

Table 3-3. Observed (O) *vs.* Expected (E) genotype for *flcry1Ab* for multiple COT67B generations as determined by TaqMan® PCR analysis

Trait	F_1		$BC1(F_1)$		$BC3(F_1)$	
	O	**E**	**O**	**E**	**O**	**E**
Positive	23	23	25	27.5	20	22.5
Negative	0	0	30	27.5	25	22.5
Total	23	23	55	55	45	45
X^2 value	All plants positive for *flcry1Ab*		0.291		0.356	

E.5. Southern Analysis for Functional Element Copy Number

The copy number of the functional genetic elements introduced into COT67B from constructs pNOV4641 and pNOV1914 and the stability of the T-DNA insert were determined by Southern blot analyses. This section describes the test material and methods used to determine the number of copies of the *flcry1Ab* gene, ACT2 promoter, and the Ubq3-*aph4* selectable marker complex present per genome in COT67B. Maps showing the locations of the restriction sites within the T-DNA insert and the probes used for the Southern analyses are included (See Figures 3-7, 9, 11, 13, 15 and 17). The results demonstrate that the intended genetic material stably integrated into the genome of COT67B as expected.

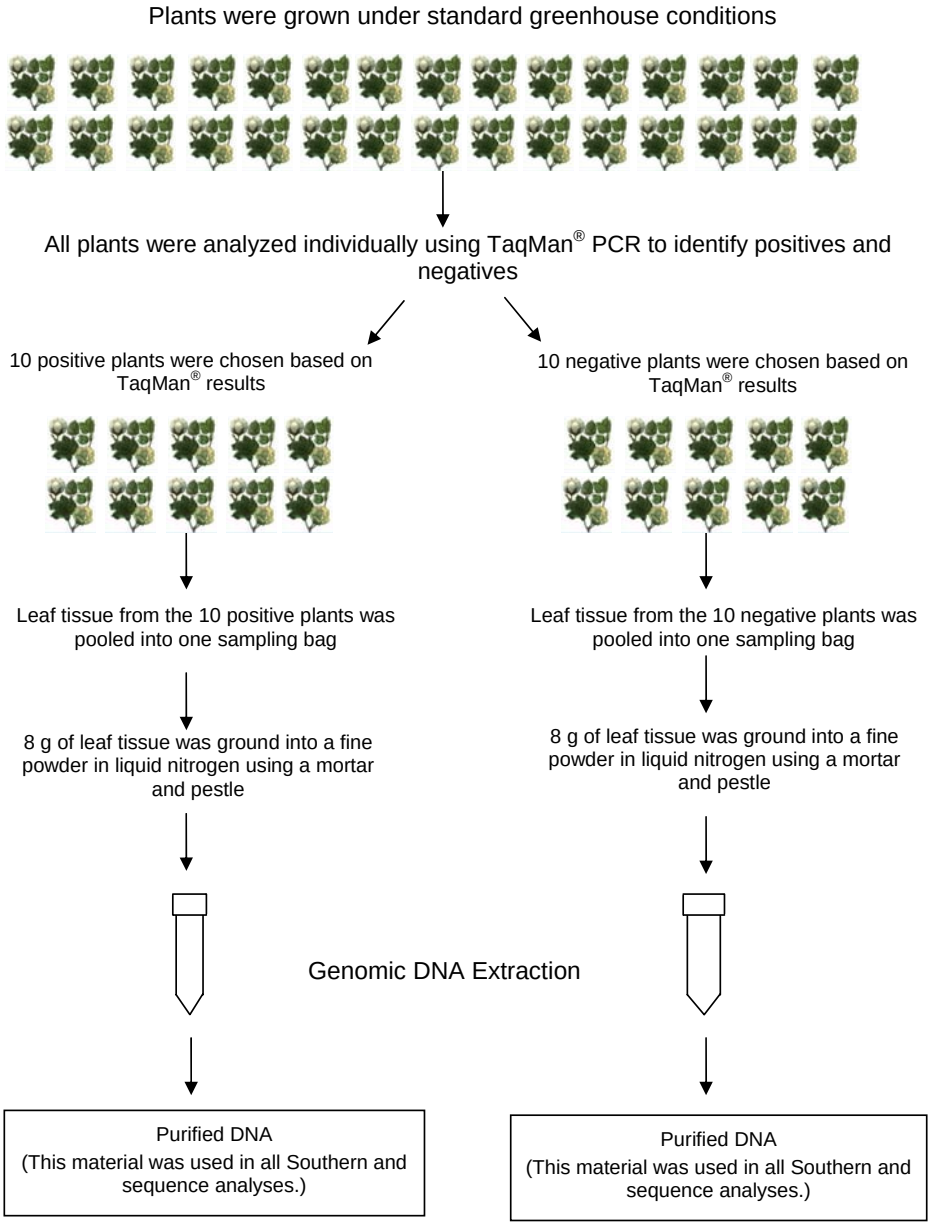
E.5.a. Test material and TaqMan® analysis

Positive and negative test materials for Southern analyses and Mendelian inheritance studies were derived from cotton seeds planted in the greenhouse and grown according to standard greenhouse practices and growth conditions. The breeding process used to develop the COT67B seed is illustrated in the pedigree chart shown in Figure 3-4.

All plants used as test materials were analyzed individually using TaqMan® PCR and DNA isolated from leaf discs. COT67B plants were confirmed positive for the *flcry1Ab* gene and negative for the *aph4* and *spec* genes, while absence of *flcry1Ab, aph4* and *spec* was confirmed for the negative control plants. All plants tested positive for the assay's internal control, the endogenous cotton *chitinase* gene, as expected.

Based on the Taqman® results, ten positive plants from the F_1, $BC1(F_1)$, and $BC3(F_1)$ generations, and ten $BC3(F_1)$ COT67B negative segregant plants were selected for molecular characterization. A schematic of the plant processing procedure is shown in Figure 3-6.

Figure 3-6. Schematic of source material used for genetic analysis



E.5.b. Southern Analyses (Restriction Enzyme Strategy and Postive Controls)

Southern analyses were performed using standard molecular biology techniques (Chomczynski, 1992).

For each functional element COT67B genomic DNA was digested with three restriction enzyme strategies to verify copy and insert number:

1. For the first enzyme digestion strategy, COT67B genomic DNA was digested with an enzyme that digests once within the T-DNA but not within the functional element being probed. This digest will result in a single unique hybridization band for each copy of the functional element present in the COT67B genome.
2. In the second enzyme scheme, another enzyme that digests once within the T-DNA but not within the functional element being probed was employed. This digest will also result in a single unique hybridization band for each copy of the functional element present.
3. Lastly, COT67B genomic DNA was digested with an enzyme(s) that released a fragment of known size. This restriction enzyme strategy gives further evidence of copy number and also demonstrates the intactness of the insert.

Positive controls on Southern blots consisted of:

1. A pNOV4641 plasmid control representing one copy equivalent based on plasmid size[6]
2. The same plasmid concentration added to the negative control DNA

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[6] Digested plasmid equal to one copy equivalent based on plasmid size was used to demonstrate a positive control for hybridization and to determine the sensitivity of the experiment.

Formulae to determine one copy equivalent based on plasmid size

((Plasmid size/(Genome size*Ploidy))*µg loaded)*1.00E+06 =	pg for 1 copy
Example	
Gossypium hirsutum genome size in bp:	2.36E+09
Ploidy[1]:	2
pNOV4641 size in bp:	10995
µg of digested COT67B DNA loaded for Southern analysis:	7.5
Calculation for pNOV4641: ((10995/(2.36E+09*2))*7.5)*1.00E+06=	17.49 pg
Calculation for pNOV1914: ((11727/(2.36E+09*2))*7.5)*1.00E+06=	18.66 pg

[1] Ploidy takes into consideration tetraploid (2n=4x)

3. A pNOV1914 plasmid control representing one copy equivalent based on plasmid size
4. The same plasmid concentration added to the negative control DNA

Negative controls were included in each Southern experiment to identify any endogenous *G. hirsutum* sequences that might cross-hybridize with the element-specific probe.

E.5.c. Results of Southern Analyses for Functional Element Copy Number

Table 3-4 shows the expected and observed hybridization bands for the Southern analyses. In addition, for each Southern blot, there is a map showing the location of the specific probe and the locations of the restriction enzyme sites used in that analysis. These can be found in Figures 3-7, 9, 11, 13, 15 and 17. The results of the corresponding Southern analyses are shown in Figures 3-8, 10, 12, 14, 16 and 18.

Table 3-4. Expected and observed hybridization band sizes in Southern analyses

Figure Reference	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Approximate Observed Band Size (kb)
GENOMIC DNA: Southern Analyses for Copy Number of Functional Elements (Figures 8, 10, 12, 14, 16, 18).						
Figure 8, Lane 2	BC3(F_1)	*Xmn*I	*flcry1Ab*	1	>4.8	6.1
Figure 8, Lane 3	Negative	*Xmn*I	*flcry1Ab*	0	None	None
Figure 8, Lane 4	BC3(F_1)	*Kpn*I	*flcry1Ab*	1	>5.5	9.0
Figure 8, Lane 5	Negative	*Kpn*I	*flcry1Ab*	0	None	None
Figure 8, Lane 6	BC3(F_1)	*Pac*I + *Asc*I	*flcry1Ab*	1	5.3	5.3
Figure 8, Lane 7	Negative	*Pac*I + *Asc*I	*flcry1Ab*	0	None	None
Figure 10, Lane 2	BC3(F_1)	*Kpn*I	ACT2	1	>5.5	9.0
Figure 10, Lane 3	Negative	*Kpn*I	ACT2	0	None	None
Figure 10, Lane 4	BC3(F_1)	*Bst*EII	ACT2	1	>3.4	4.1
Figure 10, Lane 5	Negative	*Bst*EII	ACT2	0	None	None
Figure 10, Lane 6	BC3(F_1)	*Pac*I + *Asc*I	ACT2	1	5.3	5.3
Figure 10, Lane 7	Negative	*Pac*I + *Asc*I	ACT2	0	None	None
Figure 12, Lane 2	BC3(F_1)	*Xmn*I	pNOV4641 Backbone	0	None	None
Figure 12, Lane 3	Negative	*Xmn*I	pNOV4641 Backbone	0	None	None
Figure 12, Lane 4	BC3(F_1)	*Kpn*I	pNOV4641 Backbone	0	None	None
Figure 12, Lane 5	Negative	*Kpn*I	pNOV4641 Backbone	0	None	None
Figure 12, Lane 6	BC3(F_1)	*Pac*I + *Asc*I	pNOV4641 Backbone	0	None	None
Figure 12, Lane 7	Negative	*Pac*I + *Asc*I	pNOV4641 Backbone	0	None	None
Figure 14, Lane 2	BC3(F_1)	*Hin*dIII	Ubq3-*aph4*	0	None	None
Figure 14, Lane 3	Negative	*Hin*dIII	Ubq3-*aph4*	0	None	None
Figure 14, Lane 4	BC3(F_1)	*Pme*I	Ubq3-*aph4*	0	None	None
Figure 14, Lane 5	Negative	*Pme*I	Ubq3-*aph4*	0	None	None
Figure 14, Lane 6	BC3(F_1)	*Bam*HI	Ubq3-*aph4*	0	None	None
Figure 14, Lane 7	Negative	*Bam*HI	Ubq3-*aph4*	0	None	None

Table 3-4. Continued

Figure Reference	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Approximate Observed Band Size (kb)
GENOMIC DNA: Southern Analyses for Copy Number of Functional Elements (Figures 8, 10, 12, 14, 16, 18).						
Figure 16, Lane 2	BC3(F_1)	*Hin*dIII	pNOV1914 Backbone	0	None	None
Figure 16, Lane 3	Negative	*Hin*dIII	pNOV1914 Backbone	0	None	None
Figure 16, Lane 4	BC3(F_1)	*Pme*I	pNOV1914 Backbone	0	None	None
Figure 16, Lane 5	Negative	*Pme*I	pNOV1914 Backbone	0	None	None
Figure 16, Lane 6	BC3(F_1)	*Bam*HI	pNOV1914 Backbone	0	None	None
Figure 16, Lane 7	Negative	*Bam*HI	pNOV1914 Backbone	0	None	None
Figure 18, Lane 2	F_1	*Xmn*I	*flcry1Ab*	1	>4.8	6.1
Figure 18, Lane 3	BC1(F_1)	*Xmn*I	*flcry1Ab*	1	>4.8	6.1
Figure 18, Lane 4	BC3(F_1)	*Xmn*I	*flcry1Ab*	1	>4.8	6.1
Figure 18, Lane 5	Negative	*Xmn*I	*flcry1Ab*	0	None	None
pNOV4641						
Figure 8, Lanes 8 & 10	pNOV4641	*Pac*I + *Asc*I	*flcry1Ab*	1	5.3	5.3
Figure 10, Lanes 8 &10	pNOV4641	*Pac*I + *Asc*I	ACT2	1	5.3	5.3
Figure 12, Lanes 8 &10	pNOV4641	*Pac*I + *Asc*I	pNOV4641 Backbone	1	5.7	5.7
Figure 18, Lanes 6 & 8	pNOV4641	*Xmn*I	*flcry1Ab*	1	4.9	4.9
pNOV1914						
Figure 14, Lanes 8 & 10	pNOV1914	*Bam*HI	Ubq3-*aph4*	1	2.9	2.9
Figure 16, Lanes 8 & 10	pNOV1914	*Bam*HI	pNOV1914 Backbone	1	8.9	8.9

1) Copy Number of *flcry1Ab*

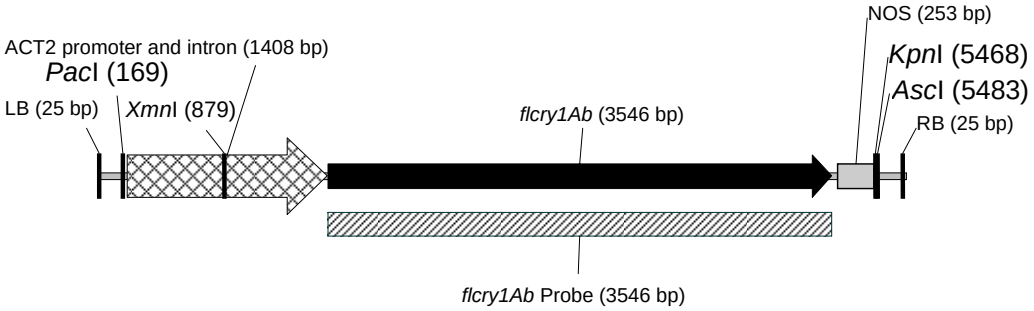
A *flcry1Ab*-specific probe was used for the *flcry1Ab* Southern analysis. A map of the T-DNA region in the transformation plasmid pNOV4641 indicating the locations of the *flcry1Ab*-specific probe and the restriction enzyme sites used in this analysis is shown in Figure 3-7. The results of this analysis are shown in Figure 3-8.

Genomic COT67B DNA digested with *Xmn*I (Lane 2) produced a single hybridization band of approximately 6.1 kb corresponding to a single copy of the *flcry1Ab* gene. Genomic COT67B DNA digested with *Kpn*I (Lane 4) produced a single hybridization band at approximately 9.0 kb corresponding to a single copy of the *flcry1Ab* gene. Genomic COT67B DNA digested with *Pac*I + *Asc*I (Lane 6) produced a single hybridization band at the expected size of approximately 5.3 kb, corresponding to a single copy of the *flcry1Ab* gene and confirming the intactness of the insert. The negative control corresponding to each digest showed no hybridization (*Xmn*I Lane 3, *Kpn*I Lane 5 and *Pac*I + *Asc*I Lane 7). The *Pac*I + *Asc*I digestion of the pNOV4641 plasmid produced a 5.3 kb band (positive control, Lanes 8 and 10) as expected.

For the *flcry1Ab*-specific probe, the restriction enzyme digests resulted in a single hybridization band in each case, demonstrating that COT67B contains a single copy of *flcry1Ab*. No unexpected bands were detected, indicating that COT67B does not contain any additional *flcry1Ab* coding regions other than that associated with the T-DNA.

Figure 3-7. Locations of *Xmn*I, *Kpn*I, *Pac*I and *Asc*I restriction sites and position of *flcry1Ab*-specific probe in the T-DNA region of transformation plasmid pNOV4641 used to create COT67B

The 5.7 kb T-DNA region of pNOV4641 used to create transformation Event COT67B is shown. The positions of the recognition sequences for the XmnI, KpnI, PacI, and AscI restriction enzymes used in the Southern blot analysis with the flcry1Ab-specific probe are indicated. The vertical arrows indicate the sites of restriction digestion. Sizes of the predicted restriction fragments, calculated from the size of the pNOV4641 linear map, are indicated.



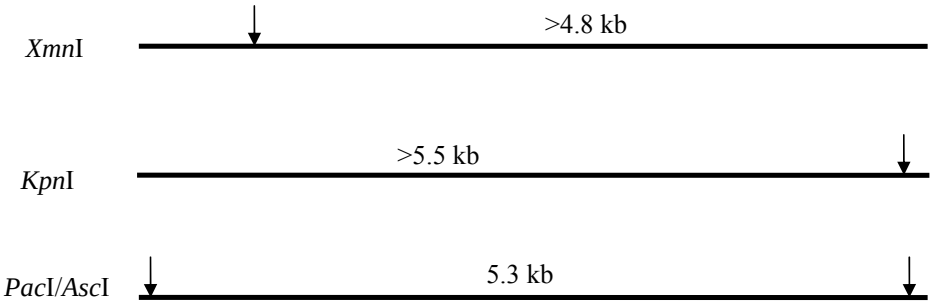
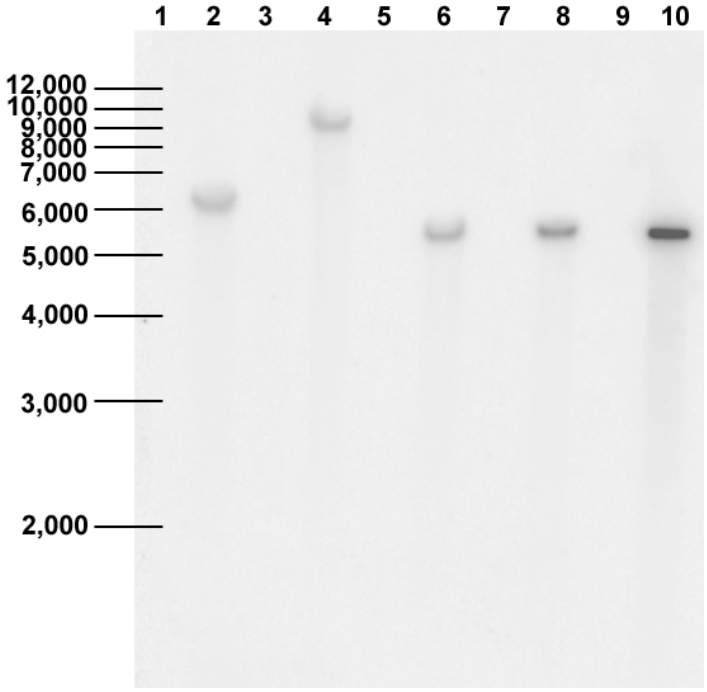


Figure 3-8. Southern analysis of COT67B with a *flcry1Ab*-specific probe

Cotton genomic DNA (7.5 µg) was digested with *Xmn*I, *Kpn*I, *Pac*I + *Asc*I restriction enzymes and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized to a *flcry1Ab*-specific probe (3546 bp).



Lane 1: Molecular Weight Marker (Kb DNA Ladder, Stratagene)
Lane 2: BC3(F_1) generation of COT67B digested with *Xmn*I
Lane 3: Negative nontransgenic from BC3(F_1) generation digested with *Xmn*I
Lane 4: BC3(F_1) generation of COT67B digested with *Kpn*I
Lane 5: Negative nontransgenic from BC3(F_1) generation digested with *Kpn*I
Lane 6: BC3(F_1) generation of COT67B digested with *Pac*I + *Asc*I
Lane 7: Negative nontransgenic from BC3(F_1) generation digested with *Pac*I + *Asc*I
Lane 8: Negative nontransgenic from BC3(F_1) generation digested with *Pac*I + *Asc*I plus 17.5 pg of *Pac*I + *Asc*I-digested pNOV4641 plasmid
Lane 9: Blank
Lane 10: 17.5 pg of *Pac*I + *Asc*I-digested pNOV4641 plasmid

2) Copy Number of ACT2 promoter

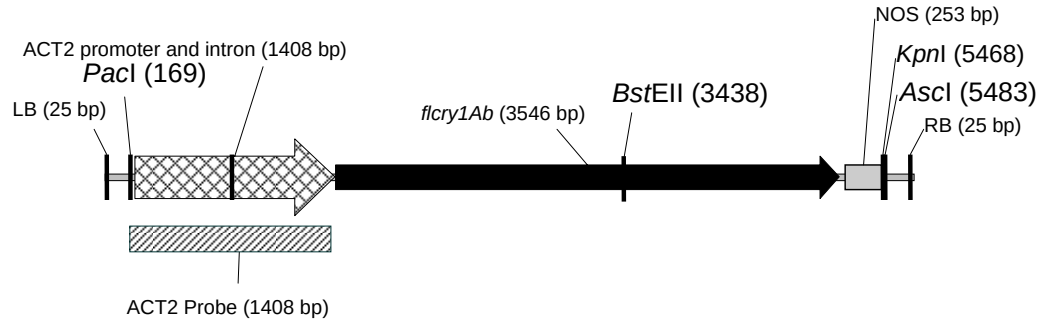
An ACT2-specific probe was used for the ACT2 promoter Southern analysis. A map of the T-DNA region in the transformation plasmid pNOV4641 indicating the locations of the ACT2-specific probe and the restriction enzyme sites used in this analysis is shown in Figure 3-9. The results of this analysis are shown in Figure 3-10.

Genomic COT67B DNA digested with *Kpn*I (Lane 2) produced a single hybridization band of approximately 9.0 kb corresponding to a single copy of the ACT2 promoter. Genomic COT67B DNA digested with *Bst*EII (Lane 4) produced a single hybridization band at approximately 4.1 kb corresponding to a single copy of the ACT2 promoter. Genomic COT67B DNA digested with *Pac*I + *Asc*I (Lane 6) produced a single hybridization band at the expected size of approximately 5.3 kb, corresponding to a single copy of the ACT2 promoter and confirming the intactness of the insert. The negative control corresponding to each digest showed no hybridization (*Kpn*I Lane 3, *Bst*EII Lane 5 and *Pac*I + *Asc*I Lane 7). The *Pac*I + *Asc*I digestion of the pNOV4641 plasmid produced a 5.3 kb band (positive control, Lanes 8 and 10) as expected.

For the ACT2-specific probe, the restriction enzyme digests resulted in a single hybridization band in each case, demonstrating that COT67B contains a single copy of the ACT2 promoter. No unexpected bands were detected, indicating that COT67B does not contain any additional ACT2 coding regions other than that associated with the T-DNA.

Figure 3-9. Locations of *Kpn*I, *Bst*EII, *Pac*I and *Asc*I restriction sites and position of ACT2-specific probe in the T-DNA region of transformation plasmid pNOV4641 used to create COT67B

The 5.7 kb insert of pNOV4641 used to create transformation Event COT67B is shown. The positions of the recognition sequences for the KpnI, BstEII, PacI and AscI restriction enzymes used in the Southern blot analysis with the ACT2-specific probe are indicated. The vertical arrows indicate the sites of restriction digestion. Sizes of the predicted restriction fragments, calculated from the size of the pNOV4641 linear map, are indicated.



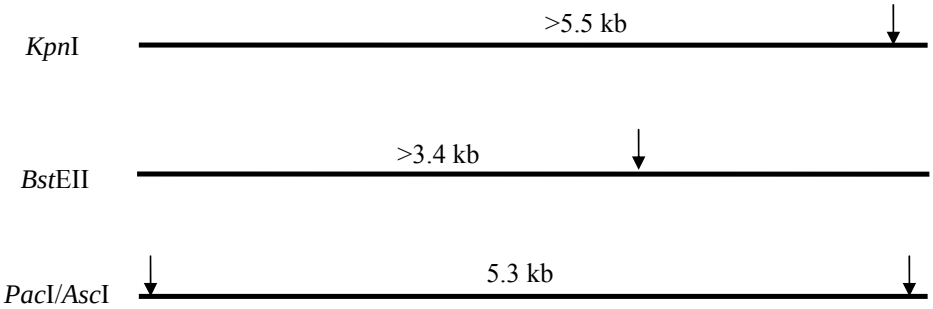
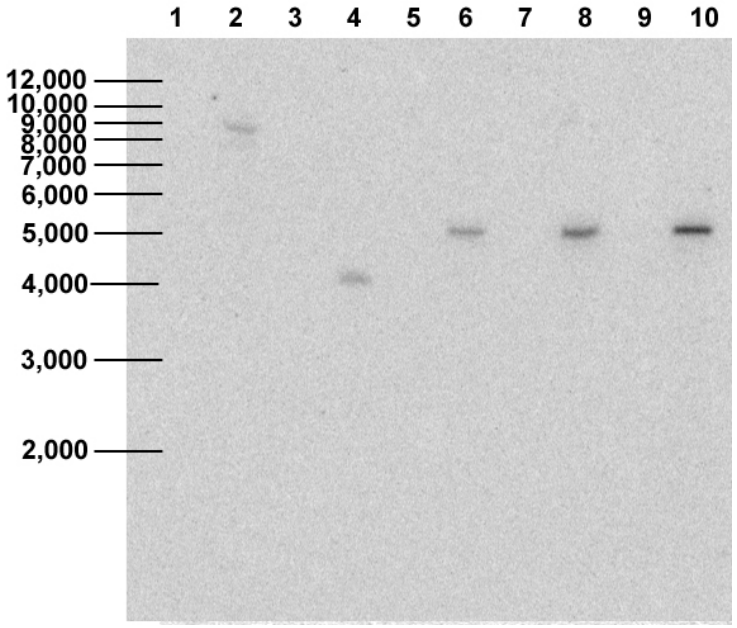


Figure 3-10. Southern analysis of COT67B with an ACT2-specific probe

Cotton genomic DNA (7.5 µg) was digested with KpnI, BstEII, PacI + AscI restriction enzymes and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized to an ACT2-specific probe (1408 bp).



Lane 1: Molecular Weight Marker (Kb DNA Ladder, Stratagene)
Lane 2: BC3(F_1) generation of COT67B digested with *Kpn*I
Lane 3: Negative nontransgenic from BC3(F_1) generation digested with *Kpn*I
Lane 4: BC3(F_1) generation of COT67B digested with *Bst*EII
Lane 5: Negative nontransgenic from BC3(F_1) generation digested with *Bst*EII
Lane 6: BC3(F_1) generation of COT67B digested with *Pac*I + *Asc*I
Lane 7: Negative nontransgenic from BC3(F_1) generation digested with *Pac*I + *Asc*I
Lane 8: Negative nontransgenic from BC3(F_1) generation digested with *Pac*I + *Asc*I plus 17.5 pg of *Pac*I + *Asc*I-digested pNOV4641 plasmid
Lane 9: Blank
Lane 10: 17.5 pg of *Pac*I + *Asc*I-digested pNOV4641 plasmid

3) Plasmid pNOV4641 Backbone Sequences

A map of the transformation plasmid pNOV4641 indicating the locations of the pNOV4641 backbone-specific probe and the restriction enzymes sites used in this analysis is shown in Figure 3-11. The results of this analysis are shown in Figure 3-12.

Genomic COT67B DNA was digested with *Xmn*I (Lane 2), *Kpn*I (Lane 4) and *Pac*I + *Asc*I (Lane 6). The blot was hybridized with a probe covering the entire backbone region of pNOV4641. No detectable hybridization bands were observed in the COT67B genomic samples (Lanes 2, 4 and 6) or in the negative controls (Lanes 3, 5 and 7). The *Pac*I + *Asc*I digestion of the pNOV4641 plasmid produced an expected 5.7 kb band (positive control, Lanes 8 and 10). No hybridization bands were detected for the genomic samples, demonstrating that COT67B does not contain any backbone sequences from the transformation plasmid pNOV4641.

Figure 3-11. Locations of *Xmn*I, *Kpn*I, *Pac*I, and *Asc*I restriction enzyme sites and the position of backbone-specific probe in the transformation plasmid pNOV4641

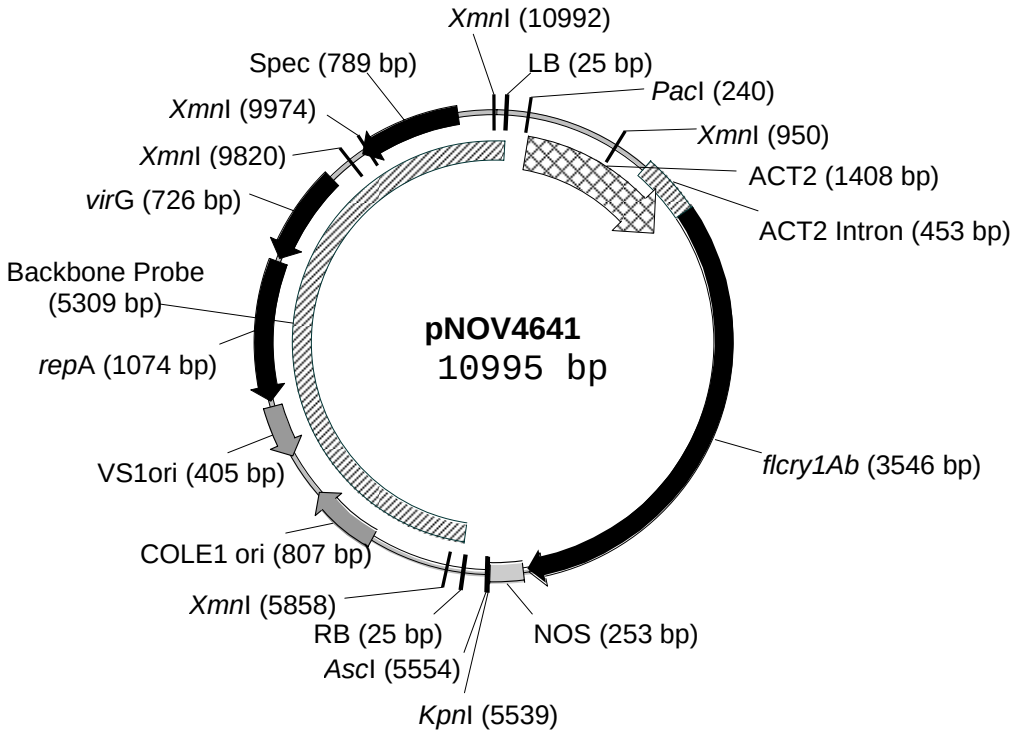
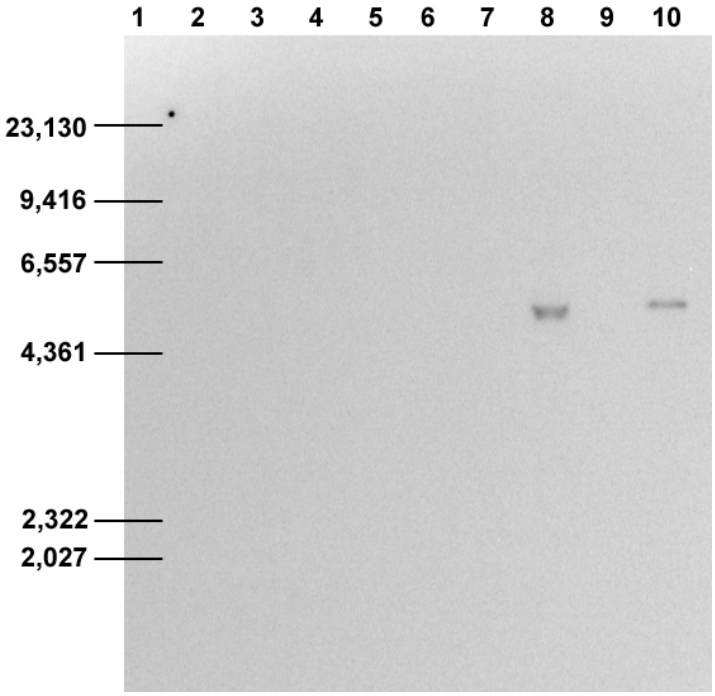


Figure 3-12. Southern analysis of COT67B with pNOV4641 backbone-specific probe

Cotton genomic DNA (7.5 µg) was digested with *Xmn*I, *Kpn*I, *Pac*I + *Asc*I restriction enzymes and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized to a pNOV4641 backbone-specific probe (5309 bp).



Lane 1: Molecular Weight Marker (Lambda DNA-*Hin*dIII Digest
Lane 2: BC3(F_1) generation of COT67B digested with *Xmn*I
Lane 3: Negative nontransgenic from BC3(F_1) generation digested with *Xmn*I
Lane 4: BC3(F_1) generation of COT67B digested with *Kpn*I
Lane 5: Negative nontransgenic from BC3(F_1) generation digested with *Kpn*I
Lane 6: BC3(F_1) generation of COT67B digested with *Pac*I + *Asc*I
Lane 7: Negative nontransgenic from BC3(F_1) generation digested with *Pac*I + *Asc*I
Lane 8: Negative nontransgenic from BC3(F_1) generation digested with *Pac*I + *Asc*I plus 17.5 pg of *Pac*I + *Asc*I-digested pNOV4641 plasmid
Lane 9: Blank
Lane 10: 17.5 pg of *Pac*I + *Asc*I-digested pNOV4641 plasmid

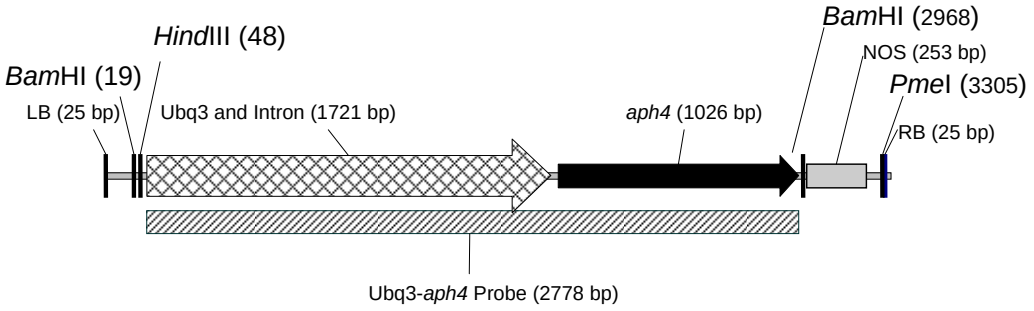
4) Ubq3 and *aph4* Sequences

A map of the transformation plasmid pNOV1914 indicating the locations of the Ubq3-*aph4*-specific probe and the restriction enzyme sites used in this analysis is shown in Figure 3-13. The results of this analysis are shown in Figure 3-14.

Genomic COT67B DNA was digested with *Hin*dIII (Lane 2), *Pme*I (Lane 4) and *Bam*HI (Lane 6). The blot was hybridized with a probe covering the Ubq3 promoter and *aph4* sequences from pNOV1914. No detectable hybridization bands were observed in the COT67B genomic samples (Lanes 2, 4 and 6) or in the negative controls (Lanes 3, 5 and 7). The *Bam*HI digestion of the pNOV1914 plasmid produced an expected 2.9 kb band (positive control, Lanes 8 and 10). No hybridization bands were detected for the genomic samples, demonstrating that COT67B does not contain any Ubq3 promoter or *aph4* sequences from the transformation plasmid pNOV1914.

Figure 3-13. Locations of *Hin*dIII, *Pme*I, and *Bam*HI restriction sites and position of Ubq3-*aph4*-specific probe in the T-DNA region of transformation plasmid pNOV1914 used to create COT67B

The 3.3 kb insert of pNOV1914 used to create transformation Event COT67B is shown. The positions of the recognition sequences for the HindIII, PmeI, and BamHI restriction enzymes used in the Southern blot analysis with the Ubq3-aph4-specific probe are indicated. The vertical arrows indicate the sites of restriction digestion. Sizes of the predicted restriction fragments, calculated from the size of the pNOV1914 linear map, are indicated.



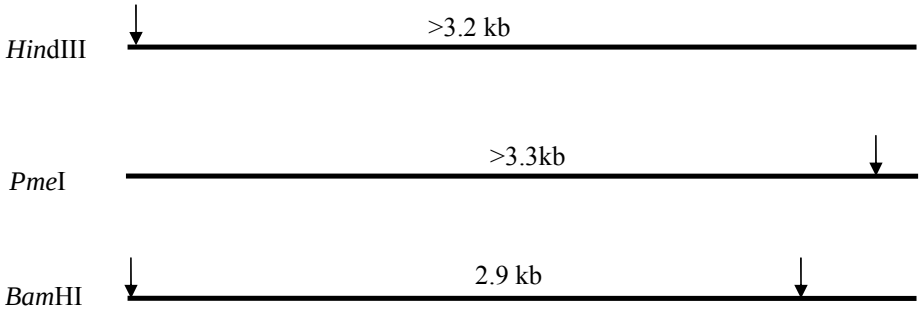
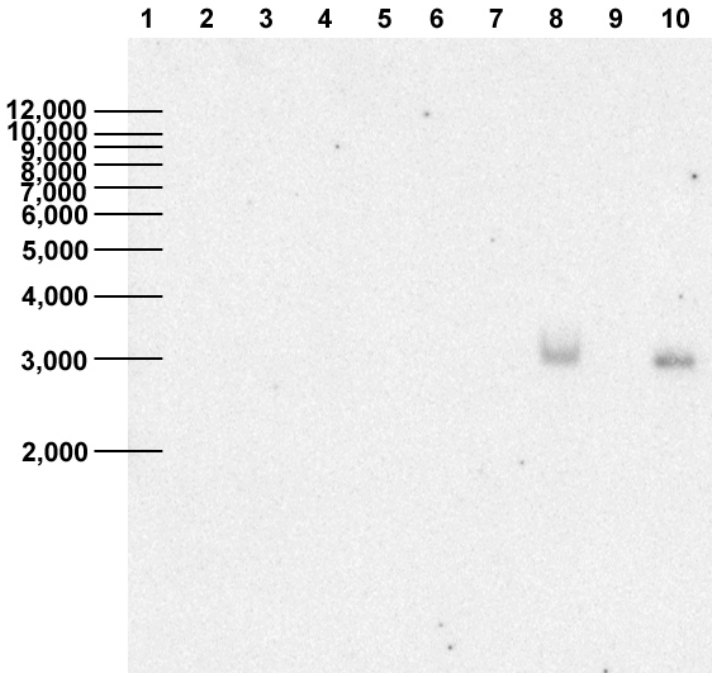


Figure 3-14. Southern analysis of COT67B with Ubq3-*aph4*-specific probe

Cotton genomic DNA (7.5 µg) was digested with *Hind*III, *Pme*I, and *Bam*HI restriction enzymes and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized to a Ubq3-*aph4*-specific probe (2778 bp).



Lane 1: Molecular Weight Marker (Kb DNA Ladder, Stratagene)
Lane 2: BC3(F_1) generation of COT67B digested with *Hin*dIII
Lane 3: Negative nontransgenic from BC3(F_1) generation digested with *Hin*dIII
Lane 4: BC3(F_1) generation of COT67B digested with *Pme*I
Lane 5: Negative nontransgenic from BC3(F_1) generation of COT67B digested with *Pme*I
Lane 6: BC3(F_1) generation of COT67B digested with *Bam*HI
Lane 7: Negative nontransgenic from BC3(F_1) generation digested with *Bam*HI
Lane 8: Negative nontransgenic from BC3(F_1) generation digested with *Bam*HI plus 18.7 pg of *Bam*HI-digested pNOV1914 plasmid
Lane 9: Blank
Lane 10: 18.7 pg of *Bam*HI-digested pNOV1914 plasmid

5) Plasmid pNOV1914 Backbone Sequences

A map of the transformation plasmid pNOV1914 indicating the locations of the pNOV1914 backbone-specific probe and the restriction enzyme sites used in this analysis is shown in Figure 3-15. The results of this analysis are shown in Figure 3-16.

Genomic COT67B DNA was digested with *Hin*dIII (Lane 2), *Pme*I (Lane 4) and *Bam*HI (Lane 6). The blot was hybridized with a probe covering the entire backbone region of pNOV1914. No detectable hybridization bands were observed in the COT67B genomic samples (Lanes 2, 4 and 6) or in the negative controls (Lanes 3, 5 and 7). The *Bam*HI digestion of the pNOV1914 plasmid produced an expected 8.9 kb band (positive control, Lanes 8 and 10). No hybridization bands were detected for the genomic samples, demonstrating that COT67B does not contain any backbone sequences from the transformation plasmid pNOV1914.

Figure 3-15. Locations of *Hin*dIII, *Pme*I, and *Bam*HI restriction sites and the position of the backbone-specific probe in the transformation plasmid pNOV1914

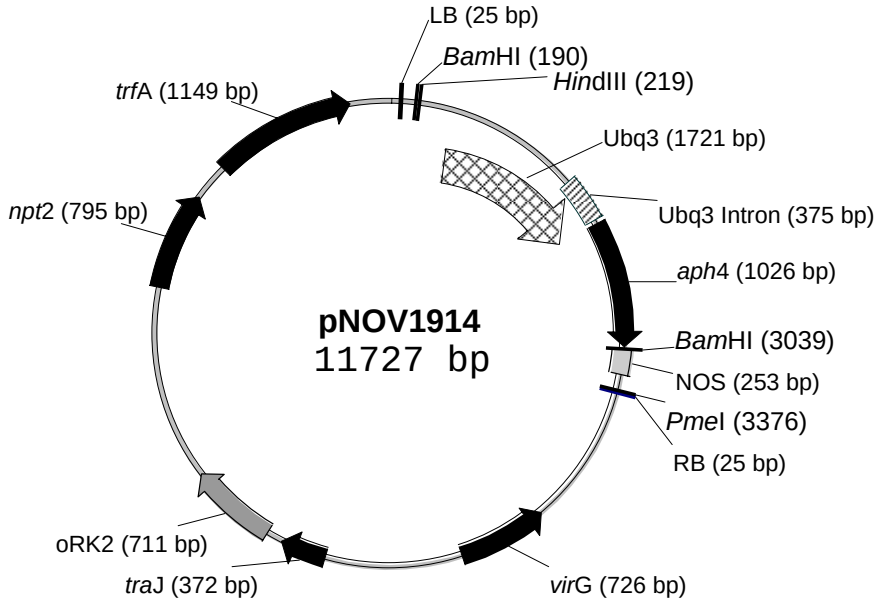
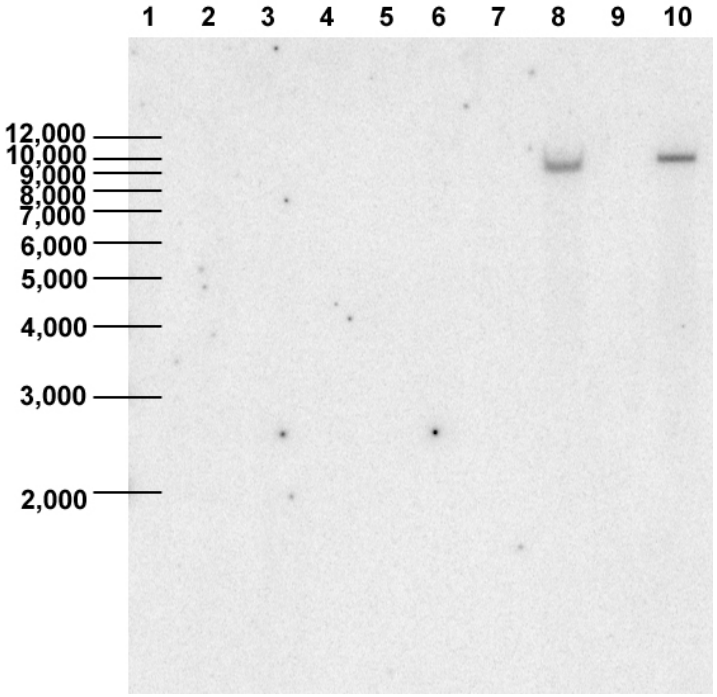


Figure 3-16. Southern analysis of COT67B with pNOV1914 backbone-specific probe

Cotton genomic DNA (7.5 µg) was digested with *Hin*dIII, *Pme*I, and *Bam*HI restriction enzymes and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized to a pNOV1914 backbone-specific probe (8383 bp).



Lane 1: Molecular Weight Marker (Kb DNA Ladder, Stratagene)
Lane 2: BC3(F_1) generation of COT67B digested with *Hin*dIII
Lane 3: Negative nontransgenic from BC3(F_1) generation digested with *Hin*dIII
Lane 4: BC3(F_1) generation of COT67B digested with *Pme*I
Lane 5: Negative nontransgenic from BC3(F_1) generation digested with *Pme*I
Lane 6: BC3(F_1) generation of COT67B digested with *Bam*HI
Lane 7: Negative nontransgenic from BC3(F_1) generation digested with *Bam*HI
Lane 8: Negative nontransgenic from BC3(F_1) generation digested with *Bam*HI plus 18.7 pg of *Bam*HI-digested pNOV1914 plasmid
Lane 9: Blank
Lane 10: 18.7 pg of *Bam*HI-digested pNOV1914 plasmid

6) Summary of Southern analyses of the functional elements

Southern analyses of the functional elements demonstrated that:

1. COT67B contains a single copy of the *flcry1Ab* gene,
2. The integrity of the insert is maintained,
3. COT67B contains no pNOV4641 or pNOV1914 plasmid backbone sequences, and
4. COT67B contains no Ubq3 promoter or *aph4* gene sequences.

E.6. Generational Stability Southern Analysis with flcry1Ab-specific Probe

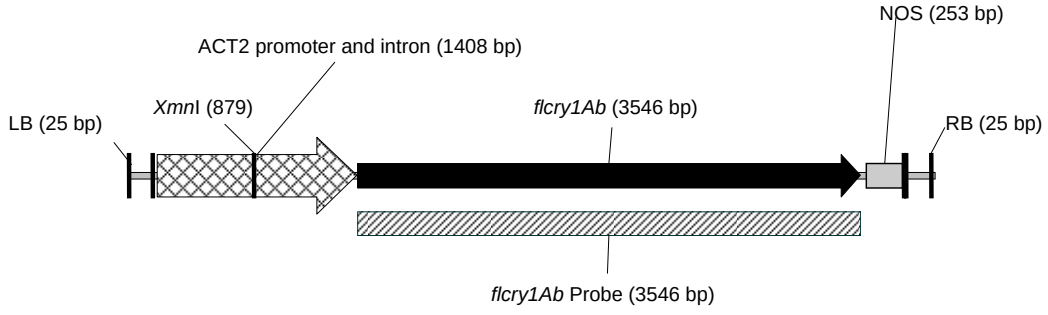
Southern analysis was performed on F_1, BC1F1 and BC3(F_1) plants as outlined for Southern analyses in the materials and methods found in Appendix 1. Cotton genomic DNA (7.5 µg) was digested with *Xmn*I restriction enzyme and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized with a *flcry1Ab*-specific probe (3546 bp).

A map of the T-DNA region in the transformation plasmid pNOV4641 indicating the locations of the *flcry1Ab*-specific probe and the *Xmn*I restriction enzyme sites used in this analysis is shown in Figure 3-17. The results of this analysis are shown in Figure 3-18.

Genomic COT67B DNA from F_1, BC1(F_1) and BC3(F_1) generations (see Figure 3-4 for pedigree) digested with XmnI (Lanes 2, 3 and 4) produced a single hybridization signal of approximately 6.1 kb corresponding to the single copy of the *flcry1Ab* gene present in COT67B. The negative control (negative segregant of BC3(F_1); Lane 5) showed no hybridization as expected. The *Xmn*I digestion of the pNOV4641 plasmid produced a 4.9 kb band (positive control, Lanes 6 and 8) as expected. The hybridization patterns for F_1, BC1(F_1) and BC3(F_1) of COT67B in this Southern analysis were identical, demonstrating the stability of the *flcry1Ab* cassette over multiple generations.

Figure 3-17. Locations of restriction sites and position of the 3546 bp *flcry1Ab*-specific probe

The 5.7 kb insert of pNOV4641 used in the transformation of COT67B is shown. The positions of the recognition sequences for the restriction enzymes used in the Southern analysis with the *flcry1Ab*-specific probe are indicated. The vertical arrow indicates the site of restriction digestion. Size of the predicted restriction fragment, calculated from the size of the pNOV4641 linear map, is indicated.



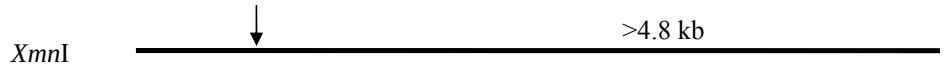
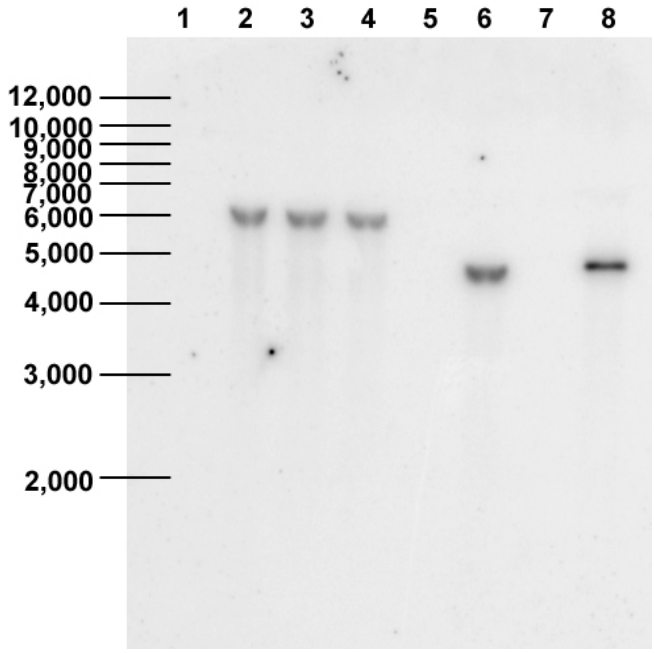


Figure 3-18. Generational stability southern analysis of COT67B with the 3546 bp *flcry1Ab*-specific probe

Cotton genomic DNA (7.5 µg) was digested with *Xmn*I restriction enzyme and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized with a *flcry1Ab*-specific probe (3546 bp).



Lane 1: Molecular weight markers (Kb DNA Ladder, Stratagene)
Lane 2: F_1 generation of COT67B digested with *Xmn*I
Lane 3: BC1(F_1) generation of COT67B digested with *Xmn*I
Lane 4: BC3(F_1) generation of COT67B digested with *Xmn*I
Lane 5: Negative nontransgenic from BC3(F_1) generation digested with XmnI
Lane 6: Negative nontransgenic from BC3(F_1) generation digested with *Xmn*I plus 17.5 pg of *Xmn*I-digested pNOV4641 DNA
Lane 7: Blank
Lane 8: 17.5 pg *Xmn*I-digested pNOV4641 DNA

E.7. Summary of Genetic Analysis

Sequence analysis of the entire T-DNA insert present in COT67B demonstrated that:

1. The intactness of the insert and the contiguousness of the functional elements has been maintained.
2. Some truncation occurred at the RB and LB ends of the T-DNA during the transformation process that resulted in COT67B; however, these deletions have no effect on the functionality of the T-DNA insert.

Data from Southern analyses demonstrated that:

1. COT67B contains a single intact T-DNA insert as demonstrated by a robust restriction enzyme strategy.
2. A single copy of the full-length *cry1Ab* gene (*flcry1Ab*) and a single copy of the ACT2 promoter are present in the T-DNA insert in COT67B.
3. COT67B does not contain the selectable marker gene, hygromycin B phosphotransferase (*aph4*), or the Ubq3 promoter sequence from the transformation plasmid pNOV1914.
4. COT67B does not contain any of the backbone sequences from the transformation plasmids pNOV4641 or pNOV1914.
5. The insert is stable over several generations of COT67B, segregating according to Mendelian genetics as a single locus trait.

Sequence analysis of cotton genome sequences flanking the T-DNA insert demonstrated that:

1. The COT67B T-DNA insert does not disrupt any known endogenous *G. hirsutum* gene.
2. No novel open reading frames span either junction between the COT67B T-DNA insert and *G. hirsutum* genomic sequence.

F. Conclusions of Characterization of the Genetic Material in COT67B

Data in this chapter demonstrates that the *flcry1Ab* insecticide gene and the regulatory elements required for the functioning of the gene were effectively and stably introduced into COT67B. The introduction of the genetic material was accomplished using standard transformation methodology designed to transfer only the intended genes and the data confirms that the insertion of the T-DNA occurred in a manner such that the integrity of the surrounding genome remained intact. The tumor inducing plasmid in the *Agrobacterium* strain was removed (disarmed) prior to use as a transformation vector and an antibiotic was used during the tissue culture phase to eliminate extracellular *Agrobacterium* from the cotton tissues. Further, the use of two constructs to separately deliver the insecticide and selectable marker genes enabled extraneous DNA (selectable

marker gene and vector backbone sequences) to be eliminated through conventional plant breeding. Consequently, no adverse effect arising from transformation methodology is predicted. Based on the results of the genetic analysis of COT67B, there is no reason to expect that unintended or adverse effects will increase the plant pest or weediness potential of cotton or cause harm to cotton, other plants, or the environment.

CHAPTER 4. CHARACTERIZATION OF THE INTRODUCED FLCRY1AB PROTEIN

This chapter summarizes the evaluation of FLCry1Ab protein. The studies summarized here and outlined in greater detail in Appendices 2, 3A, B, C, D, and E demonstrate that FLCry1Ab protein is produced both *in planta* by COT67B cotton plants and in a microbial over-expression system as expected based on the analysis of the introduced genetic material presented in Chapter 3. Moreover, FLCry1Ab is expressed in COT67B tissues and whole plants. This is consistent with the intended function of the genetic sequences introduced to regulate expression, eg., the actin2 constitutive promoter.

The data and information in this chapter includes: 1) the description, characterization and mode of action of the *B. thuringiensis* Cry proteins in general and the FLCry1Ab protein in COT67B, in particular, 2) evidence for equivalence of the microbially produced protein used for safety studies to the protein produced *in planta* from COT67B, 3) the levels of FLCry1Ab protein in COT67B tissues and whole plants and, 4) a summary of the food and feed safety assessment. Sequence alignments illustrating the similarity of the FLCry1Ab protein in COT67B to other Cry proteins are shown in Appendix 2. Detailed descriptions of the materials and methods for the studies conducted to characterize FLCry1Ab in COT67B cotton plants and in the microbial test substance are found in Appendix 3A ad 3B. Likewise, materials, methods and results for quantification of levels of FLCry1Ab in COT67B tissues and whole plants are found in Appendix 3C; those demonstrating stability of FLCry1Ab over multiple generations are found in Appendix 3D; Appendix 3E contains methods and results for quantification of FLCry1Ab protein levels in the processed cottonseed products, meal, oil and linters.

The Cry1Ab protein in COT67B is referred to as 'FLCry1Ab' throughout this petition. It is recognized, however, that studies in which ELISA was used to measure the concentrations of Cry protein (using a polyclonal anti-Cry1Ab antibody) cannot distinguish between intact, full-length Cry1Ab and smaller immunoreactive derivatives. Therefore, the concentrations of 'FLCry1Ab' measured by ELISA and described herein do not necessarily reflect solely the concentration of FLCry1Ab, but may include smaller Cry1Ab polypeptides.

A. The FLCry1Ab Protein

A.1. A Brief Description of *B. thuringiensis* Cry Proteins and Mode of Action

Crickmore *et al.* (1998 and 2004) identified at least 44 primary classes of Bt Cry proteins (Cry1 – Cry44) ranging in molecular mass size from 25 kDa to over 130 kDa. The authors also devised a nomenclature that incorporates four hierarchical ranks consisting (in descending order) of an Arabic numeral, uppercase letter, lowercase letter, and an Arabic numeral (e.g., Cry1Ab1 protein). Thus, proteins with highly homologous amino acid sequences are grouped together:

1. <45% homology differ in primary rank (e.g., Cry1, Cry2, etc),
2. >45% but <78% homology differ in secondary rank (e.g., Cry1A, Cry1B),
3. >78% but <95% homology differ in tertiary rank (e.g., Cry1Ac, Cry1Ab), and
4. >95% homology differ in quaternary rank (e.g., Cry1Ab1, Cry1Ab2) and are considered allelic variants.

In practice, the primary rank correlates with specific insecticidal activity; for example, Cry1, Cry2, Cry3, and Cry4 Bt proteins are toxic to lepidopteran, lepidopteran/dipteran, coleopteran, and dipteran pests, respectively (Bravo, 1997; Höfte and Whitely, 1989).

A generalized mode of action for Cry proteins has been described by English and Slatin (1992). It includes ingestion of the crystals by insects, solubilization of the crystals in the insect midgut, and proteolytic processing of the released Cry protein by digestive enzymes, sometimes with partial digestion activating the toxin. The activated protein diffuses through the peritrophic membrane of the insect to the midgut epithelium. There it binds to specific high-affinity receptors on the surface of the midgut epithelium of target insects (Hoffman et al., 1988a and 1988b; Van Rie et al., 1989 and 1990; Wolfersberger et al., 1986). Pores are formed in the membrane, leading to leakage of intracellular contents (e.g., K^+) into the gut lumen and water into the epithelial gut cells (Sacchi et al., 1986). The larval gut epithelial cells swell because of osmotic pressure and lyse. The gut becomes paralyzed as a consequence of changes in electrolytes and pH, causing the larval insect to stop eating and die (Broderick, *et al.* 2006).

Strong receptor binding followed by membrane alterations are criticals step in the mechanism of action for Cry proteins. Irreversible binding of these proteins to midgut receptors appears to be correlated with insect susceptibility to the toxin (Schnepf et al., 1998). This observation is relevant to assessing the safety of Cry proteins for humans since Shimada *et al.* (2006) found weak, and possibly nonspecific binding of Cry1Ab to mammalian intestinal epithelial cell receptors. However, there was no disruption of membrane integrity. (Sacchi et al., 1986; Shimada et al., 2006). This would explain, at least in part, the absence of any reported adverse effects for *B.t.* products in humans.

A.2. Description of the FLCry1Ab Protein in COT67B

Syngenta's COT67B was transformed with a synthetic full-length c*ry1Ab* gene originally derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (Btk). The native or wild-type *cry1Ab* gene is a product of genetic recombination of the *B. thuringiensis kurstaki* HD-1 genes, *cry1Aa* and *cry1Ac*. It is well documented that mutations resulting in deletions of stretches of DNA are common during genetic recombination. Such was the case for the native *cry1Ab* gene (Geiser[7] et al., 1986). The loss of DNA resulted in a

[7] M. Geiser was employed by the former Ciba-Geigy Corp., a legacy company of Syngenta.

diminished capacity for native *cry1Ab* to encode Cry1Ab protein in fermentative cultures of *B. thuringiensis* under the customary fermentation temperatures. It was subsequently discovered that this inefficiency was attributable to the absence of 26 amino acids encoded by the stretch of DNA lost during the formation of c*ry1ab*. Syngenta "repaired" the gene by replacing the deleted coding region with the functional coding region from *cry1Aa*. This "full-length" version of the gene was used in transformation to produce COT67B and is referred to as full-length *cry1Ab* (*flcry1Ab*). The gene encodes the same full-length Cry1Ab protein produced by *B. thuringiensis kurstaki* HD-1, except for the additional 26 amino acids in the C-terminal portion of the protein. This 26 amino acid sequence is referred to as the 'Geiser motif" by Syngenta scientists. The additional amino acid sequence has no apparent functionality in plants, and is not contained in the region of the protein responsible for insecticidal activity (Geiser and Moser, 1991; Koziel, *et al.* 1997).

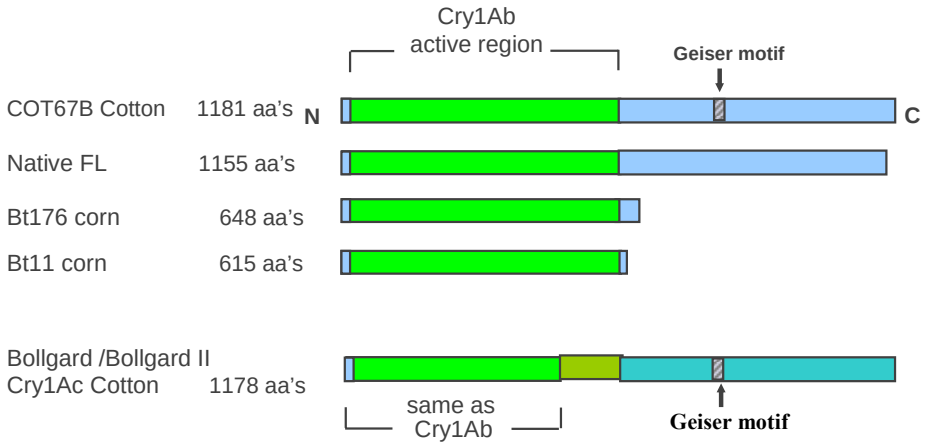
Although the Geiser motif is absent from native Cry1Ab protein, the motif and highly homologous sequences are present in essentially all other native full-length Cry1 proteins including those contained in several EPA-registered commercial *B. thuringiensis* microbial insecticides (e.g., Cry1Aa, Cry1Ac, Cry1Ca, Cry1Da as found in *B. thuringiensis.kurstaki*. and/or *B. thuringiensis aizawai* products) and plant incorporated protectants (Bollgard and Bollgard II cottons) (See Chapter 4, Section B.).

The Cry1Ab protein is also present in a number of *B. thuringiensis* corn plant-incorporated protectants registered by the EPA since 1996 and re-registered in 2001 and 2006. The FDA completed food and feed safety consultations for these products and the EPA, through its statutory authority under the Federal Food Drug and Cosmetic Act, established a permanent exemption from the requirement of a tolerance for the Cry1Ab protein and the genetic material necessary for its production in all plants (40 CFR 180.1173).

The schematic in Figure 4-1 compares the relative sizes of Cry1Ab proteins found in pesticide products that have been registered. Event Bt11-derived corn (615 amino acids) and Event 176-derived corn (648 amino acids) produce truncated Cry1Ab proteins that encompass slightly more than the active insecticidal region of the protein, whereas the insecticidal region represents roughly one-half of the full-length protoxin present in native Cry1Ab (1155 amino acids) and that encoded in COT67B (1181 amino acids). The "Cry1Ac" protein produced in Bollgard® and Bollgard II® cotton is the result of fusing a 5' portion of a *cry1Ab* gene to the majority of the *cry1Ac* gene. The portion of the *cry1Ab* gene used encodes an N-terminal amino acid sequence (466 amino acids in length) that is highly homologous to the N-terminal amino acid sequence encoded by the *cry1Ac* gene (Monsanto, 1994). Sequence comparisons for these Cry proteins are shown in Appendix 2.

Figure 4-1. Comparison of Cry1Ab proteins of various amino acid (aa) lengths in EPA-registered Bt plant incorporated protectants ('Native FL'; full-length), Bollgard® Cotton, COT67B and Bt corn events '176' and 'Bt11'

The small shaded (hatched) area on the COT67B and Bollgard® proteins represent the 'Geiser motif.'



B. Characterization of the FLCry1Ab Protein

Large quantities of protein are required to perform toxicology, ecotoxicology, biochemical and insecticidal activity studies. Because it is very difficult to extract and purify sufficient quantities of the protein from transgenic cotton plants for the aforementioned studies, a 'test substance' containing FLCry1Ab protein was produced *via* expression of the *flcry1Ab* gene in a recombinant *Escherichia coli* (*E. Coli*) over-expression system. The test substance, designated FLCRY1AB-0103, was characterized and compared with FLCry1Ab protein from COT67B tissue. The equivalency of FLCry1Ab protein from FLCRY1AB-0103 and FLCry1Ab protein from COT67B was established and its suitability as a surrogate in invertebrate and mammalian safety studies was demonstrated.

The following section summarizes the characterization of FLCRY1AB-0103 and the studies conducted to demonstrate its biochemical and functional equivalence to the FLCry1Ab protein isolated from COT67B leaf tissue. The test substance was characterized several times over the course of 14 months: after initial production, a second time after several months of storage at -20°C, and lastly, prior to initiation of the protein equivalency study. The purity and bioactivity information presented in parts B1 and B2 of this section represents the results of the most recent recharacterization. Detailed materials and methods for protein characterization and equivalency studies are provided in Appendix 3.A. and B.

B.1. Purity of FLCRY1AB-0103 Test Substance

Purity determinations of test substances are undertaken to verify that the intended protein is intact and that it represents a proportion of the test substance in such a quantity as to be acceptable for subsequent characterization studies. This section describes the purity characteristics of test substance FLCRY1AB-0103.

FLCRY1AB-0103 was determined to contain *ca.* 98.0% protein as measured by absorbance at 280 nm. Following SDS-polyacrylamide gel electrophoresis, densitometric analysis of the Coomassie® blue-stained gel indicated that intact, FLCry1Ab represented *ca*. 78.9% of the total protein in FLCRY1AB-0103 (Appendix 3A, Figure 1). Therefore, the purity of test substance FLCRY1AB-0103 was calculated to be *ca.* 77.3% FLCry1Ab by weight (Table 4-1). Purity determinations were conducted several times over the course of the 14 months of use and storage at -20°C. During this time, the Cry1Ab protein concentration in FLCRY1AB-0103 declined somewhat due most likely to excess handling of the substance. However, consistent with the initial analysis, the most recent Western blot analysis confirmed that a dominant immunoreactive band corresponding to the predicted molecular weight of *ca*. 133.5 kDa was present, indicating that the FLCry1Ab protein had remained intact during the experimentation period (Appendix 3.A., Figure 2).

B.2. Insecticidal Activity of FLCRY1AB-0103 Test Substance

Concurrent with purity determinations, FLCRY1AB-0103 was evaluated for bioactivity against first-instar European corn borer (ECB; *Ostrinia nubilalis*) larvae to confirm that the protein retained insecticidal activity. Although there was some decrease in activity over the period use it is clear that FLCRY1AB-0103 retained high levels of insecticidal activity (Table 4-1).

Table 4-1. Characterization and Bioactivity of FLCry1Ab in Test Substance FLCRY1AB-0103

Date of Analysis	Total Protein [g protein/g FLCRY1AB-0103]	Densitometric Analysis [% FLCry1Ab/total protein]	Purity* [% Cry1Ab/ FLCRY1AB-0103]	72-hour ECB LC_{50} [ng FLCry1Ab/cm^2 diet surface] (95% Confidence Interval)
Dec 2004 (Graser, 2005)	0.947	90.4	85.6	3.7 (2.3 - 4.9)
May 2005 (Graser, 2005)	0.965	88.8	85.7	8.3 (6.0 - 10.7)
Feb 2006 (Kramer, 2006)	0.980	78.9	77.3	16.5 (12.4 - 21.0)

*Purity calculation: total protein x densitometric analysis

B.3. FLCry1Ab Protein Equivalency

A series of studies directly comparing microbially produced FLCRY1AB-0103 to FLCry1Ab protein produced *in planta* were conducted to demonstrate biochemical and functional equivalence. Two positive control plant derived protein substances were used in the studies: one, designated LPCOT67B-010, was prepared from leaf tissue; another designated IAPCOT67B-0106, was prepared from immunoaffinity column purified leaf tissue-derived protein. The negative control was prepared from nontransgenic cotton leaf tissue and designated LPCOT67B-106C. The protein equivalency studies included:

1. Western blot analysis and immunoreactivity analysis to demonstrate integrity of the proteins,
2. SDS-PAGE and peptide mass mapping analysis to support the identical nature the proteins,
3. Glycosylation analysis to show that no post-translation changes occurred,
4. FLCry1Ab bioactivity assay to demonstrate equivalent insecticidal activity, and
5. N-Terminal amino acid sequence analysis of the microbially derived protein to demonstrate that the residues correspond to those predicted for FLCry1Ab.

Results of Western blot analysis showed that FLCry1Ab from both COT67B and FLCRY1AB-0103 have the expected molecular weight of *ca.* 133.5 kDa and that both immunologically cross-react with anti-Cry1Ab antibodies (Appendix 3.B.-Figure 2). No evidence of post-translational glycosylation of FLCry1Ab from COT67B or FLCRY1AB-0103 was observed (Appendix 3.B.-Figure 3). Both the plant-and microbially derived proteins were active in ECB bioassays, with estimated LC_{50}s after 72 hours of 1.3 ng FLCry1Ab/cm^2 (95% confidence interval = 0.9 – 1.9 ng/cm^2 diet surface) and 5.2 ng FLCry1Ab/cm^2 (95% confidence interval = 4.0 – 6.6 ng/cm^2 diet surface), respectively (Appendix 3.B. - Table 1 and Table 2). Results of N-terminal amino acid sequence analysis of FLCry1Ab in test substance FLCRY1AB-0103 confirmed the predicted sequence (Appendix 3.B.-Figure 1). In addition, peptide mass mapping analysis of the plant- and microbially derived proteins provided 16% and 30% coverage, respectively, of the predicted amino acid sequence of FLCry1Ab and confirmed the identity of the insecticidal protein from both sources (Appendix 3.A.-Figure 4 and-Figure 5).

In summary, it can be stated that the *flcry1ab* gene encodes a protein of the size and characteristics predicted by two types of sequence analyses. The protein is bioactive and does not appear to be glycosylated. Data to support these conclusions were obtained with FLCry1Ab from two sources, plant derived and microbially derived. Protein characterization demonstrated that FLCry1Ab protein from both sources were biochemically and functionally equivalent. Although small declines in purity and bioactivity of the protein in test substance FLCRY1AB-0103 were observed, the data demonstrated that microbially produced FLCRY1AB-0103 is fit for use in studies used to assess the safety of FLCry1Ab.

B.4. Quantification and Stability of FLCry1Ab Protein Produced in COT67B

The information provided in this section establishes that FLCry1Ab protein is expressed in various tissues and whole plants of COT67B as expected based on the genetic elements present in the T-DNA insert of construct pNOV4641. The levels of FLCry1Ab protein in the various tissues were quantified to establish margins of exposure for nontarget organism (NTO) studies. The details and implications of these studies are found in Chapter 7, Environmental Consequences of Introduction. The results of those studies gave no indication that FLCry1Ab would harm beneficial organisms or contribute to a situation of increased weediness or plant pest potential.

The level of FLCry1Ab protein in various COT67B tissues was estimated using enzyme-linked immunosorbent assay (ELISA). The materials and methods for the ELISA analysis, field locations and a description of the tissue types and growth stages analyzed are detailed in Appendix 3.C. To produce the tissues for analysis, COT67B and nontransgenic Coker 312 cotton plants were planted at four field locations during the 2004 growing season. The sites were located in the major cotton-growing region of the United States. At each location, five plants each of COT67B and Coker 312 were harvested at five developmental time points: squaring, *ca.* 4 weeks post emergence; 1st white bloom, *ca.* 9 weeks post emergence; peak bloom, *ca.* 13 weeks post emergence; 1st open boll, *ca.* 15 weeks post emergence and pre-harvest, *ca.* 22 weeks post emergence. The levels of FLCry1Ab protein in COT67B young leaves, old leaves and root tissues at squaring, 1st white bloom, peak bloom and 1st open boll are presented on a dry and fresh weight basis in Table 4-2 and Table 4-3, respectively. These data, corrected for extraction efficiency are provided in Appendix 3.C.-Tables 2.b. and 3.b. The concentration of FLCry1Ab in flowers, bolls, seed and whole plants is presented on a dry and fresh weight basis in Table 4-4. These data corrected for extraction efficiency are also provided in Appendix 3.C-Table 4. Levels of FLCry1Ab in pollen, nectar and fiber are presented in Table 4-5. The limit of quantitation (LOQ) and limit of detection (LOD) for the various tissue types are provided in Appendix 3.C-Tables 6 and 7, respectively.

Quantifiable concentrations of FLCry1Ab protein were detected, as expected, in all COT67B plant tissues except fiber and nectar. The concentrations of FLCry1Ab were generally similar between the four locations where COT67B was planted for each tissue type at each time point. Where the concentration of FLCry1Ab appeared variable, there were no consistent trends to indicate that plants grown in a given location had relatively higher or lower FLCry1Ab concentrations. FLCry1Ab concentrations in most Coker 312 tissue samples were either below the limit of detection or below the limit of quantification.

In addition to quantifying the levels of FLCry1Ab protein in various tissues, ELISA analysis was used to assess FLCry1Ab stability over multiple generations of COT67B (Pence, 2006). Plants from three generations (F_1, BC1(F_1), and BC4(F_1)) were grown under standard greenhouse conditions. The mean FLCry1Ab concentrations measured in leaves of the F_1, BC1(F_1), and BC4(F_1) generations of COT67B were 68.17, 66.27, and 54.16 µg/gdw, respectively (Table-4-6). The consistency of FLCry1Ab concentrations

reflects the inherent stability of transgenic protein expression across multiple generations of COT67B cotton. See Appendix 3.D. for detailed methods and results.

Table 4-2. FLCry1Ab concentrations in young and old leaves and roots on a dry weight (dwt) basis during development of COT67B plants

Tissue Type[1]	Developmental Stage			
	Squaring	1st White Bloom	Peak Bloom	1st Open Boll
	Mean ug/g dwt ± SD [2] (range) [3]			
Young Leaves	231.87 ± 24.84 (192.58 – 277.58)	201.14[4] (195.02 – 207.49)	115.63 ± 32.71 (76.20 – 154.71)	62.79 ± 20.61 (48.22 – 77.36)
Old Leaves	175.04 ± 55.18 (93.76 – 246.7)	195.39 ± 16.55 (162.17 – 267.27)	187.97 ± 60.74 (118.49 – 268.02)	148.23 ± 78.25 (70.81 – 251.31)
Roots	42.59 ± 17.58 (21.90 – 69.36)	22.04 ± 2.06 (19.33 – 24.93)	18.40 ± 6.88 (11.98 – 28.98)	9.50 ± 3.60 (4.66 – 13.63)

[1] Tissue types are described in Appendix 3.C. Limits of detection and quantitation are described in Appendix 3.C.-Tables 6 and 7.

[2] The mean and standard deviation (SD) were calculated across sites and replicates (n=4 for most tissue types; however see Appendix 3.C. for a further description of the field locations and growth stages sampled and analyzed).

[3] Minimum and maximum values were determined for each tissue type across sites.

[4] SD not applicable; data from one location only

Table 4-3. FLCry1Ab concentrations in young and old leaves and roots on a fresh weight (fwt) basis during development of event COT67B plants

Tissue Type[1]	Developmental Stage			
	Squaring	1st White Bloom	Peak Bloom	1st Open Boll
	Mean µg/g fwt ± SD [2] (range) [3]			
Young Leaves	65.72 ± 32.64 (34.44 – 107.88)	43.31[4] (41.99 – 44.67)	27.35 ± 4.52 (24.40 – 34.05)	15.09 ± 5.85 (10.95 – 19.22)
Old Leaves	36.64 ± 8.71 (22.32 – 48.85)	42.29 ± 4.97 (33.78 – 59.52)	41.66 ± 10.67 (28.65 – 54.17)	34.72 ± 17.07 (16.33 – 56.34)
Roots	8.42 ± 2.33 (4.46 – 12.23)	6.87 ± 0.31 (5.29 – 8.07)	6.74 ± 1.73 (5.19 – 9.63)	3.74 ± 1.21 (2.00 – 5.12)

[1] Tissue types are described in Appendix 3.C. Limits of detection and quantitation are described in Appendix 3.C.-Tables 6 and 7.

[2] The mean and standard deviation (SD) were calculated across sites and replicates (n=4 for most tissue types; however see Appendix 3.C. for a further description of the field locations and growth stages sampled and analyzed).

[3] Minimum and maximum values were determined for each tissue type across sites.

[4] SD not applicable; data from one location only.

Table 4-4. FLCry1Ab concentrations in whole plants, seeds, flowers and bolls of COT67B plants on a dry (dwt) and fresh (fwt) weight basis

Tissue Type[1]	Mean µg/g dwt ± SD [2] (range) [3]	Mean µg/g fwt ± SD [2] (range) [3]
Whole Plant (pre-harvest)	30.24 ± 13.08 (15.84 – 54.56)	10.85 ± 3.84 (6.99 – 19.41)
Seed (pre-harvest)	18.38 ± 6.34 (8.69 – 25.03)	14.68 ± 5.58 (6.64 – 22.06)
Bolls (1st open boll)	34.83 ± 9.80 (20.63 – 47.21)	6.88 ± 2.17 (3.84 – 9.20)
Flowers (peak bloom) [4]	119.36 ± 16.88 (101.33 – 139.87)	18.95 ± 2.68 (16.09 – 22.21)

[1] Tissue types are described in Appendix 3.C. Limits of detection and quantitation are described in Appendix 3.C.-Tables 6 and 7.

[2] The mean and standard deviation (SD) were calculated across sites and replicates (n=4 for most tissue types, however see Appendix 3.C. for a further description of the field locations and growth stages sampled and analyzed).

[3] Minimum and maximum values were determined for each tissue type across sites.

[4] Flowers were collected at a single location; 6 replicate samples were used to determine the mean and standard deviation.

Table 4-5. FLCry1Ab concentrations in pollen, nectar and fiber of COT67B plants grown at Winnsboro, Louisiana

Tissue Type	µg /g sample[1]
Pollen[2]	4.28[3]
Nectar[4]	<0.0002
Fiber[5]	<0.02 (<0.01 – <0.07)

[1] Unless otherwise noted.

[2] Pollen values are reported on a per gram sample basis (air-dried overnight, as collected).

[3] The Cry1Ab concentration in pollen is 5.45 µg Cry1Ab/g sample when corrected for extraction efficiency.

[4] Nectar values are reported on a per milliliter nectar basis (as collected).

[5] Five samples were collected from the Winnsboro, LA field site. One sample was above the LOD but below the LOQ (<0.07 µg Cry1Ab/g sample), which is likely due to boll or seed contamination from the ginning process.

Table 4-6. FLCry1Ab concentrations in leaf tissue from multiple generations of COT67B

Generation	Mean µg Cry1Ab/gdw ± SD (range)	Mean µg Cry1Ab/gfw ± SD (range)
F_1	68.17 ± 9.64 (54.90—81.62)	19.68 ± 2.23 (17.28–22.42)
BC1(F_1)	66.27 ± 16.88 (41.01—81.44)	17.62 ± 1.76 (14.84–19.63)
BC4(F_1)	54.16 ± 12.08 (39.36—67.39)	16.60 ± 2.29 (14.35–20.31)

N = 5 (samples analyzed).

Near-isogenic, nontransgenic samples were all <LOD and are not shown in the table.

B.5. Levels of FLCry1Ab Protein in Processed Fractions

In addition to quantifying the levels of FLCry1Ab produced in various tissues over multiple generations, cottonseed linters, defatted toasted cottonseed meal and once-refined cottonseed oil were produced from COT67B. These products were analyzed by ELISA to quantify FLCry1Ab in the various processed fractions. The concentration of FLCry1Ab in the fuzzy seed used to produce the processed fractions was also determined (de Fontes and Hill, 2006). A quantifiable level of FLCry1Ab was found in the fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B plants. The concentrations of FLCry1Ab determined for the fuzzy seed, linters, and defatted toasted cottonseed meal were 25.05 µg FLCry1Ab/g, 9.65 µg FLCry1Ab/g, and 47.50 µg FLCry1Ab/g, respectively (Table 4-7). FLCry1Ab was not detectable in the once-refined oil from COT67B [limit of detection = 0.003 µg FLCry1Ab/ml]. See Appendix 3.E. for detailed methods and results.

The primary food uses of cotton are refined cottonseed oil and cottonseed "linters." Refined cottonseed oil is highly processed using heat, solvent and alkali treatments. Linters consist of essentially 100% pure cellulose fibers and are subjected to heat and solvent extraction. Given the relatively low levels of FLCry1Ab in processed cottonseed fractions and the fact that refined cottonseed oil and cotton fiber contain little to no protein of any kind, the potential for human exposure to FLCry1Ab from food products containing COT67B by-products is expected to be minimal.

Table 4-7. FLCry1Ab concentrations in COT67B fuzzy seed and processed cottonseed products

Fuzzy Seed and Processed Cottonseed Product	Mean µg FLCry1Ab/g ± S. D. (range)	Mean µg FLCry1Ab/g Corrected for Extraction Efficiency ± S. D. (range)
COT67B		
Fuzzy seed	18.31 ± 1.40 (16.84 - 19.63)	25.05 ± 1.92 (23.04 - 26.85)
Linters[1]	7.79 ± 4.87 (3.10 - 12.83)	9.65 ± 6.03 (3.84 - 15.90)
Defatted toasted meal	32.87 ± 3.09 (29.54 - 35.65)	47.50 ± 4.47 (42.69 - 51.52)
Once-refined oil	<LOD[2]	<LOD
Coker 312		
Fuzzy seed	<LOD	<LOD
Linters	<LOD	<LOD
Defatted toasted meal	<LOD	<LOD
Once-refined oil	<LOD	<LOD

Except where noted otherwise, three replicate samples were used to determine the means and standard deviations.

[1] Six samples were used to determine the mean and standard deviation of linters.

[2] <LOD = All values for the sample were below the limit of detection for the ELISA, and range & standard deviation could not be calculated.

B.6. Mammalian Safety Assessment Summary

B.6.a. Toxicological Assessment

1. An extensive bioinformatics search was performed to determine whether any proteins in the database showed significant amino acid sequence homology to FLCry1Ab, indicating they may be closely related to FLCry1Ab, and whether any proteins with significant sequence homology to FLCry1Ab are known to be toxins. The FLCry1Ab query sequence showed no significant sequence homology to any proteins identified as, or known to be, toxins other than delta-endotoxins, including other Cry proteins.
2. An acute oral toxicity study in the mouse demonstrated that FLCry1Ab administered as a single dose of *ca*. 1830 mg FLCry1Ab/kg body weight via test substance FLCRY1AB-0103 had no adverse effects on body weight, food consumption, hematology parameters, blood clinical chemistry parameters, organ weights, macroscopic pathology or microscopic pathology and is therefore considered to be nontoxic.
3. Effective July 22, 2004, EPA established a permanent exemption from the requirement of a tolerance for Cry1Ab in all plants (40 CFR 180.1173), http://www.epa.gov/fedrgstr/EPA-PEST/2004/April/Day-23/p9136.htm)

B.6.b. Allergenic Potential Assessment

1. Bacteria have no history of allergenicity (Taylor and Hefle, 2001; FAO/WHO, 2001). Additionally, despite decades of widespread use of *Bt* insecticides on food crops, there have been no reports of oral allergies to these preparations, and the US EPA has stated that it "…was not aware of any report of *Bt* being an allergen" (US EPA 2000, 2001, 2005).
2. An extensive bioinformatics search was performed to determine whether the amino acid sequence of FLCry1Ab shows homology with proteins known or suspected to be allergens. The results of these analyses revealed no significant amino acid sequence homology to known or putative allergenic proteins.
3. The susceptibility of FLCry1Ab to proteolytic degradation in simulated mammalian gastric fluid (SGF) was investigated and the data support the conclusion that FLCry1Ab expressed in transgenic cotton plants will be readily digested under typical mammalian gastric conditions.
4. The effect of temperature on the stability of FLCry1Ab was determined by incubating test substance FLCRY1AB-0103 for 30 minutes at a range of temperatures (25, 37, 65 and 95ºC) followed by bioassay against *O. nubilalis* larvae (Graser and Mims, 2006). There was no significant effect on bioactivity against *O. nubilalis* at 25 and 37ºC. Incubation of the test substance at 65°C for 30 minutes substantially reduced insecticidal activity and incubation at 95°C for 30 minutes resulted in a complete loss of

bioactivity. Hence, it is expected that typical processing procedures will result in a loss of bioactivity.

5. FLCry1Ab in microbially produced and plant extracted test substances was analyzed to test for glycosylation. Neither the microbially produced nor the plant-derived FLCry1Ab protein was glycosylated (Graser and Li, 2006).
6. The lack of observed toxicity to rodents acutely exposed to a high oral dose of FLCry1Ab indicates that any residues that *might* be present in food products containing COT67B by-products will not pose a safety concern.
7. The weight of evidence indicates that FLCry1Ab is not likely to be a food allergen. A substantial body of data exists to conclude that there is a reasonable certainty that no harm will result to the U.S. population, including sensitive subpopulations, from exposure to FLCry1Ab and the genetic material necessary for its production in cotton.

A detailed assessment of the mammalian safety of the FLCry1Ab protein in COT67B has been provided to the Environmental Protection Agency in support of a FIFRA Sec. 3 registration for a stacked cotton product comprising COT67B and COT102, which was previously deregulated in July 2005. A Cry1Ab protein similar to FLCry1Ab in COT67B is produced in a Syngenta corn product, Event Bt11 corn. Although Bt11 corn produces a truncated Cry1Ab protein, it has the same active insecticidal region as does the FLCry1Ab produced in COT67B (Figure 4-1, see also Sections 4.A.1 and 4.A.2., this Chapter). Data supporting the mammalian safety of Cry1Ab in support of the current registration of the Cry1Ab protein in Bt11 corn are contained in reports previously reviewed by the EPA. The reviews of these data are summarized in the Agency's Biopesticides Registration Action Document dated Oct. 15, 2001. These data lend additional support for the human safety of Cry1Ab.

(http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/2-id_health.pdf.).

C. Conclusion for FLCry1Ab Protein Characterizaton

Based on the data and information in this chapter it can be concluded that the *flcry1ab* gene and the donor sequences necessary for gene regulation function as expected to encode the FLCry1Ab protein *in planta* in COT67B and in a recombinant microbial over-expression system. The FLCry1Ab protein produced from both sources was shown to be biochemically and functionally equivalent demonstrating efficacy and lending validity to the use of the microbially derived FLCry1Ab protein in studies to demonstrate safety to mammals, other plants and the environment. The FLCry1Ab protein in COT67B was detected in a range of plant tissues consistent with known constitutive promoter expression and at levels determined to pose negligible dietary exposure. Results of mammalian safety studies provided no evidence that FLCry1Ab is toxic or allergenic or would pose a health risk to mammals. Thus, it has been demonstrated that the genetic and regulatory elements in COT67B produce the intended FLCry1Ab protein in the manner expected in COT67B cotton plants and in a microbial over-expression system.

Further, the expression of FLCry1Ab protein in tissues and whole COT67B plants occurs as expected and at levels anticipated for genes driven by constitutive promoters. Hence, the FLCry1AB protein in COT67B is expected to act solely as an insecticidal toxin specific to lepidopteran insects and therefore is highly unlikely to contribute to a situation of increased weediness or plant pest potential.

CHAPTER 5. PHENOTYPIC, AGRONOMIC AND ECOLOGICAL ASSESSMENT OF COT67B

Information in this section is used to evaluate the differences between the way the transgenic plant and its nontransgenic counterparts interact with the environment in managed and unmanaged ecosystems. Such information is useful in assessing the likelihood that deregulating COT67B will be harmful to the environment, either directly, indirectly or cumulatively.

All available phenotypic data can support crop familiarity (i.e., knowledge and experience with the crop, the trait, the receiving environment, and the interaction of these factors) (Hokanson *et al.*, 1999) to evaluate any contribution to pest potential. For example, cotton has been grown and studied for centuries and its biology, life history and pest potential is well documented. In addition, USDA APHIS, and numerous regulatory agencies world-wide have evaluated the potential environmental impact of insect resistant cotton varieties developed through biotechnology. This body of knowledge firmly establishes familiarity with the species and trait and serves as a baseline for the variability common to either conventional cotton or biotechnology-derived insect resistant varieties.

Measurement of phenotypic characteristics and environmental interactions provide information and data for a comparative assessment of ecological risk (pest potential) between a biotechnology-derived crop and an appropriate control in both managed and unmanaged ecosystems. When a difference is measured, a tiered approach is used to assess whether a difference is biologically meaningful. As such, evaluation of phenotypic characteristics is specific to the biology of the crop using replicated plots at multiple locations with appropriate controls, which may include conventional cultivars as references. When no statistically significant differences (typically $p \leq 0.05$) in phenotypic characteristics are detected between the transgenic plant and the appropriate control, a conclusion of no contribution to pest potential can be made. If a statistically significant difference in a characteristic is detected, the magnitude of the difference is considered (relative to the known ranges of values for the species), and its effect on pest potential assessed to determine if it is biologically meaningful. Detected differences in a characteristic are considered alone and in the context of 1) whether or not trends were observed over locations; 2) whether differences were detected in other measured characteristics; 3) whether the differences enhance any inherent pest potential of the crop; and 4) potential effects if the trait is transferred to a wild or weedy species.

Agronomic and phenotypic evaluations were conducted at 22 locations in 2004, 2005 and 2006 throughout the U.S. cotton belt to determine whether unintended phenotypic effects or ecological interactions may have resulted from the insertion of the *flcry1Ab* gene in COT67B relative to its null isoline (hereafter referred to as COT67B(-) in this section) and the conventional cultivar used for transformation, Coker 312. Experienced agronomists, breeders, and field scientists at locations representative of the environments and agronomic conditions in which COT67B is expected to be grown performed these evaluations. The specific plant characteristics, number of locations, year(s) evaluated, and USDA notification numbers under which the field trials were conducted are provided in Table 5-1.

Table 5-1. Plant growth and phenotypic characteristics of Event COT67B, COT67B(-) and Coker 312 evaluated in the U.S. during 2004, 2005 and 2006

Characteristic	Number of Locations	Year(s) Evaluated	USDA Notification Numbers
Growth Habit[1]			
Plant height	15	2005, 2006	05-034-02n, 06-039-16n
Total nodes	15	2005, 2006	05-034-02n, 06-039-16n
Height to node ratio	22	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n
Vegetative nodes	15	2005, 2006	05-034-02n, 06-039-16n
Node of the 1st fruiting branch	6	2004	04-041-01n
Number of fruiting nodes	15	2005, 2006	05-034-02n, 06-039-16n
Germination and Emergence			
Field emergence / Final stand	13	2005, 2006	05-034-02n, 06-039-16n
4 day germination	12	2005, 2006	05-034-02n, 06-039-16n
7/9 day germination	12	2005, 2006	05-034-02n, 06-039-16n
Cool germination	12	2005, 2006	05-034-02n, 06-039-16n
Abnormal germination	12	2005, 2006	05-034-02n, 06-039-16n
Cool abnormal germination	12	2005, 2006	05-034-02n, 06-039-16n
Vigor	12	2005, 2006	05-034-02n, 06-039-16n
Hard seed	8	2006	06-039-16n
Flowering Period			
Days to first flower	15	2005, 2006	05-034-02n, 06-039-16n
Days to 50% flower	3	2004	04-041-01n
Nodes above white flower	15	2005, 2006	05-034-02n, 06-039-16n

Table 5-1. Continued

Characteristic	Number of Locations	Year(s) Evaluated	USDA Notification Numbers
Reproductive Potential			
Days to 50% open boll	10	2004, 2006	04-041-01n, 06-039-16n
Bolls per plant	9	2006	06-039-16n
Vegetative bolls per plant	9	2006	06-039-16n
Days to first open boll	3	2004	04-041-01n
Seed per plant	13	2005, 2006	05-034-02n, 06-039-16n
Seed per plant (g)	13	2005, 2006	05-034-02n, 06-039-16n
Seed per acre	13	2005, 2006	05-034-02n, 06-039-16n
Percent lint turnout	16	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n,
Fuzzy seed per acre	13	2005, 2006	05-034-02n, 06-039-16n
Seed index (grams per 100 seeds)	13	2005, 2006	05-034-02n, 06-039-16n
Seed cotton per acre	18	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n
Lint yield per acre	13	2005, 2006	05-034-02n, 06-039-16n
Fiber Quality			
Length	17	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n
Strength	17	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n
Micronaire	17	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n
Uniformity	17	2004, 2005, 2006	04-041-01n, 05-034-02n, 06-039-16n
Elongation	15	2005, 2006	05-034-02n, 06-039-16n
Maturity	15	2005, 2006	05-034-02n 06-039-16n

[1] Evaluations performed in 2004 at bloom and pre-harvest, in 2005 at square, early and late bloom and in 2006 at square, early and late bloom and pre-harvest

A. Experimental Comparators

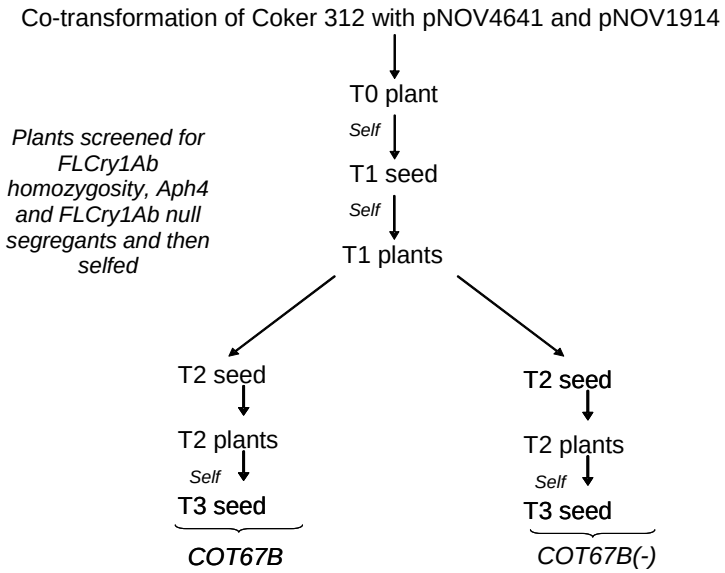
Event COT67B was selected from approximately one hundred independent transformation events based on its insecticidal efficacy, acceptable molecular profile and suitable agronomic and phenotypic characteristics. The initial plants obtained from the transformation process were designated the T0 generation and all subsequent generations of COT67B were derived from this plant. T0 plants were selfed, giving rise to T1 seed.

During the initial transformation process, the *flcry1Ab* and the antibiotic-resistance selectable marker genes (*aph4*) were introduced on separate constructs. This

transformation strategy allowed them to insert independently into the cotton genome, so they would also segregate independently during selfing and breeding. Therefore, T1 COT67B plants were segregating for both the *flcry1Ab* and *aph4* genes. The T1 plants were screened for those homozygous for the transgene of interest, but lacking the *aph4* gene. Coincidentally, COT67B(-) null segregants lacking both the *flcry1Ab* and *aph4* genes were also identified. These plants were selfed to give rise to T2 seed. The T2 plants were then selfed to produce the T3 seed used in these studies. A schematic of the production of COT67B and COT67B(-) seed is shown in Figure 5-1.

In addition to COT67B(-), Coker 312, the nontransgenic parental variety used to develop COT67B was included as a second comparator in these studies. Coker 312 is a United States Protected Variety (PVP 7200100) currently owned by the SeedCo Corporation of Lubbock, TX. The Coker Pedigreed Seed Company initially released Coker 312 in 1974, which was developed from a cross of Coker 100 with D&PL-15. Coker 312 was chosen because of its amenability to tissue culture and molecular transformation techniques.

Figure 5-1. Summary of derivation of COT67B and COT67B(-) seed used for 2004 agronomic trials.



B. Experimental Program

Field evaluations were performed with COT67B, COT67B(-) and Coker 312 at twenty-two locations combined over 2004, 2005 and 2006. During that period thirty-five plant characteristics were evaluated and more than 2,500 individual data points collected. The amount of data collected differed between the 2004 and 2005 and 2006 field seasons as individual transformation event selections were performed in 2004. COT67B was identified as the lead event in 2005 and consequently, the field protocols and

characteristics evaluated were similar to those evaluated in 2006. Severe weather in 2004 and 2005, and a later than ideal planting in 2005 compared to 2006 prevented the collection of data at some locations and plant growth stages. However, the ability to perform meaningful comparisons between COT67B and its nontransgenic comparators was not affected.

The data reported in this section are presented by the year collected, the experimental program including methods employed, characteristics evaluated and the results summarized across locations. Individual location data are presented in Apendices 4.A., B., and C.

These data, in total, demonstrate that no unintended phenotypic effects or ecological interactions resulted from the insertion of the *flcry1Ab* gene in COT67B relative to its null isoline COT67B(-) and the conventional cultivar used for transformation, Coker 312. Consequently, COT67B poses no more a plant is risk either directly to the cotton ecosystem, indirectly to agriculture as a whole, or cumulatively, taking into consideration incremental past, present and reasonably foreseeable future impacts resulting from the deregulation and unconfined planting of COT67B compared to cotton varieties currently cultivated.

C. 2004 Experimental Program

The agronomic and phenotypic characteristics of COT67B plants were first evaluated in the field in 2004. The relative growth rate, seed cotton yield and fiber quality characteristics of COT67B, COT67B(-) and Coker 312 were compared.

C.1. Experimental Methods

C.1.a. Field trial locations and experimental design

Data were collected from trials planted at seven locations distributed across the southern and southeastern cotton growing regions of the U.S. However, due to adverse weather and/or field conditions all parameters were not assessed at every location. Table 5-2 identifies the location, date of planting and data collected at each location. These trials were planted under USDA notification 04-041-01n.

At each location, seed were planted in plots comprising two, 40 foot rows spaced 38 inches apart, with approximately three plants per foot. Two rows of a different cotton variety were planted as a buffer between each plot, so that each trial comprised a single continuous canopy of cotton. Four replicate plots of each entry were planted at each location. The plots were arranged in a randomized complete block design, but with randomization constrained so COT67B and COT67B(-) plots were always adjacent to each other to allow a more sensitive comparison between these treatments. A number of other entries were also present in these trials; however, the analysis presented here was restricted to the three genotypes of interest, Coker 312, COT67B and COT67B(-).

Table 5-2. Locations of 2004 COT67B, COT67B(-) and Coker 312 agronomic and phenotypic evaluation trials and data collected.

Location	Planting Date	Data Collected[1]
Leland, MS	5/11/2004	H:N(1), H:N(2), N, F, B, Y, LT, FQ
Winnsboro, LA (1)	5/27/2004	H:N(1), H:N(2), N, F, B, Y, FQ
Winnsboro, LA (2)	5/30/2004	H:N(1), H:N(2), N, Y, LT, FQ
Vero Beach, FL	6/15/2004	H:N(1), H:N(2), N, F
Newport, AR	5/26/2004	H:N(1), H:N(2), N, Y
Quitman, GA	6/11/2004	H:N(1)
Bossier City, LA	5/24/2004	H:N(1), H:N(2), N, B, Y, LT

[1] H:N(1) = height to node ratio at early flowering, H:N(2) = height to node ratio at pre-harvest, N = node of first fruiting branch, F = days to 50% flowering, B = days to 50% open boll, Y = yield, LT = % lint turnout, FQ = fiber quality

C.2. Agronomic Practices

Before planting, all trial seed were treated with Dynasty CST at a rate of 34 gai / 100 kg seed and with Cruiser at a rate of 0.34 mg ai/seed to protect against seedling fungal diseases and early season sucking insect pests, respectively.

Standard agronomic practices for cotton production at each location were used to maintain the plants. Weeds were controlled by standard agricultural methods, including mechanical cultivation, hand-weeding, use of residual herbicides at planting and use of post-directed herbicide applications. All trials were treated with commercially available insecticides as needed to control all insect pests, including lepidoptera. Treatments were made to ensure the intrinsic agronomic and phenotypic characteristics of the plants were observed without being confounded by potentially different responses of the plants to insect attack.

C.2.a. Relative growth rates and plant development

Height-to-node ratios were recorded at two developmental stages, early flowering and pre-harvest (after boll maturation). At each time point, five plants were selected at random from each plot. The height of each plant (in inches) was measured, and the total number of main stem nodes present on the plant counted. The height-to-node ratio was derived by dividing the height by the number of nodes on each plant, and the mean height-to-node ratio for all five plants in the plot was calculated.

The node position of the first fruiting branch was also determined pre-harvest after boll maturation. Five plants were sampled at random per plot and the node that gave rise to the first fruiting branch on each plant was recorded. The mean node position of the first fruiting branch was then calculated.

The number of days to 50% flowering was determined when 50% of the plants in each plot had at least one white flower. Similarly, the number of days to 50% open boll was determined when 50% of the harvestable bolls in each plot were open.

C.2.b. Seed cotton yield and percent lint turnout

At the end of the growing season, each two row plot was harvested and the seed cotton weighed. The yield of seed cotton per acre was calculated by multiplying the yield from each plot by the appropriate scaling factor. For example, trials planted with 38" row spacing comprise 13,762 running feet per acre. Thus, the yield per acre is calculated by multiplying the yield per plot (2 x 40 ft rows) by 13,762 divided by 80. Each batch of seed cotton was then ginned to separate the lint from the cotton seed. The lint turnout is expressed as the weight of lint after ginning as a percentage of the weight of seed cotton before ginning.

C.2.c. Fiber quality characteristics

Ginned COT67B, COT67B(-) and Coker 312 lint samples were submitted to High Volume Instrumentation (HVI) testing for fiber characteristic and quality evaluations. Many of these qualities are genetically controlled while some are heavily influenced by environment but all are important in the evaluation of the agronomic suitability of COT67B and potential unintended effects resulting from the insertion of the *flcry1Ab* gene.

The fiber quality parameters evaluated in 2004 were:

- Micronaire - measure of the surface area of cotton lint fibers (Hake *et al.* 1996).
- Maturity - calculated from HVI generated micronaire values.
- Length - measured length of the fibers to the nearest 32nd of an inch (Hake *et al.* 1996).
- Strength - relative strength of one tex of cotton fibers, with a tex being the mass in grams of 1000 meters of fiber (Hake *et al*. 1996).
- Uniformity - estimation of the short fiber content in a given cotton fiber sample, the parameter is calculated as the ratio of the average length of all fibers divided by the average length of the longer half of the fibers (Hake *et al.* 1996).

C.3. Statistical Analysis

Data were subjected to analysis of variance across location. The statistical significance of an event effect was determined using a standard F-test. An F-test probability of $p \leq 0.05$ indicates that the difference between events was statistically significant at the customary 0.05 level of probability. An F-test was also used to assess the significance of the location x event interaction. A statistically significant interaction suggests that the effect of event varied from one location to another.

C.4. Results of 2004 Experimental Program

C.4.a. Relative growth rates and plant development

Table 5-3 presents the mean height-to-node ratios at early flowering and preharvest after boll maturation, the position of first fruiting branch, and days to 50% flowering and 50% open boll of COT67B, COT67B(-) isoline and Coker 312 at each location assessed. In all cases, there was no evidence of a location x genotype interaction i.e. the relative performance of the genotypes did not differ according to location (Appendix 4.A.). Consequently, to maximize the sensitivity of the comparisons between genotypes, the mean for each genotype was calculated across all locations. In all cases, COT67B, COT67B(-) and Coker 312 were not significantly different for the growth characteristics measured.

Table 5-3. Comparison of plant growth characteristics of COT67B, COT67B(-) and nontransgenic cotton cultivar Coker 312 at multiple growth stages and locations in 2004

Genotype	H:N Ratio (early flowering)[1]	H:N Ratio (pre-harvest)[2]	Node of First Fruiting Branch[2]	Days to 50% Flowering[3]	Days to 50% Open Boll[4]
Coker 312	1.86	2.28	5.24	60.92	127.25
COT67B	1.88	2.29	4.95	61.58	129.17
COT67B(-)	1.83	2.26	4.70	61.92	128.42
LSD ($p \leq 0.05$)	NS	NS	NS	NS	NS
Treatment Probability (F)	0.576	0.766	0.074	0.171	0.40

[1] Data representative of all seven locations

[2] Data representative of all locations except Quitman, GA due to adverse weather and field conditions at time of evaluation

[3] Data representative of Leland, MS, Winnsboro, LA and Bossier City, LA due to adverse weather and field conditions at time of evaluation at other locations

[4] Data representative of Leland, MS and Winnsboro, LA due to adverse weather and field conditions at time of evaluation at the other locations

C.5. Seed Cotton Yield and Percent Lint Turnout

Table 5-4 represents the across location mean seed cotton yield and mean percent lint turnout for COT67B, COT67B(-) and Coker 312. There was no evidence of a genotype x location interaction for seed cotton yield (Appendix 4.A.). Therefore, the mean data for each genotype across all locations was calculated. The yield of seed cotton from Coker 312 was significantly ($p \leq 0.05$) higher than either COT67B or COT67B(-) but there was no significant difference in yield between COT67B and COT67B(-).

Analysis of percent lint turnout identified a significant genotype x location interaction for Coker 312 but not for the other treatments (Appendix 4.A.). Consequently, the mean

Coker 312 data were excluded from the across location analysis, which revealed no evidence of any genuine difference between COT67B and COT67B(-) (Table 5-4).

C.6. Fiber Quality Characteristics

Table 5-5 presents the comparison of fiber micronaire, length, strength and uniformity for COT67B, COT67B(-), and Coker 312. There was no evidence for a genotype x location interaction for fiber micronaire or strength, but there was for length and uniformity data (Appendix 4.A.). For both of these characteristics the significance of the interaction was largely a consequence of the low levels of plot-to-plot variation in the data rather than a major inconsistency in performance from one location to another. Consequently, averaging over locations was considered meaningful (Appendix 4.A.).

There were no significant differences between genotypes for across location mean fiber micronaire, length or uniformity (Table 5-5). However, although the fiber strength of COT67B was significantly different from COT67B(-), neither genotype was significantly different from Coker 312.

Table 5-4. Comparison of mean seed cotton yield (lbs/acre) and percent lint turnout of COT67B, COT67B(-) and Coker 312 at multiple locations in 2004

Genotype	Yield of Seed Cotton (lbs/ acre)[1]	Percent Lint Turnout [2]
Coker 312	2660.68a[3]	43.39[4]
COT67B	2391.99b	42.25
COT67B(-)	2236.86b	42.70
Treatment Probability (F)	0.03	0.394

[1] Yield data was not taken at Vero Beach, FL or Quitman, GA

[2] Percent lint turnout was not determined at Winnsboro, LA, Vero Beach, FL, Newport AR or Quitman, GA

[3] Means followed by different letters are significantly different at $p \leq 0.05$

[4] Coker 312 mean data were excluded from the across location analysis

Table 5-5. Comparison of cotton fiber characteristics of COT67B, COT67B(-) and nontransgenic cotton cultivar Coker 312 grown at Leland, MS and Winnsboro, LA sites in 2004

Genotype	Micronaire	Length (inches)	Strength (g/tex)	Fiber Uniformity Index
Coker 312	4.42	1.21	28.99 AB[1]	82.75
COT67B	4.24	1.21	29.7 A	82.83
COT67B null isoline	4.23	1.21	28.76 B	82.75
LSD ($p \leq 0.05$)	NS	NS	NS	NS
Treatment Probability (F)	0.073	0.762	0.034	0.942

[1] Means followed by different letters are significantly different at $p \leq 0.05$

D. 2005 Experimental Program

Field trials of COT67B, COT67B(-) and Coker 312 were conducted in 2005 at locations in Georgia, Mississippi, North Carolina, South Carolina and Texas. These locations are representative of the various environments and insect pressures of the U.S. cotton belt where the predominance of COT67B will be grown. The results from these evaluations are consistent with those of 2004 and lend further support to the conclusion that COT67B represents no greater a plant pest risk than either of its comparators.

D.1. Experimental Methods

D.1.a. Field trial locations and experimental design

Data were collected from trials planted at seven locations distributed across the southern and southeastern cotton growing regions of the U.S. However, for logistical or other reasons (adverse weather, conditions, field conditions, etc.), all parameters were not assessed at every location. Table 5-6 identifies the locations, date of planting and data collected. These trials were planted under USDA notification 05-034-02n.

At each location, seed were planted in plots comprising two, 35-foot rows spaced 38 inches apart, with approximately 3.5 to 4 plants per row foot. Four replicate plots of each entry were planted at each location and arranged in a randomized complete block design. The entire trial was reproductively isolated from other cotton through the planting of at least sixteen border rows of conventional cotton variety DP491. A number of other

entries were also present in these trials; however, the analysis presented here was restricted to COT67B, COT67B(-) and Coker 312.

Table 5-6. Locations of 2005 COT67B, COT67B(-) and Coker 312 agronomic and phenotypic evaluation trials and data collected

Location	Planting Date	Data Collected[1]
Estill, SC	6/16/2005	PM, Yield, SP, SG
Hartsville, SC	6/15/2005	PM, Yield, SP, SG
Red Springs, NC	6/14/2005	PM
Winterville, MS	6/18/2005	PM, Yield, SP, SG,
Tifton, GA	6/17/2005	PM, Yield, SP, SG
Verona, MS	6/16/2005	PM
Haskell, TX	6/20/2005	PM

[1] PM = plant mapping; SP = seed productivity and SG = seed germination

D.1.b. Agronomic practices

Before planting, all trial seed were treated with Dynasty CST at a rate of 34 g ai / 100 kg seed and with Cruiser at a rate of 0.34 mg ai/seed to protect against seedling fungal diseases and early season sucking insect pests, respectively.

Typical management inputs including fertilizer, residual herbicides and growth regulators (mepiquat chloride) were applied as needed. Some local modifications were made to management depending on location, trial type, and/or local custom as needed. All trials were treated with commercially available insecticides as needed to control all insect pests, including Lepidoptera. Treatments were made to ensure the intrinsic agronomic and phenotypic characteristics of the plants were observed without being confounded by potentially different responses of the plants to insect attack.

D.1.c. Relative growth rates and plant development

Event COT67B, COT67B(-) and Coker 312 plants were mapped and monitored. Plant monitoring was performed at three phenotypic stages during the 2005 season; early square, early bloom, and late bloom at all locations except Haskell, TX where early square measurements were not possible. The plant characteristics evaluated at each growth stage are summarized in Table 5-7. Plant stands were measured by sampling the center 10 feet of each plot row for total emergence and plant establishment. Twenty plants per tested line per location (5 plants per plot/rep with 4 replicates per location) were sampled for all plant monitoring measurements.

Table 5-7. Phenotypic growth stages of COT67B, COT67B(-) and Coker 312 and characteristics evaluated at each location in 2005.

Growth Stage	Plant characteristic evaluated (unit)	2005 Trial Locations
Emergence	Plants per row foot (#)	Estill, SC Hartsville, SC Tifton, GA Winterville, MS
Early square	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Height to node ratio	Estill, SC Hartsville, SC Red Springs, NC Tifton, GA Verona, MS Winterville, MS
Early bloom	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Nodes above white flower (#) Date of first flower (date) Height to node ratio	Estill, SC Hartsville, SC Red Springs, NC Tifton, GA Verona, MS Winterville, MS Haskell, TX
Late bloom	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Nodes above white flower (#) Height to node ratio	Estill, SC Hartsville, SC Red Springs, NC Tifton, GA Verona, MS Winterville, MS Haskell, TX

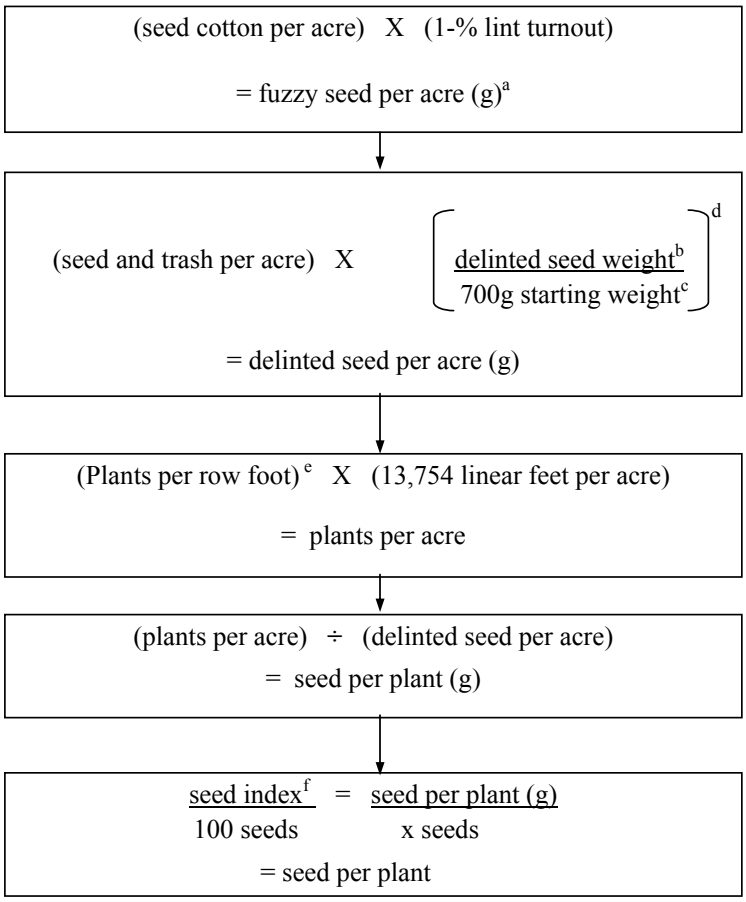
D.1.d. Seed cotton yield and percent lint turnout

At the end of the growing season, each two row plot was machine harvested and the seed cotton weighed. The yield of seed cotton per acre was calculated by multiplying the yield from each plot by the appropriate scaling factor. For example, trials were planted with 38" row spacing comprise 13,762 running feet per acre. Thus, the yield per acre was calculated by multiplying the yield per plot (2 x 35 ft rows) by (13,762 ÷ 70). Each batch of seed cotton was then ginned to separate the lint from the cotton seed. Lint turnout is expressed as the weight of lint after ginning as a percentage of the weight of seed cotton before ginning.

D.1.e. Seed productivity

Estimates were made of seed productivity per plant for COT67B, COT67B(-) and Coker 312 using the data for seed cotton yield, lint yield, lint turnout, de-linting turnout, final plant stand estimates and seed index measurements from the Estill and Hartsville, SC, Tifton GA and Winterville, MS trials. The formulae for calculating seed per plant using these data is presented in Figure 5-2.

Figure 5-2. Formulae for calculating seed productivity per plant using the data for seed cotton yield, lint yield, lint turnout, delinting turnout, final plant stand and seed index measurements



[a] fuzzy seed includes leaf and other plant material adhering to fibers left on seed after ginning
[b] weight of seed after acid delinting removes remaining fibers and plant material
[c] 700g = weight of fuzzy seed before acid delinting
[d] delinted seed weight ÷ 700g fuzzy seed starting weight = delinting turnout
[e] plants per row foot calculated from actual stand count
[f] seed index = weight of 100 acid delinted seeds

D.1.f. Seed germination and viability

Seed germination and seedling vigor are important characteristics to assess whether COT67B seed has the potential to be more persistent in the environment than COT67B(-) and Coker 312. Standardized germination assays of the Association of Official Seed Analysts (AOSA, 1998) are used as a baseline to measure the germination potential and vigor of cottonseed. These tests were conducted on seed collected from the Estill and Hartsville, SC, Tifton GA and Winterville, MS trials. For each of the following tests, four replicates of 50 seed per plot per location were evaluated following the methods of the Association of Official Seed Analysts (AOSA, 1998).

1. Four-day scoring on standard germination test conducted at alternating temperatures of 20°C/30°C (68°F/86°F). All seedlings longer than 1.5 inches considered germinated.
2. Seven and nine day scoring of the standard germination test; all seedlings longer than 1.5 inches considered germinated.
3. Stressed germination test at 18°C (64.4°F); a seven-day scoring of germination. All seedlings longer than 1.5 inches counted.
4. Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).
5. Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).
6. Vigor Index as calculated by 4 day warm germination + cool germ = seed index.

D.1.g. Fiber quality characteristics

Lint samples were submitted to (HVI) testing for fiber characteristic and quality evaluations as in 2004. In addition to measuring fiber micronaire, maturity, length and strength, elongation (the ratio of the elongation of a cotton fiber at a breaking load (Reily 1997)) was also evaluated.

D.1.h. Statistical analysis

Statistical analyses employed were determined by the type of data to be analyzed. All analyses were conducted using JMP software (SAS institute, 2001) by subjecting the data to typical ANOVA procedures via the appropriate model(s). Means were separated using LSD (Gomez and Gomez; 1984.)

D.2. Results of 2005 Experimental Program

D.2.a. Relative growth rates and plant development

Event COT67B, COT67B(-) and Coker 312 plants were monitored and mapped during early square, early bloom, and late bloom growth stages. As indicated above, early square measurements were taken at all locations but Haskell, TX. The data was analyzed first as randomized complete blocks to obtain within location means and then, where data balance allowed, an across location analysis was conducted. The across location analysis for each of the three growth stages is presented in Table 5-8 through Table 5-10. The data analyzed by location is attached in Appendix 4.B.

D.2.b. In summary:

Early square (Table 5-8) – Across six locations no statistically significant differences ($P \leq 0.05$) were measured in height, height to node ratio and total number of nodes, vegetative nodes and fruiting nodes when comparing COT67B, to either COT67B(-) or Coker 312.

Early bloom (Table 5-9) - Across seven locations, COT67B was not significantly different ($P \leq 0.05$) from COT67B(-) for any of the characteristics measured and significantly different from Coker 312 for only the number of total nodes and the number of vegetative nodes. Although statistically significant, this difference was small and has little biological meaning in terms of plant weed or pest potential.

Late bloom (Table 5-10) - Across seven locations, COT67B was significantly different ($P \leq 0.05$) from COT67B(-) for total nodes and nodes above white flower and significantly different from Coker 312 for total nodes, number of vegetative nodes and number of nodes above white flower. Although statistically significant, these differences were also small and likely have little biological meaning in terms of plant pest or weed potential.

Table 5-8. Across location analysis of early square plant mapping information for COT67B, COT67b(-) and Coker 312 evaluated at six locations during 2005

Genotype	Height	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	18.1	9.4	4.7	4.6	1.94
COT67B(-)	17.2	9.3	4.6	4.7	1.83
Coker 312	18.1	9.5	4.8	4.8	1.90
LSD ($p \leq 0.05$)	NS[1]	NS	NS	NS	NS
Treatment Probability (F)	0.133	0.521	0.104	0.761	0.189

[1] NS = Not significant

Table 5-9. Across location analysis of early bloom plant mapping information for COT67B, its null isoline and Coker 312 evaluated at seven locations during 2005[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Date of First Flower	Height to Node Ratio
COT67B	29.3	13.9b	4.6b	9.3	6.8	7-Aug	2.13
COT67B(-)	28.2	13.5b	4.6b	9.0	6.8	7-Aug	2.10
Coker 312	29.1	14.2a	4.8a	9.4	6.8	6-Aug	2.07
LSD ($p \leq 0.05$)	NS[2]	0.5	0.2	NS	NS	NS	NS
Treatment Probability (F)	0.317	0.034	0.007	0.203	0.990	0.549	0.480

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Table 5-10. Across location analysis of late bloom plant mapping information for COT67B, its null isoline and Coker 312 evaluated at seven locations during 2005[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Height to Node Ratio
COT67B	33.3	15.6b	4.6b	11.0	2.8b	2.13
COT67B(-)	33.5	16.1a	4.8ab	11.3	3.4a	2.08
Coker 312	35.0	16.2a	4.9a	11.3	3.3a	2.16
LSD ($p \leq 0.05$)	NS[2]	0.4	0.2	NS	0.3	NS
Treatment Probability (F)	0.136	0.024	0.032	0.236	0.004	0.342

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

D.2.c. Seed cotton yield, percent lint turnout and seed productivity

Data for seed cotton yield, lint yield, lint turnout, de-linting turnout, final plant stand estimates and seed index measurements from the Estill and Hartsville, SC, Tifton GA and Winterville, MS trials were used to estimate seed productivity for COT67B, COT67B(-) and Coker 312. These data are presented in Table 5-11. The analysis of these characteristics failed to identify a significant interaction between the three genotypes other than for the across location seed index comparison. However, COT67B was not significantly different from its null segregant, COT67B(-). Consequently, from these data, it can be concluded that the insertion of the *flcry1Ab* gene and expression of the

FLCry1Ab protein did not alter COT67B plants in comparison to its negative isoline (COT67B(-)) or Coker 312.

Table 5-11. Across location analysis of lint yield and seed productivity for COT67B, COT67B(-) and Coker 312 evaluated at four locations during 2005[1]

Genotype	Seed Cotton/ Acre (lbs)	% Lint Turnout	Lint/ Acre (lbs)	Delinted Weight (g)	% Delinting Turnout	Plants/ Row Foot	Seed Index (g)	Seed/ Plant (g)	Seed/ Plant
COT67B	1405	31.06	433	551	78.7	3.13	7.74b	8.08	105.49
COT67B (-)	1459	30.81	446	548	78.3	2.94	8.07b	8.98	111.17
Coker 312	1392	30.05	422	542	77.5	2.98	8.60a	8.68	101.15
LSD ($p \leq 0.05$)	NS[2]	NS	NS	NS	NS	NS	0.51	NS	NS
Variety p	0.6123	0.1710	0.06461	0.6636	0.6636	0.2041	0.0021	0.3769	0.3532

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

D.3. Seed Germination and Viability

Results of the seed germination evaluations from 2005 are presented in Table 5-12. There were no statistically significant differences across locations for the six seed evaluations performed. Although the number of abnormal seed by location was statistically significantly different, there was no consistent trend between genotypes. In addition to these evaluations, there was no significant difference in plant stand (plants per row foot) as indicated in Table 5-11. Consequently, from these data, there is no evidence to indicate the insertion of the *flcry1Ab* gene and expression of the FLCry1Ab protein increased the germination potential for COT67B, which could lead to greater persistence or competitiveness than either its negative isoline or Coker 312.

Table 5-12. Across location nalysis of seed germination and viability characteristics for COT67B, COT67B(-) and Coker 312 seed collected from four locations during 2005[1]

Genotype	4-Day Germ.[2]	7 and 9-Day Germ.[3]	Cool[4]	Abnormal[5]	Cool Abnormal[6]	Vigor Index[7]
COT67B	50.88	57.81	37.53	12.84	15.47	88.40
COT67B(-)	56.06	63.28	45.25	13.53	17.97	101.31
Coker 312	52.41	60.41	39.25	15.19	19.09	91.65
LSD ($p \leq 0.05$)	NS[8]	NS	NS	NS	NS	NS
Variety p	0.3961	0.3954	0.091	0.2203	0.113	0.1801

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] 4-day count on standard germination test conducted at alternating temperatures of 20°C/30°C; 68°F/86°F; all seedlings longer than 1.5 inches counted.

[3] 7/9 day scoring of the standard germination test; all seedlings longer than 1.5 inches counted.

[4] Stressed germination at 18°C (64.4°F); 7-day reading of germination; all seedlings longer than 1.5 inches counted.

[5] Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[6] Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[7] Vigor Index as calculated by 4 day warm germination + cool germ = seed index.

[8] NS = Not significant

D.4. Fiber Quality Characteristics

Table 5-13 presents the comparison of fiber micronaire, length, strength, uniformity and elongation for COT67B, COT67B(-), and the nontransgenic line Coker 312. There was no evidence for a genotype x location interaction for fiber micronaire or strength, but there was for length and uniformity data (Appendix 4.B). For both of these characteristics the significance of the interaction was largely a consequence of the very low levels of plot-to-plot variation in the data rather than a major inconsistency in performance from one location to another. Consequently, averaging over locations was considered meaningful (Appendix 4.B.). In addition, the range of fiber length and uniformity for the three genotypes evaluated falls within the range for these characteristics reported by the USDA Agricultural Marketing Service for upland cotton grown in the U.S. (AMS, 2006).

There were no significant differences between genotypes for across location mean fiber micronaire, length or uniformity (Table 5-13). Although the fiber strength of COT67B was significantly different from its null isoline, neither genotype was significantly different from the nontransgenic line Coker 312. Therefore, these differences are not considered biologically meaningful.

Table 5-13. Across location analysis of fiber quality characteristics for COT67B, its null isoline and Coker 312 evaluated at six locations during 2005[1]

Genotype	Maturity	Micronaire	Length (inches)	Strength (g/tex)	Fiber Uniformity Index	% Elongation
COT67B	85.00	4.05	1.19a	31.19	83.93a	12.35
COT67B(-)	84.60	4.00	1.13b	30.97	83.00b	12.56
Coker 312	84.45	3.99	1.17a	30.93	83.50ab	12.67
LSD ($p \leq 0.05$)	NS[2]	NS	0.023	NS	0.623	NS
Trtmnt Prob (F)	0.11	0.3524	<0.0001	0.57	0.0059	0.4716

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

E. 2006 Experimental Program

Field trials of COT67B, COT67B(-) and Coker 312 were conducted in 2006 at locations in Alabama, Georgia, Louisiana, Mississippi, North Carolina, South Carolina and Texas (Table 5-14). These locations are representative of the various environments and insect pressures of the U.S. cotton where the predominance of COT67B will be grown. The results from these evaluations are consistent with those of 2004 and 2005 and lend further support to the conclusion that COT67B represents no greater a plant pest risk than either of its comparators.

Table 5-14. Locations of 2006 COT67B, COT67B(-) and Coker 312 agronomic and phenotypic evaluation trials

Location	Planting Date	Data Collected[1]
Belle Mina, AL	5/18/2006	PM, Yield, SP, SG
Tifton, GA	6/8/2006	PM, Yield, SP, SG
Alexandria, LA	5/18/2006	Yield, SP
Verona, MS	5/23/2006	Yield, SP, SG
Winterville, MS	6/18/2006	PM, Yield, SP, SG
Red Springs, NC	6/14/2006	PM, Yield, SP, SG
Estill, SC	5/18/2006	PM, Yield, SP, SG
Hartsville, SC	5/23/2006	PM, Yield, SP, SG
Haskell, TX	5/19/2006	PM
College Station, TX	5/18/2006	PM, Yield, SP, SG

[1] PM = plant mapping; SP = seed productivity and SG = seed germination

E.1. Experimental Methods

The methods employed and data collected in the 2005 experimental program described on in Section D. above were followed for the evaluations conducted in 2006 with few exceptions as identified in the following sections.

These trials were planted under USDA notification 06-039-16n.

E.2. Results of 2006 Experimental Program

E.2.a. Relative growth rates and plant development

In 2005, COT67B, COT67B(-) and Coker 312 plants were monitored and mapped during early square, early bloom and late bloom growth stages. In 2006, an additional, later season (pre-harvest) evaluation was performed to collect data regarding the number of reproductive and vegetative bolls per plant and date of 50% open boll (Table 5-15). As in 2005, twenty COT67B, COT67B(-) and Coker 312 plants per location (5 plants per replicate and 4 replicates per location) were sampled for all plant monitoring measurements. The number of days to 50% open boll was estimated by assessing when 50% of the harvestable bolls in each plot were open. The data was analyzed first as randomized complete blocks to obtain within location means and then, where data balance allowed, an across location analysis was conducted. The across location analysis for each of the four growth stages is presented in Table 5-16 through Table 5-19. The data analyzed by location is attached in Appendix 4.C.

Table 5-15. Phenotypic growth stages of COT67B, COT67B(-) and Coker 312 and characteristics evaluated in 2006 at eight locations

Growth Stage	Plant characteristic evaluated (unit)	2006 Trial Locations
Emergence	Plants per row foot (#)	Belle Mina, AL Tifton, GA Winterville, MS Red Springs, NC Estill, SC Hartsville, SC Haskell, TX College Station, TX
Early square	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Height to node ratio	Belle Mina, AL Tifton, GA Winterville, MS Red Springs, NC Estill, SC Hartsville, SC Haskell, TX College Station, TX
Early bloom	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Nodes above white flower (#) Date of first flower (date) Height to node ratio	Belle Mina, AL Tifton, GA Winterville, MS Red Springs, NC Estill, SC Hartsville, SC Haskell, TX College Station, TX
Late bloom	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Nodes above white flower (#) Height to node ratio	Belle Mina, AL Tifton, GA Winterville, MS Red Springs, NC Estill, SC Hartsville, SC Haskell, TX College Station, TX
Pre-harvest	Plant height (inches) Total nodes (#) Vegetative nodes (#) Fruiting nodes (#) Height to node ratio Bolls per plant Date of 50% open boll Vegetative bolls per plant	Belle Mina, AL Tifton, GA Winterville, MS Red Springs, NC Estill, SC Hartsville, SC Haskell, TX College Station, TX

E.2.b. In summary

Across location analysis of early square data (Table 5-16) showed no statistically significant differences ($p \leq 0.05$) were measured in height, height to node ratio and total number of nodes, vegetative nodes and fruiting nodes when comparing COT67B, to either COT67B(-) or Coker 312 across eight locations.

Across location analysis of early bloom data (Table 5-17) showed no statistically significant differences ($p \leq 0.05$) were measured between COT67B and COT67B(-) for the six characteristics measured (total nodes, fruiting nodes and nodes above white flower). COT67B was statistically different from Coker 312 only in plant height. Although statistically significant, this difference was small and has no biological meaning in terms of plant weed or pest potential.

Across location analysis of late bloom data (Table 5-18) showed that COT67B was significantly different ($p \leq 0.05$) from COT67B(-) only for nodes above white flower and significantly different from Coker 312 for plant height, number of vegetative nodes and height to node ratio. Although statistically significant, these differences were also small and likely have no biological meaning in terms of plant weed or pest potential.

Across location analysis of pre-harvest data (Table 5-19) showed statistically significant differences ($p \leq 0.05$) between COT67B and COT67B(-) for plant height, height to node ratio and date to 50% open boll. Statistically significant differences between COT67B and Coker 312 were observed for number of vegetative bolls, height to node ratio and date to 50% open boll. These differences were again small and likely have no biological meaning in terms of plant weed or pest potential. In addition, the results demonstrate the tendency for Coker 312 to be more vegetative than COT67B in growth habit as measured by plant height, height to node ratio, and the allocation of nodes to vegetative vs. reproductive growth.

Table 5-16. Across location analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at eight locations during 2006

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	14.1	9.8	4.4	5.4	1.41
COT67B(-)	14.4	10.0	4.4	5.6	1.40
Coker 312	14.2	10.1	4.5	5.6	1.39
LSD ($p \leq 0.05$)	NS	NS	NS	NS	NS[1]
Treatment Probability (F)	0.740	0.092	0.116	0.256	0.897

[1] NS = Not significant

Table 5-17. Across location analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at eight locations during 2006[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	23.5b	13.8	4.3	9.5	1.69	6.4ab	Jul-13
COT67B(-)	23.9b	13.7	4.3	9.4	1.73	6.2b	Jul-12
Coker 312	24.8a	14.0	4.4	9.5	1.76	6.5a	Jul-13
LSD ($p \leq 0.05$)	0.8	NS[2]	NS	NS	NS	0.2	NS
Treatment Probability (F)	0.019	0.063	0.106	0.577	0.059	0.009	0.236

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Table 5-18. Across location analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at eight locations during 2006[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	28.4b	16.2	4.4b	11.9	1.73b	3.4a
COT67B(-)	27.8b	15.9	4.4b	11.6	1.73b	3.1b
Coker 312	29.8a	16.3	4.7a	11.6	1.82a	3.4a
LSD ($p \leq 0.05$)	1.3	NS[2]	0.1	NS	0.08	0.3
Treatment Probability (F)	0.015	0.266	<.0001	0.343	0.044	0.044

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Table 5-19. Across location final (pre-harvest) plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at six locations during 2006[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Date of 50% Open Boll	Vegetative Bolls/ Plant
COT67B	31.6ab	18.5	4.5	14.0	1.71b	11.3a	29-Sep. a	6.3b
COT67B(-)	30.6b	18.6	4.4	14.2	1.64b	8.8b	27-Sep. b	4.4b
Coker 312	32.5a	18.1	4.5	13.6	1.78a	11.2a	25 Sep. c	11.8a
LSD ($p \leq 0.05$)	1.4	NS[2]	NS	NS	0.06	1.1	1	3.4
Treatment Probability (F)	0.047	0.377	0.585	0.248	0.001	0.000	0.001	0.001

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

E.2.c. Seed cotton yield and percent lint turnout

Machine picked yields were recorded before (seed cotton) and after (lint) ginning from all locations and are presented in Table 5-20 and Table 5-21. The results for the yields of seed cotton and lint per acre demonstrate a statistically significant difference between COT67B and COT67B(-) and Coker 312. These results, which did not occur in 2005, are likely a maturity/determinancy effect, which has a high likelihood of being influenced by breeding and selection. Furthermore, with regard to percent lint turnout, COT67B was significantly different than Coker 312 but not from COT67B(-).

Table 5-20. Across location seed cotton yield, lint yield and percent lint turnout for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

Genotype	Seed Cotton Yield/ Acre (lbs)	Lint Yield/ Acre (lbs)	Percent Lint Turnout
COT67B	1858b	613b	32.8a
COT67B(-)	2173a	707a	32.6a
Coker 312	2184a	692a	31.7b
LSD ($p \leq 0.05$)	175	56	0.58
Treatment Probability (F)	0.0006	0.0028	0.0003

[1] Means followed by different letters are significantly different at $p \leq 0.05$

E.3. Seed Productivity

Estimates were made for various parameters evaluating the relative seed productivity of COT67B, COT67B(-) and Coker 312. Data shown in Table 5-21 were generated using lint turnout, seed counts, and final established plant stand using the formulae presented in Figure 5-2.

Note: Seed cotton yield, lint yield and percent turnout are the same as that reported in Table 5-20. This data is included in Table 5-21 solely to facilitate review.

In 2005, no significant difference was identified in either the location or across location analyses for delinted seed weight. Consequently, the average delinting turnout (78%) was used in the 2006 calculations of seed productivity (Table 5-11).

In summary:

As described above, COT67B, differed statistically from COT67B(-) and Coker 312 in terms of lint yield (Table 5-20 and Table 5-21). However, COT67B was typically intermediate of the two control genotypes for the other plant and seed productivity characteristics measured (Table 5-21). Consequently, it can be concluded from these results that insertion and expression of the *flcry1Ab* gene in COT67B did not result in an unintended increase in plant survival or seed production and thus, does not pose any more of a plant pest risk than nontransgenic cotton genotypes with regard to these characteristics.

Table 5-21. Across location analysis of COT67B, COT67B(-) and Coker 312 seed productivity parameters evaluated at nine locations during the summer of 2006[1,2]

Genotype	Seed Cotton/ Acre (lbs)	% Lint Turnout	Lint/ Acre[1] (lbs)	Plants/ Row Foot	Seed Index	Seed/ Plant(g)	Seed/ Plant
COT67B	1858b	32.8a	613b	3.53b	9.01a	10.15b	111a
COT67B(-)	2173a	32.6a	707a	3.81a	8.65b	8.47c	97b
Coker 312	2184a	31.7b	692a	3.22c	8.95a	10.96a	121a
LSD ($p \leq 0.05$)	175	0.58	56	0.24	0.12	1.18	13
Variety p	0.0006	0.003	0.0028	<0.0001	0.0023	0.0004	0.0026

[1] See Figure 5-2 for formulae to calculate seed per plant

[2] Means followed by different letters are significantly different at $p \leq 0.05$

E.4. Fiber Quality Characteristics

Lint samples were submitted to High Volume Instrumentation (HVI) testing for fiber characteristics/quality evaluation as in 2005. Table 5-22 presents the fiber quality characters for COT67B and COT67B(-) and Coker 312 on average across nine locations. These data were analyzed across locations while statistically preserving the blocking effects within locations, which allows an LSMEAN for each parameter to be presented. For the fiber characteristics measured COT67B was either not significantly different from COT67B(-) or intermediate to it and Coker 312. The following summarizes these results.

1. Maturity - COT67B and COT67B(-) demonstrated significantly higher fiber maturity than Coker 312 across testing locations.

2. Micronaire - COT67B and COT67B(-) demonstrated significantly lower fiber micronaire than Coker 312 across testing locations.
3. Length - COT67B and COT67B(-) had significantly shorter fiber than Coker 312 across testing locations. This characteristic, is heavily influenced by varietal background and breeding.
4. Strength - Coker 312 demonstrated significantly stronger fiber than either COT67B or COT67B(-), which were statistically similar.
5. Uniformity - Both COT67B and COT67B(-) demonstrated lower uniformity than Coker 312.
6. Elongation - Statistically significant differences in fiber elongation were observed between COT67B, COT67B(-) and Coker 312. Although statistically significant, these differences were small and within the range typically seen for cotton varieties commercially available (Riley, 1997) and is therefore, not considered biologically meaningful.

Table 5-22. Across location analysis of fiber quality characteristics for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

Across Locations[1]						
Genotype	**Maturity**	**Micronaire**	**Length (inches)**	**Strength (g/tex)**	**Length Uniformity Index**	**% Elongation**
COT67B	86.9b	4.43b	1.15b	31.48b	83.20b	10.75a
COT67B(-)	87.1b	4.41b	1.16b	31.54b	83.23b	10.45b
Coker 312	87.6a	4.53a	1.18a	32.30a	83.94a	10.16c
LSD ($p \leq 0.05$)	0.28	0.08	0.013	0.64	0.20	0.19
Treatment Probability (F)	<0.0001	0.0070	<0.0001	0.0212	0.0006	0.0031

[1] Means followed by different letters are significantly different at $p \leq 0.05$

E.5. Seed Germination, Viability and Dormancy

Seed germination, viability and dormancy characteristics was measured using a battery of seven germination tests typically performed by the cottonseed industry. The same germination tests were performed in 2005 (Table 5-12) but in 2006, an evaluation of the percentage hard seed was also performed to further address the potential for dormancy. Delinted seed from eight locations was identity preserved by genotype, location, and plot/replicate to permit a statistically valid comparison of the seed qualities evaluated.

Across all locations there were no statistically significant differences between COT67B and Coker 312 for any of seven quality parameters measured (Table 5-23). Only in the nine-day standard germination test did a statistically significant difference appear between COT67B and COT67B(-) but while this difference is statistically significant, it

is relatively small and likely biologically irrelevant as COT67B was not significantly different from Coker 312.

Table 5-23. Across location analysis of germination, viability and dormancy characteristics for COT67B, COT67B(-) and Coker 312 seed collected from eight locations during 2006[1]

Genotype	4-Day[2]	9-Day[3]	Cool[4]	Abnormal[5]	Cool Abnormal[6]	Vigor Index[7]	Hard Seed[8]
COT67B	79.2	83.3b	68.3	8.8	14.5	162.5	4.6
COT67B(-)	78.1	81.0c	66.5	10.2	15.5	159.1	4.4
Coker 312	80.6	84.2a	68.2	9.1	13.9	164.7	4.7
LSD ($p \leq 0.05$)	NS[9]	2.2	NS	NS	NS	NS	NS
Variety p	0.1294	0.0185	0.3035	0.2338	0.3040	0.0525	0.9005

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] 4-day count on standard germination test conducted at alternating temperatures of 20°C/30°C; 68°F/86°F; all seedlings longer than 1.5 inches counted.

[3] 7/9 day scoring of the standard germination test, all seedlings longer than 1.5 inches counted.

[4] Stressed germination at 18°C (64.4°F); 7-day reading of germination; all seedlings longer than 1.5 inches counted.

[5] Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[6] Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[7] Vigor Index as calculated by 4 day warm germination + cool germ = seed index.

[8] Hard seed have not imbibed water and are hard to cut with a razor.

[9] NS = Not significant

F. Summary of 2004, 2005 and 2006 Agronomic and Phenotypic Evaluations

Field evaluations were performed with COT67B, COT67B(-) and Coker 312 at twenty-two locations combined over 2004, 2005 and 2006. During that period thirty-five plant characteristics were evaluated and more than 2,500 individual data points collected. Statistically significant differences between COT67B and the control genotypes were few, not consistent across locations and years and therefore, not considered biologically meaningful. Therefore, there is no evidence that insertion of the Cry1Ab transgene into event COT67B altered the growth habit or fiber quality of the plant. Consequently, COT67B poses no more a plant pest risk directly to the cotton ecosystem, indirectly to agriculture as a whole or cumulatively taking into consideration incremental past, present and reasonably foreseeable future impacts resulting from the deregulation and unconfined planting of COT67B compared to cotton varieties currently cultivated.

G. Pollen Morphology

Lighter or smaller pollen can be distributed further via wind and a higher frequency of fertility may increase the plants ability to transfer its genetic information to other cotton plants. Both of these characteristics have the potential to increase the range and competitiveness of COT67B compared to nontransgenic counterparts. Consequently, the size, shape, fertility and weight of pollen grains derived from COT67B and Coker 312 cotton plants were evaluated to determine whether the insertion of the *flcry1Ab* gene into Coker 312 and expression of the FLCry1Ab protein by COT67B may have unexpectedly altered these characteristics. To evaluate size, shape and fertility, fresh pollen was collected from four replicated samples of ten pollen grains collected from extruded anthers of COT67B and Coker 312 cotton plants growing in the Syngenta greenhouse in Research Triangle Park, NC. To assess potential differences in the weight of pollen grains derived from these genotypes, 16 replicate samples of the pollen collected from five flowers each of COT67B and Coker 312 were weighed.

Pollen grains were stained for starch content using an iodine/ potassium iodide solution (Lugol solution, Sigma-Aldrich). Photographs were then taken under 100X magnification to assess the extent of starch staining, size and shape. Pollen grain size was measured using a Wild Heerbrugg #310345 microscope micrometer. The weight of pollen grains was measured on a Mettler AE240 balance.

Pollen shape, size and the extent of mature pollen grains in the population capable of fertilizing cotton stigmas are presented in Figures 5-3 and 5-4 As assessed by starch staining, no differences were observed in the shape or level of maturity between pollen grains shed from COT67B compared to those of Coker 312 cotton plants. Table 5-24 provides the pollen grain size (diameter), percent viable pollen and average weight of pollen grains collected from COT67B and Coker 312 cotton plants. It can be concluded from these data and observations that the insertion of the *flcry1Ab* gene and expression of the FLCry1Ab protein altered neither the size, shape, fertility nor weight of COT67B pollen, which could lead to greater persistence or competitiveness than the conventional cultivar Coker 312 from which it was derived.

Table 5-24. Comparison of COT67B and Coker 312 pollen grains collected from cotton plants grown in the greenhouse in Research Triangle Park, NC (n=10)

Genotype	Average Pollen Diameter (um)	Viable Pollen (%)	Average Pollen Weight
COT67B	102 ± 4.11	100	0.019 ± 0.013
Coker 312	103 ± 4.15	100	0.030 ± 0.020

Figure 5-3. Photograph of COT67B pollen at 100X magnification (100 µM = 1 bar)

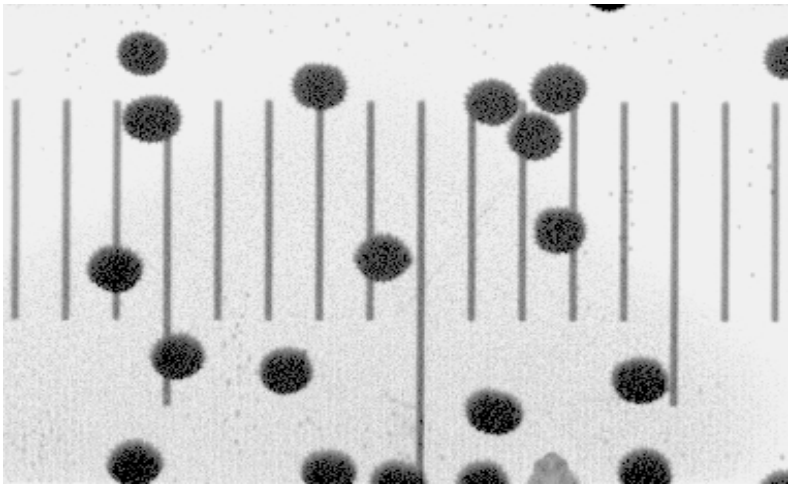
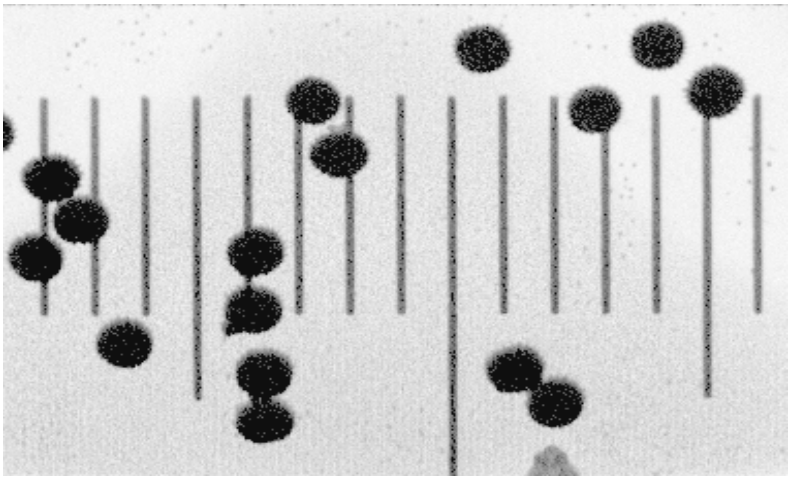


Figure 5-4. Photograph of control pollen at 100X magnification (100 µM = 1 bar)



H. Compositional and Nutritional Characteristics of COT67B Seed

H.1. Introduction

Compositional analysis of COT67B seed was performed in order to investigate whether the levels of various key nutrients or antinutrients were similar to conventional counterparts, and within the normal range of variation reported for cotton. For transgenic plants without purposefully altered nutritional properties, the nutritional evaluation is part of the "weight-of-evidence" approach for evaluating whether there were any unanticipated consequences of the genetic modification and is used as part of the assessment of plant pest risk and weediness potential.

Whole cottonseed ('fuzzy seed') can be a valuable source of protein, fat and energy in animal feed. Consequently, it is valuable to analyze whether the levels of important nutritional parameters for feed use such as proximates, amino acids, fatty acids and minerals have been altered due to genetic modification in comparison to conventional cotton varieties.

Cottonseed also contains the anti-nutrients gossypol and cyclopropenoid fatty acids, which limit the amount of cottonseed that can be used in feed rations (OECD 2004). Gossypol is a terpenoid phytoalexin that is present in seeds, foliage and roots of cotton plants, and protects plants from insect damage and disease. Gossypol toxicity can occur in non-ruminant animals or immature cattle if the amount of cottonseed meal contained in the animal feed exceeds recommended levels. Gossypol occurs in both a free and bound form in cotton but is toxic only in the free form (OECD 2004). Cottonseed also contains measurable amounts of the cyclopropenoid fatty acids malvalic, sterculic and dihydrosterculic acid. Cyclopropenoid fatty acids are considered to be antinutritional compounds, known to inhibit the desaturation of stearic acid to oleic acid in the human or animal body, resulting in alterations of membrane permeability and an increase in the melting point of fats.

Cottonseed oil is the major cotton product used for human consumption, but constitutes only 5-6% of the total vegetable oil produced in the US. Small quantities of cottonseed by products (meal and linters) are also used in some human foods (OECD 2004).

H.2. Experimental Methods

H.2.a. Plant material

Cotton plants were grown in 2004, according to local agronomic practices for cotton production, at four locations: Bossier City, Louisiana, Winnsboro, Louisiana, Leland, Mississippi and Newport, Arizona, which are representative of the U.S cotton belt.

At each location, four replicate plots of COT67B and Coker 312, were planted in randomized complete blocks. Cottonseed was harvested from each plot and ginned. A sample of whole (fuzzy) cottonseed from each plot was ground and kept frozen until analysis.

H.2.b. Compositional analysis

Analytes measured were based on recommendations of the Organisation for Economic Co-operation and Development (OECD 2004) for comparative assessment of compositional considerations for new varieties of cotton, and included quantification of the major constituents of cottonseed, (protein, carbohydrates, fat, ash and fiber), minerals (calcium, phosphorus), amino acids and fatty acids. The anti-nutrients typically found in cottonseed, gossypol and cyclopropenoid fatty acids (malvalic, sterculic and dihydrosterculic acid), were also measured.

H.2.c. Statistical analysis

The data for each analyte was subjected to an analysis of variance across locations using the model

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

where Y_{ijk} is the observed response for entry i at location j block k, U is the overall mean, T_i is the genotype effect (transgenic vs. nontransgenic), L_j is the location effect, $B(L)_{jk}$ is the effect of block within location, LT_{ij} is the location x genotype interaction effect and e_{ijk} is the residual error.

The statistical significance of any differences in the quantities of an analyte measured between COT67B and Coker 312 was determined using an F-test. An F-test probability of ≤ 0.05 indicates that the difference between the genotypes was statistically significant.

An F-test was also used to assess the significance of the location x genotype interaction. An F-test probability of ≤ 0.05 suggests that the effect of the genotype was not consistent across locations and therefore a comparison of genotypes averaged across locations may not be valid. In that instance individual location means were analyzed.

H.3. Results

The outcome of the analysis of each analyte or group of analytes is discussed below and the data are summarized in Table 5-25 through Table 5-31. It is important to note that the location x genotype interaction was not significant for any analyte measured with the exception of Vitamin E.

H.3.a. Proximates

There were no statistically significant differences observed between the COT67B and Coker 312 proximate composition (Table 5-25). The average levels of these analytes were within the range of values reported in the International Life Science Institute (ILSI) Crop Composition Database (ILSI 2006) and OECD (2004).

H.3.b. Minerals

There was no statistically significant difference in the level of phosphorus for both COT67B and Coker 312 (Table 5-26). A statistically significant difference in the level of calcium was observed, however the magnitude of the difference was small and was within the range reported by ILSI (2006) and OECD (2004).

H.3.c. Amino Acids

There were no statistically significant differences in amino acid levels of COT67B and Coker 312 (Table 5-27 and Table 5-28) and the average levels of amino acids were within the range of natural variation as reported by ILSI (2006) and OECD (2004).

H.3.d. Fatty Acids

Several of the fatty acids were below the limit of quantitation (<LOQ) in the cottonseed samples of both genotypes. The mean quantifiable fatty acid levels of COT67B and Coker 312 cottonseed are shown in Table 5-29. Statistically significant differences were observed in levels of palmitic, stearic and oleic acids, although the magnitude of the differences were very small. Levels of these and other measurable fatty acids were within the ranges reported by ILSI (2006) and OECD (2004).

H.3.e. Anti-Nutrients

Levels of the anti-nutrients, gossypol (total and free) and the cyclopropenoid fatty acids (sterculic, malvalic, and dihydrosterculic acid), are shown in Table 5-30. A statistically significant difference was observed only in dihydrosterculic acid levels. Levels of all cyclopropenoid fatty acids measured, including dihydrosterculic acid, as well as total and free gossypol, were within the ranges reported by ILSI (2006) and OECD (2004).

H.3.f. Vitamin E

Levels of vitamin E are shown in Table 5-31. A statistically significant genotype-by-location interaction was noted, which suggests that the effect of genotype was not consistent across locations, hence, the comparison of genotypes averaged across locations may not be valid for vitamin E, therefore the individual location means are provided (Table 5-31). Most importantly, although vitamin E levels were statistically different between COT67B and Coker 312, the average levels of vitamin E in both COT67B and Coker 312 cotton seed at all locations were within the ranges reported by ILSI (2006), thus, no adverse consequences due to differences in vitamin E levels are anticipated.

H.4. Summary of compositional analysis

Key nutritional and anti-nutritional components of cottonseed from COT67B and nontransgenic Coker 312 were measured and compared. Among the 41 different analytes measured, 35 showed no statistically significant differences between the two cotton lines. Importantly, the mean values of all analytes measured fell within the range of natural variation published in the literature. Therefore, the data supports the conclusion that

products derived from COT67B seed do not have any biologically meaningful differences in terms of plant pest risk from nontransgenic cotton. COT67B seed is compositionally similar to the conventional cotton variety, Coker 312, and is as safe and nutritious for food and feed use.

Table 5-25. Proximate composition of COT67B seed (% dry weight) collected at four locations during the summer of 2004

Data from ILSI Crop Composition Database version 3.0, and OECD consensus document included for comparison

		Moisture % fw	Protein	Fat	Ash	Carbohydrates	ADF	NDF	TDF
Coker 312		4.9	18.7	17.0	4.3	60.1	52.6	56.4	49.8
COT67B		5.1	19.5	18.5	4.3	57.7	52.2	58.0	48.4
F-test Probability for Genotype ($p \leq 0.05$)		0.333	0.550	0.069	0.613	0.208	0.882	0.530	0.434
ILSI (2006)	average	8.7	20.30	18.2	4.20	57.1	47.20	52.30	54.20
	range	7.3 - 11.8	11.7 - 28.3	9.2 - 24.6	3.2 - 6.2	47.4 - 74.4	31.50 - 66.9	38.10 - 71.4	41.5 - 74.5
	N	18	65	47	65	65	65	65	47
OECD (2004)	range	4.0 - 9.9	21.8 - 34.2	15.4 - 36.3	3.8 - 5.0	NA*	29.0 - 40.1	40.0 - 54.8	

All values are for whole (fuzzy) cottonseed

* NA = OECD calculates values for carbohydrates differently than ILSI and Syngenta; values not comparable

NO CBI

Table 5-26. Calcium and Phosphorus composition of COT67B seed (ppm dry weight) collected at four locations during the summer of 2004

Data from ILSI Crop Composition Database version 3.0, and OECD consensus document included for comparison

		Calcium	Phosphorus
Coker 312		1447	6376
COT67B		1522	6347
F-test Probability for Genotype (p≤0.05)		***0.042***	0.913
ILSI (2006)	average	1292.2 mg/kg	6057.5 mg/kg
	range	877.6 - 1857.9	3089.5 - 8342.5
	N	65	65
OECD (2004)	range	1050 – 3300 mg/kg	5600 – 8600 mg/kg

all values are for whole (fuzzy) cottonseed
ppm = mg/kg

NO CBI

Table 5-27. Amino acid composition of COT67B seed (% dry weight) collected at four locations during the summer of 2004

Data from ILSI Crop Composition Database version 3.0, and OECD consensus document included for comparison

		Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
Coker 312		14.78	4.97	7.50	30.4	6.04	6.70	6.40	2.64	6.83
COT67B		15.01	4.85	7.79	30.7	6.10	6.77	6.47	2.53	7.03
F-test Probability for Genotype ($p \leq 0.05$)		0.786	0.734	0.464	0.856	0.857	0.843	0.847	0.440	0.608
ILSI (2006)	average	17.06 mg/g	5.81 mg/g	8.23 mg/g	35.46 mg/g	6.88 mg/g	7.71 mg/g	7.24 mg/g	3.08 mg/g	8.09 mg/g
	range	10.01 - 23.25	3.37 - 8.11	5.03 - 10.70	19.58 - 49.89	4.10 - 9.39	4.44 - 10.42	4.19 - 9.57	1.59 - 4.26	4.52 - 11.29
	N	65	65	65	65	65	65	65	65	65
OECD (2004)	range (%)	2.09 - 3.55	0.74 - 1.21	0.94 - 1.63	4.33 - 8.16	0.82 - 1.39	0.93 - 1.58	0.85 - 1.51	0.38 - .86	1.01 - 1.67

All values are for whole (fuzzy) cottonseed

NO CBI

Table 5-28. Amino acid composition of COT67B seed (% dry weight) collected at four locations during the summer of 2004

Data from ILSI Crop Composition Database version 3.0, and OECD consensus document included for comparison

		Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
Coker 312		2.39	5.03	9.39	4.40	8.17	4.57	7.51	16.56
COT67B		2.33	5.21	9.58	4.41	8.32	4.65	7.69	16.65
F-test Probability for Genotype ($p \leq 0.05$)		0.674	0.570	0.734	0.956	0.773	0.781	0.677	0.931
ILSI (2006)	average	2.84 mg/g	5.86 mg/g	10.72 mg/g	4.76 mg/g	9.44 mg/g	5.22 mg/g	8.50 mg/g	19.36 mg/g
	range	1.52 - 3.87	3.45 - 7.90	6.27 - 14.40	3.16 - 6.69	5.36 - 13.05	3.09 - 6.99	5.24 - 10.96	10.51 - 27.88
	N	65	65	65	65	65	65	65	65
OECD (2004)	range (%)	0.35 - 0.54	0.71 - 1.17	1.27 - 2.23	0.48 - 1.17	1.13 - 2.03	0.62 - 1.03	1.01 - 1.65	2.38 - 4.40

All values are for whole (fuzzy) cottonseed

NO CBI

Table 5-29. Fatty acid composition[1] of COT67B seed (% of total fatty acids)[2] collected at four locations during the summer of 2004

Data from ILSI Crop Composition Database version 3.0 included for comparison

		14:0 Myristic	16:0 Palmitic	16:1 Palmitoleic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic
Coker 312		0.70	24.23	0.56	2.32	15.17	56.35	0.22
COT67B		0.68	23.84	0.54	2.39	15.59	56.27	0.23
F-test Probability for Genotype ($p \leq 0.05$)		0.226	***<0.001***	0.131	***0.002***	***<0.001***	0.498	0.566
ILSI (2006)	average	0.73	24.04	0.59	2.41	15.7	56.0	0.34
	range	0.53 - 0.99	21.1 - 26.9	0.46 - 0.89	2.15 - 3.32	13.4 - 20.0	48.9 - 60.7	0.16 - 0.62
	N	49	65	49	65	65	65	65

[1] All values are for whole (fuzzy) cottonseed

[2] All other fatty acids measured were <LOQ (0.01 - 0.02 % FW)

NO CBI

Table 5-30. Gossypol and cyclopropenoid fatty acid levels in COT67B seed (% dry weight) collected at four locations during the summer of 2004

Data from ILSI Crop Composition Database version 3.0, and OECD consensus document included for comparison

		Total Gossypol	Free Gossypol	Sterculic acid	Malvalic acid	Dihydrosterculic acid
Coker 312		0.579 %	0.492 %	0.297 % total FA	0.490 % total FA	0.174 % total FA
COT67B		0.642 %	0.536 %	0.297 % total FA	0.470 % total FA	0.151 % total FA
F-test Probability for Genotype ($p \leq 0.05$)		0.183	0.339	0.984	0.095	*<0.001*
ILSI (2006)	average	1.05 %	0.59 %	0.42 % total FA	0.30 % total FA	0.16 % total FA
	range	0.23 - 1.39	0.46 - 1.99	0.17 - 0.66	0.18 - 0.66	0.11 - 0.24
	N	65	34	62	62	59
OECD (2004)	range	0.51 - 1.43 %	0.47 - 0.70 %	0.13 - 0.70 % total FA	0.17 - 0.61 % total FA	0.11 - 0.50 % total FA

All values are for whole (fuzzy) cottonseed

NO CBI

Table 5-31. Vitamin E levels in COT67B seed (mg/g dry weight) collected at four locations during the summer of 2004

Location	Treatment	Vitamin E α-Tocopherol (mg/g DW)
Bossier City, LA	Coker 312	0.159
	COT67B	0.162
Winnsboro, LA	Coker 312	0.093
	COT67B	0.146
Leland, MS	Coker 312	0.143
	COT67B	0.121
Newport, AZ	Coker 312	0.162
	COT67B	0.195
Coker 312	Average	0.139
COT67B	Average	0.156
F-test Probability for Genotype[1] ($p \leq 0.05$)		***0.016***
F-test Probability for Location by Genotype Interaction[1] ($p \leq 0.05$)		***0.004***
ILSI (2006)	Average	0.1123
	Range	0.0821 - 0.2252
	N	18

[1] Statistically significant F-test probabilities (<0.05) indicated in italics

NO CBI

CHAPTER 6. CULTIVATION PRACTICES

A. Intended Cultivation Area

A.1. Regions Where the Plant Will be Grown

Cotton is grown in 17 states, stretching from Virginia to California, covering more than 12 million acres or about 19,000 square miles[8] In the last five years, cotton acreage in the Mid-South States of Arkansas, Louisiana, Mississippi, and Tennessee have ranged from 910,000 to 1.17 million acres, 500,000 to 630,000 acres, 1.1 million to 1.2 million acres, and 530,000 to 700,000 acres, respectively (NASS 2006). Texas is the largest cotton producing state in the U.S. with almost six million acres harvested in 2005. Because of its vast size, there are several distinct cotton production regions in Texas with varying production and variety requirements. These areas range from pine forests in the east, across blackland prairies, gulf prairies and marshes, post oak savannas, and rolling and high plains, to mountains and desert valleys and plateaus in the west. (Gould 1975). It is anticipated that COT67B will be grown in the many of the same environments as other commercial cotton varieties, particularly those in the mid-South and Southeast U.S and Texas.

A.2. New Ecosystems Where the Plant Will be Grown

The introduced Lepidopteran resistant trait in COT67B is not intended to confer any competitive advantage in terms of weediness or to extend the range of cultivation outside of existing cultivation areas.

B. Cultivation Practices

B.1. Standard Cultivation Practices for Cotton

Cotton is an intensively managed commodity crop. In the spring, farmers prepare for planting in several ways. Producers who plant using no-till or conservation tillage methods use special equipment designed to plant the seed through the litter that covers the soil surface. Producers who employ conventional tillage practices, plow or "list" the land into rows forming firm seed-beds for planting.

Seeding is done with mechanical planters which cover as many as 10 to 24 rows at a time. The planter opens a small trench or furrow in each row, drops in the right amount of seed, covers them and packs the earth on top of them. The seed is planted at uniform intervals in either small clumps ("hill-dropped") or singularly ("drilled"). Cultivators are used to uproot weeds and grass, which compete with the cotton plant for soil nutrients, sunlight

[8] http://www.cotton.org/econ/world/index.cfm

and water. Producers in south Texas plant cotton as early as February. In Missouri and other northern parts of the Cotton Belt, they plant as late as June.

About two months after planting, flower buds called squares appear on the cotton plants. In another three weeks, the blossoms open. Their petals change from creamy white to yellow, then pink and finally, dark red. After three days, they wither and fall, leaving green pods which are called cotton bolls. Inside the boll moist fibers grow and push out from the newly formed seeds. As the boll ripens, it turns brown. The fibers continue to expand. Finally, they split the boll apart and the cotton bursts forth.

Cotton grows slowly in the spring and can be shaded out easily by weeds. If weeds begin to overpower the seedling cotton, drastic reductions in yield can result. Later in the season, cotton leaves fully shade the ground and suppress mid-to-late season weeds. For these reasons, weed control is focused on providing a 6 to 8-week weed-free period directly following planting. Producers employ close cultivation and planters that place the cottonseed deep into moist soil, leaving weed seeds in high and dry soil. Herbicides or cultivation controls weeds between the rows.

Cotton diseases have been contained largely through the use of resistant cotton varieties. Rotation to non-host crops such as grain or corn also breaks the disease cycle. Nematodes, while not truly a disease, cause the plant to exhibit disease-like symptoms. Nematodes are microscopic worm-like organisms that attack cotton's roots causing the plant to stop growing, and as a result, causes reduced yield. Crop rotation is the primary method of managing for nematodes.

Throughout the world, cotton has proven vulnerable to the attack of many insect species. In the United States, the cotton industry has consistently relied heavily on insecticide use strategies to manage arthropod pests (Leonard *et al*. 1999). However, the overall availability of novel insecticides has decreased due to difficulties in the discovery of new chemistry, the significant cost of registration and re-registration, cancellation of uses, and the development of insect resistance to insecticides. The lack of effective products against specific pests has caused producers to increase the doses and application frequency of available insecticides to obtain satisfactory control. Frequently, the use of products in such a manner induces economic outbreaks of other cotton insect pests by disrupting native beneficial arthropod populations that limit pest populations.

While harvesting is one of the final steps in the production of cotton crops, it is one of the most important. The crop must be harvested before weather can damage or completely ruin its quality and reduce yield. Cotton is machine harvested in the U.S., beginning in July in south Texas and in October in more northern areas of the Belt. Stripper harvesters, used chiefly in Texas and Oklahoma, have rollers or mechanical brushes that remove the entire boll from the plant. In the rest of the Belt, spindle pickers are used. These cotton pickers pull the cotton from the open bolls using revolving barbed spindles that entwine the fiber and release it after it has separated from the boll. All harvesting systems use air to convey and elevate the seed cotton into a storage bin referred to as a basket. Once the basket is full, the stored seed cotton is dumped into a boll buggy, trailer or module builder. Today, nearly all cotton is stored in modules. Modules allow the cotton to be

stored without loosing yield or quality prior to ginning. Specially designed trucks pick up modules of seed cotton from the field and move them to the gin. From the gin, fiber and seed go different ways. The ginned fiber, now called lint, is pressed together and made into dense bales weighting about 500 pounds. To determine the value of cotton, samples are taken from each bale and classed according to fiber length (staple), strength, micronaire, color and cleanness. Producers usually sell their cotton to a local buyer or merchant who, in turn, sells it to a textile mill either in the United States or a foreign country. The seed usually is sold by the producer to the gin. The ginner either sells for feed or to an oil mill where the linters are removed in an operation very much like ginning. Linters are baled and sold to the paper, batting and plastics industries, while the seed is processed into cottonseed oil, meal and hulls.

http://www.cotton.org/pubs/cottoncounts/fieldtofabric/harvest.cfm

B.2. Cultivation Practices for Event COT67B

No changes to agronomic practices typically applied in the management of conventional cotton are required for COT67B. Specifically, no increases in pesticides and fertilizers are required and nor are changes in cultivation, planting, harvesting or volunteer control necessary.

B.3. Insect Resistance Management Plan for FLCry1Ab in COT67B

The availability of novel lepidopteran-resistance traits in cotton lessens the selection pressure for pest adaptation to existing pest control methods (Gould, 2003; Appendix 2). 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. Consequently, the utility of the novel trait should be protected through the implementation of an appropriate and effective insect resistance management (IRM) program.

Resistance management for Bt cotton has become well established since the first Bt cotton was commercially grown in 1996 (EPA 1998, 2001, 2002, 2004, 2006). 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-Bt 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 Bt cotton (EPA 1998, 2001).

The FLCry1Ab protein in COT67B is similar in efficacy against key target pests to the Cry1Ac protein produced in existing commercial Bt cotton (Bollgard®). Thus, the refuge options for Bollgard will also be effective for FLCry1Ab cotton. In addition, Syngenta will deploy COT67B only in a breeding stack with Vip3A Event COT102 to extend the spectrum of activity of the cotton in the commercial product VipCot™. fSyngenta applied to EPA for a FIFRA Section 3 registration for COT102 x COT67B on December 15, 2006. The Vip3A protein is characterized by a range of properties that very clearly

distinguish it from the FLCry1Ab and Bt cry proteins expressed by the other PIP cotton varieties available to US growers (Estruch et al. 1996; Lee et al. 2003, 2006; Appendix 2).

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 (Roush 1998; Caprio 1998; and Zhao et al. 2003. In addition, a bioeconomic model by Livingston et al. (2004) predicts that the addition of a second protein to an existing single protein variety decreases the risk of resistance to the initial protein, while increasing the risk of resistance to the new protein. Computer models developed by Dr. Mike Caprio of Mississippi State University indicate that this is likely to be true for the stack of FLCry1Ab and Vip3A in insect species that are sensitive to both proteins. Therefore, it is also likely that deployment of Cry1Ab stacked with Vip3A will slow the development of resistance to the insecticidal proteins deployed in VipCot™, which will preserve its durability and that of the Bt proteins contained in Bollgard, Bollgard II® and WideStrike® cotton varieties. This in turn will help preserve the durability of other control tools by expanding the range of pest management options available and reducing the dependence on any one.

CHAPTER 7. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION OF COT67B

A. Introduction

The purpose of this chapter is to assess the environmental safety of COT67B, and in particular whether it poses a plant pest risk. The environmental safety of commercial cultivation of COT67B cotton is considered in two parts: the likelihood that it will be toxic to nontarget organisms, including species beneficial to agriculture and endangered and threatened species; and the likelihood that it will become a serious weed of agriculture or non-agricultural habitats. A fundamental assumption of this chapter is that the cultivation of nontransgenic cotton poses no currently unacceptable environmental risks, and hence if it can be shown that COT67B does not increase those risks significantly, COT67B can be regarded as environmentally safe and not a plant pest. A detailed risk analysis is included as Appendix 5.

B. The Safety of COT67B to Nontarget Organisms

Studies of composition and nutritional quality (Chapter 5) corroborate the hypothesis that COT67B is equivalent to conventional cotton, apart from the presence of full-length Cry1Ab (FLCRY1AB protein). Estimates of numerous compositional parameters in field-grown COT67B and near-isogenic, nontransgenic cotton were compared, and most showed no statistically significant difference associated with the presence of the *flcry1Ab* transgene. For analytes that differed significantly between COT67B and the null-isoline, the concentration of the analyte in COT67B was within the range found in other cotton varieties. The results of the compositional analysis demonstrate that any risk from toxicity of COT67B to nontarget organisms will arise from FLCry1Ab.

The risk of FLCry1Ab to nontarget organisms was evaluated by testing the hypothesis that the no observable adverse effect concentration (NOAEC) of FLCry1Ab is greater than the expected environmental concentration (EEC) of the protein to which nontarget organisms would be exposed *via* cultivation of COT67B (*i.e.*, EEC/NOAEC $\leq$ 1) . Corroboration of this hypothesis would indicate minimal risk of toxicity from FLCry1Ab in COT67B. The biology of cotton and the inherent degradability of FLCry1Ab in soil indicate that exposure of nontarget organisms to FLCry1Ab will be limited to cotton fields during and immediately after cultivation of COT67B; therefore, the hypothesis that NOAEC/EEC $\leq$ 1 is corroborated for organisms that do not occur in cotton fields because, in effect, the EEC is zero.

The risk to nontarget organisms that occur in cotton fields was assessed in five categories: above-ground arthropods, soil-dwelling invertebrates, pollinators, wild mammals and wild birds. In addition, the risk to farmed fish was assessed because cottonseed meal is used in the manufacture of fish feed. For each category of organism a "worst-case" EEC was estimated by assuming that organisms consume a diet comprising 100% tissue of COT67B expressing the highest mean concentration of FLCry1Ab. "Realistic" EECs

were also estimated by making reasonable, but still conservative, assumptions about the actual diets of organisms in cotton fields.

The effects of FLCry1Ab or truncated Cry1Ab (trCry1Ab) on representative indicator species of each category of nontarget organism were tested in the laboratory. Most studies exposed test species to diet incorporating a microbial test substance containing purified FLCry1Ab from an *E. coli* expression system and which had been shown to be a suitable surrogate for FLCry1Ab produced in COT67B. Exposure of nontarget organisms to test substances containing trCry1Ab was also considered because of the close similarity of the insecticidal regions of FL and trCry1Ab. In all studies, there were no observable adverse effects, and therefore the NOAEC was the concentration of FLCry1Ab or trCry1Ab in the diet.

Risk to nontarget organisms was evaluated as the hazard quotient (HQ = EEC/NOAEC). The HQs for worst-case and realistic EECs are presented in Table 1. Re-characterization of the microbial test substance suggested some loss of bioactivity, but not concentration, during storage. It is possible that studies carried out subsequent to the previous test substance characterization were affected by this loss of activity. For these studies, two HQs are presented: HQ_c based on the concentration of FLCry1Ab, and HQ_{ba} based on worst-case assumptions about the bioactivity of FLCry1Ab in the test diets.

Table 7-1. Hazard quotients for nontarget organisms (NTOs) exposed to FLCry1Ab via COT67B

Test Species	NTO Group Represented	Worst-case EEC	Realistic EEC
Ladybeetle	Above-ground arthropods	$HQ_{c*} \leq 0.1420$	$HQ_c \leq 0.0184$
		$HQ_{ba*} \leq 0.6391$	$HQ_{ba} \leq 0.0826$
Flower bug	Above-ground arthropods	$HQ_c \leq 0.1414$	$HQ_c \leq 0.0183$
		$HQ_{ba} \leq 0.6365$	$HQ_{ba} \leq 0.0823$
Rove beetle	Soil dwellers	$HQ_c \leq 0.0141$	$HQ_c \leq 0.0002$
		$HQ_{ba} \leq 0.0634$	$HQ_{ba} \leq 0.0008$
Springtail	Soil dwellers	$HQ \leq 0.8244$	$HQ \leq 0.0099$
Honeybee	Pollinators	$HQ \leq 0.0708$	$HQ \leq 0.0708$
Bobwhite quail	Wild birds	$HQ \leq 0.0630$	$HQ \leq 0.0058$
Mouse	Wild mammals	$HQ \leq 0.0050$	$HQ \leq 0.0004$
Catfish	Farmed fish	$HQ \leq 1.84$	$HQ \leq 0.92$
		$HQ_{ba} \leq 8.28$	$HQ_{ba} \leq 4.14$

[*] c = concentration
[**] ba = bioactivity

Most HQs are less than 1 even under worst-case assumptions of exposure and bioactivity of the test substance; thus the risk hypothesis is corroborated and the risk of toxicity of COT67B to nontarget organisms is minimal (Appendix 5.F.1). It should be remembered that the NOAEC is the single concentration used in the study and no adverse effects were observed in any study, therefore, the HQs are maxima. The HQs for farmed fish do not demonstrate a risk to fish, merely that under some assumptions, the exposure to FLCry1Ab in the study was less than the conservative estimates of exposure *via* feed.

C. Risk of Increased Weediness Potential of COT67B

COT67B may pose a plant pest risk if it shows greater weediness potential than conventional cotton. The abundance and diversity of wild plants could be reduced if feral populations of COT67B, or hybrids of COT67B with wild species, establish and spread into semi-natural or natural habitats; organisms that rely on these wild plants for food or shelter could also be harmed. If COT67B is more likely to be a volunteer weed than conventional cotton, the yield of other crops may be affected. Volunteers reduce crop yield directly through competition, and indirectly by acting as "green bridges" for pests and pathogens, and are therefore regarded as plant pests.

The risks to wildlife and agricultural productivity from weedy cotton populations are low: volunteer cotton populations are easily managed, and feral populations occur rarely in the US cotton belt. Agronomic studies tested the hypothesis that the weediness potential of COT67B is unchanged with respect to conventional cotton. No differences were detected between COT67B and nontransgenic cotton in growth, reproduction or interactions with pests and diseases, other than the intended effect of protection from lepidopteran pests. The main controls on feral populations of cotton are poor seed dispersal, competition from other plants and lack of water, therefore insect resistance is unlikely to affect weediness potential. In addition, cultivation of COT67B does not require different fertilizer or herbicide application, tillage, planting or harvesting from existing commercial cotton varieties. Therefore, the ease of management of volunteer cotton is likely to be unchanged by the introduction of COT67B cotton.

D. Summary Environmental Risk Assessment

Nontarget organisms are highly unlikely to suffer toxicity from exposure to COT67B. The composition of COT67B is not significantly different from nontransgenic cotton, apart from the presence of FLCry1Ab. Laboratory studies indicate that FLCry1Ab in COT67B is unlikely to be toxic to any nontarget organism. Hence, risks to the abundance and diversity of nontarget organisms from exposure to toxic substances in COT67B are minimal.

Conventional cotton rarely forms self-sustaining populations outside cultivation. Expression of FLCry1Ab is unlikely to increase the potential of cotton to become weedy, and field trials indicate no consistent effect of the presence of *flcry1Ab*, or unintended effects of transformation are likely to increase weediness potential. In addition, the likelihood of weediness evolving as the result of gene flow from COT67B is low because populations of feral cotton or wild relatives do not occur near areas of cotton cultivation.

Hence, the risks to wild plants from invasions of feral or wild populations derived from COT67B, and the risks to yields of crops potentially affected by volunteer cotton, are minimal.

In summary, no significant plant pest risk from the proposed cultivation of COT67B has been identified.

CHAPTER 8. ADVERSE CONSEQUENCES

Syngenta knows of no study results or observations associated with COT67B that would be anticipated to result in adverse consequences to the quality of the human environment, directly, indirectly, or cumulatively, including endangered species, unique geographic areas, critical habitats, public health and safety (including children and minorities), genetic diversity of cotton, farmer or consumer choice, insect resistance or the economy, either within or outside the U.S. COT67B cotton plants produce a similar insectidical Cry1Ab protein as a number of other deregulated products and offers additional choice for protection from feeding damage caused by lepidopteran pests. As such, COT67B is expected to produce the same beneficial effects as previously deregulated Bt cotton products also registered by the EPA as PIPs and which are commercially available, which include additional grower choice, increased competition and extended useful life of Bt cotton technology generally. The information provided in this section addresses potential beneficial and adverse consequences of deregulation of COT67B cotton to show that the introduction of COT67B will not significantly affect the quality of the human environment. The direct, indirect, and cumulative impact of deregulating of COT67B is analyzed within the framework of the factors listed by the Council on Environmental Quality (CEQ).

A. Granting the Petition for Determination of NonRegulated Status for COT67B Would Not Significantly Affect the Quality of the Human Environment

The National Environmental Policy Act ("NEPA"), 42 U.S.C. §§ 4321 *et seq*., requires that agencies undertaking a major Federal action "significantly affecting the quality of the human environment" provide a detailed statement of the environmental impact of the proposed action, any adverse environmental effects that cannot be avoided, and alternatives to the action." 42 U.S.C. § 4332 (C). Where the significance of an action is uncertain, agencies may use an Environmental Assessment ("EA") to identify, analyze and evaluate the impacts of the proposed action. The EA will satisfy the NEPA obligation where it provides sufficient evidence and analysis to support a "finding of no significant impact ("FONSI").

The test of whether an action "significantly" affects the environment requires considerations of both context and intensity, (40 C.F.R. § 1508.27). The term "context" refers to the setting within which the proposed action takes place. *Id.; see also Coliseum Square Ass'n v. Jackson*, 465 F.3d 215, 239-41 (5th Cir. 2006). The term "intensity" refers to "the severity of the impact" and includes factors such as effects on human health, cumulative impacts and effects on endangered species.

The data and information submitted by Syngenta in support of its request for a determination of nonregulated status is sufficient under NEPA for APHIS' preparation of an EA to support a FONSI. The context within which the deregulation of COT67B will occur is one in which multiple lepidopteran-protected Bt cotton products are in use, and have been for some time. These Bt cotton products do not present plant pest risks; they do, however, provide valuable tools in the effort to control lepidopteran pests that

continue to infest cotton and have developed resistance to a number of conventional pesticides. COT67B provides beneficial marketplace diversification and is being introduced to the market with a registration that includes a number of specific and legally enforceable insect resistance management and monitoring requirements.

The same facts demonstrate the low intensity of this action. The cumulative impacts of an additional lepidopteran-protected cotton product will be insignificant. Cotton is a well-understood and managed crop; genetic diversity is carefully maintained and safeguarded; there are no adverse effects on endangered, threatened, or other non-target species; and a strong suite of measures to prevent the development of insect resistance is in place. The following discussion summarizes these facts in more detail.

A.1. Context

The context or setting of the proposed deregulation is based in the production of an intensively cultivated row crop – cotton – that is primarily grown in warm climates such as the "Cotton Belt" of the Southern United States (Virginia, Georgia, North Carolina, South Carolina, Florida, Alabama, Louisiana, Mississippi, Arkansas, Tennessee, Missouri, Kansas, Oklahoma, Texas, New Mexico, Arizona and California). Cotton sustains significant damage from several lepidopteran pests, which include the cotton bollworm and tobacco budworm. In 2004, these pests infested 11 million acres and reduced production by ca. 380,000 bales or 109.1 million dollars ($288 per 480 pound bale) (Williams 2005).

Before the introduction of Bt cotton varieties in 1996, chemical pesticides were widely used to control these pests. Organophosphate, carbamate and pyrethroid products accounted for a substantial percentage of the insecticides used. However, these three classes of pesticides require numerous safety warnings and extensive use restrictions, which raise concerns for worker safety, water contamination and other environmental risks. Bt cotton varieties offer a very effective, and environmentally benign alternative to chemical insecticides and have been extensively adopted by cotton farmers. In 2006, these varieties represented 95.5% of the cotton planted in the U.S (USDA AMS, 2006).

The US Environmental Protection Agency has consistently found that the registration of Bt PIPs are in the public interest. These findings have largely been based on a determination that Bt PIPs present less risk than conventional chemical pesticide alternatives. The Agency's view concerning Bt PIPs is well accepted and supported by the work of others. In an interesting analysis, The Center for Food and Agricultural Policy estimates that these products increased cotton production by almost 600 million pounds, improved farm income by almost $300 million, and reduced chemical pesticide use by more than 1.6 million pounds (Sankula et al. 2005) since their introduction in 1996.

Cotton event COT67B, which produces the FLCry1Ab protein, will be combined through conventional breeding with event COT102 cotton. COT102, deregulated by the USDA on July 8, 2005, produces the Vip3A protein that is both efficacious and possesses a novel mode of action from the Cry proteins produced in other Bt cotton varieties. The

combination of the FLCry1Ab and Vip3A proteins produced by COT67B and COT102, respectively in a single variety (hereafter referred to as VipCot™) through traditional breeding will offer complete protection from tobacco budworm and is expected to provide equivalent or greater control of the considerably more predominant cotton bollworm than the current Bt cotton products. Syngenta applied to EPA for a FIFRA Sec. 3 registration for VipCot™ in December 2006.

VipCot™ also presents a strong reduced risk profile. The deregulation of COT67B by USDA and registration of VipCot™ by EPA is not expected to significantly expand the number of Bt cotton acres; acreage is expected to remain relatively stable. However, VipCot™ cotton's anticipated strong market presence and unique mode of action will contribute to resistance management and the long-term viability of Bt PIP cotton technology. The significant replacement of conventional chemical insecticides by Bt PIP cotton is well documented. The introduction of VipCot™ may result in a small replacement of cotton acres currently treated with chemical insecticides, but its major benefits will be additional grower choice, increased competition and extended useful life of Bt cotton technology generally (resulting from the superior efficacy of the combined FLCry1Ab and Vip3A proteins and the unique mode of action of the latter, expressed by VipCot™).

The importance of cotton as a fiber crop, and its dependence on human management, has produced a long history of great care to protect germplasm lines of cotton. Decades prior to the introduction of transgenic cotton products, the cotton industry developed effective methods and means to maintain product segmentation and genetic purity standards. Moreover, with respect to both conventional and transgenic cotton, the ability to protect and maintain the genetic purity of breeding lines is critical to seed companies and developers of new varieties such as COT67B. Consequently, seed companies routinely apply standard breeding techniques – including physical isolation – that have proven effective at maintaining the genetic purity of breeding lines.[9]

Resistance management for Bt cotton has become well established since the first Bt cotton was commercially grown in 1996 (EPA 1998, 2001, 2002, 2004, 2006). 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-Bt cotton. Syngenta expects that the eventual EPA registration for VipCot™ cotton containing COT67B will require, as the Agency has for previously registered Bt cotton products, refuges and related measures to deter the development of pest resistance to the Bt proteins produced in these varieties. These methods have been used successfully for several years, as evidenced by the fact that there have been no documented instances of confirmed insect resistance to Bt cotton having

[9] See further discussion below at B.7.c.

developed in the field, and Syngenta is not aware of any studies demonstrating the development of insect resistance in the field.[10]

A.2. Intensity

With regard to the intensity element of the "significance" determination, CEQ regulations provide 10 factors to guide the analysis. 40 C.F.R. § 1508.27(b).[11] These factors "do not constitute categorical rules such that their presence or absence means an impact is per se significant." (*Coliseum Square Ass'n*, 465 F.3d at 240). Instead, "all that would have to be shown is that all the factors were in some way addressed and evaluated; whether this was done in factor-by-factor fashion is irrelevant." (*Spiller v. White*, 352 F.3d 235, 243, 5th Cir. 2003). Here, APHIS' EA should provide data and analysis that appropriately address and evaluate the considerations provided by the CEQ regulation.

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[10] See further discussion below at B.2.c.

[11] (1) Impacts that may be both beneficial and adverse. A significant effect may exist even if the Federal agency believes that on balance the effect will be beneficial.

(2) The degree to which the proposed action affects public health or safety.

(3) Unique characteristics of the geographic area such as proximity to historic or cultural resources, park lands, prime farmlands, wetlands, wild and scenic rivers, or ecologically critical areas.

(4) The degree to which the effects on the quality of the human environment are likely to be highly controversial.

(5) The degree to which the possible effects on the human environment are highly uncertain or involve unique or unknown risks.

(6) The degree to which the action may establish a precedent for future actions with significant effects or represents a decision in principle about a future consideration.

(7) Whether the action is related to other actions with individually insignificant but cumulatively significant impacts. Significance exists if it is reasonable to anticipate a cumulatively significant impact on the environment. Significance cannot be avoided by terming an action temporary or by breaking it down into small component parts.

(8) The degree to which the action may adversely affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or may cause loss or destruction of significant scientific, cultural, or historical resources.

(9) The degree to which the action may adversely affect an endangered or threatened species or its habitat that has been determined to be critical under the Endangered Species Act of 1973.

(10) Whether the action threatens a violation of Federal, State, or local law or requirements imposed for the protection of the environment.

B. Analysis of CEQ Intensity Factors Supporting a Finding of No Significant Impact

B.1. Impacts That May Be Both Beneficial and Adverse

40 C.F.R. § 1508.27(b)(1) emphasizes that agencies must take into account beneficial effects, as well as adverse ones. As set out below, VipCot™ is expected to produce the same beneficial effects as previously deregulated Bt cotton products also registered by the EPA as PIPs and which are commercially available, which include additional grower choice, increased competition and extended useful life of Bt cotton technology generally. However, these effects do not constitute significant impacts on the environment; in each case, these effects merely provide incremental benefits over the status quo and do not bring about any qualitative change in the environment. APHIS has already deregulated multiple lepidopteran-control cotton events, each using proteins based on those produced by *Bacillus thuringiensis* to control the same destructive pests targeted by COT67B as described in this petition. *See also*: Approval of Mycogen Seeds/Dow AgroSciences LLC Request No. 03-036-01p Seeking a Determination of Non-regulated Status for *Bt* Cry1F Insect-Resistant Cotton Event 281-24-236 (March. 9, 2004) [12]; Approval of Mycogen Seeds/Dow AgroSciences LLC Request No. 03-036-02p Seeking a Determination of Non-regulated Status for *Bt* Cry1Ac Insect-Resistant Cotton Event 3006-210-23 (March. 9, 2004)[13]; Approval of Monsanto Company Request No. 01-025-1-p Seeking a Determination of Non-regulated Status for Bt Cry2Ab Lepidopteran Resistant Cotton Line 15985 (November 22, 2002) [14]; Approval of Monsanto Company Request No. 94-38-01-p Seeking a Determination of Non-regulated Status for Bt Cry1Ac lepidopteran Resistant Cotton Lines 531, 757, 1076 (July 13, 1995) [15] and Approval of Syngenta Seeds, Inc. Request No. 03-155-01p Seeking a Determination of Non-regulated Status for Lepidopteran Insect Protected Cotton Transformation Event COT102 (January 28, 2005) [16]. COT67B as a component of VipCot™ will reduce conventional pesticide use and provide similar beneficial effects on the environment as are already occurring due to the use of preceding Lepidopteran resistant cotton events (Appendix 6, Public Interest Document). The commercial availability of COT67B or VipCot™ will simply maintain or add incrementally to these continuing benefits.

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[12] A*vailable at* http://www.aphis.usda.gov/brs/aphisdocs/03_03601p.pdf.
[13] A*vailable at* http://www.aphis.usda.gov/brs/aphisdocs/03_03602p.pdf
[14] A*vailable at* http://www.aphis.usda.gov/brs/aphisdocs/00_34201p.pdf
[15] A*vailable at* http://www.aphis.usda.gov/brs/aphisdocs/94_30801p.pdf
[16] A*vailable at* http://www.aphis.usda.gov/brs/aphisdocs/03_15501p.pdf

B.2. Effects on Public Health or Environmental Safety

B.2.a. COT67B Does Not Contain Antibiotic Resistance Marker Genes

COT67B does not present the risk of causing or spreading antibiotic resistance. Chapter 3 of this petition describes in detail the genetic construction of COT67B and demonstrates in a scientific matter that the final product does not contain any of the backbone sequences from the transformation vectors, pNOV4641 or pNOV1914 or the antibiotic resistance marker gene *aph4* that confers resistance to hygromycin.

B.2.b. COT67B Is Expected to Reduce Application of Conventional Pesticides

Previous Bt cotton varieties have resulted in reduced conventional pesticide use, as farmers find the Bt products to be particularly effective at controlling lepidopteran pests (Appendix 6). Based on this experience, it is reasonable to expect that deregulation and commercialization of COT67B will result in continued reductions in the use of conventional pesticides. As set out in Syngenta's petition, this reduction in conventional pesticide use would diminish the environmental risks of controlling lepidopteran pests, as the chemical alternatives to COT67B present numerous risks to humans and other nontarget organisms, whereas Cry1Ab presents little or no such risk (Appendix 5).

B.2.c. Insect Resistance Management Program

As a condition of its EPA registration for COT67B as a component of VipCot™, Syngenta expects that it will be required to implement an insect resistance management ("IRM") plan. This is not a hypothetical solution nor is it a response that might be implemented at an uncertain later date. A detailed and thorough system of refuge requirements and enforcement measures designed to prevent the development of insect resistance is planned and was submitted to EPA as part of Syngenta's application for a FIFRA Section 3 registration for VipCot cotton™ (Supplement 22).

The IRM program Syngenta proposes is described in detail in Supplement 22 accompanying this submission. In summary, this program is based on a combination of the plants expressing a high dose of Bt protein against key target pests and reliance on an appropriate refuge of non-transgenic plants to produce susceptible insects. Modeling results indicate that the refuge included in the IRM plan is an effective measure against the development of lepidopteran insect resistance to the FLCry1Ab or Vip3A proteins produced in VipCot™ (Caprio 2006).

The effectiveness of IRM plans is not hypothetical. IRM refuges have been required for all other commercial Bt cotton products and have been used successfully over the past decade, as evidenced by the fact that there have been no documented instances of confirmed insect resistance to Bt cotton having developed in the field, and Syngenta is not aware of any studies demonstrating the development of insect resistance in the field.

The IRM program is itself one component of an overall package of integrated pest management techniques, which include crop management, maintenance of refuge quality,

and education as to the proper use of insecticides in refuges in order to maintain overall survival of Lepidopteran pests to function effectively as refuge mating partners.

The IRM plan (Supplement 22) will be implemented using a product stewardship program. The specific refuge requirements and other stewardship practices to be used are set out in great detail in the IRM plan—again, there is nothing hypothetical about the stewardship measures for VipCot™. Growers will not have a choice whether to follow these procedures while continuing to grow VipCot™ cotton; they will be contractually bound to follow the procedures, by means of stewardship agreements, and their compliance will be monitored and enforced according to a fully documented compliance program, which is reviewed and evaluated by EPA. Syngenta will communicate these requirements to growers using a wide-ranging grower education campaign. The IRM program also requires resistance monitoring and detection; a remedial action plan to be implemented in the event of unexpected levels of lepidopteran pest damage; and an annual IRM plan review.

Thus, Syngenta is obligated to implement specific and detailed procedures aimed at preventing or significantly delaying insect resistance; it will require these procedures of growers and will monitor and enforce compliance; and data on the record indicate the effectiveness of this type of program against insect resistance.

B.2.d. Environmental Safety

The toxicity of insecticidal Cry proteins such as FLCry1Ab depend on its binding to specific receptors present in the insect mid-gut. Test results demonstrate that this specificity limits the protein's toxic effect to certain lepidopteran species. An overview of the lack of toxicity of FLCry1Ab to nontarget species is found in Chapter 7 of this petition.

B.2.e. Health and Human Safety Studies Confirm That COT67B Poses No Significant Health or Safety Concerns

Numerous health and safety studies have been conducted on COT67B and the FLCry1Ab protein, and these studies confirm that there are no significant health or safety concerns associated with this event.

The Cry1Ab protein is also present in a number of *B. thuringiensis* corn plant incorporated protectants registered by the EPA since 1996 and re-registered in 2001 and 2006. The FDA completed food and feed safety consultations for these products and the EPA, through its statutory authority under the Federal Food Drug and Cosmetic Act, established a permanent exemption from the requirement of a tolerance for the Cry1Ab protein and the genetic material necessary for its production in all plants (40 CFR 180.1173). The health and safety of children and minorities were also considered in the establishment of this exemption from the requirement of a tolerance.

B.3. Unique Characteristics of a Geographic Area

There is no indication that this action would have a significant effect on the unique characteristics of any particular geographic area. As with Bt cotton products previously deregulated and commercialized, COT67B is expected to be used throughout cotton-producing areas of the country.

B.4. Degree to Which the Effects on the Quality of the Human Environment are Likely to be Highly Controversial

There is no "controversy" as that term is used in the NEPA context regarding the use of transgenic Bt cotton varieties. "The term 'controversial' refers to the existence of a substantial dispute ... as to the size, nature, or effect of the major federal action rather than to the existence of opposition to a use." (*Heartwood, Inc. v. U.S. Forest Serv.*, 380, F.3d 428, 8th Cir. 2004). The experience with multiple lepidopteran control products in corn and cotton demonstates that there is no controversy regarding the use of COT67B. This petition contains substantial evidence demonstrating in great detail why the deregulation of COT67B would not have significant effects on the quality of the human environment. By contrast, there is no scientific evidence that contradicts the data submitted in this petition.

B.5. Degree to Which the Possible Effects on the Human Environment Are Highly Uncertain or Involve Unique or Unknown Risks

With respect to this factor, Syngenta's petition has the advantage of having been preceded by the deregulation of multiple Bt Lepidopteran resistant cotton events. *See* Approval of Mycogen Seeds/Dow AgroSciences LLC Request No. 03-036-01p Seeking a Determination of Non-regulated Status for *Bt* Cry1F Insect-Resistant Cotton Event 281-24-236 (March. 9, 2004); Approval of Mycogen Seeds/Dow AgroSciences LLC Request No. 03-036-02p Seeking a Determination of Non-regulated Status for *Bt* Cry1Ac Insect-Resistant Cotton Event 3006-210-23 (March. 9, 2004); Approval of Monsanto Company Request No. 01-025-1-p Seeking a Determination of Non-regulated Status for Bt Cry2Ab Lepidopteran Resistant Cotton Line 15985 (November 22, 2002); Approval of Monsanto Company Request No. 94-38-01-p Seeking a Determination of Non-regulated Status for Bt Cry1Ac Lepidopteran Resistant Cotton Lines 531, 757, 1076 (July 13, 1995) and Approval of Syngenta Seeds, Inc. Request No. 03-155-01p Seeking a Determination of Non-regulated Status for Lepidopteran Insect Protected Cotton Transformation Event COT102 (January 28, 2005). Experience with previous events expressing Cry1Ac, Cry1F, Cry2Ab, and Vip3A proteins serves as a guide to the expected effects of this event expressing Cry1Ab. More broadly, microbial Bt pesticides have been used for decades, and transgenic Bt cotton has been grown commercially for more than 10 years[17] – so the likely effects of Bt cotton are well known.

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[17] *See* http://www.aphis.usda.gov/brs/not_reg.html, listing Bt events as early as 1994.

To the extent that risks such as insect resistance may manifest later, these risks are known and there are preventative mechanisms in place. The IRM plan and refuge system described above and in Supplement are designed to detect the beginnings of insect resistance and swiftly counteract it.

Thus, the absence of significant uncertainty is demonstrated by large volumes of data and analysis indicating predicted effects, observations of the effects of similar products over previous decades, and plans in place to account for any deviation from expected effects.

B.6. Degree to Which the Action May Establish a Precedent or Represents a Decision in Principle About a Future Consideration

By its terms, this request for deregulation applies only to COT67B. There is no indication that APHIS intends to use this action as a decision in principle about future deregulations. Nor is there any suggestion that another federal action would be effectively decided as a result of APHIS' conclusion with regard to COT67B.

B.7. Cumulative Impacts

A cumulative impact is "the impact on the environment which results from the incremental impact of the action when added to other past, present, and reasonably foreseeable future actions regardless of what agency . . . undertakes such other actions" 40 C.F.R. § 1508.7.

The cumulative impacts section refers to the combination of an effect of the proposed action *with the effects of other federal actions*, not combinations of interactions between this event and features of the environment (such as multiple factor interactions between the Bt-based protein and other chemicals in a given Bt cotton variety). *TOMAC v. Norton*, 433 F.3d 852, 864 (D.C. Cir. 2006).[18]

APHIS has previously made determinations of nonregulated status as to other Bt cotton products. *See* Approval of Mycogen Seeds/Dow AgroSciences LLC Request No. 03-036-01p Seeking a Determination of Non-regulated Status for *Bt* Cry1F Insect-Resistant Cotton Event 281-24-236 (March. 9, 2004); Approval of Mycogen Seeds/Dow AgroSciences LLC Request No. 03-036-02p Seeking a Determination of Non-regulated Status for *Bt* Cry1Ac Insect-Resistant Cotton Event 3006-210-23 (March. 9, 2004); Approval of Monsanto Company Request No. 01-025-1-p Seeking a Determination of Non-regulated Status for Bt Cry2Ab Lepidopteran Resistant Cotton Line 15985

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[18] "Appellant appears to misunderstand the function of a cumulative impacts analysis. TOMAC construes the requirement to mean that BIA was required to consider the 'cumulative impact of all the casino's expected impacts when added together.' . . . This is not correct. The 'cumulative' impacts to which the regulation refers are those outside of the project in question; it is a measurement of the effect of the current project along with any other past, present, or likely future actions."

(November 22, 2002); Approval of Monsanto Company Request No. 94-38-01-p Seeking a Determination of Non-regulated Status for Bt Cry1Ac Lepidopteran Resistant Cotton Lines 531, 757, 1076 (July 13, 1995) and Approval of Syngenta Seeds, Inc. Request No. 03-155-01p Seeking a Determination of Non-regulated Status for Lepidopteran Insect Protected Cotton Transformation Event COT102 (January 28, 2005).

Data and analysis submitted in this petition demonstrate that COT67B is not a "tipping point" that will combine with these previous deregulations to cause a significant impact.

B.7.a. Data Submitted in the Petition Incorporate the Effects of Previous Actions

The crucial requirement is that the overall analysis takes into account the effects of other actions in assessing the predicted effects of the action at issue. "It makes sense to consider the 'incremental impact' of a project for possible cumulative effects by incorporating the effects of other projects into the background 'data base' of the project at issue, rather than by restating the results of the prior studies." (*Coalition on Sensible Transp.*, 826 F.2d at 70). Studies and testing that support Syngenta's petition took place in an environment in which numerous varieties of Bt cotton are widely used,[19] thus "incorporating the effects of other projects into the background" data. *See* Chapter 5 (noting that field trials of COT67B showed no adverse effects to non-target insects, and no enhanced susceptibility to insect pests). Accordingly, the petition's findings that COT67B will not be toxic to nontarget organisms, for example, rest on data that include cumulative effects with previous Bt cotton varieties.

B.7.b. Specialization of Cotton Cultivation Has Been Maintained Through Multiple Bt Cotton Events.

Maintaining genetic purity has been a feature of cotton cultivation for decades as part of varietal seed and specialty cotton production, and multiple Bt cotton events have not significantly affected these processes, even considering the effects of these transgenic events cumulatively.

Standards for genetic purity and seed quality are largely set by industry associations, state, national, and international institutions. Many of these standards were developed decades prior to the advent of transgenic cotton. A basic requirement for maintaining genetic purity in seed production fields is ensuring that only intended cross-pollination occurs. Many options are available for this purpose: maintaining isolation distances to prevent pollen movement from other cotton or planting border or barrier rows to intercept pollen and employing natural barriers to pollen.

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[19] *See* USDA, Agricultural Marketing Service, Cotton Program – Cotton Varieties Planted – 2006 Crop.

Seed producers also need to maintain practices that increase assurance that seed sources are not mixed during planting, harvest, and cleaning operations. Every seed company, in cooperation with their growers, has procedures in place to reduce chances for contamination during each step of seed production. Seed handling standards have been established by the Association of Official Seed Certifying Agencies (see AOSCA 2003) that form the basis for these operations, and individual seed companies also generally develop additional in-house procedures to reduce opportunities for contamination, including pre- and postharvest cleaning and inspections of harvesters, module makers used in harvest, transport vehicles, bins used for storage, and ginning facilities. Fields are rogued, off-types and weeds removed, and the fields are inspected multiple times by both company and industry staff. State Crop Improvement associations are also involved in monitoring the production of Foundation, Registered, and Certified seed, providing third-party inspections of seed fields to assess compliance with quality standards related to isolation and potential contamination from other crops, weeds, or disease.

In addition to the specialization adopted across the industry to enable varietal seed production for upland cotton, which represents 98% of U.S. cotton production, a number of other specialty cottons are produced (USDA NASS 2006). These include long staple varieties of Pima and Acala and colored and organic cotton varieties. Niche markets for colored and organic cotton products have been developed. Many farmers save their seed for future use and/or sale. In 2006, there were at least six companies offering organic and/or colored cottonseed in the U.S.:

Organica Seed Co., Wilbraham MA
Texas Organic Cotton Marketing Cooperative, Lubbock, TX
Southern Exposure Seed Exchange, Mineral, VA
Native Seeds/SEARCH, Tuscon, AZ
Reimer Seeds, http://www.reimerseeds.com/
Local Harvest, http://www.localharvest.org/

Similar to the production of conventional seed, industry quality standards for specialty cotton products have led these seed producers and growers to employ a variety of techniques to ensure that their products are not pollinated by or commingled with conventional cotton. In general, all the management practices used in conventional seed production to ensure quality standards are also employed in, and are sufficient to meet standards for, the production of specialty cotton seed.

Clearly, and for decades prior to the introduction of transgenic cotton products, the cotton industry developed effective methods and means to maintain product segmentation and genetic purity standards. As a result, these widespread practices have served to ensure that the broad adoption of transgenic cotton in the U.S. (including the sale and cultivation of multiple Bt cotton varieties over more than a decade) has had no significant impact, even in the aggregate, on the production of cotton seed and specialty cotton products.

Syngenta is not aware of any evidence that COT67B will act as a "tipping point" that will undermine the effectiveness of these methods of maintaining generic purity, which have been successful through previous transgenic events.

B.7.c. Genetic Diversity of Cotton Has Been Preserved Following Multiple Bt Cotton Events.

The adoption of multiple varieties of transgenic cotton has had no significant impact on the genetic diversity of cultivated cotton or the availability of diverse cotton germplasm resources, even considering the effects of these transgenic events cumulatively. Genetically distinct cotton varieties have always been developed for various geographies and purposes, and are continually improved by plant breeding. This has not been altered by transgenic cotton. Rather, transgene events are simply incorporated into these breeding programs, and have not obviated the continuous improvement of the base genetics that underlie the performance of modern cotton varieties.

In addition, the adoption of transgenics was preceded by efforts to identify and preserve sources of cotton genetic diversity, and to make these resources available for utilization by public and private cotton breeders. Among these efforts are the National Collection of Gossypium Germplasm, which is housed at the Southern Crops Research Laboratory, Crop Germplasm Research Unit, College Station, TX. According to the Germplasm Resources Information Network ("GRIN," http://www.ars-grin.gov/npgs/searchgrin.html), Gossypium collections were made as early as 1985. Currently, there are 10,206 accessions representing 41 species from 74 countries and/or political jurisdictions.

Other public cotton germplasm resource centers include collections at CICR (Central Institute for Cotton Research, India), Centre for Plant Biodiversity Research (Australia), and various collections residing at national programs of different countries around the world. Specific cotton germplasm is also available from individual breeders working on cotton at public institutions and universities. The deregulation of transgenic cotton events provides yet another source of genetic diversity that can be utilized in the improvement of cotton performance.

Thus, observation of the cumulative effects of numerous other transgenic cotton products indicates that the genetic diversity of cotton has been maintained in co-existence with these events. Syngenta is not aware of any evidence to suggest that COT67B will act as a "tipping point" that will doom these methods of maintaining genetic diversity, which have been successful through previous transgenic events.

B.7.d. Multiple Bt Cotton Events Have Resulted in No Documented Insect Resistance Developing in the Field.

There have been no documented instances of confirmed insect resistance to Bt cotton having developed in the field, and Syngenta is not aware of any studies showing insect resistance to Bt cotton products, despite the introduction of multiple previous events over the past decade. All commercialized Bt cotton products are subject to mandatory refuge requirements. The fact that there have been no documented instances of confirmed insect

resistance to Bt cotton in the field indicates that the use of mandatory refuges is effective in preventing or delaying the development of insect resistance to Bt, even cumulatively after multiple Bt cotton event introductions. *See* description of IRM program and supporting data and studies cited above and in Appendix 7.

Again, Syngenta is not aware of any evidence to suggest that COT67B will act as a "tipping point" that will cause the previously-effective refuge system to become ineffective.

B.8. Historical and Cultural Effects

There is no indication that this action would adversely affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or that it may cause loss or destruction of significant scientific, cultural, or historical resources.

B.9. Effects on Endangered Species

Endangered or threatened moths and butterflies may conceivably be sensitive to FLCry1Ab protein, given that the protein is selectively toxic to certain lepidopteran species. There are current 19 butterfly, 2 moth and 3 skipper species, respectively, federally listed (US Fish and Wildlife Service, 2007) as endangered or threatened. While it is not possible, due to their status, to directly test listed endangered species for sensitivity to FLCry1Ab protein, it can be concluded that larvae of these species will not be exposed to FLCry1Ab expressed in cotton. In general, these species occur on very limited acreage and are endangered or threatened because of habitat destruction and/or reduced availability of the single or few species of host plants that will support larval survival for a specific species. None of the listed species feeds directly on cotton plants and their potential for exposure to cotton pollen is negligible. Cotton is primarily self-pollinated, although some insect pollination occurs. There is minimal potential for cotton pollen to become windborne or drift, although small amounts of cotton pollen may be dispersed by pollinators.

In its reassessment of the environmental safety of Bt cotton, the EPA concluded that, although three endangered or threatened lepidopteran species occur in cotton-growing counties of California (Quino Checkerspot butterfly and Kern Primrose Sphinx moth) and North Carolina (St. Francis' Satyr butterfly), the larvae of these species do not feed on cotton and will not be exposed to the Bt protein because their habitats do not overlap with cotton fields and the amounts of cotton pollen (if any) that might be deposited on their host plants would be negligible and have no impact (US EPA, 2001). These conclusions for Cry1Ac Bt cotton will also be applicable to the FLCry1Ab protein produced in COT67B.

B.10. Compliance with Law

There has been no suggestion that this action would violate Federal, State, or local law or requirements imposed for the protection of the environment, nor is there any evidence that would so indicate.

C. Economic Effects[20]

C.1. Increased Competition for Bt Cotton Control Products

Prior to the commercialization of COT67B in VipCot™ cotton, there are three participants in the market for Bt cotton Lepidopteran pest control products: Mycogen Seeds/Dow AgroSciences LLC with Events 281-24-236 and 3006-210-23 (Widestrike®), and Monsanto with Event 531 (Bollgard®) and Event 15985 (Bollgard II®). Market research results described in Appendix 6 indicate that cotton farmers perceive competition in this market as limited, and believe that they would benefit from another choice in the form of VipCot™. Increasing competition in a concentrated market is likely to help consumers by reducing prices and increasing the range of available product options (*See*, *e.g.*, *Eastman Kodak Co. v. Image Technical Servs., Inc.*, 504 U.S. 451, 483-85 1992). Thus, the availability of VipCot™ cotton containing COT67B would also provide competitive benefits to the market.

C.2. Farmer and Consumer Choice

As described above, cotton cultivation has long included effective methods of maintaining genetic purity, and mechanisms are in place to protect the genetic diversity of cotton. Specialization of cotton cultivation, in production of specialty cotton such as organic, has required genetic purity procedures, and cotton growers have utilized these methods effectively to prevent undesired gene flow. This is despite the planting of 6,577 acres of organic cotton in 2005 (OTA, 2006), which represented just 0.04% of cotton produced in the country (USDA NASS 2006).

Syngenta is aware of no studies showing that these methods have been any less effective at preventing gene flow from transgenic cotton varieties, or that specialty cottons, have become less available since transgenic cotton has come into general use. Furthermore, there is no indication on the record that COT67B will alter this co-existence between organic/specialty cotton and the widespread use of transgenic cotton varieties. Accordingly, there is no reason to believe that COT67B will in any way limit farmer or consumer choice.

[20] Subsection C. does not correspond to factors listed in 40 C.F.R. § 1508.27. This is an additional issue relating to the significance of this action, which has been considered by some courts in reviewing NEPA compliance.

C.3. Effects on the Export Market

Syngenta's stewardship agreements with growers will include a term requiring growers to divert this product away from export markets where cotton seed or its products have not yet received regulatory approval for import ("channeling"). Syngenta will communicate these requirements to growers using a wide-ranging grower education campaign. As noted in the context of the IRM program, these procedures are not hypothetical.

In addition, the ability to channel particular types of cotton for particular uses, such as the export market, is demonstrated by the continuing success of the specialty cotton market. Use of identity preservation ("IP") measures has enabled growers to maintain a wide variety of specialized cotton products, including Pima, Acala, colored and organic cotton. As set out above, these practices have continued successfully long after the introduction of numerous varieties of transgenic cotton.

D. Summary

Syngenta is seeking deregulation of cotton Event COT67B under APHIS regulation 7 CFR part 340.6. The National Environmental Policy Act (NEPA). 42 U.S.C. 4321 *et seq*. requires agencies undertaking such actions to provide a detailed statement of the environmental impact of the proposed action, any adverse environmental effects that cannot be avoided, and alternatives to the action (42 U.S.C. 4332). Where the significance of an action is uncertain, agencies may use an EA to identify, analyze and evaluate the impacts of the proposed action. The EA will satisfy the NEPA obligation where it provides sufficient evidence and analysis to support a FONSI. Factors to assess significance of a proposed action as listed by the Council on Environmental Quality are addressed in this Chapter. Based on the analysis of data and information provided in this petition, Syngenta believes an EA is sufficient to evaluate the impact of deregulation of COT67B and that a conclusion of no significant impact is warranted thereby satisfying the requirements of NEPA 42 U.S.C. 4321 *et seq*.

CHAPTER 9. REFERENCES

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APPENDIX 1. MATERIALS AND METHODS FOR MOLECULAR ANALYSIS OF COT67B

A. TaqMan® PCR

All plants were individually analyzed using TaqMan® PCR (Ingham *et al.*, 2001) for the presence of the *flcry1b, aph4* and *spec* genes. For each individual plant, leaf discs were taken, and DNA was isolated using the Wizard Genomic DNA Purification kit (Promega Cat. No. A1120). COT67B plants were confirmed positive for the *flcry1Ab* gene and negative for the *aph4* and *spec* genes, while absence of all three genes was confirmed for the negative control plants. All plants were TaqMan® PCR positive for the assay's internal control, the endogenous cotton *chitinase* gene, as expected.

A.1. Genomic DNA Extraction

Genomic DNA used for molecular analyses was isolated from the pooled leaf tissue using the method from Thomas *et al*. (1993).

The following buffers were used for genomic DNA extraction:

Extraction Buffer A: 0.25 M NaCl, 0.2 M Tris, pH 8.0, 50 mM EDTA, 0.1% v/v 2-mercaptoethanol, 2.5% w/v polyvinylpyrollidone (PVP-40)

Extraction Buffer B: 0.5 M NaCl, 0.2 M Tris pH 8.0, 50 mM EDTA, 1% v/v 2-mercaptoethanol, 2.5% PVP-40, 3% sarkosyl, 20% ethanol

DNA was extracted by first grinding the leaf tissue into a fine powder using a mortar and pestle under liquid nitrogen. Eight grams of plant tissue from each COT67B generation and eight grams of plant tissue from each negative control were ground and placed into separate 50 ml conical tubes. A 25 ml volume of Extraction Buffer A was added to each tube, and samples were gently mixed and centrifuged for 10 minutes at 4657 x *g*. The supernatant was discarded and 6 ml of Extraction Buffer B was added to each sample. The samples were mixed and incubated for 30-60 minutes at 65ºC. Using a sterile loop, the samples were mixed once during the incubation period. After the incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added to each sample. The samples were mixed gently by inversion and centrifuged for 20 minutes at 4657 x *g*. The aqueous layer was collected in a clean 50 ml conical tube, and a 0.1 volume of 3 M NaOAc was added and mixed. Next, a 0.7 volume of 100% isopropanol was added, and the samples were mixed by inversion and centrifuged for five minutes at 4657 x g. The aqueous layer was decanted and the pellet was allowed to air dry briefly. The pellet was resuspended in 1000 µl of 1X TE overnight at 4ºC.

After complete resuspension, the samples were transferred to microcentrifuge tubes and 6µl of RNase A (10 mg/ml) was added and incubated at 37ºC for 30 minutes. The samples were then centrifuged at 9800 x *g* for 10 minutes. The supernatant was removed

and placed into a new tube, and a 0.5 volume of 7.5 M NH_4OAc was added. The tubes were centrifuged for 10 minutes at 19,600 x *g*. The supernatant was removed and placed in a new tube. A 0.7 volume of isopropanol was added and the samples were mixed by inversion to precipitate the DNA. The samples were centrifuged for 10 minutes at 19,600 x *g* to pellet the DNA. The supernatant was decanted and the pellet was washed twice in 500 µl of 70% ethanol, spinning for 5 minutes at 19,600 x *g* after each wash. The supernatant was decanted and the pellet air dried briefly. Finally, the pellet was resuspended in approximately 500 µl of 1X TE at 65ºC. The isolated DNA was stored at 4ºC.

A.2. DNA Quantification

The concentration of the DNA samples was measured using the QuantiT™ PicoGreen® (Molecular Probes, Cat. No. 11111) technology with a Turner Biosystems TBS-380 Fluorometer following the manufacturer's instructions. A Lambda DNA standard was used to calibrate the instrument prior to quantitation.

B. T-DNA Insert Sequencing

The nucleotide sequence of the entire T-DNA insert in COT67B was determined to demonstrate overall integrity of the insert, contiguousness of the functional elements, and to detect any individual base-pair changes, should they have occurred post-transformation. The COT67B insert was amplified from DNA derived from the BC3(F_1) generation (Chapter 3, Figure 3-4) as two individual overlapping fragments (Chapter 3, Figure 3-4). PCR amplification was carried out using Advantage™ cDNA PCR Polymerase (Clonetech, Cat. No.639105). Each PCR product was individually cloned into pCR®-XL-TOPO plasmid (Invitrogen, Cat. No. K4700-20) and three separate clones for each PCR product were identified and sequenced. Sequencing was carried out using the ABI3730XL analyzer using ABI BigDye® 1.1 or Big Dye 3.1 dGTP (for GC-rich templates) chemistry. Sequence analysis was done using the Phred, Phrap, and Consed package from the University of Washington and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998). The final consensus sequence was determined by combining the sequence data from the six individual clones (three for each PCR product) to generate one consensus sequence of the COT67B insert. Sequence alignment was performed using the ClustalW program with the following parameters: scoring matrix blosum 55, gap opening penalty 15, gap extension penalty 6.66 (Thompson *et al.*, 1994).

C. Southern Analyses

Southern analyses were performed using standard molecular biology techniques (Chomczynski, 1992). Genomic DNA (7.5 µg) was digested with the appropriate restriction enzymes overnight at the optimal temperature for each enzyme. Additional enzyme was added to each reaction the following morning, and the reaction was allowed to continue for approximately four hours. Digested DNA was loaded onto 1% agarose gels and bands were separated electrophoretically in 1X TAE buffer. Following a 10 minute depurination in 0.25 N HCl, DNA was denatured in 0.5 M NaOH and 1.5 M NaCl

for 30 minutes. The DNA was then transferred to a Zeta-Probe® GT membrane (Bio-Rad, Cat. No. 162-0195) *via* alkaline transfer for one hour using a Boekel/Appligene Vacuum Blotter (Boekel, Cat. No. 230600). The membranes were briefly rinsed in 2X SSC. The DNA was then crosslinked to the membrane using a Stratalinker® UV Crosslinker (Stratagene, Cat. No. 400071) with the "auto crosslink" setting.

Element-specific, full-length PCR-generated probes were labeled with dCTP-^{32}P *via* random priming using the Megaprime™ DNA labeling system (Amersham Biosciences, Cat. No. RPN1607). For all element-specific probes 5-25 ng of DNA was used for labeling; 5 ng was used for DNA molecular weight marker probes. Unincorporated isotope was removed using Micro Bio-Spin® Chromatography Columns (Bio-Rad, Cat. No.732-6223). Membranes were incubated in prehybridization solution [PerfectHyb™ Plus Hybridization Buffer, Calf Thymus DNA (100 µg/mL)] for approximately one hour at 65ºC. Radiolabeled probe was added to the prehybridization solution, and the membranes were incubated for a minimum of three hours. Hybridization was carried out at 65°C, followed by multiple washes in 2X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS. The membranes were then subjected to autoradiography.

Appendix 1-Figure 1. Deduced amino acid sequence for the full-length Cry1Ab protein produced in COT67B

```
   1   MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51   VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101   EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151   QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201   GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251   DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301   NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351   VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401   YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451   PMFSWIHRSA EFNNIIPSSQ ITQIPLTKST NLGSGTSVVK GPGFTGGDIL
 501   RRTSPGQIST LRVNITAPLS QRYRVRIRYA STTNLQFHTS IDGRPINQGN
 551   FSATMSSGSN LQSGSFRTVG FTTPFNFSNG SSVFTLSAHV FNSGNEVYID
 601   RIEFVPAEVT FEAEYDLERA QKAVNELFTS SNQIGLKTDV TDYHIDQVSN
 651   LVECLSDEFC LDEKKELSEK VKHAKRLSDE RNLLQDPNFR GINRQLDRGW
 701   RGSTDITIQG GDDVFKENYV TLLGTFDECY PTYLYQKIDE SKLKAYTRYQ
 751   LRGYIEDSQD LEIYLIRYNA KHETVNVPGT GSLWPLSAPS PIGKCGEPNR
 801   CAPHLEWNPD LDCSCRDGEK CAHHSHHFSL DIDVGCTDLN EDLGVWVIFK
 851   IKTQDGHARL GNLEFLEEKP LVGEALARVK RAEKKWRDKR EKLEWETNIV
 901   YKEAKESVDA LFVNSQYDRL QADTNIAMIH AADKRVHSIR EAYLPELSVI
 951   PGVNAAIFEE LEGRIFTAFS LYDARNVIKN GDFNNGLSCW NVKGHVDVEE
1001   QNNHRSVLVV PEWEAEVSQE VRVCPGRGYI LRVTAYKEGY GEGCVTIHEI
1051   ENNTDELKFS NCVEEEVYPN NTVTCNDYTA TQEEYEGTYT SRNRGYDGAY
1101   ESNSSVPADY ASAYEEKAYT DGRRDNPCES NRGYGDYTPL PAGYVTKELE
1151   YFPETDKVWI EIGETEGTFI VDSVELLLME E
```

Appendix 1-Figure 2. *Gossypium hirsutum* genomic sequence flanking the 5' region of the COT67B T-DNA insert (700bp)

```
  1  TAAGGTCGTT AAAGGTGAAT TTCATATTTG ATTAAAAACA TGTTTTCAAA GTGTTTTTCA
     ATTCCAGCAA TTTCCACTTA AAGTATAAAC TAATTTTTGT ACAAAAGTTT CACAAAAAGT

 61  ACTTATTTTA ACATTATTAT AATGTTTATA AAGTTACTTT GAAGTTTCAT TAAGGAATTC
     TGAATAAAAT TGTAATAATA TTACAAATAT TTCAATGAAA CTTCAAAGTA ATTCCTTAAG

121  TCGAGTTTTA ATAAATTTTT GTCATAATAG CCAAAAAGTG TCGAAAAATT TTGATACCTG
     AGCTCAAAAT TATTTAAAAA CAGTATTATC GGTTTTTCAC AGCTTTTTAA AACTATGGAC

181  GAAACAGGTA GTTTTGTATA GTTTAACGGT TGGGAATTAG TCCTAGATGA ATATGCAGAT
     CTTTGTCCAT CAAAACATAT CAAATTGCCA ACCCTTAATC AGGATCTACT TATACGTCTA

241  GAATGGAACT TGTTTCATAC AAAAGGGGTA AGTGTAGATT GAGGTATTTG CGTTTTAAGT
     CTTACCTTGA ACAAAGTATG TTTTCCCCAT TCACATCTAA CTCCATAAAC GCAAAATTCA

301  TTGTTATGTT AAAGGGTTCA GTTACATGAT AATGTAGTTT GGGCATGTAT TTTAAATGGT
     AACAATACAA TTTCCCAAGT CAATGTACTA TTACATCAAA CCCGTACATA AAATTTACCA

361  TTAAGTGAGT CCTTGGTTGT CACTTGACTA TGTGTATCAT GTGATTGTTG TGCGTGAAGC
     AATTCACTCA GGAACCAACA GTGAACTGAT ACACATAGTA CACTAACAAC ACGCACTTCG

421  CTCTGATTCA TTAGAGCCTT CTACAAGTTG AAGGAAGAGC TAGTTCGAGC TTTTGGTTTT
     GAGACTAAGT AATCTCGGAA GATGTTCAAC TTCCTTCTCG ATCAAGCTCG AAAACCAAAA

481  CTGTGAAAGT TGATATTTTG TTAGTGTTTT GGAATCACTG CTCGTAGATG ATTCATGGTG
     GACACTTTCA ACTATAAAAC AATCACAAAA CCTTAGTGAC GAGCATCTAC TAAGTACCAC

541  TAATTGGGAA TTTAGATGTA GCCTAAACCC CTACTCTATA TTCTCAAAGT AAGTGTTCTT
     ATTAACCCTT AAATCTACAT CGGATTTGGG GATGAGATAT AAGAGTTTCA TTCACAAGAA

601  ATGCCTATGA TTAAATGTGA TATATGTATG CTTGTGAAAT TATGAAATTA TGAGCATATA
     TACGGATACT AATTTACACT ATATACATAC GAACACTTTA ATACTTTAAT ACTCGTATAT

661  TGAGATGCTA TGACATATGC TATAAGCATT TGATAACTTG
     ACTCTACGAT ACTGTATACG ATATTCGTAA ACTATTGAAC
```

Appendix 1-Figure 3. *Gossypium hirsutum* genomic sequence flanking the 3' region of the COT67B T-DNA insert (700 bp)

```
  1  ATAATGTGCT AATGATGTGA ATATGCAGTG CTTATGTGCG GAAAATCAAT AAGTAAATAT
     TATTACACGA TTACTACACT TATACGTCAC GAATACACGC CTTTTAGTTA TTCATTTATA

 61  GTGATAACAA TGTGGATATT TTGGCCTTGT GCTATTATGA GACCGTTGGA TATAGTTGGC
     CACTATTGTT ACACCTATAA AACCGGAACA CGATAATACT CTGGCAACCT ATATCAACCG

121  ATGCCATAGG GTTGTGAGTA CTCATCTTTG TGATGTTGTT TATGGGGCGT TGGGGCCCAA
     TACGGTATCC CAACACTCAT GAGTAGAAAC ACTACAACAA ATACCCCGCA ACCCCGGGTT

181  GGACAATTTT TGGAAAGATA AGGGAATGTG AGCTAAGCTT AATTCACCGG GATATGTGTG
     CCTGTTAAAA ACCTTTCTAT TCCCTTACAC TCGATTCGAA TTAAGTGGCC CTATACACAC

241  TTTGGTGTGC TGGAGAGTGT TAACTATATG CTTCACTTAT GGGACATGTA CGACACTATG
     AAACCACACG ACCTCTCACA ATTGATATAC GAAGTGAATA CCCTGTACAT GCTGTGATAC

301  AGTCAATATT GGTCTGTTGG TGATCCATGT ATTCGATGTG TGGTGATAGG GTCCACATTA
     TCAGTTATAA CCAGACAACC ACTAGGTACA TAAGCTACAC ACCACTATCC CAGGTGTAAT

361  TATTTCATAT CCTCAAGAGC CAAACTATCA TAAAACATGA CTGAAAGTGA CTAAATGTGA
     ATAAAGTATA GGAGTTCTCG GTTTGATAGT ATTTTGTACT GACTTTCACT GATTTACACT

421  TTAAAATGTG TTGTAGTATA TGCTTAAATA TTCATGTGAT TAATGTGTAA ATATTCATGA
     AATTTTACAC AACATCATAT ACGAATTTAT AAGTACACTA ATTACACATT TATAAGTACT

481  AAGATGATAA AATGTGTTAA ACATGACATA GGAGTAGAAG ATGTTATGAT TATATTGCAT
     TTCTACTATT TTACACAATT TGTACTGTAT CCTCATCTTC TACAATACTA ATATAACGTA

541  GTTTGCTTTG TTGATGCATA ATGATTTGTT TGCGTAGTGG TTGTTTTCAC CATTCACTGA
     CAAACGAAAC AACTACGTAT TACTAAACAA ACGCATCACC AACAAAAGTG GTAAGTGACT

601  GCTTGTTAAG CTCACGCACT CCTTTTTAAT CATTACAGAT AATTAGTGCC GGTGTGAGTG
     CGAACAATTC GAGTGCGTGA GGAAAAATTA GTAATGTCTA TTAATCACGG CCACACTCAC

661  GTATGGTCTC GAGGGGTGAT CCAAGCCAGA CATTTAGTTG
     CATACCAGAG CTCCCCACTA GGTTCGGTCT GTAAATCAAC
```

Appendix 1-Figure 4. Alignment of the COT67B T-DNA insert with the T-DNA insert within the pNOV4641 plasmid

```
                          LB
COT67B Insert     (1) ------------------------------------GACAACTTAATA
pNOV4641          (1) TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATA


COT67B Insert    (13) ACACATTGCGGATACGGCCATGCTGGCCGCCCGGGCACCGGTAAATTTCC
pNOV4641         (51) ACACATTGCGGATACGGCCATGCTGGCCGCCCGGGCACCGGTAAATTTCC


COT67B Insert    (63) TGCAGGGCTAGCAGATCTCTCGAGGTTTAAACGGGCCCACGCGTGCGGCC
pNOV4641        (101) TGCAGGGCTAGCAGATCTCTCGAGGTTTAAACGGGCCCACGCGTGCGGCC


                                                ACT2 promoter and intron
COT67B Insert   (113) GCTCCGGATTCGAATTAATTAACGTACGAAGCTTGCATGCCTGCAGGTCG
pNOV4641        (151) GCTCCGGATTCGAATTAATTAACGTACGAAGCTTGCATGCCTGCAGGTCG


                          ACT2 promoter and intron
COT67B Insert   (163) ACAAAATTTAGAACGAACTTAATTATGATCTCAAATACATTGATACATAT
pNOV4641        (201) ACAAAATTTAGAACGAACTTAATTATGATCTCAAATACATTGATACATAT


                          ACT2 promoter and intron
COT67B Insert   (213) CTCATCTAGATCTAGGTTATCATTATGTAAGAAAGTTTTGACGAATATGG
pNOV4641        (251) CTCATCTAGATCTAGGTTATCATTATGTAAGAAAGTTTTGACGAATATGG


                          ACT2 promoter and intron
COT67B Insert   (263) CACGACAAAATGGCTAGACTCGATGTAATTGGTATCTCAACTCAACATTA
pNOV4641        (301) CACGACAAAATGGCTAGACTCGATGTAATTGGTATCTCAACTCAACATTA


                          ACT2 promoter and intron
COT67B Insert   (313) TACTTATACCAAACATTAGTTAGACAAAATTTAAACAACTATTTTTTATG
pNOV4641        (351) TACTTATACCAAACATTAGTTAGACAAAATTTAAACAACTATTTTTTATG


                          ACT2 promoter and intron
COT67B Insert   (363) TATGCAAGAGTCAGCATATGTATAATTGATTCAGAATCGTTTTGACGAGT
pNOV4641        (401) TATGCAAGAGTCAGCATATGTATAATTGATTCAGAATCGTTTTGACGAGT
```

Appendix 1-Figure 4. Continued

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                                ACT2 promoter and intron
COT67B Insert   (413) TCGGATGTAGTAGTAGCCATTATTTAATGTACATACTAATCGTGAATAGT
pNOV4641        (451) TCGGATGTAGTAGTAGCCATTATTTAATGTACATACTAATCGTGAATAGT

                                ACT2 promoter and intron
COT67B Insert   (463) GAATATGATGAAACATTGTATCTTATTGTATAAATATCCATAAACACATC
pNOV4641        (501) GAATATGATGAAACATTGTATCTTATTGTATAAATATCCATAAACACATC

                                ACT2 promoter and intron
COT67B Insert   (513) ATGAAAGACACTTTCTTTCACGGTCTGAATTAATTATGATACAATTCTAA
pNOV4641        (551) ATGAAAGACACTTTCTTTCACGGTCTGAATTAATTATGATACAATTCTAA

                                ACT2 promoter and intron
COT67B Insert   (563) TAGAAAACGAATTAAATTACGTTGAATTGTATGAAATCTAATTGAACAAG
pNOV4641        (601) TAGAAAACGAATTAAATTACGTTGAATTGTATGAAATCTAATTGAACAAG

                                ACT2 promoter and intron
COT67B Insert   (613) CCAACCACGACGACGACTAACGTTGCCTGGATTGACTCGGTTTAAGTTAA
pNOV4641        (651) CCAACCACGACGACGACTAACGTTGCCTGGATTGACTCGGTTTAAGTTAA

                                ACT2 promoter and intron
COT67B Insert   (663) CCACTAAAAAAACGGAGCTGTCATGTAACACGCGGATCGAGCAGGTCACA
pNOV4641        (701) CCACTAAAAAAACGGAGCTGTCATGTAACACGCGGATCGAGCAGGTCACA

                                ACT2 promoter and intron
COT67B Insert   (713) GTCATGAAGCCATCAAAGCAAAAGAACTAATCCAAGGGCTGAGATGATTA
pNOV4641        (751) GTCATGAAGCCATCAAAGCAAAAGAACTAATCCAAGGGCTGAGATGATTA

                                ACT2 promoter and intron
COT67B Insert   (763) ATTAGTTTAAAAATTAGTTAACACGAGGGAAAAGGCTGTCTGACAGCCAG
pNOV4641        (801) ATTAGTTTAAAAATTAGTTAACACGAGGGAAAAGGCTGTCTGACAGCCAG

                                ACT2 promoter and intron
COT67B Insert   (813) GTCACGTTATCTTTACCTGTGGTCGAAATGATTCGTGTCTGTCGATTTTA
pNOV4641        (851) GTCACGTTATCTTTACCTGTGGTCGAAATGATTCGTGTCTGTCGATTTTA
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Appendix 1-Figure 4. Continued

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                                ACT2 promoter and intron
COT67B Insert   (863) ATTATTTTTTTGAAAGGCCGAAAATAAAGTTGTAAGAGATAAACCCGCCT
pNOV4641        (901) ATTATTTTTTTGAAAGGCCGAAAATAAAGTTGTAAGAGATAAACCCGCCT


                                ACT2 promoter and intron
COT67B Insert   (913) ATATAAATTCATATATTTTCCTCTCCGCTTTGAATTGTCTCGTTGTCCTC
pNOV4641        (951) ATATAAATTCATATATTTTCCTCTCCGCTTTGAATTGTCTCGTTGTCCTC


                                ACT2 promoter and intron
COT67B Insert   (963) CTCACTTTCATCAGCCGTTTTGAATCTCCGGCGACTTGACAGAGAAGAAC
pNOV4641       (1001) CTCACTTTCATCAGCCGTTTTGAATCTCCGGCGACTTGACAGAGAAGAAC


                                ACT2 promoter and intron
COT67B Insert  (1013) AAGGAAGAAGACTAAGAGAGAAAGTAAGAGATAATCCAGGAGATTCATTC
pNOV4641       (1051) AAGGAAGAAGACTAAGAGAGAAAGTAAGAGATAATCCAGGAGATTCATTC


                                ACT2 promoter and intron
COT67B Insert  (1063) TCCGTTTTGAATCTTCCTCAATCTCATCTTCTTCCGCTCTTTCTTTCCAA
pNOV4641       (1101) TCCGTTTTGAATCTTCCTCAATCTCATCTTCTTCCGCTCTTTCTTTCCAA


                                ACT2 promoter and intron
COT67B Insert  (1113) GGTAATAGGAACTTTCTGGATCTACTTTATTTGCTGGATCTCGATCTTGT
pNOV4641       (1151) GGTAATAGGAACTTTCTGGATCTACTTTATTTGCTGGATCTCGATCTTGT


                                ACT2 promoter and intron
COT67B Insert  (1163) TTTCTCAATTTCCTTGAGATCTGGAATTCGTTTAATTTGGATCTGTGAAC
pNOV4641       (1201) TTTCTCAATTTCCTTGAGATCTGGAATTCGTTTAATTTGGATCTGTGAAC


                                ACT2 promoter and intron
COT67B Insert  (1213) CTCCACTAAATCTTTTGGTTTTACTAGAATCGATCTAAGTTGACCGATCA
pNOV4641       (1251) CTCCACTAAATCTTTTGGTTTTACTAGAATCGATCTAAGTTGACCGATCA


                                ACT2 promoter and intron
COT67B Insert  (1263) GTTAGCTCGATTATAGCTACCAGAATTTGGCTTGACCTTGATGGAGAGAT
pNOV4641       (1301) GTTAGCTCGATTATAGCTACCAGAATTTGGCTTGACCTTGATGGAGAGAT
```

Appendix 1-Figure 4. Continued

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                                ACT2 promoter and intron
COT67B Insert  (1313) CCATGTTCATGTTACCTGGGAAATGATTTGTATATGTGAATTGAAATCTG
pNOV4641       (1351) CCATGTTCATGTTACCTGGGAAATGATTTGTATATGTGAATTGAAATCTG


                                ACT2 promoter and intron
COT67B Insert  (1363) AACTGTTGAAGTTAGATTGAATCTGAACACTGTCAATGTTAGATTGAATC
pNOV4641       (1401) AACTGTTGAAGTTAGATTGAATCTGAACACTGTCAATGTTAGATTGAATC


                                ACT2 promoter and intron
COT67B Insert  (1413) TGAACACTGTTTAAGGTTAGATGAAGTTTGTGTATAGATTCTTCGAAACT
pNOV4641       (1451) TGAACACTGTTTAAGGTTAGATGAAGTTTGTGTATAGATTCTTCGAAACT


                                ACT2 promoter and intron
COT67B Insert  (1463) TTAGGATTTGTAGTGTCGTACGTTGAACAGAAAGCTATTTCTGATTCAAT
pNOV4641       (1501) TTAGGATTTGTAGTGTCGTACGTTGAACAGAAAGCTATTTCTGATTCAAT


                                ACT2 promoter and intron
COT67B Insert  (1513) CAGGGTTTATTTGACTGTATTGAACTCTTTTTGTGTGTTTGCAGCTCATA
pNOV4641       (1551) CAGGGTTTATTTGACTGTATTGAACTCTTTTTGTGTGTTTGCAGCTCATA

                       ACT2 promoter and intron
                                                 flcry1Ab
COT67B Insert  (1563) AAAAGGATCCAACAATGGACAACAACCCCAACATCAACGAGTGCATCCCC
pNOV4641       (1601) AAAAGGATCCAACAATGGACAACAACCCCAACATCAACGAGTGCATCCCC


                                      flcry1Ab
COT67B Insert  (1613) TACAACTGCCTGAGCAACCCCGAGGTGGAGGTGCTGGGCGGCGAGCGCAT
pNOV4641       (1651) TACAACTGCCTGAGCAACCCCGAGGTGGAGGTGCTGGGCGGCGAGCGCAT


                                      flcry1Ab
COT67B Insert  (1663) CGAGACCGGCTACACCCCCATCGACATCAGCCTGAGCCTGACCCAGTTCC
pNOV4641       (1701) CGAGACCGGCTACACCCCCATCGACATCAGCCTGAGCCTGACCCAGTTCC


                                      flcry1Ab
COT67B Insert  (1713) TGCTGAGCGAGTTCGTGCCCGGCGCCGGCTTCGTGCTGGGCCTGGTGGAC
pNOV4641       (1751) TGCTGAGCGAGTTCGTGCCCGGCGCCGGCTTCGTGCTGGGCCTGGTGGAC
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Appendix 1-Figure 4. Continued

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                                  flcry1Ab
COT67B Insert  (1763) ATCATCTGGGGCATCTTCGGCCCCAGCCAGTGGGACGCCTTCCTGGTGCA
pNOV4641       (1801) ATCATCTGGGGCATCTTCGGCCCCAGCCAGTGGGACGCCTTCCTGGTGCA

                                  flcry1Ab
COT67B Insert  (1813) GATCGAGCAGTTGATAAACCAACGCATAGAGGAATTCGCCCGCAACCAGG
pNOV4641       (1851) GATCGAGCAGTTGATAAACCAACGCATAGAGGAATTCGCCCGCAACCAGG

                                  flcry1Ab
COT67B Insert  (1863) CCATCAGCCGCCTGGAGGGCCTGAGCAACCTGTACCAAATCTACGCCGAG
pNOV4641       (1901) CCATCAGCCGCCTGGAGGGCCTGAGCAACCTGTACCAAATCTACGCCGAG

                                  flcry1Ab
COT67B Insert  (1913) AGCTTCCGCGAGTGGGAGGCCGACCCCACCAACCCCGCCCTGCGCGAGGA
pNOV4641       (1951) AGCTTCCGCGAGTGGGAGGCCGACCCCACCAACCCCGCCCTGCGCGAGGA

                                  flcry1Ab
COT67B Insert  (1963) GATGCGCATCCAGTTCAACGACATGAACAGCGCCCTGACCACCGCCATCC
pNOV4641       (2001) GATGCGCATCCAGTTCAACGACATGAACAGCGCCCTGACCACCGCCATCC

                                  flcry1Ab
COT67B Insert  (2013) CCCTGTTCGCCGTGCAGAACTACCAGGTGCCCCTGCTGAGCGTGTACGTG
pNOV4641       (2051) CCCTGTTCGCCGTGCAGAACTACCAGGTGCCCCTGCTGAGCGTGTACGTG

                                  flcry1Ab
COT67B Insert  (2063) CAGGCCGCCAACCTGCACCTGAGCGTGCTGCGCGACGTCAGCGTGTTCGG
pNOV4641       (2101) CAGGCCGCCAACCTGCACCTGAGCGTGCTGCGCGACGTCAGCGTGTTCGG

                                  flcry1Ab
COT67B Insert  (2113) CCAGCGCTGGGGCTTCGACGCCGCCACCATCAACAGCCGCTACAACGACC
pNOV4641       (2151) CCAGCGCTGGGGCTTCGACGCCGCCACCATCAACAGCCGCTACAACGACC

                                  flcry1Ab
COT67B Insert  (2163) TGACCCGCCTGATCGGCAACTACACCGACCACGCCGTGCGCTGGTACAAC
pNOV4641       (2201) TGACCCGCCTGATCGGCAACTACACCGACCACGCCGTGCGCTGGTACAAC
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Appendix 1-Figure 4. Continued

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                                  flcry1Ab
COT67B Insert  (2213) ACCGGCCTGGAGCGCGTGTGGGGTCCCGACAGCCGCGACTGGATCAGGTA
pNOV4641       (2251) ACCGGCCTGGAGCGCGTGTGGGGTCCCGACAGCCGCGACTGGATCAGGTA

                                  flcry1Ab
COT67B Insert  (2263) CAACCAGTTCCGCCGCGAGCTGACCCTGACCGTGCTGGACATCGTGAGCC
pNOV4641       (2301) CAACCAGTTCCGCCGCGAGCTGACCCTGACCGTGCTGGACATCGTGAGCC
                                  flcry1Ab
COT67B Insert  (2313) TGTTCCCCAACTACGACAGCCGCACCTACCCCATCCGCACCGTGAGCCAG
pNOV4641       (2351) TGTTCCCCAACTACGACAGCCGCACCTACCCCATCCGCACCGTGAGCCAG

                                  flcry1Ab
COT67B Insert  (2363) CTGACCCGCGAGATTTACACCAACCCCGTGCTGGAGAACTTCGACGGCAG
pNOV4641       (2401) CTGACCCGCGAGATTTACACCAACCCCGTGCTGGAGAACTTCGACGGCAG

                                  flcry1Ab
COT67B Insert  (2413) CTTCCGCGGCAGCGCCCAGGGCATCGAGGGCAGCATCCGCAGCCCCCACC
pNOV4641       (2451) CTTCCGCGGCAGCGCCCAGGGCATCGAGGGCAGCATCCGCAGCCCCCACC

                                  flcry1Ab
COT67B Insert  (2463) TGATGGACATCCTGAACAGCATCACCATCTACACCGACGCCCACCGCGGC
pNOV4641       (2501) TGATGGACATCCTGAACAGCATCACCATCTACACCGACGCCCACCGCGGC

                                  flcry1Ab
COT67B Insert  (2513) GAGTACTACTGGAGCGGCCACCAGATCATGGCCAGCCCCGTCGGCTTCAG
pNOV4641       (2551) GAGTACTACTGGAGCGGCCACCAGATCATGGCCAGCCCCGTCGGCTTCAG

                                  flcry1Ab
COT67B Insert  (2563) CGGCCCCGAGTTCACCTTCCCCCTGTACGGCACCATGGGCAACGCTGCAC
pNOV4641       (2601) CGGCCCCGAGTTCACCTTCCCCCTGTACGGCACCATGGGCAACGCTGCAC

                                  flcry1Ab
COT67B Insert  (2613) CTCAGCAGCGCATCGTGGCACAGCTGGGCCAGGGAGTGTACCGCACCCTG
pNOV4641       (2651) CTCAGCAGCGCATCGTGGCACAGCTGGGCCAGGGAGTGTACCGCACCCTG

                                  flcry1Ab
COT67B Insert  (2663) AGCAGCACCCTGTACCGTCGACCTTTCAACATCGGCATCAACAACCAGCA
pNOV4641       (2701) AGCAGCACCCTGTACCGTCGACCTTTCAACATCGGCATCAACAACCAGCA
```

Appendix 1-Figure 4. Continued

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                                    flcry1Ab
COT67B Insert  (2713) GCTGAGCGTGCTGGACGGCACCGAGTTCGCCTACGGCACCAGCAGCAACC
pNOV4641       (2751) GCTGAGCGTGCTGGACGGCACCGAGTTCGCCTACGGCACCAGCAGCAACC

                                    flcry1Ab
COT67B Insert  (2763) TGCCCAGCGCCGTGTACCGCAAGAGCGGCACCGTGGACAGCCTGGACGAG
pNOV4641       (2801) TGCCCAGCGCCGTGTACCGCAAGAGCGGCACCGTGGACAGCCTGGACGAG

                                    flcry1Ab
COT67B Insert  (2813) ATCCCCCCTCAGAACAACAACGTGCCACCTCGACAGGGCTTCAGCCACCG
pNOV4641       (2851) ATCCCCCCTCAGAACAACAACGTGCCACCTCGACAGGGCTTCAGCCACCG

                                    flcry1Ab
COT67B Insert  (2863) TCTGAGCCACGTGAGCATGTTCCGCAGTGGCTTCAGCAACAGCAGCGTGA
pNOV4641       (2901) TCTGAGCCACGTGAGCATGTTCCGCAGTGGCTTCAGCAACAGCAGCGTGA

                                    flcry1Ab
COT67B Insert  (2913) GCATCATCCGTGCACCTATGTTCAGCTGGATTCACCGCAGTGCCGAGTTC
pNOV4641       (2951) GCATCATCCGTGCACCTATGTTCAGCTGGATTCACCGCAGTGCCGAGTTC

                                    flcry1Ab
COT67B Insert  (2963) AACAACATCATCCCCAGCAGCCAGATCACCCAGATCCCCCTGACCAAGAG
pNOV4641       (3001) AACAACATCATCCCCAGCAGCCAGATCACCCAGATCCCCCTGACCAAGAG

                                    flcry1Ab
COT67B Insert  (3013) CACCAACCTGGGCAGCGGCACCAGCGTGGTGAAGGGCCCCGGCTTCACCG
pNOV4641       (3051) CACCAACCTGGGCAGCGGCACCAGCGTGGTGAAGGGCCCCGGCTTCACCG

                                    flcry1Ab
COT67B Insert  (3063) GCGGCGACATCCTGCGCCGCACCAGCCCCGGCCAGATCAGCACCCTGCGC
pNOV4641       (3101) GCGGCGACATCCTGCGCCGCACCAGCCCCGGCCAGATCAGCACCCTGCGC

                                    flcry1Ab
COT67B Insert  (3113) GTGAACATCACCGCCCCCCTGAGCCAGCGCTACCGCGTCCGCATCCGCTA
pNOV4641       (3151) GTGAACATCACCGCCCCCCTGAGCCAGCGCTACCGCGTCCGCATCCGCTA
```

Appendix 1-Figure 4. Continued

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                                  flcry1Ab
COT67B Insert  (3163) CGCCAGCACCACCAACCTGCAGTTCCACACCAGCATCGACGGCCGCCCCA
pNOV4641       (3201) CGCCAGCACCACCAACCTGCAGTTCCACACCAGCATCGACGGCCGCCCCA

                                  flcry1Ab
COT67B Insert  (3213) TCAACCAGGGCAACTTCAGCGCCACCATGAGCAGCGGCAGCAACCTGCAG
pNOV4641       (3251) TCAACCAGGGCAACTTCAGCGCCACCATGAGCAGCGGCAGCAACCTGCAG

                                  flcry1Ab
COT67B Insert  (3263) AGCGGCAGCTTCCGCACCGTGGGCTTCACCACCCCCTTCAACTTCAGCAA
pNOV4641       (3301) AGCGGCAGCTTCCGCACCGTGGGCTTCACCACCCCCTTCAACTTCAGCAA

                                  flcry1Ab
COT67B Insert  (3313) CGGCAGCAGCGTGTTCACCCTGAGCGCCCACGTGTTCAACAGCGGCAACG
pNOV4641       (3351) CGGCAGCAGCGTGTTCACCCTGAGCGCCCACGTGTTCAACAGCGGCAACG

                                  flcry1Ab
COT67B Insert  (3363) AGGTGTACATCGACCGCATCGAGTTCGTGCCCGCCGAGGTGACCTTCGAG
pNOV4641       (3401) AGGTGTACATCGACCGCATCGAGTTCGTGCCCGCCGAGGTGACCTTCGAG

                                  flcry1Ab
COT67B Insert  (3413) GCCGAGTACGACCTGGAGAGGGCTCAGAAGGCCGTGAACGAGCTGTTCAC
pNOV4641       (3451) GCCGAGTACGACCTGGAGAGGGCTCAGAAGGCCGTGAACGAGCTGTTCAC

                                  flcry1Ab
COT67B Insert  (3463) CAGCAGCAACCAGATCGGCCTGAAGACCGACGTGACCGACTACCACATCG
pNOV4641       (3501) CAGCAGCAACCAGATCGGCCTGAAGACCGACGTGACCGACTACCACATCG

                                  flcry1Ab
COT67B Insert  (3513) ACCAGGTGAGCAACCTGGTGGAGTGCTTAAGCGACGAGTTCTGCCTGGAC
pNOV4641       (3551) ACCAGGTGAGCAACCTGGTGGAGTGCTTAAGCGACGAGTTCTGCCTGGAC

                                  flcry1Ab
COT67B Insert  (3563) GAGAAGAAGGAGCTGAGCGAGAAGGTGAAGCACGCCAAGCGCCTGAGCGA
pNOV4641       (3601) GAGAAGAAGGAGCTGAGCGAGAAGGTGAAGCACGCCAAGCGCCTGAGCGA
```

Appendix 1-Figure 4. Continued

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                                  flcry1Ab
COT67B Insert  (3613) CGAGCGCAACCTGCTGCAGGACCCCAACTTCCGCGGCATCAACCGCCAGC
pNOV4641       (3651) CGAGCGCAACCTGCTGCAGGACCCCAACTTCCGCGGCATCAACCGCCAGC

                                  flcry1Ab
COT67B Insert  (3663) TGGACCGCGGCTGGCGAGGCAGCACCGATATCACCATCCAGGGCGGCGAC
pNOV4641       (3701) TGGACCGCGGCTGGCGAGGCAGCACCGATATCACCATCCAGGGCGGCGAC

                                  flcry1Ab
COT67B Insert  (3713) GACGTGTTCAAGGAGAACTACGTGACCCTGCTGGGCACCTTCGACGAGTG
pNOV4641       (3751) GACGTGTTCAAGGAGAACTACGTGACCCTGCTGGGCACCTTCGACGAGTG

                                  flcry1Ab
COT67B Insert  (3763) CTACCCCACCTACCTGTACCAGAAGATCGACGAGAGCAAGCTGAAGGCCT
pNOV4641       (3801) CTACCCCACCTACCTGTACCAGAAGATCGACGAGAGCAAGCTGAAGGCCT

                                  flcry1Ab
COT67B Insert  (3813) ACACCCGCTACCAGCTGCGCGGCTACATCGAGGACAGCCAGGACCTGGAA
pNOV4641       (3851) ACACCCGCTACCAGCTGCGCGGCTACATCGAGGACAGCCAGGACCTGGAA

                                  flcry1Ab
COT67B Insert  (3863) ATCTACCTGATCCGCTACAACGCGAAGCACGAGACCGTGAACGTGCCCGG
pNOV4641       (3901) ATCTACCTGATCCGCTACAACGCGAAGCACGAGACCGTGAACGTGCCCGG

                                  flcry1Ab
COT67B Insert  (3913) CACCGGCAGCCTGTGGCCCCTGAGCGCCCCCAGCCCCATCGGCAAGTGCG
pNOV4641       (3951) CACCGGCAGCCTGTGGCCCCTGAGCGCCCCCAGCCCCATCGGCAAGTGCG

                                  flcry1Ab
COT67B Insert  (3963) GGGAGCCGAATCGATGCGCTCCGCACCTGGAGTGGAACCCGGACCTAGAC
pNOV4641       (4001) GGGAGCCGAATCGATGCGCTCCGCACCTGGAGTGGAACCCGGACCTAGAC

                                  flcry1Ab
COT67B Insert  (4013) TGCAGCTGCAGGGACGGGGAGAAGTGCGCCCACCACAGCCACCACTTCAG
pNOV4641       (4051) TGCAGCTGCAGGGACGGGGAGAAGTGCGCCCACCACAGCCACCACTTCAG
```

Appendix 1-Figure 4. Continued

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                                    flcry1Ab
COT67B Insert  (4063) CCTGGACATCGACGTGGGCTGCACCGACCTGAACGAGGACCTGGGCGTGT
pNOV4641       (4101) CCTGGACATCGACGTGGGCTGCACCGACCTGAACGAGGACCTGGGCGTGT

                                    flcry1Ab
COT67B Insert  (4113) GGGTGATCTTCAAGATCAAGACCCAGGACGGCCACGCCCGCCTGGGCAAT
pNOV4641       (4151) GGGTGATCTTCAAGATCAAGACCCAGGACGGCCACGCCCGCCTGGGCAAT

                                    flcry1Ab
COT67B Insert  (4163) CTAGAGTTCCTGGAGGAGAAGCCCCTGGTGGGCGAGGCCCTGGCCCGCGT
pNOV4641       (4201) CTAGAGTTCCTGGAGGAGAAGCCCCTGGTGGGCGAGGCCCTGGCCCGCGT

                                    flcry1Ab
COT67B Insert  (4213) GAAGCGTGCTGAGAAGAAGTGGCGCGACAAGCGCGAGAAGCTGGAGTGGG
pNOV4641       (4251) GAAGCGTGCTGAGAAGAAGTGGCGCGACAAGCGCGAGAAGCTGGAGTGGG
                                    flcry1Ab
COT67B Insert  (4263) AGACCAACATCGTGTACAAGGAGGCCAAGGAGAGCGTGGACGCCCTGTTC
pNOV4641       (4301) AGACCAACATCGTGTACAAGGAGGCCAAGGAGAGCGTGGACGCCCTGTTC

                                    flcry1Ab
COT67B Insert  (4313) GTGAACAGCCAGTACGACCGCCTGCAGGCCGACACCAACATCGCCATGAT
pNOV4641       (4351) GTGAACAGCCAGTACGACCGCCTGCAGGCCGACACCAACATCGCCATGAT

                                    flcry1Ab
COT67B Insert  (4363) CCACGCCGCCGACAAGCGCGTGCACAGCATTCGCGAGGCCTACCTGCCCG
pNOV4641       (4401) CCACGCCGCCGACAAGCGCGTGCACAGCATTCGCGAGGCCTACCTGCCCG

                                    flcry1Ab
COT67B Insert  (4413) AGCTGAGCGTGATCCCCGGTGTGAACGCCGCCATCTTCGAGGAACTCGAG
pNOV4641       (4451) AGCTGAGCGTGATCCCCGGTGTGAACGCCGCCATCTTCGAGGAACTCGAG

                                    flcry1Ab
COT67B Insert  (4463) GGCCGCATCTTCACCGCCTTCAGCCTGTACGACGCCCGCAACGTGATCAA
pNOV4641       (4501) GGCCGCATCTTCACCGCCTTCAGCCTGTACGACGCCCGCAACGTGATCAA
```

Appendix 1-Figure 4. Continued

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                                  flcry1Ab
COT67B Insert  (4513) GAACGGCGACTTCAACAACGGCCTGAGCTGCTGGAACGTGAAGGGCCACG
pNOV4641       (4551) GAACGGCGACTTCAACAACGGCCTGAGCTGCTGGAACGTGAAGGGCCACG

                                  flcry1Ab
COT67B Insert  (4563) TGGACGTGGAGGAGCAGAACAACCACCGCAGCGTGCTGGTGGTGCCCGAG
pNOV4641       (4601) TGGACGTGGAGGAGCAGAACAACCACCGCAGCGTGCTGGTGGTGCCCGAG

                                  flcry1Ab
COT67B Insert  (4613) TGGGAGGCCGAGGTGAGCCAGGAGGTGCGCGTGTGCCCCGGCCGCGGCTA
pNOV4641       (4651) TGGGAGGCCGAGGTGAGCCAGGAGGTGCGCGTGTGCCCCGGCCGCGGCTA

                                  flcry1Ab
COT67B Insert  (4663) CATCCTGCGCGTGACCGCCTACAAGGAGGGCTACGGCGAGGGCTGCGTGA
pNOV4641       (4701) CATCCTGCGCGTGACCGCCTACAAGGAGGGCTACGGCGAGGGCTGCGTGA

                                  flcry1Ab
COT67B Insert  (4713) CCATCCACGAGATCGAGAACAACACCGACGAACTCAAGTTCAGCAACTGC
pNOV4641       (4751) CCATCCACGAGATCGAGAACAACACCGACGAACTCAAGTTCAGCAACTGC

                                  flcry1Ab
COT67B Insert  (4763) GTGGAGGAGGAGGTTTACCCCAACAACACCGTGACCTGCAACGACTACAC
pNOV4641       (4801) GTGGAGGAGGAGGTTTACCCCAACAACACCGTGACCTGCAACGACTACAC

                                  flcry1Ab
COT67B Insert  (4813) CGCGACCCAGGAGGAGTACGAAGGCACCTACACCTCTCGCAACAGGGGTT
pNOV4641       (4851) CGCGACCCAGGAGGAGTACGAAGGCACCTACACCTCTCGCAACAGGGGTT

                                  flcry1Ab
COT67B Insert  (4863) ACGACGGCGCCTACGAGTCCAACAGCTCCGTGCCAGCTGACTACGCCAGC
pNOV4641       (4901) ACGACGGCGCCTACGAGTCCAACAGCTCCGTGCCAGCTGACTACGCCAGC

                                  flcry1Ab
COT67B Insert  (4913) GCCTACGAGGAGAAAGCCTACACCGACGGTAGACGCGACAACCCATGTGA
pNOV4641       (4951) GCCTACGAGGAGAAAGCCTACACCGACGGTAGACGCGACAACCCATGTGA
```

Appendix 1-Figure 4. Continued

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                                        flcry1Ab
COT67B Insert  (4963) GAGCAACAGAGGCTACGGCGACTACACCCCCCTGCCCGCTGGATACGTGA
pNOV4641       (5001) GAGCAACAGAGGCTACGGCGACTACACCCCCCTGCCCGCTGGATACGTGA

                                        flcry1Ab
COT67B Insert  (5013) CCAAGGAGCTGGAGTACTTCCCCGAGACCGACAAGGTGTGGATCGAGATT
pNOV4641       (5051) CCAAGGAGCTGGAGTACTTCCCCGAGACCGACAAGGTGTGGATCGAGATT

                                        flcry1Ab
COT67B Insert  (5063) GGCGAGACCGAGGGCACCTTCATCGTGGACAGCGTGGAGCTGCTGCTGAT
pNOV4641       (5101) GGCGAGACCGAGGGCACCTTCATCGTGGACAGCGTGGAGCTGCTGCTGAT

                      flcry1Ab
COT67B Insert  (5113) GGAGGAGTAGTAGATCCATCTGCAGATGAGCTCTAGATCCCCGAATTTCC
pNOV4641       (5151) GGAGGAGTAGTAGATCCATCTGCAGATGAGCTCTAGATCCCCGAATTTCC

                                   NOS
COT67B Insert  (5163) CCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTT
pNOV4641       (5201) CCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTT

                                   NOS
COT67B Insert  (5213) GCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCA
pNOV4641       (5251) GCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCA

                                   NOS
COT67B Insert  (5263) TGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTA
pNOV4641       (5301) TGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTA

                                   NOS
COT67B Insert  (5313) TGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATA
pNOV4641       (5351) TGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATA

                                   NOS
COT67B Insert  (5363) TAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACT
pNOV4641       (5401) TAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACT
```

Appendix 1-Figure 4. Continued

```
                        NOS
COT67B Insert  (5413) AGATCGGGAATTGGGTACCGAGCTCGAATTCGGCGCGCCCAATTGATTTA
pNOV4641       (5451) AGATCGGGAATTGGGTACCGAGCTCGAATTCGGCGCGCCCAATTGATTTA


COT67B Insert  (5463) AATGGCCGCTGCGGCCAATTCCTGCAGCGTTGCGGTTCTGTCAGTTCCAA
pNOV4641       (5501) AATGGCCGCTGCGGCCAATTCCTGCAGCGTTGCGGTTCTGTCAGTTCCAA


COT67B Insert  (5513) ACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTCATAACGTGACTCC
pNOV4641       (5551) ACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTCATAACGTGACTCC


COT67B Insert  (5563) CTTAATTCTCCGCTCATGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAA
pNOV4641       (5601) CTTAATTCTCCGCTCATGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAA


                                        RB
COT67B Insert  (5613) CTATCAGTGTTT------------------------
pNOV4641       (5651) CTATCAGTGTTTGACAGGATATATTGGCGGGTAAAC
```

APPENDIX 2. ALIGNMENTS OF CRY PROTEIN SEQUENCES

Appendix 2-Figure 1: Alignment of protein sequence of COT67B with similar Cry protein sequences

Gray background represents sequence identities, dashes represent gaps. The ‘Geiser’-motif is indicated in bold

```
                      1                                                  50
       Bt11-seq   (1)MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
      Bt176-seq   (1)MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
     COT67B-seq   (1)MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
Native FLCry1Ab   (1)MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
Cry1Ac-Bollgard   (1)MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
                      51                                                 100
       Bt11-seq  (51)VPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRL
      Bt176-seq  (51)VPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRL
     COT67B-seq  (51)VPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRL
Native FLCry1Ab  (51)VPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRL
Cry1Ac-Bollgard  (51)VPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRL
                     101                                                 150
       Bt11-seq (101)EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV
      Bt176-seq (101)EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV
     COT67B-seq (101)EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV
Native FLCry1Ab (101)EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV
Cry1Ac-Bollgard (101)EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV
                     151                                                 200
       Bt11-seq (151)QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI
      Bt176-seq (151)QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI
     COT67B-seq (151)QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI
Native FLCry1Ab (151)QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI
Cry1Ac-Bollgard (151)QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI
                     201                                                 250
       Bt11-seq (201)GNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNY
      Bt176-seq (201)GNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNY
     COT67B-seq (201)GNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNY
Native FLCry1Ab (201)GNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNY
Cry1Ac-Bollgard (201)GNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNY
                     251                                                 300
       Bt11-seq (251)DSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
      Bt176-seq (251)DSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
     COT67B-seq (251)DSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
Native FLCry1Ab (251)DSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
Cry1Ac-Bollgard (251)DSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
                     301                                                 350
```

Appendix 2-Figure 1: Continued

```
       Bt11-seq (301)NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRI
      Bt176-seq (301)NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRI
     COT67B-seq (301)NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRI
Native FLCry1Ab (301)NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRI
Cry1Ac-Bollgard (301)NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRI
                     351                                               400

       Bt11-seq (351)VAQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAV
      Bt176-seq (351)VAQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAV
     COT67B-seq (351)VAQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAV
Native FLCry1Ab (351)VAQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAV
Cry1Ac-Bollgard (351)VAQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAV
                     401                                               450
       Bt11-seq (401)YRKSGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRA
      Bt176-seq (401)YRKSGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRA
     COT67B-seq (401)YRKSGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRA
Native FLCry1Ab (401)YRKSGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRA
Cry1Ac-Bollgard (401)YRKSGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRA
                     451                                               500
       Bt11-seq (451)PMFSWIHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDIL
      Bt176-seq (451)PMFSWIHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDIL
     COT67B-seq (451)PMFSWIHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDIL
Native FLCry1Ab (451)PMFSWIHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDIL
Cry1Ac-Bollgard (451)PMFSWIHRSAEFNNIIASDSITQIPAVKGNFLFNGSVIS-GPGFTGGDLV
                     501                                               550
        Bt11-seq 501)RRTSPGQISTLRVNITAP-----LSQRYRVRIRYASTTNLQFHTSIDGRP
      Bt176-seq (501)RRTSPGQISTLRVNITAP-----LSQRYRVRIRYASTTNLQFHTSIDGRP
     COT67B-seq (501)RRTSPGQISTLRVNITAP-----LSQRYRVRIRYASTTNLQFHTSIDGRP
Native FLCry1Ab (501)RRTSPGQISTLRVNITAP-----LSQRYRVRIRYASTTNLQFHTSIDGRP
Cry1Ac-Bollgard (500)RLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRVRYASVTPIHLNVNWGNSS
                     551                                               600
       Bt11-seq (546)INQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGN
      Bt176-seq (546)INQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGN
     COT67B-seq (546)INQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGN
Native FLCry1Ab (546)INQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGN
Cry1Ac-Bollgard (550)IFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGN---IVGVRNFSGTA
                     601                                               650
       Bt11-seq (596)EVYIDRIEFVPAEVTFEAEY----------------------------
      Bt176-seq (596)EVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHI
     COT67B-seq (596)EVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHI
Native FLCry1Ab (596)EVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHI
Cry1Ac-Bollgard (597)GVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHI
                     651                                               700
```

Appendix 2-Figure 1: Continued

```
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (646)DQV------------------------------------------------
     COT67B-seq (646)DQVSNLVECLSDEFCLDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQ
Native FLCry1Ab (646)DQVSNLVECLSDEFCLDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQ
Cry1Ac-Bollgard (647)DQVSNLVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQ
                     701                                             750
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq (696)LDRGWRGSTDITIQGGDDVFKENYVTLLGTFDECYPTYLYQKIDESKLKA
Native FLCry1Ab (696)LDRGWRGSTDITIQGGDDVFKENYVTLLGTFDECYPTYLYQKIDESKLKA
Cry1Ac-Bollgard (697)PERGWGGSTGITIQGGDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKA
                     751                                             800
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq (746)YTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGTGSLWPLSAPSPIG**KC**
Native FLCry1Ab (746)YTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGTGSLWPLSAPSPIG--
Cry1Ac-Bollgard (747)FTRYQLRGYIEDSQDLEIYSIRYNAKHETVNVPGTGSLWPLSAQSPIG**KC**
                     801                                             850
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq (796)**GEPNRCAPHLEWNPDLDCSCRDGE**KCAHHSHHFSLDIDVGCTDLNEDLGV
Native FLCry1Ab (794)------------------------KCAHHSHHFSLDIDVGCTDLNEDLGV
Cry1Ac-Bollgard (797)**GEPNRCAPHLEWNPDLDCSCRDGE**KCAHHSHHPSLDIDVGCTDLNEDLGV
                     851                                             900
       Bt11-seq (616)-----------------------------------------------
      Bt176-seq (649)-----------------------------------------------
     COT67B-seq (846)WVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEW
Native FLCry1Ab (820)WVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEW
Cry1Ac-Bollgard (847)WVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEW
                     901                                             950
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq (896)ETNIVYKEAKESVDALFVNSQYDRLQADTNIAMIHAADKRVHSIREAYLP
Native FLCry1Ab (870)ETNIVYKEAKESVDALFVNSQYDRLQADTNIAMIHAADKRVHSIREAYLP
Cry1Ac-Bollgard (897)ETNIVYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLP
                     951                                            1000
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq (946)ELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGH
Native FLCry1Ab (920)ELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGH
Cry1Ac-Bollgard (947)ELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGH
                     1001                                           1050
```

Appendix 2-Figure 1: Continued

```
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq (996)VDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCV
Native FLCry1Ab (970)VDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCV
Cry1Ac-Bollgard (997)VDVEEQNNQRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCV
                     1051                                          1100
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq(1046)TIHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRG
Native FLCry1Ab(1020)TIHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRG
Cry1Ac-Bollgard(1047)TIHEIENNTDELKFSNCVEEEIYPNNTVTCNDYTVNQEEYGGAYTSRNRG
                     1101                                          1150
       Bt11-seq (616)------------------------------------------------
      Bt176-seq (649)------------------------------------------------
     COT67B-seq(1096)YDGAYESNSSVPADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYV
Native FLCry1Ab(1070)YDGAYESNSSVPADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYV
Cry1Ac-Bollgard(1097)YNEAP---S-VPADYASVYEEKSYTDGRRENPCEPNRGYRDYTPLPVGYV
                     1151                          1186
       Bt11-seq   (616) -----------------------------------
      Bt176-seq   (649) -----------------------------------
     COT67B-seq  (1146) TKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
Native FLCry1Ab  (1120) TKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
Cry1Ac-Bollgard  (1143) TKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
```

APPENDIX 3.A. MATERIAL, METHODS AND RESULTS FOR FLCRY1AB-0103 TEST SUBSTANCE CHARACTERIZATION

This section contains a detailed description of the materials, methods and results of the studies conducted to characterize the FLCry1Ab protein purified from recombinant *E. coli* culture.

A. Material and Methods

A.1. Test Substance FLCRY1AB-0103

Prior to this study, test substance FLCRY1AB-0103 was prepared by expressing a full-length *cry1Ab* gene in an *E. coli* over-expression system. This gene is identical to that present in COT67B, and encodes a full-length Cry1Ab protein modified with a 26-amino acid Geiser motif. The full-length *cry1Ab* gene was linked to the bacterial *tac* promoter (a hybrid of the *E. coli trp* and *lac* promoters) in a pDGFMichigan vector and transformed into *E. coli* strain DH5α.

Test substance FLCRY1AB-0103 was prepared by Syngenta Protein Science (Jealott's Hill, Bracknell, Berkshire, UK) as follows: *E. coli* cells were disrupted in 50 mM Tris buffer (pH 8.0) and centrifuged, and the resulting pellet was washed twice with 50 mM Tris buffer (pH 7.5). Inclusion bodies containing Cry1Ab were solubilized using 50 mM sodium carbonate buffer (pH 10) and insoluble material was removed by centrifugation. The supernatant containing Cry1Ab was concentrated, desalted into 25 mM ammonium bicarbonate buffer (pH 10) and lyophilized. The resulting lyophilized protein powder was designated test substance FLCRY1AB-0103 and received February 28, 2003 by Regulatory Science, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA, where it was stored desiccated at *ca.* -20°C. Additional details of the preparation of test substance FLCRY1AB-0103 are described in a separate report (Attenborough, 2003), a copy of which is maintained with the initial report study file.

A.2. Protein Quantitation

Total protein in test substance FLCRY1AB-0103 was quantified spectrophotometrically by determining the absorption at 280 nm (A_{280} method). The A_{280} method is based on the absorption of the aromatic amino acids tryptophan and tyrosine at 280 nm. The extinction coefficient at 280 nm varies with the abundance of these amino acids in the target protein. A Genesys 6 spectrophotometer (Thermo Electron Corporation, Madison WI, USA) was used to measure absorption of FLCRY1AB-0103 at 280 nm and the extinction coefficient of full-length Cry1Ab was calculated with Vector NTI® software version 9 (Invitrogen, San Diego, CA USA). The absorbance at 280 nm was multiplied by the correlation factor for the extinction coefficient, to give an approximate total protein concentration.

A.3. Densitometric Analysis

For purity determinations, aliquots of FLCRY1AB-0103 (*ca.* 3 – 6 µg Cry1Ab per lane) were subjected to SDS-PAGE using a NuPAGE® 4-12% Bis-Tris gel. Mark12™ molecular weight standards (Invitrogen; San Diego, CA, USA) were used to establish approximate molecular weights. After electrophoretic separation, protein bands were stained with Coomassie® blue (Sigma Chemical; St. Louis, MO, USA), and the distribution of the visible protein bands estimated by densitometric analysis (Gelbase/GelBlot, Version 2.1).

A.4. Purity Determination

The purity of test substance FLCRY1AB-0103 was calculated from the total sample weight and the total protein as determined by the A_{280} method, in conjunction with the densitometry data.

A.5. Immunoreactivity and Molecular Weight Determination

The integrity (intactness) of Cry1Ab in test substance FLCRY1AB-0103 was investigated using Western blot analysis. Dilutions prepared in NuPAGE sample buffer containing 5, 15 and 30 ng Cry1Ab from test substance FLCRY1AB-0103 were subjected to SDS-PAGE using a NuPAGE 4-12% Bis-Tris gels. SeeBlue Plus2® molecular weight standard (Invitrogen; San Diego, CA, USA) was used to establish the approximate molecular weight of FLCry1Ab. After electroblotting, the membrane was incubated with immunoaffinity-purified rabbit anti-Cry1Ab polyclonal antibodies raised against *B.t.k.* crystal proteins. Donkey anti-rabbit IgG linked to alkaline phosphatase (Jackson; West Grove, PA, USA), diluted 1:3,000, was used to bind to the primary antibodies and visualized by development with alkaline phosphatase substrate solution. The Western blot was examined for the presence of intact immunoreactive FLCry1Ab (*ca*. 133.5 kDa) and other immunoreactive FLCry1Ab fragments.

A.6. Insecticidal Activity Assays

The test solution was prepared by dissolving FLCRY1AB-0103 in 50 mM Tris-HCl (pH 9.5) buffer containing 2 mM EDTA, and bioactivity of Cry1Ab in the test substance was assessed in an insect feeding assay using freshly hatched first-instar European Corn Borer (ECB). The bioassay was conducted in Petri dishes (47 mm diameter, Millipore). Each dish contained 5 ml insect diet (General Purpose Insect Diet from Bio-Serv, Inc.; Frenchtown, NJ, USA) overlaid with 100 µl test solution containing concentrations ranging from *ca*. 0.2 to 400 ng Cry1Ab/cm^2 diet surface. Each treatment consisted of three replicate dishes of 10 ECB larvae/dish. ECB diet alone, and ECB diet treated with buffer (using the same volume as applied with the test substance treatments), were used as negative control treatments. The bioassay dishes were maintained at ambient temperature and humidity and mortality was assessed after 72 hours.

B. Results

B. 1. FLCry1Ab Quantitation and Purity Determination

FLCRY1AB-0103 was determined to contain 98.0% protein as measured by absorption at 280 nm. Densitometric analysis indicated that FLCry1Ab represented *ca*. 78.9% of the total protein in FLCRY1AB-0103 (Figure 1). The overall purity of the test substance was therefore determined to be 77.3% w/w FLCry1Ab (Table 3.A.1).

B.2. Immunoreactivity and Molecular Weight Determination

Western blot analysis of test substance FLCRY1AB-0103 revealed a dominant immunoreactive band (Figure 2) corresponding to the predicted molecular weight for intact, full-length Cry1Ab of *ca.* 133.5 kDa. (Figure 1*).*

B.3. Insecticidal Activity

The results of the ECB bioassay are summarized in Tables 3A.1 and 2. Test substance FLCRY1AB-0103 was bioactive, with an LC_{50} of 16.5 ng Cry1Ab /cm^2 (95% confidence interval: 12.4 - 21.0 ng/cm^2 diet surface) after 72 hours (Table 3.A.1). No mortality was observed in the negative controls using ECB diet alone and very low mortality (3%) was observed in the ECB diet treated with buffer.

B.4. Sample Stability

The initial purity of test substance FLCRY1AB-0103 was determined in December 2004 to be *ca*. 85.6% Cry1Ab and upon re-analysis in May 2005 (Graser, 2005) the purity was determined to be *ca*. 85.7% (Table 3.A.1). The third analysis of test substance FLCRY1AB-0103 represented in the present study determined that the purity was 77.3%, a decrease in Cry1Ab concentration of *ca*. 9.7% since the initial characterization *ca*. 14 months prior.

A comparison of Western blot analyses from the initial test substance characterization in December 2004 (Figure 3.A.2A; Graser, 2005), the May 2005 re-characterization (Figure 3A.2B; Graser, 2005) and the present study (Figure 3.A.2C) showed no apparent loss in Cry1Ab immunoreactivity or integrity since the initial characterization of FLCRY1AB-0103.

The 72-hour LC_{50} of 16.5 ng Cry1Ab/cm^2 diet surface (95% confidence interval: 12.4 - 21.0 ng Cry1Ab/cm^2) estimated in the present study, indicated that test substance FLCRY1AB-0103 retained substantial insecticidal activity but may have declined in activity since the initial bioassay (LC_{50} of 3.7 ng Cry1Ab/cm^2 diet surface, 95% confidence interval: 2.3 - 4.9 ng Cry1Ab/cm^2) in December 2004 and the subsequent re-characterization in May 2005 (LC_{50} of 8.3 ng Cry1Ab/cm^2 diet surface, 95% confidence interval: 6.0 - 10.7 ng Cry1Ab/cm^2) (Tables 3.A.1 and 2).

Based on the data presented in this study, it can be concluded that test substance FLCRY1AB-0103 retained a substantial concentration of intact, bioactive Cry1Ab,

although small declines in apparent purity and bioactivity may have occurred since its initial characterization *ca*. 14 months prior.

Appendix 3.A.-Table 1. Re-characterization of Cry1Ab in test substance FLCRY1AB-0103

Date of Analysis	**Total Protein** [g protein/g FLCRY1AB-0103]	**Densitometric Analysis** [% Cry1Ab/total protein]	**Purity** [% Cry1Ab/ FLCRY1AB-0103]	**72-hour ECB LC_{50}** ng Cry1Ab/cm^2 diet surface (95% Confidence Interval)
Dec 2004 (Graser 2005)	0.947	90.4	85.6	3.7 (2.3 - 4.9)
May 2005 (Graser, 2005)	0.965	88.8	85.7	8.3 (6.0 - 10.7)
Feb 2006 (present study)	0.980	78.9	77.3	16.5 (12.4 - 21.0)

Appendix 3.A.-Table 2. Bioactivity of full-length Cry1Ab in test substance FLCRY1AB-0103

Bioassay against first-instar European Corn Borer.

	European Corn Borer Mortality at 72 hours (%)		
FLCRY1AB-0103[a] [ng Cry1Ab/cm^2]	**December 2004**	**May 2005**	**February 2006**
0.20	33	10	7
0.39	30	13	3
0.78	40	10	0
1.56	40	17	7
3.13	50	27	20
6.25	90	43	27
12.5	97	63	20
25.0	100	97	67
50.0	100	100	93
100.0	100	100	100
200.0	n.d.[d]	n.d.	100
400.0	n.d.	n.d.	100
Untreated control[b]	13	10	0
Buffer control[c]	13	0	3

[a] ECB diet treated with Cry1Ab from test substance FLCRY1AB-0103

[b] Untreated control diet: ECB stock diet without any treatment

[c] Buffer control diet: ECB stock diet treated with 50mM Tris-HCl (pH 9.5), 2 mM EDTA

[d] not determined

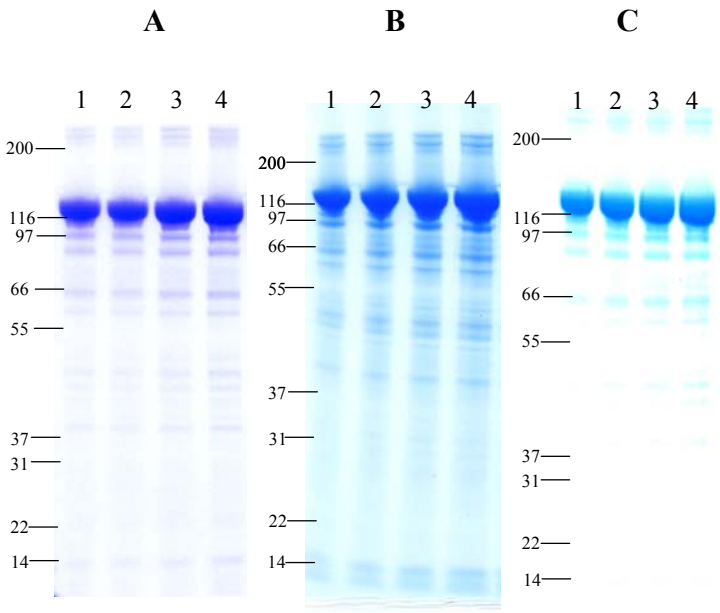
APPENDIX 3.A.-Figure 1. Purity determination of test substance FLCRY1AB-0103 by SDS-polyacrylamide gel electrophoresis

A. Initial analysis December 2004
NuPAGE 4-12% Bis-Tris gel
Lanes 1 to 4: 3.4, 4.3, 5.1 and 6.8 µg Cry1Ab, respectively, from FLCRY1AB-0103

B. Re-analysis May 2005
NuPAGE 10% Bis-Tris gel
Lanes 1 to 4: 3.4, 4.3, 5.1 and 6.9µg Cry1Ab, respectively, from FLCRY1AB-0103

C. Re-analysis February 2006
NuPAGE 4-12% Bis-Tris gel
Lanes 1 to 5: 3.1, 3.9, 4.6 and 5.4 µg Cry1Ab, respectively, from FLCRY1AB-0103

Molecular weights of Mark 12 protein standard indicated on the left side of the gels in kDa. The molecular weight of Cry1Ab corresponds to *ca*. 133.5 kDa.



Appendix 3.A.-Figure 2. Immunoreactivity of full-length Cry1Ab protein in test substance FLCRY1AB-0103 (Western blot analysis)

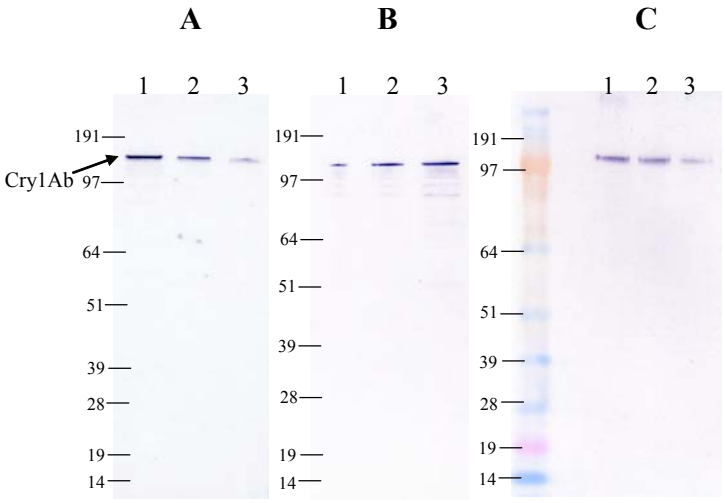
A. Initial analysis December 2004
Lanes 1, 2 and 3: 30, 15 and 5 ng Cry1Ab, respectively, from FLCRY1AB-0103

B. Re-analysis May 2005
Lanes 1, 2 and 3: 5, 15 and 30 ng Cry1Ab, respectively, from FLCRY1AB-0103

C. Re-analysis February 2006
Lanes 1, 2 and 3: 30, 15 and 5 ng Cry1Ab, respectively, from FLCRY1AB-0103

Molecular weights of SeeBlue Plus2 protein standard indicated on the left side of the blots in kDa.

The molecular weight of Cry1Ab corresponds to *ca*. 133.5 kDa.



APPENDIX 3.B. MATERIALS, METHODS AND RESULTS FOR STUDIES DEMONSTRATING EQUIVALENCY OF TEST SUBSTANCE FLCRY1AB-0103 AND *IN PLANTA*-DERIVED FLCRY1AB PROTEIN FROM COT67B

This section contains a detailed description of the materials, methods and results of the studies demonstrating equivalency of microbially and COT67B-derived FLCry1Ab protein.

A. Material and Methods

A.1. Test Substance LPCOT67B-0106

Leaf samples used for this study were from greenhouse-grown COT67B leaves. Young leaves from plants 7 to 12 weeks old were collected and frozen at -80 ± 10°C, and subsequently ground into fine powder and lyophilized. Lyophilized leaf powder was re-suspended in cotton extraction buffer containing 100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 1 mM DTT, 0.05 % Tween® 20, 0.2% L-ascorbic acid, 1 mM AEBSF and 1 mM leupeptin. The mixture was incubated for 30 min on ice with gentle intermittent mixing and then extracted using an Autogizer® homogenizer (Tomtec; Hamden, CT, USA). The extract was centrifuged for 15 min at 3,000 x g, and the supernatant was desalted with gel filtration *via* Sephadex® G-25 (PD-10 columns, Amersham Biosciences; Piscataway, NJ, USA) and eluted in a buffer containing 50 mM Tris (pH 9.5) and 2 mM EDTA. The resulting test substance was designated LPCOT67B-0106.

A.2. Test Substance IAPCOT67B-0106

Young leaves from greenhouse-grown COT67B plants as described above were collected, frozen at -80°C ± 10°C, subsequently ground into fine powder and lyophilized. Lyophilized leaf powder was resuspended in extraction buffer containing 100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 2 mM DTT, 0.1% Tween® 20, 0.2% L-ascorbic acid, 10% XAD-4, 1% PVPP and 1 tablet/50 ml Complete® protease inhibitor (Roche Diagnostics, Mannheim, Germany). The mixture was incubated for 30 min on ice with gentle intermittent mixing and then homogenized three times using a standard laboratory blender for 20 seconds. The homogenate was incubated with additional 1% PVPP under gentle stirring for another 10 min and centrifuged for 25 min at *ca*.17,000 x g. The supernatant was dialyzed three times (5 hr each) in column equilibration buffer containing 50 mM sodium bicarbonate and 100 mM sodium chloride, and subsequently loaded onto an equilibrated immunoaffinity column, with rabbit anti-Cry1Ab antibodies bound to the matrix. Cry1Ab was eluted in 100 mM CAPS buffer, pH 11, neutralized and concentrated by ultra-filtration and stored at 4 to 8°C before further use. The resulting test substance, designated IAPCOT67B-0106, was used as the source of plant-derived, immunopurified Cry1Ab for Western blot, glycosylation and peptide map mapping analysis.

A.3. Control Substance LPCOT67B-0106C

Control substance LPCOT67B-0106C was prepared from leaf material obtained from a nontransgenic near-isogenic control cotton variety (Coker 312), in a similar manner as described for Test Substance LPCOT67B-0106. Both test substance LPCOT67B-0106 and the control substance LPCOT67B-0106C were prepared fresh for the insect bioassay. Aliquots of each were stored at -20 ± 8°C in SDS-PAGE sample buffer for subsequent Western blot analysis.

A.4. Test Substance FLCRY1AB-0103

Test substance FLCRY1AB-0103 was prepared from pooled batches of *E. coli* cell paste by Syngenta Ltd. (Jealott's Hill, Bracknell, Berkshire, UK) as described in Graser, 2005. FLCry1Ab was isolated from disrupted *E. coli* cells in 50 mM Tris buffer (pH 8.0). The extract was centrifuged and the resulting pellet was washed twice with 50 mM Tris buffer (pH 7.5) and centrifuged. FLCry1Ab was solubilized from inclusion bodies using 50 mM sodium carbonate buffer (pH 10) and insoluble material was removed by centrifugation. The supernatant containing FLCry1Ab was concentrated, desalted into 25 mM ammonium bicarbonate buffer (pH 10) and lyophilized. The resulting lyophilized protein powder was designated test substance FLCRY1AB-0103. The test substance was sent on dry ice to Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA, where it was stored at -20 ± 8°C until further use. Test Substance FLCRY1AB-0103 was characterized in detail in previous studies (Graser, 2005; Kramer, 2006) and determined to contain 77.3% FLCry1Ab by weight.

A.5. FLCry1Ab Quantitation

The concentration of FLCry1Ab in test substances IAPCOT67B-0106 and LPCOT67B-0106 and control substance LPCOT67B-0106C was determined using sandwich ELISA (Tijssen, 1985). 96-well Nunc MaxiSorp™ plates (Fisher, GA, USA) were coated overnight at 2-8°C with monoclonal mouse antibodies to HO4[21] (Mab 70; generated at Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK) in a buffer containing 35 mM sodium bicarbonate and 15 mM sodium carbonate, pH 9.5 (100 µl/well). The plates were then washed five times with PBS containing 0.05% Tween® 20 and incubated in blocking buffer (PBS plus 1% nonfat milk) for at least 30 min. The plates were then washed again as described above and incubated with the indicated extracts for 2 hr at 19 ± 1°C. After washing, the plates were incubated with rabbit 3 anti-Cry1Ab antisera diluted in ELISA dilution buffer (blocking buffer plus 0.05% Tween® 20) for 1 hr at 19 ± 1°C. The plates were washed and incubated with goat-anti-Rabbit IgG conjugated with HRP antibodies (Sigma; MO, USA) in ELISA dilution buffer for 1 hr at 19 ± 1°C. The plates were washed again and incubated in TMB substrate (Sigma) solution containing 0.006% hydrogen peroxide at room temperature for 30 min in the dark for color development. The reaction was stopped by addition of 3 M

[21] HO4 is a hybrid protein comprised of the 1st and 2nd domains of Cry1Ab and the 3rd domain of Cry1C

sulfuric acid and absorbance was measured at 450 nm with a Tecan Sunrise™ plate reader (Tecan; US, NC, USA). Test substance FLCRY1AB-0103 was used for the FLCry1Ab reference standard curve.

A.6. Total Protein Determination

Total protein in test substance LPCOT67B-0106 and control substance LPCOT67B-0106C was quantified using the BCA™ method (Hill and Straka, 1988) with bovine serum albumin (BSA) used for the reference protein standard.

A.7. Molecular Weight Determination and Immunoreactivity Analysis

The integrity of FLCry1Ab in test substances FLCRY1AB-0103, IAPCOT67B-0106 and LPCOT67B-0106 was investigated by Western blot analysis. Western blot analysis relies upon immunoreactivity of FLCry1Ab with anti-Cry1Ab antibodies, and is capable of detecting nanogram quantities of immunoreactive protein and polypeptides. An aliquot containing *ca*. 40 ng FLCry1Ab protein prepared in Laemmli sample buffer (Laemmli, 1970) from the described test substances was subjected to SDS-PAGE using NuPAGE® 4-12% Bis-Tris polyacrylamide gradient gel and MES running buffer (Invitrogen; San Diego, CA, USA). An equivalent amount of control substance LPCOT67B-0106C, as measured by total protein concentration, was also included in the analysis as a negative control. SeeBlue® Plus2 molecular weight standard (Invitrogen) was used to establish approximate molecular weights. After electroblotting, the membrane was probed with immunoaffinity-purified rabbit antibodies generated against the native full-length Cry1Ab from *Bacillus thuringiensis* subsp. *kurstaki* HD-1. Donkey anti-rabbit IgG linked to alkaline phosphatase (Jackson; West Grove, PA, USA), diluted 1:3,000 in TBST buffer, was used to bind to the primary antibodies and was visualized by development with alkaline phosphatase substrate solution. The Western blot was examined for the presence of intact immunoreactive FLCry1Ab and immunoreactive polypeptides.

A.8. Insecticidal Activity

Test solutions for the bioassay were prepared in 50 mM Tris-HCl, pH 9.5, buffer containing 2 mM EDTA from test substances FLCRY1AB-0103 and LPCOT67B-0106. Bioactivity of Cry1Ab was assessed in insect feeding assays using freshly hatched first-instar *Ostrinia nubilalis.* The bioassays were conducted in Petri dishes (Fisher Scientific, Cat. # PD10-047-05) containing 5 mL insect diet (General Purpose Lepidoptera Diet from Bio-Serv, Inc.; Frenchtown, NJ, USA) overlaid with 200 µl test solution, for final concentrations ranging from *ca.* 0.11 to 100 ng Cry1Ab/cm^2 diet surface. Each treatment consisted of three replicate Petri dishes, with 10 larvae per dish. Insect diets treated with MilliQ® purified (deionized) water alone, insect diets treated with buffer alone, and insect diets treated with control substance LPCOT67B-0106C at a concentration of total protein equivalent to test substance LPCOT67B-0106, were used as negative controls. The Petri dishes were maintained at room temperature under ambient laboratory conditions. Mortality was assessed after *ca.* 72 hours and is reported for each treatment as the percent mortality among 30 larvae.

A.9. Glycosylation Analysis

To determine whether FLCry1Ab in test substances IAPCOT67B-0106 and FLCRY1AB-0103 was glycosylated, approximately 2.2 µg of Cry1Ab protein were analyzed using the DIG Glycan Detection Kit (Roche Diagnostics GmbH; Mannheim, Germany), in accordance with the manufacturer's instructions. Transferrin, a glycosylated protein, was used as a positive control and creatinase, a nonglycosylated protein, was used as a negative control. Samples were separated by SDS-PAGE using a NuPAGE® 4-12% Bis-Tris polyacrylamide gradient gel and MES running buffer (Invitrogen) and electro-blotted to PVDF membrane (Invitrogen). While on the membrane, glycan moieties were oxidized using periodate, labeled with digoxigenin, and detected with an anti-digoxigenin antibody coupled to alkaline phosphatase.

A.10. Peptide Mass Mapping Analysis

Aliquots containing 1 - 6 µg FLCry1Ab from test substances IAPCOT67B-0106 and FLCRY1AB-0103 were subjected to SDS-PAGE using a 4-12% Bis-Tris polyacrylamide gradient gel and MES running buffer (Invitrogen). The gel was stained with Coomassie® (GelCode® Blue Stain Reagent, Pierce; Rockford, IL, USA) and was sent to Syngenta Analytical Sciences (Jealott's Hill, Bracknell, Berkshire, UK) for peptide mass mapping analysis. The protein band corresponding to the molecular weight of full-length Cry1Ab (*ca.* 133.5 kDa) was excised from the gel and the protein was reduced, alkylated with iodoacetamide and digested with trypsin. The mass analysis of the FLCry1Ab-derived peptides was performed on a quadrupole time-of-flight mass spectrometer (Q-TOF2, Micromass; Manchester, UK), fitted with an EPCAS upgrade and connected to a capillary HPLC instrument (Waters CapLC™; Hertfordshire, UK). Peptide masses were identified by matching the detected peptide masses to a protein database using Mascot™ software (Matrix Sciences Ltd.; London, UK).

A.11. N-Terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequence of FLCry1Ab in test substance FLCRY1AB-0103 was determined for comparison with the predicted amino acid sequence encoded by the synthetic full-length *cry1Ab* gene. Test substance FLCRY1AB-0103 was subjected to SDS-PAGE followed by electroblotting of the protein to a PVDF membrane (Invitrogen). After staining the membrane with Amido black, the corresponding full-length Cry1Ab band (molecular weight *ca.* 133.5 kDa) was excised and sent to Proseq, Inc. Protein Sequencing Services (Boxford, MA, USA) for N-terminal amino acid sequence analysis. ProSeq's methods were developed specifically for proteins immobilized on PVDF membrane and optimized for automated Edman-based chemistry (Brauer *et al.*, 1984). The N-terminal sequencing was performed by ProSeq using non-GLP and is therefore an exception to 40 CFR Part 160.

A.12. Statistical Methods

The LC_{50} values determined in the *Ostrinia nubilalis* bioassay were calculated using the EPA Probit Analysis Program, Version 1.5. Statistical analyses were not required for other parameters evaluated in this study.

B. Results

B.1. Molecular Weight Determination and Immunoreactivity Analysis

Western blot analysis (Figure 2) of the microbial test substance FLCRY1AB-0103 as well as plant-derived test substances LPCOT67B-0106 and IAPCOT67B-0106 revealed immunoreactive bands consistent with the predicted molecular weight of *ca.* 133.5 kDa (lanes 3, 4 and 5). Lower molecular weight immunoreactive bands were also present, especially in the immunoaffinity purified test substance IAPCOT67B-0106, representing most likely degradation products of the full-length FLCry1Ab as a result of the purification process. As expected, no immunoreactive response was present in the control leaf protein preparation, LPCOT67B-0106C (Figure 2, lane 2), containing the same amount of total protein (34 µg protein) as determined in the LPCOT67B-0106 extract (Figure 2, lane 2).

B.2. Insecticidal Activity

The results of the insect bioassays of the microbially and plant-derived FLCry1Ab are summarized in Table 1. Both test substances LPCOT67B-0106 and FLCRY1AB-0103 were highly potent in the *Ostrinia nubilalis* bioassays, with an estimated LC_{50} after 72 hours of 1.3 ng Cry1Ab/cm^2 (95% confidence interval = 0.9 - 1.9 ng/cm^2 diet surface) and 5.2 ng Cry1Ab /cm^2 (95% confidence interval = 4.0 - 6.6 ng/cm^2 diet surface), respectively. The freshly prepared plant-derived protein gave a somewhat higher bioactivity in comparison to the microbial test substance. No mortality was observed using the control test substance LPCOT67B-0106C, (using the same amount of total protein, 12.6 µg protein/cm^2, as used in the highest LPCOT67B-0106 dose), the negative controls using insect diet treated with water, or the insect diet treated with buffer (Table 2).

B.3. Glycosylation Analysis

Twenty-five nanograms of the positive control protein transferrin generated a clearly visible band (Figure 3, lane 3) in the glycosylation assay. Transferrin has a molecular weight of *ca*. 80,000 and contains *ca*. 5% glycan moieties by weight. This corresponds to *ca*. 25 glucose equivalents/molecule (with a calculated molecular weight for the glucose moiety of 162). Of the 25 ng transferrin loaded on the gel, 1.25 ng could therefore be attributed to glycan moieties and was clearly detectable. Approximately 2200 ng of FLCry1Ab from both plant and microbial sources was loaded. Detection of 1.25 ng glycan corresponds to *ca*. 0.0568% by weight (1.25 ng/2200 ng), or *ca*. 0.47 glucose equivalents/FLCry1Ab molecule. In other words, FLCry1Ab bands in lanes 6 and 7 would be stained as strongly as 25 ng of transferrin in lane 3 to indicate a glycosylation (one glucose unit) of every second Cry1Ab molecule. No bands representing

glycosylated FLCry1Ab were visible in the sample prepared from test substance FLCRY1AB-0103 upon DIG Glycan analysis (Figure 3.B.3, lane 7). A very faint band was detected in the lane loaded with plant derived test substance IAPCOT67B-0106 (Figure 3.B.2, lane 6), but this band had a higher molecular weight than that predicted for Cry1Ab. Therefore, the results of the DIG Glycan analysis indicate that neither the microbially nor the plant-derived FLCry1Ab proteins are glycosylated.

B.4. Peptide Mapping

The analysis of the plant-derived FLCry1Ab by peptide mass mapping identified 14 peptides, equivalent to coverage of 16% of the total predicted full-length FLCry1Ab amino acid sequence, as shown in Figure 4. The analysis of the microbially derived Cry1Ab identified 18 peptides, equivalent to coverage of 30% of the total predicted full-length FLCry1Ab amino acid sequence, as shown in Figure 5. Furthermore, the identified peptides were representative of regions throughout the sequence of the full-length Cry1Ab protein (including peptides close to the N- and C-terminus) and therefore strongly support the identity of the purified proteins from both sources (COT67B and test substance FLCRY1AB-0103) as full-length Cry1Ab.

B.5. N-Terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequence for Cry1Ab in test substance FLCRY1AB-0103 confirmed the predicted amino acid sequence, as shown below.

Appendix 3.B.-Figure 1. N-terminal amino acid sequence for Cry1Ab in test substance FLCRY1AB-0103

Predicted sequence:	**MDNNPNINECIP**
Cry1Ab from FLCRY1AB-0103	MDNNPNINECIP

Appendix 3.B.-Table 1. Bioactivity of Cry1Ab from Recombinant *E. coli* (Test Substance FLCRY1AB-0103) and from Event COT67B Cotton (Test Substance LPCOT67B-0106) in Diet Surface Bioassays with First-Instar *Ostrinia nubilalis* Larvae

Test Substance	LC_{50} [ng Cry1Ab/cm^2] (95% Confidence Intervals)
FLCRY1AB-0103	5.2 (4.0 – 6.6)
LPCOT67B-0106	1.3 (0.9 – 1.9)

Appendix 3.B.-Table 2. Bioactivity of Cry1Ab from Recombinant *E. coli* (Test Substance FLCRY1AB-0103) and from Event COT67B Cotton (Test Substance LPCOT67B-0106) in Diet Surface Bioassays with First-Instar *Ostrinia nubilalis* Larvae.

FLCRY1AB-0103		LPCOT67B-0106	
[ng Cry1Ab/cm^2][a]	Mortality at 72h [%]	[ng Cry1Ab/cm^2][a]	Mortality at 72h [%]
0.8	0	0.1	7
1.6	17	0.2	23
3.1	27	0.4	33
6.3	77	0.9	33
12.5	80	1.7	43
25	87	3.5	83
50	93	6.9	77
100	100	13.9	90
Water control[b]	0		
Buffer control[c]	0		
LPCOT67B-0106C[d]	0		

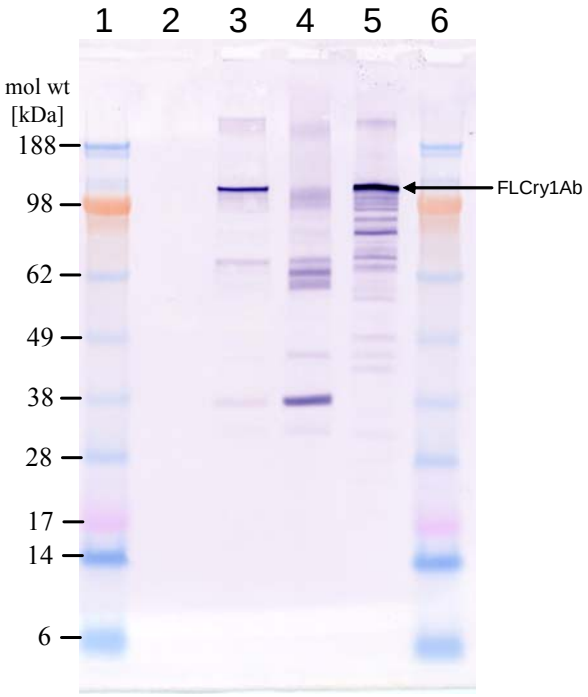
[a]Insect diet treated with Cry1Ab from test substances FLCRY1AB-0103 or LPCOT67B-0106 dissolved in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (200 µl/dish)

[b]Water control diet: Insect diet treated with water (50 µl/dish)

[c]Buffer control diet: Insect diet treated with 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (200 µl/dish)

[d]Insect diet treated with control substance LPCOT67B-0106C in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA, at same total protein concentration as applied in test substance LPCOT67B-0106 treatment.

Appendix 3.B.-Figure 2. Immunoreactivity of FLCry1Ab from Event COT67B (Test Substances LPCOT67B-0106 and IAPCOT67B-0106) and Recombinant *E. coli* (Test Substance FLCRY1AB-0103)

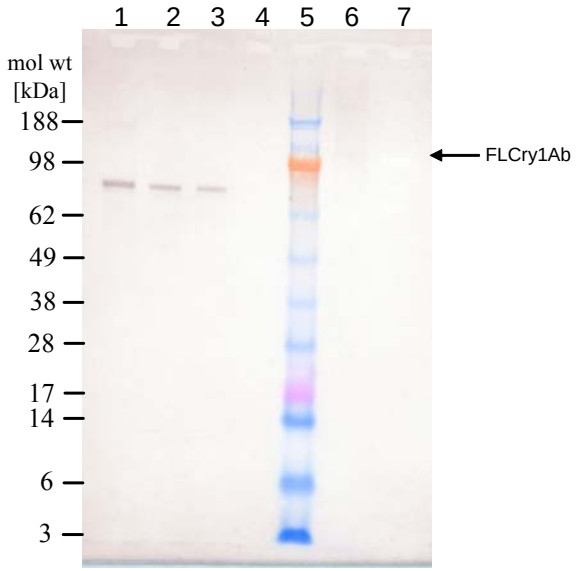


Lanes 1 and 6: Molecular weight standard SeeBlue® Plus2
Lane 2: 34 µg total protein, LPCOT67B-0106C
Lane 3: 40 ng Cry1Ab from LPCOT67B-0106
Lane 4: 40 ng Cry1Ab from IAPCOT67B-0106
Lane 5: 40 ng Cry1Ab from FLCRY1AB-0103

The molecular weight of Cry1Ab corresponds to *ca.* 133.5 kDa.

Appendix 3.B.-Figure 3. Glycosylation Analysis of FLCry1Ab Expressed in Event COT67B (Test Substance IAPCOT67B-0106) and Recombinant *E. coli* (Test Substance FLCRY1AB-0103)

Immunoaffinity-purified FLCry1Ab from COT67B (test substance IAPCOT67B-0106) and Cry1Ab from *E. coli* (test substance FLCRY1AB-0103), were analyzed for the presence of glycosyl residues using the DIG Glycan Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany).



Lanes 1, 2 and 3: 100, 50, 25 ng transferrin (positive control), respectively
Lane 4: 2 µg creatinase (negative control)
Lane 5: Molecular weight standard SeeBlue® Plus2
Lane 6: 2.2 µg Cry1Ab from IAPCOT67B-0106
Lane 7: 2.3 µg Cry1Ab from FLCRY1AB-0103

The molecular weight of Cry1Ab corresponds to *ca.* 133.5 kDa.

Appendix 3.B.-Figure 4. Predicted Amino Acid Sequence and Sequence Identified by Peptide Mass Mapping Analysis of FLCry1Ab from Event COT67B (Test Substance IAPCOT67B-1016)

Identified FLCry1Ab protein fragments derived after tryptic cleavage are in bold and underlined.

```
   1 MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51 VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101 EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151 QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201 GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251 DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301 NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351 VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401 YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451 PMFSWIHRSA EFNNIIPSSQ ITQIPLTKST NLGSGTSVVK GPGFTGGDIL
 501 RRTSPGQIST LRVNITAPLS QRYRVRIRYA STTNLQFHTS IDGRPINQGN
 551 FSATMSSGSN LQSGSFRTVG FTTPFNFSNG SSVFTLSAHV FNSGNEVYID
 601 RIEFVPAEVT FEAEYDLERA QKAVNELFTS SNQIGLKTDV TDYHIDQVSN
 651 LVECLSDEFC LDEKKELSEK VKHAKRLSDE RNLLQDPNFR GINRQLDRGW
 701 RGSTDITIQG GDDVFKENYV TLLGTFDECY PTYLYQKIDE SKLKAYTRYQ
 751 LRGYIEDSQD LEIYLIRYNA KHETVNVPGT GSLWPLSAPS PIGKCGEPNR
 801 CAPHLEWNPD LDCSCRDGEK CAHHSHHFSL DIDVGCTDLN EDLGVWVIFK
 851 IKTQDGHARL GNLEFLEEKP LVGEALARVK RAEKKWRDKR EKLEWETNIV
 901 YKEAKESVDA LFVNSQYDRL QADTNIAMIH AADKRVHSIR EAYLPELSVI
 951 PGVNAAIFEE LEGRIFTAFS LYDARNVIKN GDFNNGLSCW NVKGHVDVEE
1001 QNNHRSVLVV PEWEAEVSQE VRVCPGRGYI LRVTAYKEGY GEGCVTIHEI
1051 ENNTDELKFS NCVEEEVYPN NTVTCNDYTA TQEEYEGTYT SRNRGYDGAY
1101 ESNSSVPADY ASAYEEKAYT DGRRDNPCES NRGYGDYTPL PAGYVTKELE
1151 YFPETDKVWI EIGETEGTFI VDSVELLLME E
```

Appendix 3.B.-Figure 5. Predicted Amino Acid Sequence and Sequence Identified by Peptide Mass Mapping Analysis of FLCry1Ab from Test Substance FLCRY1AB-0103

Identified FLCry1Ab protein fragments derived after tryptic cleavage are in bold and underlined.

```
   1 MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51 VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101 EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151 QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201 GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251 DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301 NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351 VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401 YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451 PMFSWIHRSA EFNNIIPSSQ ITQIPLTKST NLGSGTSVVK GPGFTGGDIL
 501 RRTSPGQIST LRVNITAPLS QRYRVRIRYA STTNLQFHTS IDGRPINQGN
 551 FSATMSSGSN LQSGSFRTVG FTTPFNFSNG SSVFTLSAHV FNSGNEVYID
 601 RIEFVPAEVT FEAEYDLERA QKAVNELFTS SNQIGLKTDV TDYHIDQVSN
 651 LVECLSDEFC LDEKKELSEK VKHAKRLSDE RNLLQDPNFR GINRQLDRGW
 701 RGSTDITIQG GDDVFKENYV TLLGTFDECY PTYLYQKIDE SKLKAYTRYQ
 751 LRGYIEDSQD LEIYLIRYNA KHETVNVPGT GSLWPLSAPS PIGKCGEPNR
 801 CAPHLEWNPD LDCSCRDGEK CAHHSHHFSL DIDVGCTDLN EDLGVWVIFK
 851 IKTQDGHARL GNLEFLEEKP LVGEALARVK RAEKKWRDKR EKLEWETNIV
 901 YKEAKESVDA LFVNSQYDRL QADTNIAMIH AADKRVHSIR EAYLPELSVI
 951 PGVNAAIFEE LEGRIFTAFS LYDARNVIKN GDFNNGLSCW NVKGHVDVEE
1001 QNNHRSVLVV PEWEAEVSQE VRVCPGRGYI LRVTAYKEGY GEGCVTIHEI
1051 ENNTDELKFS NCVEEEVYPN NTVTCNDYTA TQEEYEGTYT SRNRGYDGAY
1101 ESNSSVPADY ASAYEEKAYT DGRRDNPCES NRGYGDYTPL PAGYVTKELE
1151 YFPETDKVWI EIGETEGTFI VDSVELLLME E
```

APPENDIX 3.C. MATERIAL, METHODS AND RESULTS FOR QUANTIFICATION OF FLCRY1AB PROTEIN IN EVENT COT67B TISSUES AND WHOLE PLANTS

This section contains a detailed description of the materials, methods and results of the studies conducted to quantify the FLCry1Ab protein in COT67B tissues

A. Material and Methods

A.1. Source of Plants for Evaluation of FLCry1Ab Concentrations

Using standard local agronomic procedures**,** plants representing one transgenic cotton line derived from COT67B and a near-isogenic, nontransgenic control line (Coker 312) were field grown concurrently in 2004 at the following locations:

- Newport, Arkansas, USA (NAR)
- Quitman, Georgia, USA (QGA)
- Winnsboro, Louisiana, USA (WLA)
- Leland, Mississippi, USA (LMS)

At each location, five plants from the COT67B transgenic line, plus five plants from the Coker 312 control line, were harvested at each of five developmental time points (listed below). Sampling times varied depending on environmental conditions.

- Squaring, *ca.* 4 weeks post emergence
- 1st White Bloom, *ca.* 9 weeks post emergence
- Peak Bloom, *ca.* 13 weeks post emergence
- 1st Open Boll, *ca.* 15 weeks post emergence
- Pre-harvest, *ca.* 22 weeks post emergence

For each stage, whole plants were shipped overnight on ice packs to the Syngenta Biotechnology, Inc. Regulatory Science Laboratory, Research Triangle Park, North Carolina, USA (SBI). Upon receipt, each of five plants was separated into parts as indicated in Table 3.C.1. (except pollen, nectar and flowers which are described in Source of Pollen, Flowers and Nectar for Evaluation of FLCry1Ab Concentrations). Young leaves from all five transgenic and near-isogenic, nontransgenic plants were pooled together to make one young leaf sample per location per time point to ensure sufficient sample size for analysis. For the other tissues, one near-isogenic, nontransgenic plant was used and the other four were discarded. After weighing the samples, they were stored at –80 ± 10ºC (except for the fiber/seed samples) until the tissue was directly analyzed or processed further for extraction and analysis. The fiber/seed samples taken at pre-harvest were kept at room temperature until ginning (between six and seven months) to avoid a freeze/thaw cycle. The fiber/seed samples collected from NAR were damp and were put into the greenhouse drying room for 7 days to prevent the fiber from molding, after which time they were stored at room temperature until ginning. At pre-harvest, only four plants collected at NAR had open bolls from which fiber/seed samples could be taken, not five. Samples were processed and extracted

as described below in Plant Tissue Processing and Tissue Extraction and quantitatively analyzed for FLCry1Ab by ELISA.

A.2. Source of Pollen, Flowers and Nectar for Evaluation of FLCry1Ab Concentrations

Flower, nectar and pollen samples from WLA were collected and pooled from a minimum of 100 cotton plants at peak bloom. The pollen, nectar and flower samples were shipped overnight to SBI on ice packs. The pollen sample was air-dried overnight at room temperature at SBI as this was not done prior to shipping. Samples were stored at –80 ± 10ºC until direct analysis or further processing of the tissues for extraction and analysis (described below in Plant Tissue Processing and Tissue Extraction).

A.3. Plant Tissue Processing

Whole plants and individual parts (except pollen, nectar and fiber) were reduced to a fine powder using either a Grindomix Knife Mill (Model GM200, #20-251-0003, Retsch, Inc., Newtown, Pennsylvania, USA) or a Retsch Cutting Mill (Model SM1, #20-709-0009, Retsch, Inc.) or a combination of the two. All processing was done in the presence of dry ice. Each ground sample was mixed well to ensure homogeneity. Processed samples were stored at –80 ± 10ºC until lyophilization. One powdered seed sample from NAR was lost due to the storage tube breaking in the freezer; therefore, only three of the four seed samples were lyophilized. Once lyophilized, equal amounts of the transgenic samples (except for young leaves which were pooled when the samples were collected) were pooled to create one composite sample for each tissue type at each time point. Lyophilized samples were stored at –80 ± 10ºC.

A.4. Tissue Extraction

FLCry1Ab was extracted from lyophilized tissue samples (i.e., all tissues except pollen, nectar and fiber). At least three replicates of each transgenic composite tissue sample were analyzed. The negative control samples were analyzed at least one time. For each sample analyzed, an aliquot of *ca.* 0.1 g of the powdered lyophilized material was transferred into a 15-ml polypropylene tube, suspended in 3 ml extraction buffer (100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 0.2% L-ascorbic acid, 0.05% Tween-20, 1 mM AEBSF, 1 mM DTT, 1 µM leupeptin, pH 9.5), incubated on wet ice for 30 min. and homogenized using an Autogizer® homogenizer (Tomtec; Hamden, Connecticut, USA; 6 cycles, setting 4). After centrifugation for *ca*. 15 min at *ca.* 10,000 x *g* at *ca.* 4ºC, the resultant supernatants were used for FLCry1Ab analysis by ELISA.

One aliquot of pollen was extracted for each pooled transgenic and nontransgenic sample. Pollen extracts were prepared by suspending *ca.* 0.1 g pollen (air-dried overnight) in 3 ml extraction buffer (100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 0.2% L-ascorbic acid, 0.05% Tween-20, 1 mM AEBSF, 1 mM DTT, 1 µM leupeptin, pH 7.5) and incubating the samples on ice for 30 min. The pollen suspensions were disrupted by five passages through a French pressure cell at *ca.* 15,000 psi. After centrifugation for *ca*. 15 min at *ca.* 10,000 x *g* at *ca.* 4ºC, the resultant supernatants were

used for FLCry1Ab analysis by ELISA. Nectar samples were analyzed directly in the ELISA (not extracted).

Fiber from five individual transgenic plants and one nontransgenic plant was extracted. Fiber extracts were prepared by suspending *ca.* 0.1 g of the fiber material into 15-ml polypropylene tubes, adding 3 ml extraction buffer (100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 0.2% L-ascorbic acid, 0.05% Tween-20, 1 mM AEBSF, 1 mM DTT, 1 µM leupeptin, pH 9.5). The suspensions were incubated on wet ice for 30 min and homogenized using a Polytron® homogenizer (Brinkmann Instruments, Inc.; Westbury, New York, USA). After centrifugation for *ca*. 15 min at *ca.* 10,000 x *g* at *ca.* 4ºC, the resultant supernatants were used for FLCry1Ab analysis by ELISA.

A.5. FLCry1Ab Quantification

The extracts, prepared as described above, and nectar samples (as received) were quantitatively analyzed for FLCry1Ab by ELISA. Either Costar EIA or Nunc Maxisorp™ plates were coated overnight at 2-8ºC with monoclonal mouse antibody generated against HO4[22] (HO4 Mab 70; generated at Syngenta Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK) diluted to 2 µg ml in carbonate/bicarbonate buffer (34.9 mM $NaHCO_3$, 15.0 mM Na_2CO_3, pH 9.5). The antibody was removed by manually flicking the plates into a sink and tapping them on paper towels to remove residual solution. The plates were blocked with blocking buffer (PBS[23] + 1% milk) for at least 30 min at room temperature. Plates were washed five times using an automated plate washer (ELx405; Bio-tek Instruments, Inc., Winooski, Vermont, USA) and triplicate samples of each tissue extract and standard [appropriate dilutions prepared in ELISA dilution buffer (PBS + 0.05% Tween-20 + 1% milk)] were applied (total volume was 100 µl per well). Following incubation for approximately two hours at 18-22ºC, the plates were washed five times and 100 µl of polyclonal rabbit antisera generated against full-length FLCry1Ab (FLCry1Ab Rb 3 antisera; CTL; diluted 1:10,000 in ELISA dilution buffer) was added to each well. The plates were incubated for approximately 1 hr at 18–22ºC and then washed five times prior to the addition of 100 µl of goat anti-rabbit IgG-HRP conjugated antibody (Sigma; St. Louis, MO, USA; diluted 1:10,000 in ELISA dilution buffer, except for the pollen/nectar assays for which it was diluted 1:20,000) per well. After incubation for approximately 1 hr at 18–22ºC, the plates were washed five times and TMB substrate was added. Color was allowed to develop for approximately 30 min at room temperature in the dark and the reaction stopped by the addition of 3M H_2SO_4 (50 µl per well). Absorbance at 450 nm was measured using a Tecan Sunrise® multi-well plate reader (Tecan; Research Triangle Park, NC, USA). The results were analyzed using the DeltaSoft Curve fitting software program (BioMetallics, Inc.; Princeton, NJ, USA). The four parameters algorithm was used to generate a curve.

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[22] HO4 is a hybrid protein comprised of the the 1st and 2nd domains of Cry1Ab and the 3rd domain of Cry1C.

[23] PBS = 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2

Temperature and incubation times were important as the ELISA was not designed to go to equilibrium. Standard curves, prepared from microbially produced full-length FLCry1Ab, were included on each plate. Data points were considered acceptable if the mean "Delta" OD value obtained lay within the linear range of the standards. Only analyses in which the coefficient of variance was less than 10% were accepted. If, due to technical error, one of the three aliquots did not yield a reliable absorbance value, then the mean absorbance of the remaining duplicate aliquots of the sample extract was used. FLCry1Ab values were calculated as follows:

> % dry weight = dry weight (g) ÷ fresh weight (g) × 100
>
> µg FLCry1Ab/gdw = ng FLCry1Ab/ml (from ELISA) × DF × volume of extraction buffer(ml) ÷ g of tissue extracted ÷ 1000
>
> µg FLCry1Ab/gfw = µg FLCry1Ab/gdw × % dry weight

The LOQ of the ELISA was estimated based on the lowest concentration of pure reference protein lying on the linear portion of the standard curve. Values were calculated as follows:

> LOQ (µg FLCry1Ab/gdw) = lowest concentration on the linear portion of standard curve (ng FLCry1Ab/ml) × DF where negative control was below the LOQ × volume of buffer used in extraction (ml) ÷ g of tissue extracted ÷ 1000

The LOD of the ELISA was estimated based on the OD + 2 standard deviations of the lowest concentration of pure full-length FLCry1Ab protein used in the standard curve. Values were calculated as follows:

> LOD (µg FLCry1Ab/gdw) = OD + 2 S.D. of lowest concentration used in the standard curve (expressed as ng FLCry1Ab/ml) × DF where negative control was below the LOD × volume of buffer used in extraction (ml) ÷ g of tissue extracted ÷ 1000

A.6. Extraction Efficiency of FLCry1Ab Protein

Extraction efficiency measurements were performed to estimate the relative amount of FLCry1Ab extracted during routine procedures, compared to that which remained associated with the post-extraction solids. Tissues (except for pollen) were extracted in triplicate as described in Tissue Extraction. One aliquot of pollen was used to determine extraction efficiency. The insoluble material was then collected and re-extracted twice more, while retaining the supernatant for analysis each time. Percent extraction efficiency was calculated as follows:

> ng FLCry1Ab/ml 1st Extraction ÷ (ng FLCry1Ab/ml 1st extraction + 2nd extraction + 3rd extraction) × 100

ELISA values were corrected for extraction efficiency as follows:

> µg FLCry1Ab/gdw (corrected for extraction efficiency) = µg FLCry1Ab/gdw ÷ % extraction efficiency

µg FLCry1Ab/gfw (corrected for extraction efficiency) = µg FLCry1Ab/gdw (corrected for extraction efficiency) × % dry weight

Source of Data for Estimations of Amounts of FLCry1Ab per-Acre and per-Hectare

Estimates of the quantities of FLCry1Ab protein in COT67B plants on a per-acre and a per-hectare basis were calculated as follows, using the values measured for whole plants described above:

g FLCry1Ab/acre = g FLCry1Ab/gfw (corrected for extraction efficiency) × average weight of whole plants (g) × 50,000 plants/acre[24]

Similar calculations were also made using a value of 123,500 plants/hectare[3].

B. Results

B.1. FLCry1Ab Concentrations at Various Cotton Developmental Stages

Quantifiable concentrations of FLCry1Ab protein were detected in most of the COT67B plant tissues analyzed (Table 2a, Table 2b, Table 3a, Table 3b, Table 4, Table 5). Results are presented as the means of the three replicate extractions of each pooled sample, except as indicated in the tables. The FLCry1Ab data presented below has been corrected for extraction efficiency. FLCry1Ab concentrations in most near-isogenic, nontransgenic control samples were either <LOD or <LOQ. The negative control seed sample from QGA was determined to have a low level of FLCry1Ab (0.24 µg/gdw) that was likely due to contamination during processing or extraction. The LOQs and LODs for the various tissue types can be found in Table 6.

Across all growth stages, mean FLCry1Ab concentrations (averaged across locations) measured in young leaves, old leaves and roots of COT67B plants ranged from *ca.* 87.70 – 323.84 µg/gdw, *ca.* 194.02 – 255.74 µg/gdw, and *ca.* 12.61 – 56.56 µg/gdw, respectively (Table 2b). Data that has not been corrected for extraction efficiency can be found in Table 2a. The corresponding values expressed on a fresh-weight basis are shown in Table 3a and Table 3b.

Mean FLCry1Ab concentrations measured in bolls collected at 1st open boll averaged *ca.* 45.24 µg/gdw across locations (Table 4); the corresponding values on a fresh weight basis are also shown, as well as data that has not been corrected for extraction efficiency). Mean FLCry1Ab concentrations measured in whole plants collected at pre-harvest averaged *ca.* 42.87 µg/gdw across locations. Mean FLCry1Ab concentrations measured in seed collected at pre-harvest averaged *ca.* 25.17 µg/gdw across locations.

Flowers, pollen and nectar were collected from WLA at peak bloom. Mean FLCry1Ab concentrations in flowers were determined to be *ca.* 161.74 µg/gdw. The concentration

[24] Based on estimated average planting density for cotton in the United States.

of FLCry1Ab in the pooled pollen sample (air-dried overnight) was determined to be *ca.* 5.45 µg/g sample (Table 5). FLCry1Ab was not detectable in nectar (LOD = 0.0002 µg/ml sample).

The mean FLCry1Ab concentration in fiber samples collected from WLA at pre-harvest was <0.02 µg/g sample. Four of the five fiber samples were <LOD. The fifth sample was <LOQ which was likely due to boll or seed contamination from the ginning process.

B.2. Estimated Total FLCry1Ab Protein per-Acre and per-Hectare

Assuming a planting density of 50,000 plants/acre (123,500 plants/hectare), estimates of mean FLCry1Ab concentrations in the transgenic plants on a per-acre (and per-hectare) basis at pre-harvest ranged from *ca.* 46.49 g FLCry1Ab/acre (114.84 g FLCry1Ab/hectare) at WLA to *ca.* 182.59 g FLCry1Ab/acre (451.01 g FLCry1Ab/hectare) at LMS (Table 7). Values have been corrected for extraction efficiency.

B.3. FLCry1Ab Extraction Efficiency

The apparent extraction efficiency of FLCry1Ab across Event COT67B plant tissues ranged from 70.7% in whole plants to 77.0% in bolls (Table 8). Extraction efficiency of FLCry1Ab from pollen averaged 78.5%.

Appendix 3.C.-Table 1. Summary of Cotton Plant Parts Retained and Analyzed at Each Developmental Stage

Tissue	Stage				
	Squaring	**1st White Bloom**	**Peak Bloom**	**1st Open Boll**	**Pre-Harvest**
Young Leaves[1]	**x**	**x**	**x**	**x**	
Roots	**x**	**x**	**x**	**x**	
Old Leaves[2]	**x**	**x**	**x**	**x**	
Bolls[3]				**x**	
Seed[4]					**x**
Fiber[5]					**x**
Pollen[6]			**x**		
Flowers[6]			**x**		
Nectar[6]			**x**		
Whole Plants[7]					**x**

[1] Small, shiny leaves approximately 2 inches (50 mm) or less; pooled sample of plants received, at collection.
[2] All leaves except for the Young Leaves.
[3] Fiber and Seed tissue removed.
[4] Fuzzy seed ground. Any fiber tissue removed after grinding and before extraction.
[5] Analyzed from WLA only.
[6] Pooled sample collected from 2004 field trials.
[7] Whole Plants consist of all tissue except for the fiber and seed.

Appendix 3.C.-Table 2A. FLCry1Ab Concentrations in Leaves and Roots on a Dry Weight Basis During Development of Event COT67B Plants

Tissue	Location*	Developmental Stage			
		Squaring	**1st White Bloom**	**Peak Bloom**	**1st Open Boll**
		Mean µg FLCry1Ab/gdw ± S. D. (range)			
Young Leaves	**NAR**	258.29 ± 13.54[a] (247.73 – 277.58)	201.14 ± 6.72[a] (195.02 – 207.49)	154.71[b]	77.36[b]
	QGA	---[c]	---	107.81[b]	NS[d]
	WLA	209.01 ± 9.70[e] (192.58 – 221.75)	---	76.20[b]	---
	LMS	228.31 ± 18.38[a] (207.12 – 251.55)	X[f]	123.80[b]	48.22[b]
Average Across Locations ± SD		**231.87 ± 24.84**	**201.14**	**115.63 ± 32.71**	**62.79 ± 20.61**
Old Leaves	NAR	232.69 ± 12.12[g] (212.50 – 246.70)	213.56 ± 32.84[g] (186.59 – 267.27)	260.84 ± 11.89 (247.11 – 268.02)	232.79 ± 16.03 (223.47 – 251.31)
	QGA	105.02 ± 8.44[g] (93.76 – 114.63)	191.43 ± 18.46[g] (171.67 – 218.14)	211.06 ± 4.19 (206.88 – 215.25)	NS
	WLA	160.83 ± 4.72[h] (153.98 – 170.61)	181.18 ± 19.45[g] (162.17 – 210.02)	122.39 ± 6.58 (118.49 – 129.98)	133.53 ± 9.34 (127.58 – 144.29)
	LMS	201.63 ± 20.48[g] (171.25 – 224.26)	X	157.58 ± 17.33 (143.38 – 176.90)	78.38 ± 10.70 (70.81 – 90.61)
Average Across Locations ± SD		**175.04 ± 55.18**	**195.39 ± 16.55**	**187.97 ± 60.74**	**148.23 ± 78.25**
Roots	NAR	63.20 ± 5.39 (59.31 – 69.36)	22.44 ± 2.77 (19.33 – 24.64)	18.98 ± 1.95 (17.75 – 21.23)	12.70 ± 1.53 (10.94 – 13.63)
	QGA	21.90 ± 3.63 (17.73 – 24.34)	23.88 ± 1.05 (22.84 – 24.93)	27.89 ± 1.13 (26.72 – 28.98)	NS
	WLA	36.55 ± 8.84[g] (28.01 – 47.53)	19.81 ± 4.17 (15.78 – 24.11)	12.64 ± 0.68 (11.98 – 13.33)	10.20 ± 0.64 (9.47 – 10.61)
	LMS	48.72 ± 5.59 (43.53 – 54.64)	X	14.08 ± 0.64 (13.34 – 14.46)	5.59 ± 1.16 (4.66 – 6.90)
Average Across Locations ± SD		**42.59 ± 17.58**	**22.04 ± 2.06**	**18.40 ± 6.88**	**9.50 ± 3.60**

N = 3 replicate samples used to determine the mean and standard deviations, unless otherwise noted
* NAR = Newport, AR; QGA = Quitman, GA; WLA = Winnsboro, LA; LMS = Leland, MS
[a] 4 replicate samples used to determine the mean and standard deviation
[b] One sample was analyzed; therefore, no mean or standard deviation could be determined
[c] --- = No tissue available for analysis once procedure optimized
[d] NS = No samples analyzed due to incorrect storage of samples before shipping
[e] 7 replicate samples used to determine the mean and standard deviation
[f] X = TaqMan® PCR tests confirmed the plants received were not COT67B
[g] 6 replicate samples used to determine the mean and standard deviation
[h] 9 replicate samples used to determine the mean and standard deviation

Appendix 3.C.-Table 2B. FLCry1Ab Concentrations in Leaves and Roots on a Dry Weight Basis During Development of Event COT67B Plants (Corrected for Extraction Efficiency)

Tissue	Location*	Developmental Stage			
		Squaring	1st White Bloom	Peak Bloom	1st Open Boll
		Mean µg FLCry1Ab/gdw ± S. D. (range)			
Young Leaves	NAR	360.75 ± 18.91[a] (345.99 – 387.69)	280.93 ± 9.38[a] (272.38 – 289.79)	216.07[b]	108.05[b]
	QGA	---[c]	---	150.57[b]	NS[d]
	WLA	291.91 ± 13.55[e] (268.96 – 309.71)	---	106.42[b]	---
	LMS	318.87 ± 25.67[a] (289.27 – 351.33)	X[f]	172.91[b]	67.35[b]
Average Across Locations ± SD		**323.84 ± 34.69**	**280.93**	**161.49 ± 45.69**	**87.70 ± 28.78**
Old Leaves	NAR	304.56 ± 16.80[g] (278.15 – 322.91)	279.52 ± 40.74[g] (244.23 – 349.82)	341.41 ± 15.57 (323.44 – 350.81)	304.70 ± 20.99 (292.50–328.94)
	QGA	137.46 ± 9.89[g] (122.72 – 150.04)	250.56 ± 26.49[g] (224.69 – 285.52)	276.26 ± 5.48 (270.78 – 281.74)	NS
	WLA	210.51 ± 6.18[h] (201.54 – 223.31)	237.14 ± 23.39[g] (212.27 – 274.90)	160.19 ± 8.61 (155.10 – 170.13)	174.77 ± 12.22 (166.98–188.86)
	LMS	263.92 ± 24.50[g] (224.15 – 293.53)	X	206.26 ± 22.26 (187.68 – 231.54)	102.59 ± 14.00 (92.69 – 118.61)
Average Across Locations ± SD		**229.11 ± 72.23**	**255.74 ± 21.66**	**246.03 ± 79.50**	**194.02 ± 102.42**
Roots	NAR	83.93 ± 7.16 (78.77 – 92.10)	29.79 ± 3.68 (25.67 – 32.72)	25.20 ± 2.60 (23.57 – 28.20)	16.86 ± 2.03 (14.52– 18.11)
	QGA	29.08 ± 4.82 (23.55 – 32.33)	31.71 ± 1.39 (30.33 – 33.11)	37.03 ± 1.50 (35.49 – 38.49)	NS
	WLA	48.54 ± 11.74[g] (37.20 – 63.12)	26.31 ± 5.54 (20.96 – 32.02)	16.79 ± 0.90 (15.91 – 17.70)	13.55 ± 0.85 (12.57 – 14.09)
	LMS	64.70 ± 7.43 (57.80 – 72.56)	X	18.70 ± 0.85 (17.71 – 19.20)	7.43 ± 1.55 (6.18 – 9.16)
Average Across Locations ± SD		**56.56 ± 23.34**	**29.27 ± 2.74**	**24.43 ± 9.14**	**12.61 ± 4.79**

N = 3 replicate samples used to determine the mean and standard deviations, unless otherwise noted
* NAR = Newport, AR; QGA = Quitman, GA; WLA = Winnsboro, LA; LMS = Leland, MS
[a] 4 replicate samples used to determine the mean and standard deviation
[b] One sample was analyzed; therefore, no mean or standard deviation could be determined
[c] --- = No tissue available for analysis once procedure optimized
[d] NS = No samples analyzed due to incorrect storage of samples before shipping
[e] 7 replicate samples used to determine the mean and standard deviation
[f] X = TaqMan® PCR tests confirmed the plants received were not COT67B
[g] 6 replicate samples used to determine the mean and standard deviation
[h] 9 replicate samples used to determine the mean and standard deviation.

Appendix 3.C.-Table 3a. FLCry1Ab Concentrations in Leaves and Roots on a Fresh Weight Basis During Development of Event COT67B Plants

Tissue	Location*	Developmental Stage			
		Squaring	1st White Bloom	Peak Bloom	1st Open Boll
		Mean µg FLCry1Ab/gfw ± S. D. (range)			
Young Leaves	NAR	57.50 ± 3.01[a] (55.14 – 61.79)	43.31 ± 1.45[a] (41.99 – 44.67)	34.05[b]	19.22[b]
	QGA	---[c]	---	25.98[b]	NS[d]
	WLA	101.68 ± 4.72[e] (93.69 – 107.88)	---	24.40[b]	---
	LMS	37.97 ± 3.06[a] (34.44 – 41.83)	X[f]	24.96[b]	10.95[b]
Average Across Locations ± SD		**65.72 ± 32.64**	**43.31**	**27.35 ± 4.52**	**15.09 ± 5.85**
Old Leaves	NAR	46.07 ± 2.54[g] (42.08 – 48.85)	47.56 ± 6.93[g] (41.55 – 59.52)	52.72 ± 2.40 (49.94 – 54.17)	52.19 ± 3.59 (50.10 – 56.34)
	QGA	25.00 ± 1.80[g] (22.32 – 27.29)	37.67 ± 3.98[g] (33.78 – 42.93)	48.82 ± 0.97 (47.85 – 49.79)	NS
	WLA	38.47 ± 1.13[h] (36.83 – 40.81)	41.65 ± 4.11[g] (37.28 – 48.28)	33.61 ± 1.81 (32.54 – 35.69)	33.90 ± 2.37 (32.39 – 36.63)
	LMS	37.00 ± 3.43[g] (31.42 – 41.15)	X	31.49 ± 3.46 (28.65 – 35.34)	18.07 ± 2.47 (16.33 – 20.90)
Average Across Locations ± SD		**36.64 ± 8.71**	**42.29 ± 4.97**	**41.66 ± 10.67**	**34.72 ± 17.07**
Roots	NAR	11.14 ± 0.95 (10.46 – 12.23)	7.22 ± 0.89 (6.22 – 7.93)	6.45 ± 0.66 (6.03 – 7.21)	4.77 ± 0.57 (4.11 – 5.12)
	QGA	5.51 ± 0.91 (4.46 – 6.13)	6.75 ± 0.30 (6.45 – 7.05)	9.27 ± 0.38 (8.88 – 9.63)	NS
	WLA	8.97 ± 2.17[g] (6.87 – 11.66)	6.63 ± 1.40 (5.29 – 8.07)	5.77 ± 0.31 (5.46 – 6.08)	4.06 ± 0.25 (3.77 – 4.22)
	LMS	8.04 ± 0.92 (7.18 – 9.02)	X	5.48 ± 0.25 (5.19 – 5.63)	2.40 ± 0.50 (2.00 – 2.96)
Average Across Locations ± SD		**8.42 ± 2.33**	**6.87 ± 0.31**	**6.74 ± 1.73**	**3.74 ± 1.21**

N = 3 replicate samples used to determine the mean and standard deviations, unless otherwise noted

* NAR = Newport, AR; QGA = Quitman, GA; WLA = Winnsboro, LA; LMS = Leland, MS

[a] 4 replicate samples used to determine the mean and standard deviation

[b] One sample was analyzed; therefore, no mean or standard deviation could be determined

[c] --- = No tissue available for analysis once procedure optimized

[d] NS = No samples analyzed due to incorrect storage of samples before shipping

[e] 7 replicate samples used to determine the mean and standard deviation

[f] X = TaqMan® PCR tests confirmed the plants received were not COT67B

[g] 6 replicate samples used to determine the mean and standard deviation

[h] 9 replicate samples used to determine the mean and standard deviation

Appendix 3.C.-Table 3b. FLCry1Ab Concentrations in Leaves and Roots on a Fresh Weight Basis During Development of Event COT67B Plants (Corrected for Extraction Efficiency)

Tissue	Location*	Developmental Stage			
		Squaring	1st White Bloom	Peak Bloom	1st Open Boll
		Mean µg FLCry1Ab/gfw ± S. D. (range)			
Young Leaves	NAR	80.30 ± 4.21[a] (77.02 – 86.30)	60.48 ± 2.02[a] (58.64 – 62.39)	47.56[b]	26.85[b]
	QGA	---[c]	---	36.29[b]	NS[d]
	WLA	142.01 ± 6.59[e] (130.85 – 150.67)	---	34.08[b]	---
	LMS	53.03 ± 4.27[a] (48.11 – 58.43)	X[f]	34.86[b]	15.29[b]
Average Across Locations ± SD		**91.78 ± 45.59**	**60.48**	**38.20 ± 6.31**	**21.07 ± 8.18**
Old Leaves	NAR	60.30 ± 3.33[g] (55.07 – 63.94)	62.25 ± 9.07[g] (54.39 – 77.91)	69.00 ± 3.15 (65.37 – 70.90)	68.31 ± 4.70 (65.58 – 73.75)
	QGA	32.73 ± 2.36[g] (29.22 – 35.72)	49.31 ± 5.21[g] (44.22 – 56.19)	63.90 ± 1.27 (62.63 – 65.17)	NS
	WLA	50.35 ± 1.48[h] (48.21 – 53.42)	54.52 ± 5.38[g] (48.80 – 63.20)	43.99 ± 2.36 (42.59 – 46.72)	44.38 ± 3.10 (42.40 – 47.95)
	LMS	48.43 ± 4.50[g] (41.13 – 53.86)	X	41.21 ± 4.53 (37.50 – 46.26)	23.66 ± 3.23 (21.37 – 27.35)
Average Across Locations ± SD		**47.95 ± 11.41**	**55.36 ± 6.51**	**54.52 ± 13.97**	**45.45 ± 22.35**
Roots	NAR	14.80 ± 1.26 (13.89 – 16.24)	9.59 ± 1.18 (8.27 – 10.54)	8.56 ± 0.88 (8.01 – 9.58)	6.33 ± 0.76 (5.45 – 6.80)
	QGA	7.32 ± 1.21 (5.93 – 8.14)	8.96 ± 0.39 (8.57 – 9.36)	12.31 ± 0.50 (11.80 – 12.79)	NS
	WLA	11.91 ± 2.88[g] (9.13 – 15.48)	8.81 ± 1.85 (7.02 – 10.72)	7.66 ± 0.41 (7.26 – 8.07)	5.39 ± 0.34 (5.00 – 5.61)
	LMS	10.68 ± 1.23 (9.54 – 11.97)	X	7.28 ± 0.33 (6.89 – 7.47)	3.19 ± 0.66 (2.66 – 3.93)
Average Across Locations ± SD		**11.18 ± 3.09**	**9.12 ± 0.42**	**8.95 ± 2.30**	**4.97 ± 1.61**

N = 3 replicate samples used to determine the mean and standard deviations, unless otherwise noted
* NAR = Newport, AR; QGA = Quitman, GA; WLA = Winnsboro, LA; LMS = Leland, MS
[a] 4 replicate samples used to determine the mean and standard deviation
[b] One sample was analyzed; therefore, no mean or standard deviation could be determined
[c] --- = No tissue available for analysis once procedure optimized
[d] NS = No samples analyzed due to incorrect storage of samples before shipping
[e] 7 replicate samples used to determine the mean and standard deviation
[f] X = TaqMan® PCR tests confirmed the plants received were not COT67B
[g] 6 replicate samples used to determine the mean and standard deviation
[h] 9 replicate samples used to determine the mean and standard deviation

Appendix 3.C.-Table 4. FLCry1Ab Concentrations in Whole Plants, Seeds, Flowers and Bolls of Event COT67B Plants on a Dry and Fresh Weight Basis

				Corrected for Extraction Efficiency	Corrected for Extraction Efficiency
Tissue	**Location**	**Mean µg FLCry1Ab/gdw ± S. D. (range)**	**Mean µg FLCry1Ab/gfw ± S. D. (range)**	**Mean µg FLCry1Ab/gdw ± S. D. (range)**	**Mean µg FLCry1Ab/gfw ± S. D. (range)**
Whole Plant (Pre-harvest)	NAR	37.39 ± 3.82 (33.92 – 41.48)	11.52 ± 1.18 (10.45 – 12.77)	52.89 ± 5.40 (47.98 – 58.67)	16.29 ± 1.66 (14.78 – 18.07)
	QGA	44.88 ± 8.49 (38.69 – 54.56)	15.97 ± 3.02 (13.77 – 19.41)	63.48 ± 12.01 (54.72 – 77.17)	22.59 ± 4.27 (19.47 – 27.46)
	WLA	17.07 ± 2.03 (15.84 – 19.41)	8.58 ± 1.02 (7.96 – 9.76)	24.14 ± 2.87 (22.41 – 27.46)	12.14 ± 1.44 (11.27 – 13.80)
	LMS	21.63 ± 1.13 (20.65 – 22.87)	7.32 ± 0.38 (6.99 – 7.74)	30.59 ± 1.60 (29.21 – 32.34)	10.35 ± 0.54 (9.88 – 10.94)
Average Across Locations ± SD		**30.24 ± 13.08**	**10.85 ± 3.84**	**42.87 ± 18.50**	**15.34 ± 5.43**
Seed (Pre-harvest)	NAR*	21.42 ± 2.05 (19.08 – 22.87)	15.50 ± 1.48 (13.80 – 16.55)	29.35 ± 2.81 (26.14 – 31.33)	21.23 ± 2.03 (18.91 – 22.67)
	QGA	20.25 ± 1.34 (18.71 – 21.04)	16.22 ± 1.07 (14.99 – 16.85)	27.75 ± 1.83 (25.63 – 28.82)	22.22 ± 1.47 (20.53 – 23.09)
	WLA[a]	22.83 ± 1.38 (21.44 – 25.03)	20.12 ± 1.22 (18.90 – 22.06)	31.28 ± 1.89 (29.37 – 34.29)	27.57 ± 1.67 (25.88 – 30.22)
	LMS	9.00 ± 0.27 (8.69 – 9.20)	6.88 ± 0.21 (6.64 – 7.03)	12.33 ± 0.38 (11.90 – 12.60)	9.42 ± 0.29 (9.09 – 9.63)
Average Across Locations ± SD		**18.38 ± 6.34**	**14.68 ± 5.58**	**25.17 ± 8.68**	**20.11 ± 7.65**
Bolls (1st Open Boll)	NAR	40.94 ± 5.47 (37.16 – 47.21)	7.97 ± 1.07 (7.24 – 9.20)	53.17 ± 7.10 (48.26 – 61.31)	10.36 ± 1.38 (9.40 – 11.94)
	QGA	NS[b]	NS	NS	NS
	WLA	40.03 ± 3.28 (37.58 – 43.76)	8.27 ± 0.68 (7.77 – 9.05)	51.99 ± 4.26 (48.80 – 56.83)	10.75 ± 0.88 (10.09 – 11.75)
	LMS	23.53 ± 2.57 (20.63 – 25.55)	4.38 ± 0.48 (3.84 – 4.76)	30.56 ± 3.34 (26.79 – 33.18)	5.69 ± 0.62 (4.99 – 6.18)
Average Across Locations ± SD		**34.83 ± 9.80**	**6.88 ± 2.17**	**45.24 ± 12.73**	**8.93 ± 2.81**
Flowers (Peak Bloom)	WLA[a]	119.36 ± 16.88 (101.33 – 139.87)	18.95 ± 2.68 (16.09 – 22.21)	161.74 ± 22.87 (137.30–189.52)	25.68 ± 3.63 (21.80 – 30.10)

N = 3 unless otherwise noted

NAR = Newport, AR; QGA = Quitman, GA; WLA = Winnsboro, LA; LMS = Leland, MS

***** Pooled seed sample from NAR consists of three plants, not five

[a] 6 replicate samples were used to determine the mean and standard deviation

[b] NS = No samples analyzed due to incorrect storage of samples before shipping

Appendix 3.C.-Table 5. FLCry1Ab Concentrations in Pollen, Nectar, and Fiber of Event COT67B Plants

Tissue	Location	µg FLCry1Ab/g sample*
Pollen[1]	WLA	4.28^
Nectar[2]	WLA	<0.0002
Fiber[3]	WLA	<0.02 (<0.01 – <0.07)

* Unless otherwise noted

^ The FLCry1Ab concentration in pollen is 5.45 µg FLCry1Ab/g sample when corrected for extraction efficiency

[1] Pollen values are reported on a per g sample basis (air-dried overnight, as collected)

[2] Nectar values are reported on a per ml nectar basis (as collected)

[3] Five samples collected from WLA. One sample was <LOQ (<0.07 µg FLCry1Ab/g sample) which is likely due to boll or seed contamination from the ginning process

Appendix 3.C.-Table 6. Approximate Limits of Quantitation for FLCry1Ab in Various Tissue Types of Event COT67B Plants

	Developmental Stage				
Tissue	**Squaring**	**1st White Bloom**	**Peak Bloom**	**1st Open Boll**	**Pre-harvest**
	Limits of Quantitation µg FLCry1Ab/gdw* (µg FLCry1Ab/gfw*)				
Young Leaves	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.06 (0.01)	N/A[1]
Old Leaves	0.06 (0.01)	0.06 (0.01)	0.06 (0.01)	0.06 (0.01)	N/A
Roots	0.08 (0.02)	0.10 (0.03)	0.10 (0.03)	0.08 (0.03)	N/A
Flowers	---[2]	---	0.06 (0.01)	---	---
Bolls	---	---	---	N/A	0.06 (0.01)
Seed	---	---	---	N/A	0.06 (0.06)
Whole Plants	N/A	N/A	N/A	N/A	0.06 (0.02)
Pollen[3]	---	---	0.74	---	---
Nectar[4]	---	---	0.002	---	---
Fiber[5]	---	---	---	N/A	0.07

* Unless otherwise noted

[1] N/A = Not analyzed at this stage

[2] "---" = Tissue not available at this stage

[3] Pooled sample collected from a minimum of 100 plants in the field. Sample was air-dried overnight = µg FLCry1Ab/g air-dried sample

[4] Pooled sample collected from a minimum of 100 plants in the field. Analyzed as collected = µg FLCry1Ab/ml sample

[5] Tissue was analyzed as collected = µg FLCry1Ab/g sample

Appendix 3.C.-Table 7. Approximate Limits of Detection for FLCry1Ab in Various Tissue Types of Event COT67B Plants

	Developmental Stage				
Tissue	**Squaring**	**1st White Bloom**	**Peak Bloom**	**1st Open Boll**	**Pre-harvest**
	Limits of Detection µg FLCry1Ab/gdw* (µg FLCry1Ab/gfw*)				
Young Leaves	0.01 (0.002)	0.01 (0.002)	0.01 (0.003)	0.01 (0.002)	N/A[1]
Old Leaves	0.01 (0.002)	0.01 (0.002)	0.01 (0.002)	0.01 (0.002)	N/A
Roots	0.05 (0.01)	0.03 (0.01)	0.06 (0.02)	0.01 (0.003)	N/A
Flowers	---[2]	---	0.01 (0.001)	---	---
Bolls	---	---	---	N/A	0.01 (0.002)
Seed	---	---	---	N/A	0.01 (0.01)
Whole Plants	N/A	N/A	N/A	N/A	0.01 (0.003)
Pollen[3]	---	---	0.08	---	---
Nectar[4]	---	---	0.0002	---	---
Fiber[5]	---	---	---	N/A	0.01

* Unless otherwise noted

[1] N/A = Not analyzed at this stage

[2] "---" = Tissue not available at this stage

[3] Pooled sample collected from a minimum of 100 plants in the field. Sample was air-dried overnight = µg FLCry1Ab/g air-dried sample

[4] Pooled sample collected from a minimum of 100 plants in the field. Analyzed as collected = µg FLCry1Ab/ml sample

[5] Tissue was analyzed as collected = µg FLCry1Ab/g sample

Appendix 3.C.-Table 8. FLCry1Ab g/Acre and g/Hectare Estimates for Event COT67B Pre-harvest Stage Plants

Location*	Mean Plant Fresh Weight (g)	Mean g FLCry1Ab/Acre ± S.D. (range)	Mean g FLCry1Ab/Hectare ± S.D. (range)
NAR	206.13	167.88 ± 17.13 (152.30 – 186.23)	414.67 ± 42.31 (376.19 – 459.98)
QGA	99.18	112.01 ± 21.19 (96.55 – 136.16)	276.67 ± 52.33 (238.49 – 336.32)
WLA	76.62	46.49 ± 5.53 (43.16 – 52.88)	114.84 ± 13.66 (106.60 – 130.60)
LMS	352.83	182.59 ± 9.53 (174.33 – 193.02)	451.01 ± 23.55 (430.59 – 476.77)

Note: Values have been corrected for extraction efficiency

*NAR = Newport, AR; QGA = Quitman, GA; WLA = Winnsboro, LA; LMS = Leland, MS

Appendix 3C-Table 9. Extraction Efficiency of FLCry1Ab from Plant Tissues of Event COT67B Plants

	ng FLCry1Ab/ml				
Sample	**1st Extraction**	**2nd Extraction**	**3rd Extraction**	**% Extraction Efficiency**	**Average % Extraction Efficiency[1]**
Young Leaves	7328.00	1963.60	921.52	71.8	71.6
	7036.80	2294.80	682.32	70.3	
	7524.80	1958.00	835.20	72.9	
Old Leaves	5436.80	1064.00	503.52	77.6	76.4
	5432.80	1581.40	436.00	72.9	
	5698.40	1122.00	435.68	78.5	
Roots	1335.20	319.76	92.46	76.4	75.3
	1538.88	483.76	170.42	70.2	
	1611.20	336.00	81.10	79.4	
Pollen	122.55	18.70	14.86	78.5	78.5
Flowers	3571.20	775.40	362.80	75.8	73.8
	3571.20	924.20	391.08	73.1	
	3394.40	914.20	378.96	72.4	
Bolls	1273.92	261.00	107.32	77.6	77.0
	1307.52	319.44	108.84	75.3	
	1458.72	303.84	102.35	78.2	
Seed	720.30	195.00	89.78	71.7	73.0
	749.10	183.30	65.19	75.1	
	750.80	217.50	72.07	72.2	
Whole Plants	538.05	174.13	58.24	69.8	70.7
	652.86	177.38	87.17	71.2	
	528.13	153.34	62.04	71.0	

[1] Calculation: Extraction 1 / (Extraction 1 + Extraction 2 + Extraction 3) * 100

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APPENDIX 3.D. MATERIALS, METHODS AND RESULTS FOR A STUDY TO DEMONSTRATE STABILITY OF FLCRY1AB PROTEIN EXPRESSION ACROSS MULTIPLE GENERATIONS OF COT67B.

A. Materials and Methods

A.1. Source of Leaf Samples for Evaluation of FLCry1Ab Concentrations

Plants from three COT67B cotton generations (see below) were grown in the greenhouse at Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA (SBI). Plants were screened using TaqMan® PCR analysis to distinguish hemizygous plants containing the *flcry1Ab* transgene from the negative segregant plants from each generation. Leaves were collected from 5 COT67B cotton plants per generation (F_1, BC1(F_1) and BC4(F_1))when the individual plant had three to five open bolls (open boll stage). This sampling strategy was used to ensure the plants would be developmentally equivalent at the time of sampling. In addition, identical plant tissues from two near-isogenic, nontransgenic cotton plants (negative segregants) from the BC1(F_1) and BC4(F_1) generations were concurrently sampled and handled in the same manner as the COT67B cotton plants. After collection, samples were weighed and stored at –80 ± 10ºC.

Generation	Pedigree
F_1[25]	2429[26] x COT67B
BC1(F_1)	2429 x F_1
BC2(F_1)[27]	2429 x BC1F_1
BC3(F_1)[27]	2429 x BC2F_1
BC4(F_1)	2429 x BC3F_1

A.2. Plant Tissue Processing

All processing was done in the presence of dry ice or liquid nitrogen to ensure samples remained frozen. Samples from the near-isogenic, nontransgenic samples were processed first to prevent possible cross-contamination. The leaf tissue sample from each plant was reduced to a fine powder using a sterile and pre-cooled mortar and pestle. Each powdered sample was mixed thoroughly to ensure homogeneity and a sub-sample from

[25] This generation produced 100% heterozygous plants. Therefore, there were no negative segregants to use for analysis.

[26] 2429 is the designation given to the recurrent parent cotton line used to generate the generations analyzed herein.

[27] This generation was not planted or analyzed at SBI. It is only listed to show how subsequent generations were produced.

each was lyophilized for subsequent analysis. Processed samples were stored at –80 ± 10ºC until lyophilization. Lyophilized samples were stored under the same conditions.

The percent dry weight of each sample was determined by recording the weight of the sample prior to lyophilization (fresh weight). Following lyophilization, the weight of the sample was recorded (dry weight). A percent dry weight was then calculated as follows:

$$\% \text{ dry weight} = \text{dry weight (g)} \div \text{fresh weight (g)} \times 100$$

The percent dry weight is used to convert protein concentrations from gram dry-weight (gdw) to gram fresh-weight (gfw).

A.3. Tissue Extraction for FLCry1Ab Analysis

For each sample analyzed, an aliquot of *ca.* 0.1 g of the powdered lyophilized material was transferred into a 15-ml polypropylene tube, suspended in 3 ml extraction buffer (100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 0.2% L-ascorbic acid, 0.05% Tween 20™, 1 mM AEBSF, 1 mM DTT, 1 µM leupeptin, pH 9.5), incubated on wet ice for 30 min and homogenized using an Autogizer® homogenizer (Tomtec; Hamden, CT, USA; 6 cycles, setting 4). After centrifugation for 15 min at *ca.* 10,000 x *g* at *ca.* 4ºC, the supernatants were retained and used for FLCry1Ab analysis by ELISA.

A.4. FLCry1Ab Quantification

The extracts prepared as described above in Tissue Extraction for FLCry1Ab Analysis were quantitatively analyzed for FLCry1Ab protein by ELISA. Nunc MaxiSorp™ plates were coated overnight at 2-8°C with monoclonal mouse antibody generated against HO4[28] (HO4 mAb 70; generated at Syngenta Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK) diluted to 2 µg/ml in carbonate/bicarbonate buffer (34.9 mM $NaHCO_3$, 15.0 mM Na_2CO_3, pH 9.5). The antibody was removed from the plates by manually flicking the solution into a sink and tapping them on paper towels to remove residual solution. The plates were then blocked with blocking buffer (PBS[29] + 1% milk) for at least 30 min at ambient temperature. Plates were washed five times with wash buffer (PBS + 0.25% Tween 20™) using an automatic plate washer (ELx405; Biotek; Winooski, VT, USA) and dilutions of each tissue extract and appropriate serial dilutions of microbially produced full-length FLCry1Ab standard prepared in dilution buffer (PBS + 0.05% Tween 20 + 1% milk) were applied (total volume was 100 µl per well). Following incubation for approximately two hours at 18-20ºC, the plates were washed five times prior to the addition of 100 µl per well of polyclonal rabbit antisera generated against FLCry1Ab (FLCry1Ab Rb 3 antisera; CTL; diluted 1:10,000 in ELISA dilution buffer). The plates were incubated for approximately 1 hr at 18–20ºC and then

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[28] HO4 is a hybrid protein comprised of the the 1st and 2nd domains of Cry1Ab and the 3rd domain of Cry1C.
[29] PBS = 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2

washed five times prior to the addition of 100 µl per well of goat anti-rabbit IgG-HRP conjugated antibody (Sigma; St. Louis, MO, USA; diluted 1:10,000 in ELISA dilution buffer). After incubation for approximately 1 hr at 18–20ºC, the plates were washed five times prior to the addition of 100 µl per well of TMB substrate. Color was allowed to develop for approximately 30 min at ambient temperature in the dark and the reaction stopped by the addition of 3M H_2SO_4 (50 µl per well). Absorbance at 450 nm was measured using a Tecan Sunrise™ multi-well plate reader (Tecan; Research Triangle Park, NC, USA). The results were analyzed with the DeltaSoft Curve fitting software program (BioMetallics, Inc.; Princeton, NJ, USA) and a four parameter algorithm.

A.5. Calculation and Explanation of ELISA Data

Data points were considered acceptable if the mean OD value, obtained from three aliquots of each dilution of sample extract, lay within the linear range of the standards and the coefficient of variance was less than 10%. If, due to technical error, one of the three aliquots of the sample extract did not yield a reliable absorbance value, then the mean absorbance of the remaining duplicate aliquots of the sample extract was used. The mean absorbance per sample was read and plotted against the curve to give a value for the concentration of Cry1Ab protein per ml of extract (without accounting for the dilution factor). The concentration of Cry1Ab per gram of plant tissue was calculated as follows:

> ng FLCry1Ab/ml = diluted ng of FLCry1Ab/ml (obtained from ELISA printout) × dilution factor
>
> µg FLCry1Ab/gdw = ng FLCry1Ab/ml × 3 ml of extraction buffer ÷ amount extracted (g) ÷ 1000
>
> µg FLCry1Ab/gfw = µg FLCry1Ab/gdw × % dry weight

A.6. Limit of Quantitation (LOQ) and Limit of Detection (LOD) Determination

The LOQ of the ELISA was estimated based on the lowest concentration of pure FLCry1Ab protein lying on the linear portion of the standard curve and the dilution factor (DF; based on OD values from dilutions of negative control tissue extracts). Values were calculated as follows:

> LOQ (µg FLCry1Ab/gdw) = lowest concentration on the linear portion of standard curve (expressed as ng FLCry1Ab/ml) × DF × volume of buffer used in extraction (ml) ÷ g of tissue extracted ÷ 1000

The LOD of the ELISA was estimated based on the OD + 2 standard deviations of the lowest concentration of pure FLCry1Ab protein used in the standard curve and the dilution factor (DF; based on OD values from dilutions of negative control tissue extracts). Values were calculated as follows:

LOD (µg Cry1Ab/gdw) = OD + 2 S.D. of lowest concentration used in the standard curve (expressed as ng Cry1Ab/ml) × DF × volume of buffer used in extraction (ml) ÷ g of tissue extracted ÷ 1000

B. Results

The mean FLCry1Ab concentrations measured in leaves from the F_1, $BC1F_1$, and $BC4F_1$ generations of COT67B cotton were 68.17, 66.27, and 54.16 µg/gdw, respectively (Table1). As expected, FLCry1Ab was not detected in the near-isogenic, nontransgenic control samples for the generations analyzed. The LOQ and LOD can be found in Table 2.

Appendix 3.D.-Table 1. FLCry1Ab Concentrations in Leaf Tissue from Multiple Generations of COT67B Cotton

Generation	Mean µg FLCry1Ab/gdw ± SD (range)	Mean µg FLCry1Ab/gfw ± SD (range)
F_1	68.17 ± 9.64 (54.90—81.62)	19.68 ± 2.23 (17.28—22.42)
$BC1F_1$	66.27 ± 16.88 (41.01—81.44)	17.62 ± 1.76 (14.84—19.63)
$BC4F_1$	54.16 ± 12.08 (39.36—67.39)	16.60 ± 2.29 (14.35—20.31)

N = 5 (samples analyzed). Values have not been corrected for extraction efficiency.
Near-isogenic, nontransgenic samples were all <LOD and are not shown in the table.

Appendix 3.D.-Table 2. Approximate Limits of Quantitation and Detection for FLCry1Ab in Leaf Tissue Samples of COT67B Cotton

Open Boll, Leaf Tissue	
Limit of Quantitation	**Limit of Detection**
µg FLCry1Ab/gdw (µg FLCry1Ab/gfw)	**µg FLCry1Ab/gdw (µg FLCry1Ab/gfw)**
0.083 (0.024)	0.007 (0.002)

APPENDIX 3.E. MATERIALS, METHODS AND RESULTS FOR ANALYSIS FOR THE PRESENCE OF FLCRY1AB PROTEIN IN LINTERS, TOASTED COTTONSEED MEAL AND ONCE-REFINED COTTONSEED OIL FROM PROCESSED SEED OF COT67B

A. Materials and Methods

A.1. Source of Cottonseed

Cottonseed from COT67B and Coker 312 were individually pooled from field-grown plants produced during the 2004 planting season from Leland, Mississippi, USA and shipped at ambient temperature to the Regulatory Science laboratory, Syngenta Biotechnology, Inc. (SBI), Research Triangle Park, NC, USA. After ginning, fuzzy cottonseed samples of approximately 1.5 kg each from COT67B and Coker 312 were sent at ambient temperature to the Food Protein Research and Development Center, Texas A&M University, College Station, Texas, USA for processing. An aliquot of each COT67B and Coker312 fuzzy seed was retained by the Food Protein Research and Development Center at 2-8˚C for subsequent analysis.

A.2. Cottonseed Processing

Processing of the COT67B and Coker 312 cottonseed was performed under the direction of Dr. Richard Clough, at the Food Protein Research and Development Center, Texas A&M University. The primary products were linters, defatted toasted cottonseed meal, and once-refined cottonseed oil. The linters contained visible particles of cottonseed hulls which could not be separated from the linters. The fuzzy seed and the processed cottonseed products were shipped on ice packs to the Regulatory Science laboratory, SBI. The fuzzy seed, linters, and defatted toasted cottonseed meal samples were stored at –20 ± 10˚C and the once-refined cottonseed oil was stored at 2-8˚C until analyzed.

The retained fuzzy seed samples of COT67B and Coker 312 were reduced to a fine powder using a Grind Central™ grinder (Cuisinart; East Windsor, New Jersey, USA). Each ground sample was mixed well to ensure homogeneity. The fuzzy seed was stored at –20 ± 10˚C until analyzed.

A.3. FLCry1Ab Extraction

The COT67B and Coker 312 samples were extracted in triplicate. Aliquots of the fuzzy seed, linters, and defatted toasted cottonseed meal (0.1 g) or once-refined cottonseed oil (100 µl) were transferred to 15-ml polypropylene tubes, suspended in 3 ml extraction buffer [100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride, 0.2% L-ascorbic acid, 0.05% Tween-20, 1 mM AEBSF, 1 mM DTT, 1 µM Leupeptin, pH 9.5], homogenized using an Autogizer® homogenizer (Tomtec; Hamden, Connecticut, USA; 6 cycles, setting 4) and then incubated on ice for 30 min. After centrifugation for 15 min at *ca.* 10,000 x *g* at 4ºC, the supernatants were used for FLCry1Ab analysis by ELISA (Tijssen, 1985).

A.4. FLCry1Ab Quantification

The extracts from the COT67B and Coker 312 samples, prepared as described in FLCry1Ab Extraction, were quantitatively analyzed for FLCry1Ab by ELISA to determine the concentration of FLCry1Ab present in each extract. Nunc Maxisorp™ plates were coated overnight at 2-8ºC with monoclonal mouse antibody generated against HO4[30] [HO4 Mab 70; generated at Syngenta Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK] diluted to 2 µg/ml in carbonate/bicarbonate buffer (34.9 mM $NaHCO_3$, 15.0 mM Na_2CO_3, pH 9.5). After incubation overnight at 2–8ºC, the antibody was removed by manually flicking the plates into a sink and tapping them on paper towels to remove residual solution. The plates were blocked with blocking buffer (PBS[31] + 1% milk) for at least 30 min at room temperature. The plates were washed five times using an automated plate washer (ELx405; Bio-tek Instruments, Inc., Winooski, Vermont, USA) and triplicate aliquots of each sample extract and standard [appropriate dilutions prepared in ELISA dilution buffer (PBS + 0.05% Tween-20 + 1% milk)] were applied (total volume was 100 µl per well). Following incubation for approximately two hours at 18-22ºC, the plates were washed five times using an automated plate washer and 100 µl of polyclonal rabbit antisera generated against full-length FLCry1Ab (FLCry1Ab Rb 3 antisera; CTL; diluted 1:10,000 in ELISA dilution buffer) was added to each well. The plates were incubated for approximately 1 hr at 18–22ºC and then washed five times prior to the addition of 100 µl of goat anti-rabbit IgG-HRP conjugated antibody (Sigma; St. Louis, Missouri, USA; diluted 1:10,000 in ELISA dilution buffer) per well. After incubation for approximately 1 hr at 18–22ºC, the plates were washed five times and TMB substrate was added. Color was allowed to develop for approximately 30 min at room temperature in the dark and the reaction was stopped by the addition of 3 M H_2SO_4 (50 µl per well). Absorbance at 450 nm was measured using a Tecan Sunrise® multi-well plate reader (Tecan; Research Triangle Park, North Carolina, USA). The results were analyzed using the DeltaSoft curve fitting software program (BioMetallics, Inc.; Princeton, New Jersey, USA).

Dilutions of reference protein prepared from microbially produced full-length Cry1Ab were included on each plate and a four-parameter algorithm was used to generate a standard curve. Data points were considered acceptable if the mean "Delta" OD value obtained lay within the linear range of the standards and the coefficient of variance was less than 10%. FLCry1Ab values were calculated as follows:

> µg FLCry1Ab/g sample = ng FLCry1Ab/ml (from ELISA) × dilution factor × volume of extraction buffer (ml) ÷ g of sample extracted ÷ 1000

The LOQ of the ELISA (for linters and defatted toasted cottonseed meal) was estimated as follows:

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[30] HO4 is a hybrid protein comprised of the 1st and 2nd domains of Cry1Ab and the 3rd domain of Cry1C.

[31] PBS = 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2

LOQ (µg Cry1Ab/g sample) = lowest concentration of reference protein on the linear portion of standard curve (ng FLCry1Ab/ml) × volume of extraction buffer (ml) ÷ g of sample extracted ÷ 1000.

The LOD of the ELISA (for linters and defatted toasted cottonseed meal) was estimated as follows:

LOD (µg FLCry1Ab/g sample) = mean OD + two standard deviations of lowest concentration of reference protein used in the standard curve (ng FLCry1Ab/ml) × volume of extraction buffer (ml) ÷ g of sample extracted ÷ 1000.

The LOQ in the cottonseed oil was determined to be the lowest concentration of FLCry1Ab that could accurately be quantitated by ELISA, based on the recovery of known FLCry1Ab concentrations added to the Coker 312 negative control oil. The LOD was determined to be the lowest concentration of Cry1Ab that was detectable by ELISA from the once-refined cottonseed oil.

A.5. FLCry1Ab Extraction Efficiency

Extraction efficiency measurements were performed on the fuzzy seed, linters, and defatted toasted cottonseed meal to estimate the relative amount of Cry1Ab extracted, compared to that which remained associated with the post-extraction solids. Samples were extracted as described in Cry1Ab Extraction. The insoluble material was then collected and re-extracted twice more, while retaining the supernatant for analysis each time. Percent extraction efficiency was calculated as follows:

ng FLCry1Ab/ml 1st extraction ÷ (ng FLCry1Ab/ml 1st extraction + 2nd extraction + 3rd extraction) × 100

B. Results

Quantifiable concentrations of FLCry1Ab were detected in the fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B (Table 1). FLCry1Ab was not detected (<LOD) in any of the nontransgenic Coker 312 control samples or in the cottonseed oil from COT67B (Table 1). The mean FLCry1Ab concentrations determined in fuzzy seed, linters, and defatted toasted cottonseed meal were 25.05 µg Cry1Ab/g, 9.65 µg FLCry1Ab/g, and 47.50 µg FLCry1Ab/g, respectively. These values have been corrected for extraction efficiency. Table 1 provides the corresponding ELISA values as measured, prior to correcting for extraction efficiency. The LOQs and LODs for the various samples can be found in Table 2.

The extraction efficiencies for fuzzy seed, linters, and defatted toasted cottonseed meal were determined to be 73.1%, 80.7%, and 69.2%, respectively (Table 3). The extraction

efficiency values indicate that the procedure used was well optimized for FLCry1Ab extraction from fuzzy seed and processed cottonseed products.

Appendix 3.E.-Table 1. FLCry1Ab Concentrations in COT67B Fuzzy Seed and Processed Cottonseed Products

Fuzzy Seed and Processed Cottonseed Product	Mean µg Cry1Ab/g ± S. D. (range)	Mean µg Cry1Ab/g Corrected for Extraction Efficiency ± S. D. (range)
COT67B		
Fuzzy seed	18.31 ± 1.40 (16.84 - 19.63)	25.05 ± 1.92 (23.04 - 26.85)
Linters[2]	7.79 ± 4.87 (3.10 - 12.83)	9.65 ± 6.03 (3.84 - 15.90)
Defatted toasted meal	32.87 ± 3.09 (29.54 - 35.65)	47.50 ± 4.47 (42.69 - 51.52)
Once-refined oil	<LOD[1]	<LOD
Coker 312		
Fuzzy seed	<LOD	<LOD
Linters	<LOD	<LOD
Defatted toasted meal	<LOD	<LOD
Once-refined oil	<LOD	<LOD

Except where noted otherwise, three replicate samples were used to determine the means and standard deviations.

[1]<LOD = All values for the sample were below the limit of detection for the ELISA, and range & standard deviation could not be calculated.

[2] Six samples were used to determine the mean and standard deviation of linters

Appendix 3.E.-Table 2. Approximate Limits of Quantitation and Detection for FLCry1Ab in COT67B Fuzzy Seed and Processed Cottonseed Products

Fuzzy Seed and Processed Cottonseed Products	Limits of Quantitation ug FLCry1Ab/g	Limits of Detection ug FLCry1Ab/g
Fuzzy seed	0.04	0.007
Linters	0.04	0.008
Defatted toasted meal	0.05	0.009
Once-refined oil*	0.05	0.003

* The values shown are in ug/ml

Appendix 3.E.-Table 3. Efficiency of Cry1Ab Extraction from COT67B Fuzzy Seed and Processed Cottonseed Products as Determined by ELISA

	ng FLCry1Ab/ml				
Fuzzy Seed and Processed Cottonseed Products	**1st Extraction**	**1st Extraction**	**1st Extraction**	**% Extraction Efficiency[1]**	**Average % Extraction Efficiency**
	16.32	4.31	1.24	74.6	
	13.33	4.31	1.38	70.1	
Fuzzy Seed	13.96	3.78	1.02	74.4	73.1
	12.83	2.59	0.29	81.7	
	11.62	2.73	0.24	79.6	
Linters	12.212	2.66	0.25	80.8	80.7
Defatted Toasted Cottonseed Meal	31.24	8.42	03.64	72.1	
	29.58	11.61	3.54	66.1	
	26.58	8.92	2.72	69.5	69.2

[1] Calculated as follows: µg FLCry1Ab/g sample = ng FLCry1Ab/ml (from ELISA) × dilution factor × volume of extraction buffer (ml) ÷ g of sample extracted ÷ 1000

APPENDIX 4.A. 2004 AGRONOMIC EVALUATION OF COT67B

A. Introduction

Specific aspects of the biology of COT67B addressed during the 2004 field season include:

1. Relative growth rate and plant development, including height to node ratios at key developmental time points, the node of the 1st fruiting branch, and time to 50% open flowers or bolls.
2. Yield
3. Fiber quality parameters, including strength, length, uniformity and micronaire.

B. Experimental Comparators

Two comparators were used in the 2004 agronomic and phenotypic evaluations of COT67B, the COT67B null isoline and Coker 312.

The *flcry1Ab* transgene and the antibiotic-resistance selectable marker gene (*aph4*) were introduced on separate constructs, which resulted in their independent insertion into the cotton genome. Consequently, these two genes segregated independently during self-pollination and those plants homozygous for only the *flcry1Ab* transgene (COT67B) and those plants lacking the trait (COT67B(-), null-isoline) were identified.

Coker 312 is the parental cotton variety originally used in the transformation process resulting in COT67B. Coker 312 is a United States Protected Variety (PVP 7200100) currently owned by the SeedCo Corporation of Lubbock, TX.

C. Experimental Methods

C.1. Field Plantings

Prior to planting, all trial seed were treated with azoxystrobin and thiamethoxam to protect against seedling fungal diseases and early season sucking insect pests respectively. The seed were treated with Dynasty CST at a rate of 34 g ai / 100 kg seed and with Cruiser at a rate of 0.34 mg ai / seed.

Data were collected from trials planted at seven locations distributed across the southern and south-eastern cotton growing regions of the U.S. However, for logistical reasons, all parameters were not assessed at every location. Appendix 4.A.-Table 1 identifies the locations, date of planting at each location and plant characteristics evaluated at each location. All trials were planted under USDA notification number 04-041-01n.

At each location, seed were planted in plots comprising two 40 foot rows, with approximately 3 plants per foot. Two rows of a different cotton variety were planted as a

buffer between each plot, so that each trial comprised a single continuous canopy of cotton.

Appendix 4.A.-Table 1. Locations of 2004 COT67B, COT67B(-) and Coker 312 agronomic and phenotypic evaluation trials and data collected

Location	Planting Date	Data Collected[1]
Leland, MS	5/11/2004	H:N(1), H:N(2), N, F, B, Y, LT, FQ
Winnsboro, LA (1)	5/27/2004	H:N(1), H:N(2), N, F, B, Y FQ
Winnsboro, LA (2)	5/30/2004	H:N(1), H:N(2), N, Y, LT, FQ
Vero Beach, FL	6/15/2004	H:N(1), H:N(2), N, F,
Newport, AR	5/26/2004	H:N(1), H:N(2), N, Y
Quitman, GA	6/11/2004	H:N(1),
Bossier City, LA	5/24/2004	H:N(1), H:N(2), N, B, Y, LT

[1] H:N(1) = height to node ratio at early flowering, H:N(2) = height to node ratio at pre-harvest, N = node of first fruiting branch, F = days to 50% flowering, B = days to 50% open boll, Y = yield, LT = % lint turnout, FQ = fiber quality

C.2. Experimental Design

Four replicate plots of each entry were planted at each location. The plots were laid out in a randomized complete block design, but with the randomization constrained so that COT67B and COT67B(-) plots were always adjacent to each other. This design allowed a more sensitive comparison between COT67B and COT67B(-). A number of other entries were also present in these trials, so that each trial comprised a total of seven entries. However, the analysis presented here was restricted to the three genotypes of interest, COT67B, COT67B(-) and Coker 312.

C.3. Agronomic Practices

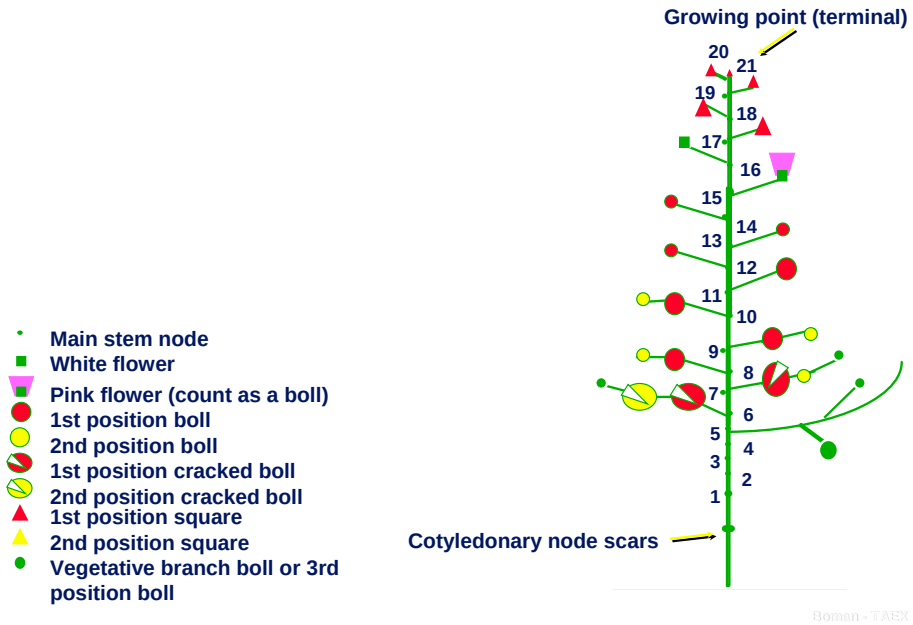
Standard agronomic practices for cotton production at each location were used to maintain the plants. Weeds were controlled by mechanical cultivation, hand-weeding, use of residual herbicides at planting and/ or use of post-directed herbicide applications. All trials were treated with commercially available insecticides as needed to control all insect pests, including Lepidoptera to ensure the intrinsic agronomic and phenotypic characteristics of the plants were observed without confounding by potentially different responses of the plants to insect feeding.

C.4. Relative Growth Rates and Plant Development

Height-to-node ratios were recorded at two developmental stages, 1) early flowering and 2) pre-harvest after boll maturation. At each time, five plants were selected at random from each plot. The height of each plant (in inches) was measured, and the total number of main stem nodes present on the plant counted. (Appendix 4A-Figure1) illustrates the

nomenclature of structures on a typical cotton plant). The height-to-node ratio was derived by dividing the height by the number of nodes on each plant, and the mean height-to-node ratio for all five plants in the plot calculated.

Appendix 4A-Figure 1. Nomenclature of structures on a cotton plant



The node position of the first fruiting branch was also determined pre-harvest after boll maturation. Five plants were sampled at random per plot and the node that gives rise to the first fruiting branch on each plant was recorded (see Appendix 4.A.-Table 1). Vegetative branches were not included. The mean node position of the first fruiting branch was then calculated.

The number of days to 50% flowering was estimated by making an assessment of when 50% of the plants in each plot had at least one white flower. Similarly, the number of days to 50% open boll was estimated by assessing when 50% of the harvestable bolls in each plot were open.

C.5. Yield

At the end of the season, each two-row plot was harvested and the seed cotton weighed. The yield of seed cotton per acre was calculated by multiplying the yield from each plot by the appropriate scaling factor. For example, trials planted with 38" row spacing comprise 13,762 running feet per acre. Thus, the yield per acre was calculated by multiplying the yield per plot (2 x 40 ft rows) by 13,762/80. Each batch of seed cotton was then processed using a Dennis Manufacturing 20 saw table-top laboratory gin to

separate the lint from the cotton seed. The lint turnout is expressed as the weight of lint after ginning as a percentage of the weight of seed cotton before ginning.

C.6. Fiber Quality Parameters

The ginned lint samples were sent to the USDA cotton classing office in Florence, South Carolina for HVI fiber analysis. HVI analysis returns data for fiber micronaire, length, strength and uniformity

C.7. Statistical Analysis

Data were subjected to analysis of variance across locations using the model

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

where Y_{ijk} is the observed response for event i in location j block k, U is the overall mean, T_i is the event effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within location, LT_{ij} is the location x event interaction effect and e_{ijk} is the residual error.

The statistical significance of the event effect was determined using a standard F-test. An F-test probability of ≤ 0.05 indicates that the difference between events was statistically significant at the customary 0.05 level. An F-test was also used to assess the significance of the location x event interaction. A statistically significant interaction suggests that the effect of event varied from one location to another.

D. Results

D.1. Relative Growth Rates and Plant Development

Appendix 4.A.-Tables 2 through 6 present the mean height-to-node ratios at early flowering and preharvest after boll maturation, the position of first fruiting branch, and days to 50% flowering and 50% open boll of COT67B, its null isoline and Coker 312 at each location assessed. As expected, there were sometimes noticeable differences in the performance of the plants grown at different locations, reflecting the different environmental conditions at different locations. However, in all cases, there was no evidence of any location x genotype interaction i.e., there was no evidence that the relative performance of the genotypes differed according to location (F-test probability for location x event interaction is always > 0.05). Accordingly, it was considered appropriate to calculate the mean data for each genotype across all locations, to maximize the sensitivity of the comparisons between the genotypes. In all cases, there was no evidence of any genuine differences among the genotypes (F-test probability for event is always > 0.05).

Appendix 4.A.-Table 2. Comparison of mean height-to-node ratios at early flowering of COT67B, COT67B(-) and Coker 312 at seven locations in summer 2004

	Location							
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Vero Beach, FL**	**Newport, AR**	**Quitman, GA**	**Winnsboro, LA (2)**	**Bossier City, LA**	**Mean**
Coker 312	2.10	1.66	1.56	0.97	3.35	1.82	1.57	1.86
COT67B	2.05	1.70	1.51	0.98	3.45	1.83	1.66	1.88
COT67B(-)	2.06	1.57	1.53	1.00	3.20	1.92	1.54	1.83
F-test probability for genotype x location interaction p≤0.05								0.91
F-test probability for genotypes p≤0.05								0.58

Appendix 4.A.-Table 3. Comparison of mean height-to-node ratios preharvest after boll maturation of COT67B, COT67B(-) and Coker 312 at six locations in summer 2004

	Location[1]						
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Vero Beach, FL**	**Newport, AR**	**Winnsboro, LA (2)**	**Bossier City, LA**	**Mean**
Coker 312	2.58	2.15	1.82	1.66	2.90	2.59	2.28
COT67B	2.50	2.13	2.01	1.65	2.88	2.62	2.29
COT67B(-)	2.41	2.04	1.85	1.57	3.15	2.53	2.26
F-test probability for genotype x location interaction p≤0.05							0.30
F-test probability for genotypes p≤0.05							0.77

[1] Data representative of all locations except Quitman, GA due to adverse weather and field conditions at time of evaluation

Appendix 4.A.-Table 4. Comparison of mean node position of the first fruiting branch of COT67B, COT67B(-) and Coker 312 at six locations in summer 2004

	Location[1]						
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Vero Beach, FL**	**Newport, AR**	**Winnsboro, LA (2)**	**Bossier City, LA**	**Mean**
Coker 312	4.80	5.05	5.50	5.05	6.05	5.00	5.24
COT67B	4.95	4.75	5.90	4.80	4.20	5.10	4.95
COT67B(-)	4.85	4.55	5.55	4.90	3.70	4.65	4.70
F-test probability for genotype x location interaction p≤0.05							0.14
F-test probability for genotypes p≤0.05							0.07

[1] Data representative of all locations except Quitman, GA due to adverse weather and field conditions at time of evaluation

Appendix 4.A.-Table 5. Comparison of mean days to 50% flowering of COT67B, COT67B(-) and Coker 312 at three locations in summer 2004

	Location[1]			
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Vero Beach, FL**	**Mean**
Coker 312	62.00	57.75	63.00	60.92
COT67B	62.00	57.25	65.50	61.58
COT67B(-)	62.50	58.25	65.00	61.92
F-test probability for genotype x location interaction p≤0.05				0.21
F-test probability for genotypes p≤0.05				0.17

[1] Data representative of Leland, MS, Winnsboro, LA and Vero Beach, FL due to adverse weather and field conditions at time of evaluation at other locations

Appendix 4.A.-Table 6. Comparison of mean days to 50% open boll of COT67B, COT67B(-) and Coker 312 at three locations in summer 2004

	Location[1,2]			
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Quitman, GA**	**Mean**
Coker 312	129.00	120.25	132.50	127.25
COT67B	129.50	123.25	134.75	129.17
COT67B(-)	129.75	120.00	135.50	128.42
F-test probability for genotype x location interaction p≤0.05				0.71
F-test probability for genotypes p≤0.05				0.40

[1] Data representative of Leland, MS, and both Winnsboro, LA locations due to adverse weather and field conditions at time of evaluation at the other locations.

D.2. Yield

Tables 4.A.7 and 8 present the mean seed cotton yield and mean percent lint turnout for COT67B, COT67B(-) and Coker 312 for each location assessed. There was no evidence of a genotype x location interaction for the seed cotton yield data, so that the mean data for each genotype across all locations were calculated. Analysis of these mean data revealed evidence of significant differences among the genotypes (F-test probability of differences among genotypes = 0.003). Significant differences are indicated by letters in Table 7. The data suggest that the yield of seed cotton from Coker 312 was significantly higher than either COT67B or COT67B(-). There was no evidence of any genuine difference between COT67B and COT67B(-).

Appendix 4.A.-Table 7. Comparison of mean seed cotton yield (lbs/acre) of COT67B, COT67B(-) and Coker 312 at five locations in summer 2004

	Location[1]					
Genotype	Leland, MS	Winnsboro, LA (1)	Newport, AR	Winnsboro, LA (2)	Bossier City, LA	Mean[2]
Coker 312	3638.45	1919.65	2265.38	2114.68	3365.25	2660.68 a
COT67B	2544.05	2021.28	2032.23	2090.90	3271.50	2391.99 b
COT67B(-)	3115.90	1519.60	1836.95	1803.85	2908.00	2236.86 b
F-test probability for genotype x location interaction p≤0.05						0.06
F-test probability for genotypes p≤0.05						0.003

[1] Yield data was not taken at Vero Beach, FL or Quitman, GA

[2] Means followed by different letters are significantly different at p<0.05 level

In the case of the percent lint turnout data, there was evidence that the relative performance of Coker 312 depended on the location at which it was grown. There was no other indication of any genotype x location interactions. Accordingly, the mean Coker 312 data were excluded from the across location analysis. This analysis revealed no evidence of any genuine difference between COT67B and COT67B(-).

Appendix 4.A.-Table 8. Comparison of mean percent lint turnout of COT67B, COT67B(-) and Coker 312 at three locations in summer 2004

	Location[1]			
Genotype	Leland, MS	Winnsboro, LA (2	Bossier City, LA	Mean[2]
Coker 312	40.55	43.93	45.69	43.39
COT67B	40.00	44.16	42.60	42.25
COT67B(-)	41.60	43.90	42.61	42.70
F-test probability for genotype x location interaction p≤0.05				0.24
F-test probability for genotypes excluding Coker 312 p≤0.05				0.39

[1] Percent lint turnout was not determined at one Winnsboro, LA location, Vero Beach, FL, Newport AR or Quitman, GA

[2] Coker 312 mean data were excluded from the across location analysis

D.3. Fiber Quality Data

Appendix 4.A.-Tables 9 through 12 present the comparison of fiber micronaire, length, strength and uniformity for COT67B,COT67B(-), and Coker 312 There was no evidence of any genotype x location interaction for the micronaire or strength data, but there was evidence for such an interaction in the length and uniformity data. However, in both cases the significance of the interaction was largely a consequence of the very low levels of plot-to-plot variation in the data rather than a consequence of any major inconsistency in performance from one location to another, and so averaging over locations was still considered meaningful. In the case of the fiber micronaire, length and uniformity data, there was no evidence of any genuine differences between any of the genotypes. In contrast, analysis of the fiber strength data provided some evidence of differences among the genotypes, specifically a small, but statistically significant increase in the fiber

strength of COT67B compared COT67B(-). Neither genotype was significantly different from Coker 312.

Appendix 4.A.-Table 9. Comparison of mean fiber micronaire values for COT67B, COT67B(-) and Coker 312 grown at grown at Leland, MS, and both Winnsboro, LA locations in summer 2004

	Location			
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Winnsboro, LA (2)**	**Mean**
Coker 312	4.28	4.48	4.50	4.42
COT67B	4.08	4.28	4.38	4.24
COT67B(-)	4.13	4.28	4.30	4.23
F-test probability for genotype x location interaction p≤0.05				0.98
F-test probability for genotypes p≤0.05				0.07

Appendix 4.A.-Table 10. Comparison of mean fiber length (inches) for COT67B, its null isoline and Coker 312 grown at grown at Leland, MS, Winnsboro, LA(1) and Quitman, GA in summer 2004

	Location			
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Winnsboro, LA (2)**	**Mean**
Coker 312	1.25	1.19	1.19	1.21
COT67B	1.25	1.21	1.16	1.21
COT67B(-)	1.25	1.21	1.17	1.21
F-test probability for genotype x location interaction p≤0.05				0.03
F-test probability for genotypes p≤0.05				0.76

Appendix 4.A.-Table 11. Comparison of mean fiber strength (grams/tex) for COT67B, its null isoline and Coker 312 grown at grown at Leland, MS, and both Winnsboro, LA locations in summer 2004

	Location			
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Winnsboro, LA (2)**	**Mean[1]**
Coker 312	30.15	28.85	27.98	28.99 ab
COT67B	31.30	29.60	28.20	29.7 a
COT67B(-)	29.53	29.00	27.75	28.76 b
F-test probability for genotype x location interaction p≤0.05				0.62
F-test probability for genotypes p≤0.05				0.03

[1] Means followed by different letters are significantly different at the 5% level

Appendix 4.A.-Table 12. Comparison of mean fiber uniformity index for COT67B, its null isoline and Coker 312 grown at grown at Leland, MS, and both Winnsboro, LA locations in summer 2004

	Location			
Genotype	**Leland, MS**	**Winnsboro, LA (1)**	**Winnsboro, LA (2)**	**Mean**
Coker 312	84.00	81.50	82.75	82.75
COT67B	84.50	82.75	81.25	82.83
COT67B(-)	84.00	82.25	82.00	82.75
F-test probability for genotype x location interaction p≤0.05				0.01
F-test probability for genotypes p≤0.05				0.94

E. Discussion

During the 2004 field season, data was collected to assess whether event COT67B had been altered from COT67B(-) or Coker 312 such that its potential to be a plant pest or weed had been increased. Field trials were conducted at seven locations representative of the U.S. cotton belt and eleven different plant growth, yield and fiber quality characteristics were evaluated. Statistically significant differences between COT67B and the control genotypes were few, not consistent across locations and not considered biologically meaningful. Therefore, there is no evidence that insertion of the *flcry1Ab* transgene into event COT67B has altered the growth habit or fiber quality of the plant and consequently, its pest/weed potential has not increased in comparison to nontransgenic cotton.

APPENDIX 4.B. SUMMARY OF 2005 COT67B AGRONOMIC TESTING RESULTS

A. Summary

During the 2005 planting season Delta and Pine Land Company evaluated the agronomic characteristics of Event COT67B. The locations chosen for the experimental program were intended to represent the various environments and insect pressures of the US cotton belt. However, at least one and in some cases two tropical weather systems passed over or near plots in Mississippi, South Carolina, and Georgia causing wind and water damage as the bolls began to open. In addition, the trials were planted several weeks later than normal for cotton in the US. Nonetheless, statistically valid comparisons between the agronomic characteristics of COT67B, its null isoline, COT67B(-) and Coker 312 could be drawn from the data. These results support the conclusion that COT67B, other than tolerance to feeding by lepidopteran insect pests, is agronomically equivalent to its null isoline and the conventional cotton variety Coker 312 and does not represent a plant pest risk.

B. Materials and Methods

Prior to planting, all seed were treated with commercial seed treatments containing both fungicides and insecticides. Fungicidal treatments consisted of Dynasty™ CST providing the following active ingredients (rates): azoxystrobin (8.82g ai/CWT), fludioxynil (1.40g ai/CWT), and mefenoxam (4.34 g ai/CWT.) Insecticidal treatments for thrips and aphid control consisted of thiomethoxam (Cruiser®@ 0.34 mg/kernel) applied to all seeds prior to planting.

All plots were planted under USDA notification number 05-034-02n between June 14 and June 18, 2005 using cone planting equipment. Specific planting dates by location were as follows: Estill, SC (06-16-05) Hartsville, SC (06-15-05) Red Springs, NC (06-14-05) Winterville, MS (06-18-05), Tifton, GA (06-17-05 and Verona, MS (06-16-05.) Seeds were packaged for planting prior to shipment to the various locations. Two-hundred seeds per packet (one row) were packaged enabling stands from 3.5 to 4 plants per row foot to be obtained in all trials. Plots consisted of two rows per plot, four replicates per variety, and finished plots were 35 feet in length with 38-inch row spacing. Trials were designed as randomized complete blocks to allow for proper statistical analysis.

All plots maintained reproductive isolation via the use of at least sixteen border rows. Border rows were planted using DP 491 in the same seed density as the test plots.

Typical management inputs including pre-plant applications of nitrogen fertilizers at rates ranging from 70-110 pounds per acre, pre-plant incorporation of residual herbicides for grass and weed control, lay-by applications of various residual herbicides and timely in-season applications of growth regulators (mepiquat chloride) were used as needed in this trial series. Some local modifications were made to management depending on location, trial type, and/or local custom as needed. Weed management consisted of a

comprehensive program of pre-plant burn-down herbicides and both pre and post-emergence in-season herbicide applications in combination with hand weeding, as needed, throughout the season.

Since agronomic evaluation was the primary goal of these plots, they were protected from insects in a manner similar to conventional cotton. Lepidopteran pests consisting primarily of *Helicoverpa zea* (Boddie) and *Spodoptera frugiperda* (J.E. Smith) were controlled at all sites using university extension thresholds and applications of pyrethroid insecticides (ex. Karate - lambda cyhalothrin), Sucking pests were effectively excluded in all locations via the use of selective sprays. Lygus/sucking pest control was achieved by using standard scouting procedures followed by timely applications of acephate, acetamiprid, and/or dicrotophos.

The sampling techniques included:

1. Plant monitoring – Stands, emergence, plant mapping as appropriate, monitoring for aberrant characteristics. Stands were measured by sampling the center 10 feet of each plot row for total emergence and plant establishment. A total of twenty plants per tested line per location (5 per plot/rep with 4 replicates per location) were sampled for all plant monitoring measurements.
2. Insect monitoring – In all locations notes were made assessing the infestations of various insect species in the plots.
3. Yield – Machine picked seed cotton yields and percent lint turnout after ginning were determined from the Estill, SC, Hartsville, SC, Winterville, MS and Tifton, GA locations.
4. Fiber Quality – The quality of the fiber was evaluated from all harvested plots to assess fiber maturity, length, strength, micronaire, uniformity and elongation.
5. Seed Productivity – Samples of undelinted, fuzzy seed were identity preserved as individual samples from each replicate, plot, and location (via post ginning seed subsamples) from the Estill, SC, Hartsville, SC, Winterville, MS and Tifton, GA locations where ginning, percent turnout and plant stand evaluations were available. These data enabled the calculation of seed produced per plant as described in.Figure 4.B 1.
6. Seed quality – Seed from the Estill, SC, Hartsville, SC, Winterville, MS and Tifton, GA locations was evaluated in several tests to assess overall seed quality and vigor. Seed germination and seedling vigor are important characteristics to assess whether COT67B seed has the potential to be more persistent in the environment than COT67B(-) and Coker 312. Standardized germination assays of the Association of Official Seed Analysts (AOSA, 1998) are used as a baseline to measure the germination potential and vigor of cottonseed. These tests were conducted on seed collected from the Estill and Hartsville, SC, Tifton GA and Winterville, MS trials. For each of the following tests, four replicates of 50 seed per plot per location were

evaluated following the methods of the Association of Official Seed Analysts (AOSA, 1998).

7. Four-day scoring on standard germination test conducted at alternating temperatures of 20°C/30°C (68°F/86°F). All seedlings longer than 1.5 inches considered germinated.
8. Seven and nine day scoring of the standard germination test; all seedlings longer than 1.5 inches considered germinated.
9. Stressed germination test at 18°C (64.4°F); a seven-day scoring of germination. All seedlings longer than 1.5 inches counted.
10. Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).
11. Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).
12. Vigor Index as calculated by 4 day warm germination + cool germ = seed index.

C. Statistical Analysis

Statistical analyses were conducted using JMP software (SAS institute, 2001) by subjecting the data to typical ANOVA procedures via the appropriate model(s). Means were separated using LSD (Gomex and Gomez; 1984.)

- Data represented in Tables 4.B.4 - 14 were analyzed first as randomized complete blocks to obtain within location analyses as needed. Where data balance allowed, an across location analysis was conducted to obtain the data presented in Tables 4.B.1 - 3.
- Data presented in Tables 4.B.15 - 21 were analyzed as single locations via ANOVA. Data are presented by location and an across location mean is presented within the data table. However, no statistics are associated with the associated mean.
- Data presented in Table 4.B.22 are from an analysis conducted from yield data collected at the Estill, SC, Hartsville, SC, Winterville, MS and Tifton, GA locations. Analysis of these data was conducted using JMP and a statistical model accounting for the blocking effects within each location, which allowed generation of an LSMEAN across locations. (Note; the Verona, MS location (which contained a subset of the tested lines) was omitted from this analysis to allow adequate balance for statistical analysis. The Red Springs, NC site was omitted because adverse weather conditions impacted the ability to appropriately analyze yield and yield data was not collected from the Haskell, TX location.)

- Data represented in Table 4.B.23 are also analyzed as blocked replicated sites. Seed remnants from each location were identity preserved and taken to the laboratory, by replicate, for further analysis. All components of the randomized complete block trial design were preserved in testing procedure, data collection, and data analysis, which allowed for an analysis accounting for blocking effects across the four locations of data. LSMEANs were generated and the results are presented in Table 4.B.23.

D. Results and Discussion

D.1. Plant Mapping and Monitoring

Plant mapping and monitoring were performed in all locations to evaluate the appropriate agronomic characters. Early square, early bloom, and late bloom plant mapping measurements were made during the season. The one exception was the Haskell, TX location at which early square measurements were not taken due to adverse field conditions. The data was analyzed across locations and appropriate LSMEANS are presented in Tables 4.B.1 through 3. Tables 4.B.4 through 14 present the actual data by location.

As stated previously, late planting and numerous tropical weather systems complicated the agronomic evaluations from 2006. However, meaningful conclusions can be drawn from the data including:

- No statistically significant differences were measured in height, total nodes #, vegetative nodes #, fruiting node #, and height to node ratios when comparing COT67B, to either COT67B(-), or Coker 312 (Table 4.B.1).
- In evaluating the early bloom data (Table 4.B.2), the same trends remain with COT67B and COT67B(-) not being significantly different. However, Coker 312 does show minor differences in height and total node number as compared to either COT67B or COT67B(-).
- In evaluating the late bloom data (Table 4.B.3) some differences begin to manifest themselves for various parameters. However, although statistically significant, these differences were small and likely have little biological meaning in terms of plant pest or weed potential.
- The plant mapping and monitoring data presented by location are presented in Tables 4.B.4 through 14. These data demonstrate few statistically significant differences between COT67B and COT67B(-), which were small and not consistent across locations. More differences between COT67B and Coker 312 were demonstrated, however, these differences were also small and demonstrate the tendency of Coker 312 to be somewhat more vegetative in its growth habit than does COT67B.

D.2. Yields

Machine picked yield for the Estill, SC, Hartsville, SC, Winterville, MS and Tifton, GA locations are presented in Table 4.B.21. Yield results were complicated considerably by the late planting and adverse tropical weather, which impacted the ability to analyze yield from the Red Springs, NC location and yield data was not collected from the Haskell, TX location. However, on average across four locations, Coker 312, COT67B, and COT67B(-) demonstrate no significant difference in yield potential.

D.3. Fiber Quality

From the ginning data, estimates of percent lint turnout were obtained (Table 4.B.22). Lint samples were submitted to High Volume Instrumentation (HVI) testing for fiber characteristics/quality evaluations. Many of these qualities are directly genetically controlled and some are heavily influenced by environment but they are all useful in the agronomic evaluation of a target transgene for negative effects. Tables 4.B.16 through 20 present the various fiber quality characters for each location individually and on average across locations. These data were analyzed within each location and a mean is presented across the six locations. Table 4.B.21 presents a summary of relevant fiber quality characteristics across six of the testing locations (the Verona, MS location (which contained a subset of the tested lines) was omitted from this analysis to allow adequate balance for statistical analysis). This data was analyzed across locations while statistically preserving the blocking effects within locations, which allowed a mean for each parameter to be presented in Table 4.B.21.

D.3.a. In summary

- Percent Lint Turnout defined as the ratio of seed, fiber, and trash weight harvested to the weight of lint actually recovered as fiber after ginning. – Table 4.B.22 – No statistically significant differences in percent lint turnout were observed across the testing locations.
- Maturity as calculated from the HVI generated micronaire values –Tables 4.B.15 and 21 – No statistically significant differences in maturity were observed across the testing locations.
- Micronaire defined as a measure of the surface area of cotton lint fibers (Hake et. al. 1996) –Tables 4.B.16 and 21 – Only in one location (Tifton, GA; Table 4.B.16) were significant differences in micronaire values documented. No statistically significant difference in micronaire was observed in the across locations analysis from Table 4.B.21.
- Length defined as the measured length of the fibers to the nearest 32nd of an inch (Hake et. al. 1996) –Tables 4.B.17 and 21 – Only in one location (Winterville, MS; Table 4.B.17) were significant differences in length measurable. However, in the across location analysis, COT67B did demonstrate shorter fiber than did either Coker 312 or COT67B(-) (Appendix 4.B.-Table 21). In the case of length as a fiber characteristic, it is heavily

influenced by varietal background and breeding efforts, so this will likely prove to not be a difficulty for COT67B as a component of a stacked product.

- Strength defined as the relative strength of one tex of cotton fibers with a tex being the mass in grams of 1000 meters of fiber (Hake et. al. 1996) –Tables 4.B.18 and 21. No statistically significant differences in strength were observed.
- Uniformity defined as an estimation of the short fiber content in a given cotton fiber sample. This is calculated as the ratio of the average length of all fibers divided by the average length of the longer half of the fibers (Hake et. al. 1996) –Tables 4.B.19 and 21. Only in one location (Winterville, MS; Table 4.B.19) did statistically valid differences in fiber uniformity become measurable. In the across location analysis (Table 4.B.21) both COT67B and COT67B(-) were similar in uniformity and both demonstrated lower uniformity than the Coker 312 comparison.
- Elongation defined as the extentisibility or alternatively, the elongation of cotton fibers at a breaking load (Reily 1997) –Tables 4.B.20 and 21. No statistically significant differences in fiber elongation were observed.

D.4. Seed productivity

Estimates were made for various parameters evaluating the relative seed productivity of the targeted regulated events. Between, COT67B, COT67B(-) and Coker 312 no statistically significant difference in seed production was observed in either seed weight generated or actual seed numbers produced (Table 4.B.22)

D.5. Seed quality

The seed generated in evaluating seed productivity were identity preserved and moved into evaluations of seed quality by tested line, location, and plot/replicate, which allowed a statistically valid comparison of the seed qualities involved. As measured by the typical battery of seed quality evaluations used by D&PL, no statistically significant differences were observed for the quality parameters measured across COT67B, COT67B(-) and Coker 312 (Table 4.B.23.)

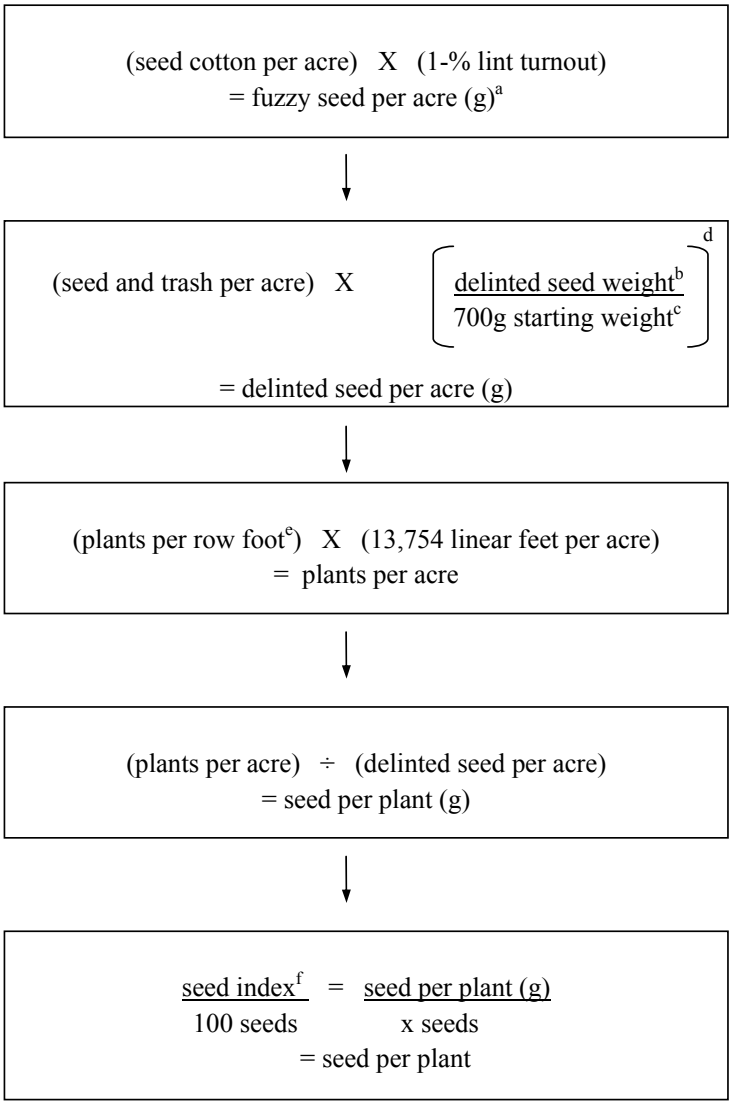
D.6. Insect Monitoring

In many locations, insect evaluations were made for several purposes. Across our testing, we observed that many of the transgenic entries demonstrated significant levels of insecticidal efficacy, particularly toward *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera spp*., *Trichoplusia ni*, and *Pseudoplusia includens*. Evaluations were also made as to potential impacts on nontarget, non-pestiferous, and/or beneficial insects. These observations were primarily in regards to *Orius spp*., various *Chrysopa spp*., and several species of beneficial Coccinellid beetles. However, no impacts on any nontarget insect species were identified at these testing locations.

E. Conclusions

Across the 2005 COT67B evaluation program no aberrant agronomic characteristics were observed that could lead to an unintended increase in plant survival or seed production and thus, COT67B does not pose any more of a plant pest risk than nontransgenic cotton genotypes with regard to these characteristics.

Appendix 4.B-Figure 1. Formulae for calculating seed productivity per plant using the data for seed cotton yield, lint yield, lint turnout, delinting turnout, final plant stand and seed index measurements



[a] fuzzy seed includes leaf and other plant material adhering to fibers left on seed after ginning
[b] weight of seed after acid delinting removes remaining fibers and plant material
[c] 700g = weight of fuzzy seed before acid delinting
[d] delinted seed weight ÷ 700g fuzzy seed starting weight = delinting turnout
[e] plants per row foot calculated from actual stand count
[f] seed index = weight of 100 acid delinted seeds

Appendix 4.B-Table 1. Across location analysis of early square plant mapping information for COT67B, COT67b(-) and Coker 312 evaluated at six locations during 2005

Genotype	Height	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	18.1	9.4	4.7	4.6	1.94
COT67B(-)	17.2	9.3	4.6	4.7	1.83
Coker 312	18.1	9.5	4.8	4.8	1.90
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.133	0.521	0.104	0.761	0.189

[1] NS = Not significant

Appendix 4.B-Table 2. Across location analysis of early bloom plant mapping information for COT67B, its null isoline and Coker 312 evaluated at seven locations during 2005[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Date of First Flower	Height to Node Ratio
COT67B	29.3	13.9ab	4.6b	9.3	6.8	7-Aug	2.13
COT67B(-)	28.2	13.5b	4.6b	9.0	6.8	7-Aug	2.10
Coker 312	29.1	14.2a	4.8a	9.4	6.8	6-Aug	2.07
LSD (p≤0.05)	NS[2]	0.5	0.2	NS	NS	NS	NS
Variety p	0.317	0.034	0.007	0.203	0.990	0.549	0.480

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 3. Across location analysis of late bloom plant mapping information for COT67B, its null isoline and Coker 312 evaluated at seven locations during 2005[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Height to Node Ratio
COT67B	33.3	15.6b	4.6b	11.0	2.8b	2.13
COT67B(-)	33.5	16.1a	4.8ab	11.3	3.4a	2.08
Coker 312	35.0	16.2a	4.9a	11.3	3.3a	2.16
LSD (p≤0.05)	NS[2]	0.4	0.2	NS	0.3	NS
Variety p	0.136	0.024	0.032	0.236	0.004	0.342

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 4. Analysis of early square plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Estill, SC (date of evaluation July 21, 2005)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height:Node Ratio
COT67B	24.7	9.4	4.7	4.7	2.61
COT67B(-)	23.7	9.7	4.6	5.2	2.44
Coker 312	24.5	9.4	4.6	4.8	2.61
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.607	0.407	0.529	0.115	0.371

[1] NS = Not significant

Appendix 4.B-Table 5. Analysis of early square plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Hartsville, SC (date of evaluation July 20, 2005)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height:Node Ratio
COT67B	16.0b	9.2ab	4.7	4.4	1.76
COT67B(-)	15.3b	9.0b	4.7	4.3	1.70
Coker 312	17.4a	9.7a	4.7	5.1	1.79
LSD (p≤0.05)	0.7	0.5	NS[1]	NS	NS
Variety p	0.001	0.024	0.835	0.062	0.203

[1] NS = Not significant

Appendix 4.B-Table 6. Analysis of early square plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Red Springs, SC (date of evaluation July 22, 2005)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height:Node Ratio
COT67B	13.7	9.5	4.7b	4.8	1.44
COT67B(-)	12.6	9.3	4.6b	4.7	1.36
Coker 312	12.4	9.5	5.1a	4.4	1.31
LSD (p≤0.05)	NS[2]	NS	0.3	NS	NS
Variety p	0.488	0.893	0.021	0.577	0.423

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 7. Analysis of early bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Estill, SC (date of evaluation August 8, 2005)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Date of First Flower	Height:Node Ratio
COT67B	33.5	12.9	4.5	8.5	6.7a	5-Aug	2.59
COT67B(-)	31.9	12.4	4.3	8.2	6.2b	4-Aug	2.57
Coker 312	32.6	13.0	4.7	8.3	6.9a	5-Aug	2.52
LSD (p≤0.05)	NS[2]	NS	NS	NS	0.2	NS	NS
Variety p	0.342	0.442	0.086	0.780	0.001	0.503	0.723

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 8. Analysis of early bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Hartsville, SC (date of evaluation August 10, 2005)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Date of First Flower	Height:Node Ratio
COT67B	29.6	13.0b	4.3	8.7	6.4	5-Aug	2.27
COT67B(-)	28.8	13.3ab	4.6	8.7	6.5	5-Aug	2.18
Coker 312	32.4	14.2a	4.6	9.6	6.9	3-Aug	2.29
LSD (p≤0.05)	NS[2]	0.9	NS	NS	NS	NS	NS
Variety p	0.248	0.038	0.329	0.052	0.176	0.582	0.672

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 9. Analysis of early bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Red Springs, NC (date of evaluation August 9, 2005)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Date of First Flower	Height:Node Ratio
COT67B	24.7	13.9	4.3	9.6	6.5	2-Aug	1.78
COT67B(-)	24.3	13.8	4.5	9.3	6.6	2-Aug	1.77
Coker 312	22.5	13.8	4.6	9.2	6.4	2-Aug	1.63
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	NS
Variety p	0.474	0.940	0.058	0.531	0.815	0.536	0.286

[1] NS = Not significant

Appendix 4.B-Table 10. Analysis of early bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Haskell, TX (date of evaluation August 23, 2005)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Date of First Flower	Height:Node Ratio
COT67B	29.5	15.8	5.5a	10.3	7.6	17-Aug	1.87
COT67B(-)	27.7	14.7	5.0b	9.7	7.8	17-Aug	1.89
Coker 312	28.9	15.9	5.5a	10.4	7.0	14-Aug	1.83
LSD (p≤0.05)	NS[2]	NS	0.3	NS	NS	NS	NS
Variety p	0.436	0.244	0.010	0.573	0.557	0.300	0.813

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 11. Analysis of late bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in in Estill, SC (date of evaluation August 25, 2005)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Height:Node Ratio
COT67B	37.6	14.8	4.5	10.2	2.6	2.56
COT67B(-)	38.7	15.5	4.6	11.0	3.1	2.49
Coker 312	40.2	15.3	4.5	10.8	3.1	2.63
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS
Variety p	0.323	0.482	0.886	0.464	0.118	0.237

[1] NS = Not significant

Appendix 4.B-Table 12. Analysis of late bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in in Hartsville, SC (date of evaluation August 29, 2005)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Height:Node Ratio
COT67B	38.9	16.9b	4.5b	12.4	3.9	2.31
COT67B(-)	39.4	17.4ab	5.0a	12.4	4.5	2.27
Coker 312	43.3	18.1a	5.3a	12.8	4.2	2.40
LSD (p≤0.05)	NS[2]	0.8	0.4	NS	NS	NS
Variety p	0.271	0.025	0.009	0.391	0.271	0.668

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 13. Analysis of late bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Red Springs, NC (date of evaluation August 24, 2005)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Height:Node Ratio
COT67B	24.9	14.8b	4.4	10.4	3.3	1.69
COT67B(-)	25.9	14.8b	4.3	10.5	3.5	1.75
Coker 312	27.2	16.0a	4.8	11.2	3.9	1.71
LSD (p≤0.05)	NS[2]	1.0	NS	NS	NS	NS
Variety p	0.385	0.044	0.104	0.303	0.351	0.837

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 14. Analysis of late bloom plant monitoring information from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trial in Haskell, TX (date of Evaluation September 16, 2005)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Nodes Above White Flower	Height:Node Ratio
COT67B	31.8	16.2b	5.2	11.0b	1.6b	1.97
COT67B(-)	30.2	16.6a	5.3	11.3a	2.4a	1.83
Coker 312	29.5	15.6c	5.0	10.6c	1.9b	1.90
LSD (p≤0.05)	NS[2]	0.2	NS	0.1	0.4	NS
Variety p	0.140	<.0001	0.125	<.0001	0.009	0.067

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 15. Relative fiber maturity from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trials

	Location						
Genotype	Estill, SC	Hartsville, SC	Red Springs, NC	Winterville, MS	Tifton, GA	Verona, MS	Average
COT67B	85.00	84.75	86.00	86.25	83.25	86.25	85.25
COT67B(-)	84.75	84.75	86.25	84.75	82.50	86	84.83
Coker 312	84.25	84.75	85.50	84.75	83.00	-	84.45
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	-
Variety p	0.5946	1	0.5853	0.1042	0.0723	0.6376	-

[1] NS = Not significant

Appendix 4.B-Table 16. Micronaire from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trials[1]

	Location						
Genotype	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Winterville, MS**	**Tifton, GA**	**Verona, MS**	**Average**
COT67B	3.88	4.25	4.43	4.28	3.60a	4.4	4.14
COT67B(-)	3.85	4.20	4.53	4.10	3.33b	4.38	4.07
Coker 312	3.88	4.30	4.23	3.98	3.55ab	-	3.99
LSD ($p \leq 0.05$)	NS[2]	NS	NS	NS	0.21	NS	-
Variety p	0.961	0.4851	0.607	0.1959	0.0392	0.7888	-

[1] Means followed by different letters are significantly different at $p \leq 0.05$
[2] NS = Not significant

Appendix 4.B-Table 17. Fiber length from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trials[1]

	Location						
Genotype	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Winterville, MS**	**Tifton, GA**	**Verona, MS**	**Average**
COT67B	1.19	1.19	1.12	1.23a	1.22	1.2	1.19
COT67B(-)	1.17	1.16	1.07	1.14b	1.13	1.2	1.15
Coker 312	1.16	1.19	1.11	1.20a	1.21	-	1.17
LSD ($p \leq 0.05$)	NS[2]	NS	NS	0.05	NS	NS	-
Variety p	0.2298	0.1878	0.1905	0.0163	0.0571	0.8361	-

[1] Means followed by different letters are significantly different at $p \leq 0.05$
[2] NS = Not significant

Appendix 4.B-Table 18. Fiber strength (g/tex) from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trials

	Location						
Genotype	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Winterville, MS**	**Tifton, GA**	**Verona, MS**	**Average**
COT67B	31.45	31.30	31.41	31.83	30.00	32.25	31.37
COT67B(-)	31.30	31.30	30.13	30.70	30.40	32.95	31.13
Coker 312	31.38	32.03	30.88	31.73	30.05	-	31.21
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	-
Variety p	0.3385	0.4095	0.1422	0.4706	0.751	0.1801	-

[1] NS = Not significant

Appendix 4.B-Table 19. Percent fiber uniformity from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trials[1]

	Location						
Genotype	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Winterville, MS**	**Tifton, GA**	**Verona, MS**	**Average**
COT67B	83.50	83.55	83.25	85.63a	83.65	84.8	84.06
COT67B(-)	83.98	83.88	82.63	82.65c	82.88	84.45	83.41
Coker 312	82.83	83.88	83.33	84.45b	83.03	-	83.50
LSD (p≤0.05)	NS[2]	NS	NS	2.12	NS	NS	-
Variety p	0.4575	0.7585	0.6956	0.0372	0.4604	0.4161	-

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.B-Table 20. Percent fiber elongation from 2005 COT67B, COT67B(-) and Coker 312 agronomic evaluation trials

	Location						
Genotype	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Winterville, MS**	**Tifton, GA**	**Verona, MS**	**Average**
COT67B	11.70	13.25	12.23	11.58	12.95	11.68	12.23
COT67B(-)	11.90	13.03	12.10	12.70	13.05	12.10	12.48
Coker 312	12.48	13.38	12.33	12.08	13.10	-	12.67
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	-
Variety p	0.4658	0.8315	0.6956	0.0698	0.7542	0.4161	-

[1] NS = Not significant

Appendix 4.B-Table 21. Across location fiber quality characteristic analysis for COT67B, COT67B(-)and Coker 312 evaluated at six locations during 2005[1]

Genotype	Maturity	Micronaire	Length (inches)	Strength (g/tex)	Fiber Uniformity Index	% Elongation
COT67B	85.00	4.05	1.19a	31.19	83.93a	12.35
COT67B(-)	84.60	4.00	1.13b	30.97	83.00b	12.56
Coker 312	84.45	3.99	1.17a	30.93	83.50ab	12.67
LSD ($p \leq 0.05$)	NS[2]	NS	0.023	NS	0.623	NS
Variety p	0.11	0.3524	<0.0001	0.57	0.0059	0.4716

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Appendix 4.B-Table 22. Lint yield and seed productivity analysis for COT67B, COT67B(-)and Coker 312 evaluated at four locations during 2005[1]

Location	Genotype	Seed Cotton/ Acre	% Lint Turn Out	Lint/ Acre	Delinted Weight	% Delinting Turnout	Plants/ RF	Seed Index	Seed/ Plant (g)	Seed/ Plant
Estill, SC	COT67B	1214	32.6	394	549	78.4	3.26	7.06	6.55	92.01
	COT67B(-)	1277	32.5	416	528	75.4	3.19	7.43	6.72	90.59
	Coker 312	1378	32.7	450	538	76.9	3.05	8.02	8.12	99.41
Hartsville, SC	COT67B	1274	33.3	417	530	75.7	2.64	7.41	8.17	109.65
	COT67B(-)	1393	33.1	453	545	77.9	2.49	8.10	9.73	120.26
	Coker 312	1503	32.3	492	555	79.3	2.65	8.74	10.05	114.76
Tifton, GA	COT67B	2029	29.6	600	546	78.0	3.25	7.53	11.28	149.85
	COT67B(-)	1911	29.0	557	555	79.3	3.35	8.09	10.73	132.25
	Coker 312	1827	28.0	515	550	78.6	2.98	8.50	11.72	137.32
Winterville, MS	COT67B	1103	28.7	320	580	82.8	3.36	8.98	6.32	70.44
	COT67B(-)	1256	28.6	359	565	80.6	2.73	8.65	8.76	101.58
	Coker 312	859	27.1	232	526	75.1	3.26	9.10	4.83	53.12
LSD (p≤0.05)		NS[2]	NS	NS	NS	NS	NS	NS	NS	NS
Loc x Var p		0.0849	0.9057	0.1163	0.1572	0.5242	0.1141	0.1141	0.1014	0.0698
Average Across Locations	COT67B	1405	31.06	433	551	78.7a	3.13	7.74	8.08	105.49
	COT67B(-)	1459	30.81	446	548	78.3a	2.94	8.07	8.98	111.17
	Coker 312	1392	30.05	422	542	77.5b	2.98	8.60	8.68	101.15
LSD (p≤0.05)		NS	NS	NS	NS	0.51	NS	NS	NS	NS
Variety p		0.6123	0.1710	0.06461	0.6636	0.0021	0.2041	0.2041	0.3769	0.3532

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

NO CBI

Appendix 4.B-Table 23. Seed quality analysis from D&PL 2005 VipCot Agronomic Evaluation Trials[1]

Location	Genotype	4-Day Germination[2]	7 and 9-Day Germination[3]	Cool Germination[4]	Abnormal Germination[5]	Cool & Abnormal Germination[6]	Vigor Index[7]
Estill, SC	COT67B	44.88	48.00	31.75	19.63	15.00	76.63
	COT67B(-)	48.25	53.50	41.00	12.38	20.50	89.25
	Coker 312	49.63	56.13	37.88	15.75	23.00	87.50
Hartsville, SC	COT67B	44.00	53.38	33.38	10.25	17.75	77.38
	COT67B(-)	52.38	59.75	35.88	20.75	19.63	88.25
	Coker 312	49.38	64.00	38.75	16.75	17.63	88.13
Tifton, GA	COT67B	57.63	66.13	43.88	11.25	13.13	101.50
	COT67B(-)	63.25	74.00	51.25	12.50	16.13	114.50
	Coker 312	47.13	54.63	35.75	21.00	21.50	82.88
Winterville, MS	COT67B	57.00	63.75	41.13	10.25	16.00	98.13
	COT67B(-)	60.38	65.88	52.88	8.50	15.63	113.25
	Coker 312	63.50	66.88	44.63	7.25	14.25	108.13
LSD (p≤0.05)		NS[8]	NS	NS	5.85	NS	NS
Loc x Var p		0.5614	0.3317	0.5723	0.0005	0.2798	0.6222
Across Location Average	COT67B	50.88	57.81	37.53	12.84	15.47	88.40
	COT67B(-)	56.06	63.28	45.25	13.53	17.97	101.31
	Coker 312	52.41	60.41	39.25	15.19	19.09	91.65
LSD (p≤0.05)		NS	NS	NS	NS	NS	NS
Variety p		0.3961	0.3954	0.091	0.2203	0.113	0.1801

[1] Means followed by different letters are significantly different at p≤0.05

[2] 4-day count on standard germination test conducted at alternating temperatures of 20°C/30°C; 68°F/86°F; all seedlings longer than 1.5 inches counted.

[3] 7/9 day scoring of the standard germination test; all seedlings longer than 1.5 inches counted.

[4] Stressed germination at 18°C (64.4°F); 7-day reading of germination; all seedlings longer than 1.5 inches counted.

[5] Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[6] Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[7] Vigor Index as calculated by 4 day warm germination + cool germ = seed index.[8] NS = Not significant

NO CBI

APPENDIX 4.C. SUMMARY OF 2006 TESTING RESULTS

A. Summary

During the 2006 planting season Delta and Pine Land Company evaluated the agronomic characteristics of Event COT67B cotton. The locations chosen for the experimental program were intended to represent the various environments and insect pressures of the U.S. cotton belt. Statistically valid comparisons between the agronomic characteristics of COT67B, its null isoline and Coker 312 that could potentially influence plant pest potential were made from the data These results support the conclusion that COT67B cotton, other than tolerance to feeding by lepidopteran insect pests, is agronomically equivalent to its null isoline and the conventional cotton variety Coker 312 and does not represent a plant pest risk.

B. Materials and Methods

Prior to planting, all seed were treated with commercial seed treatments containing both fungicides and insecticides. Fungicidal treatments consisted of Dynasty™ CST providing the following active ingredients (rates): azozystrobin (8.82g ai/CWT), fludioxynil (1.40g ai/CWT), and mefenoxam (4.34 g ai/CWT.) Insecticidal treatments for thrips and aphid control consisted of thiomethoxam (Cruiser®@ 0.34 mg/kernel) applied to all seeds prior to planting.

All plots were planted under USDA notification number 06-039-16n between May 18, 2006 and June 18, 2006 using cone planting equipment. Specific planting dates by location were as follows: Alexandria, LA (05-18-06), Belle Mina, AL (05-18-06), College Station, TX (05-18-06), Estill, SC(05-18-06) Hartsville, SC (05-23-06), Haskell, TX (05-19-06), Red Springs, NC (06-14-06), Winterville, MS(06-18-06), Tifton, GA (06-08-06) and Verona, MS (05-23-06.) Seeds were packaged for planting prior to shipment to the various locations. Two-hundred seeds per packet (one row) were packaged enabling stands from 3.5 to 4 plants per row foot to be obtained in all trials. Plots consisted of 2 rows per plot, 4 replicates per variety, and finished plots 35 feet in length with 38-inch row spacing. Trials were designed as randomized complete blocks to allow for proper statistical analysis.

All plots maintained reproductive isolation via the use of at least sixteen border rows. Border rows were planted using DP 491in the same seed density as the test plots.

Typical management inputs including pre-plant applications of nitrogen fertilizers at rates ranging from 70-110 pounds per acre, pre-plant incorporation of residual herbicides for grass and weed control, lay-by applications of various residual herbicides and timely in-season applications of growth regulators (mepiquat chloride) were used as needed in this trial series. Some local modifications were made to management depending on location, trial type, and/or local custom as needed. Weed management consisted of a comprehensive program of pre-plant burn-down herbicides and both pre and post-

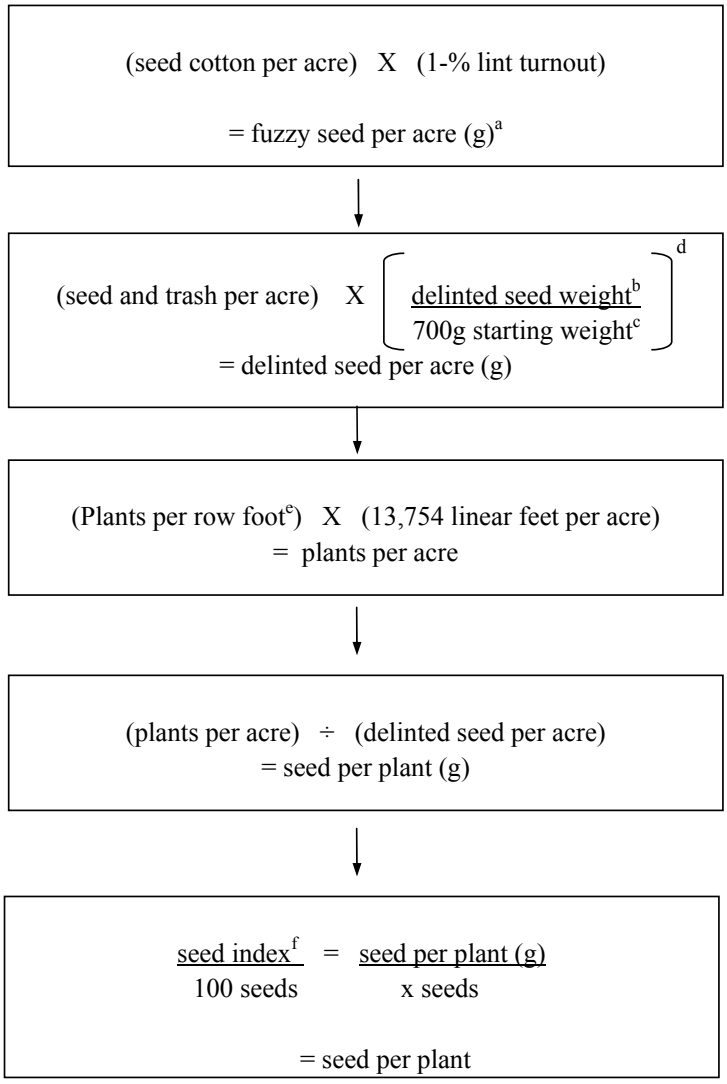
emergence in-season herbicide applications in combination with hand weeding, as needed, throughout the season.

Since agronomic evaluation was the primary goal of these plots, they were protected from insects in a manner similar to conventional cotton. Lepidopteran pests consisting primarily of *Helicoverpa zea* (Boddie) and *Spodoptera frugiperda* (J.E. Smith) were controlled at all sites using university extension thresholds and applications of pyrethroid insecticides (ex. Karate - lambda cyhalothrin), Sucking pests were effectively excluded in all locations via the use of selective sprays. Lygus/sucking pest control was achieved by using standard scouting procedures followed by timely applications of acephate, acetamiprid, and/or dicrotophos.

The sampling techniques used in various locations were to include:

1. Plant monitoring – Stands, emergence, plant mapping as appropriate, monitoring for aberrant characteristics. Stands were measured by sampling the center 10 feet of each plot row for total emergence and plant establishment. A total of twenty plants per tested line per location (5 per plot/rep with 4 replicates per location) were sampled for all plant monitoring measurements.
2. Insect monitoring – In all locations notes were made accessing the infestations of various insect species in the plots.
3. Yield – Machine picked seed cotton yields and percent lint turnout after ginning were determined from the Estill, SC, Hartsville, SC, Winterville, MS and Tifton, GA locations.
4. Fiber Quality – The quality of the fiber was evaluated from all harvested plots to assess fiber maturity, length, strength, micronaire, uniformity and elongation.
5. Seed Productivity – Samples of undelinted, fuzzy seed were identity preserved as individual samples from each replicate, plot, and location (via post ginning seed subsamples) from nine locations where ginning, percent turnout and plant stand evaluations were available. These data enabled the calculation of seed produced plant as described in Figure 4.C.1.

Appendix 4.C-Figure 1. Formulae for calculating seed productivity per plant using the data for seed cotton yield, lint yield, lint turnout, delinting turnout, final plant stand and seed index measurements



[a] fuzzy seed includes leaf and other plant material adhering to fibers left on seed after ginning

[b] weight of seed after acid delinting removes remaining fibers and plant material

[c] 700g = weight of fuzzy seed before acid delinting

[d] delinted seed weight ÷ 700g fuzzy seed starting weight = delinting turnout = 78% which is the average delinting turnout in 2005

[e] plants per row foot calculated from actual stand count

6. Seed quality – Seed from nine locations was evaluated in tests to assess overall seed quality and vigor. Seed germination and seedling vigor are important characteristics to assess whether COT67B cottonseed has the potential to be more persistent in the environment than COT67B(-) and Coker 312. Standardized germination, vigor and dormancy assays of the Association of Official Seed Analysts (AOSA, 1998) were used as a baseline to measure the germination potential and vigor of cottonseed. For each of the following tests, four replicates of 50 seed per plot per location were evaluated following the methods of the Association of Official Seed Analysts (AOSA, 1998).
    1) Four-day scoring on standard germination test conducted at alternating temperatures of 20°C/30°C (68°F/86°F). All seedlings longer than 1.5 inches considered germinated.
    2) Seven and nine day scoring of the standard germination test; all seedlings longer than 1.5 inches considered germinated.
    3) Stressed germination test at 18°C (64.4°F); a seven-day scoring of germination. All seedlings longer than 1.5 inches counted.
    4) Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).
    5) Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).
    6) Vigor Index as calculated by 4 day warm germination + cool germ = seed index.
    7) Hard seed have not imbibed water and are hard to cut with a razor

B.1. Statistical Analysis

All data in Tables 4.C.1 through 16 were subjected to various types of appropriate statistical analysis as dictated by the data type. The analysis was conducted using JMP (SAS institute, 2001) and by subjecting the data to typical ANOVA procedures via the appropriate model(s) using a statistical model accounting for the blocking effects within each location. In effect, replicates within a site remain nested within that site for analysis purposes, allowing the generation of an LSMEAN across locations. Included with this analysis are the across location LSD's. Means were separated using Least Significant Differences Test (Gomez 1984) where appropriate.

C. Results and Discussion

C.1. Plant Mapping and Monitoring

Plant mapping and monitoring were performed in all locations to evaluate the appropriate agronomic characters. Early square, early bloom, late bloom, and final plant mapping measurements were made during the season. The data were analyzed across locations and appropriate LSMEANS are presented in Tables 4.C.1 through 4. Individual location data are presented in Tables 4.C.5 through 31

The 2006 testing environment allowed for excellent evaluations of the agronomic characteristics of COT67B in comparison to COT67B(-) and Coker 312 across a wide variety of environments.

In summary:

Early square (Table 4.C.1) - no statistically significant differences ($p \leq 0.05$) were measured in height, height to node ratio and total number of nodes, vegetative nodes and fruiting nodes when comparing COT67B, to either COT67B(-) or Coker 312 across eight locations.

Early bloom (Table 4.C.2) - Across all eight locations, no statistically significant differences ($p \leq 0.05$) were measured between COT67B and COT67B(-) for the six characteristics measured (total nodes, fruiting nodes and nodes above white flower). COT67B was statistically different from Coker 312 only in plant height. Although statistically significant, this difference was small and has little biological meaning in terms of plant weed or pest potential.

Late bloom (Table 4.C.3) - COT67B was significantly different ($p \leq 0.05$) from COT67B(-) only for nodes above white flower and significantly different from Coker 312 for plant height, number of vegetative nodes and height to node ratio. Although statistically significant, these differences were also small and likely have little biological meaning in terms of plant weed or pest potential.

Pre-harvest (Table 4.C.4) – Statistically significant differences ($p \leq 0.05$) between COT67B and COT67B(-) were observed for plant height, height to node ratio and date to 50% open boll. Statistically significant differences between COT67B and Coker 312 were observed for number of vegetative bolls, height to node ratio and date to 50% open boll. These differences were again small and likely have little biological meaning in terms of plant weed or pest potential. In addition, the results demonstrate the tendency for Coker 312 to be more vegetative than COT67B in growth habit as measured by plant height, height to node ratio, and the allocation of nodes to vegetative vs. reproductive growth.

Across the entire group of trials Coker 312 demonstrates the tendency to be somewhat more vegetative in growth habit as measured by plant height, height to node ratio, and the allocation of nodes to vegetative vs. reproductive growth than do either COT67B or COT67B(-).

The plant mapping and monitoring data presented by location is presented in Tables 4.C.5 through 31. These data demonstrate few statistically significant differences between COT67B and COT67B(-) and/or Coker 312, which were small and not consistent across locations. Moreover differences between COT67B and Coker 312 demonstrated, the tendency of Coker 312 to be somewhat more vegetative in it's growth habit than COT67B.

C.2. Yields

Machine picked yields were taken in all locations and are presented in Table 32. On average across nine locations, Coker 312 and COT67B(-) demonstrate no significant difference in yield potential while COT67B shows significantly lower yields than either of the other tested lines. This is likely a maturity/determinanacy effect and has a high likelihood of being influenced by breeding and selection.

C.3. Seed Productivity

Estimates were made for various parameters evaluating the relative seed productivity of COT67B, COT67B(-) and Coker 312. The results of this testing are presented in Table 32. COT67B differed statistically from COT67B(-) and Coker 312 in terms of lint yield. However, COT67B was typically intermediate of the two control genotypes for the other plant and seed productivity characteristics measured. Consequently, it can be concluded from these results that insertion and expression of the *flcry1Ab* gene in COT67B did not result in an unintended increase in plant survival or seed production and thus, does not pose any more of a plant pest risk than nontransgenic cotton genotypes with regard to these characteristics.

C.4. Seed Quality

Seed germination, viability and dormancy characteristics was measured using a battery of seven germination tests typically performed by the cottonseed industry. The same germination tests were performed in 2005, but in 2006 an evaluation of the percentage hard seed was also performed to further address the potential for dormancy. Delinted seed from eight locations was identity preserved by genotype, location, and plot/replicate to permit a statistically valid comparison of the seed qualities evaluated.

Across all locations there were no statistically significant differences between COT67B and Coker 312 for any of seven quality parameters measured (Table 4.C.33). Only in the nine-day standard germination test did a statistically significant difference appear between COT67B and COT67B(-) but while this difference is statistically significant, it is relatively small and likely biologically irrelevant.

C.5. Fiber Quality

As plots were harvested, seed cotton sub-samples were collected for ginning. From the ginning data, we were able to obtain estimates of lint turnout. Lint samples were submitted to High Volume Instrumentation (HVI) testing for fiber characteristics/quality evaluations. Many of these qualities are directly genetically controlled and some are

heavily influenced by environment but they are all useful in the agronomic evaluation of a target transgene for negative effects. Tables 4.C.34-40 present the various lint turnout and fiber quality characters for each location individually and on average across locations.

In summary:

- Maturity - COT67B and COT67B(-)demonstrated significantly greater fiber maturity than Coker 312 across the testing locations (Tables 4.C.34 and 35).
- Micronaire - COT67B and COT67B(-) demonstrated significantly lower fiber micronaire than Coker 312 (Tables 4.C.34 and 36).
- Length - COT67B and COT67B(-), which were statistically similar, demonstrated significantly shorter fiber than Coker 312 (Tables 4.C.34 and 37).
- Strength - COT67B and COT67B(-), which were statistically similar, demonstrated significantly weaker fiber than Coker 312 (Tables 4.C.34 and 38).
- Uniformity - in the across location analysis, both COT67B and COT67B(-) were similar in uniformity and both demonstrated lower uniformity than the Coker 312 (Tables 4.C.34 and 39).
- Elongation - statistically significant differences in fiber elongation were observed as ranked from highest to lowest: COT67B, COT67B(-) and Coker 312 (Tables 4.C.34 and 40).

C.6. Insect Monitoring

In many locations, insect evaluations were made for several purposes. Across our testing, we observed that many of the transgenic entries demonstrated significant levels of insecticidal efficacy, particularly toward *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera spp*., *Trichoplusia ni*, and *Pseudoplusia includens*. Evaluations were also made as to potential impacts on non-target, non-pestiferous, and/or beneficial insects. These observations were primarily in regards to *Orius spp*., various *Chrysopa spp*., and several species of beneficial Coccinellid beetles. However, no impacts on any nontarget insect species were identified at these testing locations.

D. Conclusions

Across the 2006 COT67B evaluation program no aberrant agronomic characteristics were observed that could lead to an unintended increase in plant survival or seed production and thus, COT67B does not pose any more of a plant pest risk than nontransgenic cotton genotypes with regard to these characteristics.

Appendix 4.C-Table 1. Across location analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at eight locations during 2006

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	14.1	9.8	4.4	5.4	1.41
COT67B(-)	14.4	10.0	4.4	5.6	1.40
Coker 312	14.2	10.1	4.5	5.6	1.39
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.740	0.092	0.116	0.256	0.897

[1] NS = Not significant

Appendix 4.C-Table 2. Across location analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at eight locations during 2006[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	23.5b	13.8	4.3	9.5	1.69	6.4ab	Jul-13
COT67B(-)	23.9b	13.7	4.3	9.4	1.73	6.2b	Jul-12
Coker 312	24.8a	14.0	4.4	9.5	1.76	6.5a	Jul-13
LSD (p≤0.05)	0.8	NS[2]	NS	NS	NS	0.2	NS
Variety p	0.019	0.063	0.106	0.577	0.059	0.009	0.236

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 3. Across location analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at eight locations during 2006[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	28.4b	16.2	4.4b	11.9	1.73b	3.4a
COT67B(-)	27.8b	15.9	4.4b	11.6	1.73b	3.1b
Coker 312	29.8a	16.3	4.7a	11.6	1.82a	3.4a
LSD (p≤0.05)	1.3	NS[2]	0.1	NS	0.08	0.3
Variety p	0.015	0.266	≤.0001	0.343	0.044	0.044

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 4. Across location final (pre-harvest) plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at six locations during 2006[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Date of 50% Open Boll	Vegetative Bolls/ Plant
COT67B	31.6ab	18.5	4.5	14.0	1.71b	11.3a	29-Sep a	6.3b
COT67B(-)	30.6b	18.6	4.4	14.2	1.64b	8.8b	27-Sep b	4.4b
Coker 312	32.5a	18.1	4.5	13.6	1.78a	11.2a	25 Sep c	11.8a
LSD (p≤0.05)	1.4	NS[2]	NS	NS	0.06	1.1	1	3.4
Variety p	0.047	0.377	0.585	0.248	0.001	0.000	0.001	0.001

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 5. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Winterville, MS during 2006 (date of evaluation 7/3/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	22.2	12.1	5.1	7.0	1.85
COT67B(-)	20.7	11.9	5.0	6.9	1.74
Coker 312	21.8	12.7	5.2	7.5	1.75
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.444	0.384	0.201	0.612	0.680

[1] NS = Not significant

Appendix 4.C-Table 6. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Hartsville, SC during 2006 (date of evaluation 7/3/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	16.4	10.4	4.4	6.1	1.58
COT67B(-)	16.3	10.7	4.5	6.3	1.52
Coker 312	15.7	10.0	4.2	5.7	1.58
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.877	0.138	0.381	0.137	0.917

[1] NS = Not significant

Appendix 4.C-Table 7. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Estill, SC during 2006 (date of evaluation 6/28/06)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	13.3	9.2	4.2	5.0	1.44b
COT67B(-)	14.3	9.2	4.1	5.1	1.55a
Coker 312	13.4	9.6	4.3	5.3	1.40b
LSD (p≤0.05)	0.4	NS[2]	NS	NS	0.09
Variety p	0.002	0.189	0.058	0.266	0.019

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 8. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Red Springs, NC during 2006 (date of evaluation 7/7/06)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	12.1a	9.2b	4.2	5.0b	1.32a
COT67B(-)	9.5b	9.1b	4.1	5.0b	1.05b
Coker 312	12.1a	9.9a	4.0	5.9a	1.22a
LSD (p≤0.05)	1.3	0.4	NS[2]	0.3	0.13
Variety p	0.004	0.007	0.163	0.001	0.006

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 9. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Haskell, TX during 2006 (date of evaluation 6/22/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	6.7	7.4	4.0	3.4	0.90
COT67B(-)	7.5	7.7	4.2	3.5	0.97
Coker 312	7.6	8.0	4.0	4.0	0.96
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.182	0.526	0.308	0.414	0.212

[1] NS = Not significant

Appendix 4.C-Table 10. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Bella Mina, AL during 2006 (date of evaluation 7/7/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	13.5	10.4	4.9	5.5	1.29
COT67B(-)	14.4	10.7	4.6	6.1	1.35
Coker 312	13.9	10.6	4.5	6.1	1.30
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.746	0.748	0.112	0.052	0.748

[1] NS = Not significant

Appendix 4.C-Table 11. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at College Station, TX during 2006 (date of evaluation 7/7/06)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	16.7	11.3	4.4b	6.9	1.48
COT67B(-)	20.8	12.6	4.8b	7.8	1.65
Coker 312	18.0	11.8	5.8a	6.0	1.52
LSD (p≤0.05)	NS[2]	NS	0.8	NS	NS
Variety p	0.147	0.178	0.015	0.136	0.177

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 12. Analysis of early square plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Tifton, GA during 2006 (date of evaluation 7/11/06)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio
COT67B	11.8	8.2	4.1	4.1	1.43
COT67B(-)	11.7	8.4	4.2	4.2	1.40
Coker 312	11.7	8.2	4.2	4.0	1.43
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS
Variety p	0.972	0.776	0.748	0.792	0.629

[1] NS = Not significant

Appendix 4.C-Table 13. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Winterville, MS during 2006 (date of evaluation 7/18/06)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	36.6	16.0	5.3	10.8	2.29	7.7a	10-Jul
COT67B(-)	36.9	16.1	5.1	10.9	2.30	7.1b	8-Jul
Coker 312	37.7	16.5	5.4	11.1	2.29	7.5a	9-Jul
LSD (p≤0.05)	NS[2]	NS	NS	NS	NS	0.3	NS
Variety p	0.755	0.252	0.410	0.638	0.994	0.003	0.056

[1] Means followed by different letters are significantly different at p≤0.05
[2] NS = Not significant

Appendix 4.C-Table 14. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Hartsville, SC during 2006 (date of evaluation 7/14/06)[1]

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	19.8	12.8	4.1	8.8	1.55	7.0	10b-Jul
COT67B(-)	19.9	12.1	4.0	8.1	1.65	6.6	11b-Jul
Coker 312	19.9	12.4	4.2	8.2	1.61	7.2	12a-Jul
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	1
Variety p	0.999	0.263	0.345	0.262	0.715	0.406	0.016

[1] Means followed by different letters are significantly different at p≤0.05
[2] NS = Not significant

Appendix 4.C-Table 15. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Estill, SC during 2006 (date of evaluation 7/11/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	19.1	13.8	4.2	9.6	1.39	8.1	8-Jul
COT67B(-)	18.9	13.8	4.2	9.6	1.38	8.4	9-Jul
Coker 312	20.7	14.0	4.0	10.0	1.48	8.6	8-Jul
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	NS
Variety p	0.245	0.658	0.166	0.406	0.411	0.474	0.371

[1] NS = Not significant

Appendix 4.C-Table 16. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Red Springs, NC during 2006 (date of evaluation 7/26/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	15.7b	12.4	4.2	8.2	1.27	5.7	20-Jul
COT67B(-)	14.4b	11.8	4.2	7.7	1.23	5.5	21-Jul
Coker 312	17.0a	12.5	4.0	8.5	1.36	6.1	20-Jul
LSD (p≤0.05)	1.6	NS[1]	NS	0.6	NS	NS	NS
Variety p	0.022	0.092	0.120	0.029	0.099	0.088	0.503

[1] NS = Not significant

Appendix 4.C-Table 17. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Bella Mina, AL during 2006 (date of evaluation 7/26/06)

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	21.4	14.4	4.9	9.5	1.48	4.8	12-Jul
COT67B(-)	21.8	14.4	4.5	9.9	1.51	4.1	9-Jul
Coker 312	22.0	14.5	4.5	10.0	1.51	5.0	11-Jul
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	NS
Variety p	0.913	0.908	0.118	0.288	0.832	0.103	0.130

[1] NS = Not significant

Appendix 4.C-Table 18. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Haskell, TX during 2006 (date of evaluation 7/21/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	19.8	13.0	4.0	9.0b	1.53	3.6	7b-Jul
COT67B(-)	21.5	13.1	4.0	9.1ab	1.65	3.3	5a-Jul
Coker 312	21.5	13.4	4.0	9.4a	1.61	3.7	5a-Jul
LSD (p≤0.05)	1.4	NS[2]	NS	0.3	NS	NS	1
Variety p	0.037	0.196	0.947	0.028	0.052	0.338	0.046

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 19. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at College Station, TX during 2006 (date of evaluation 7/18/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	26.5	14.7	4.4b	10.3	1.81	7.6	11-Jul
COT67B(-)	28.5	15.1	4.8b	10.3	1.89	7.7	12-Jul
Coker 312	29.0	15.4	5.8a	9.7	1.88	7.9	14-Jul
LSD (p≤0.05)	NS[2]	NS	0.8	NS	NS	NS	NS
Variety p	0.343	0.295	0.015	0.534	0.683	0.510	0.417

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 20. Analysis of early bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Tifton, GA during 2006 (date of evaluation 8/1/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower	Date of First Bloom
COT67B	29.6	13.5	3.6	10.0a	2.20b	6.7	24-Jul
COT67B(-)	29.4	13.4	3.6	9.8ab	2.21b	6.7	24-Jul
Coker 312	30.4	13.1	3.7	9.4b	2.33a	6.3	24-Jul
LSD (p≤0.05)	NS[2]	NS	NS	0.4	0.06	NS	NS
Variety p	0.321	0.143	0.864	0.035	0.003	0.507	0.935

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 21. Analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Winterville, MS during 2006 (date of evaluation 8/1/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	43.0a	18.9	5.3a	13.6	2.28	4.6
COT67B(-)	40.6b	19.2	5.0b	14.2	2.11	4.4
Coker 312	43.5a	18.7	5.2ab	13.4	2.34	4.5
LSD (p≤0.05)	2.2	NS[2]	0.2	NS	NS	NS
Variety p	0.036	0.593	0.041	0.388	0.070	0.576

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 22. Analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Hartsville, SC during 2006 (date of evaluation 8/3/06).

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	25.7	16.0	4.1	11.9	1.61	3.3
COT67B(-)	25.1	16.1	4.0	12.1	1.56	3.0
Coker 312	25.7	16.6	4.2	12.4	1.55	3.8
LSD ($p \leq 0.05$)	NS[1]	NS	NS	NS	NS	NS
Variety p	0.900	0.485	0.244	0.514	0.860	0.276

[1] NS = Not significant

Appendix 4.C-Table 23. Analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Estill, SC during 2006 (date of evaluation 7/27/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	20.7	14.8a	4.1	10.8a	1.40c	5.1a
COT67B(-)	21.9	13.8c	4.1	9.7b	1.59b	4.3b
Coker 312	24.8	14.2b	4.2	10.0b	1.75a	4.5b
LSD ($p \leq 0.05$)	NS[2]	0.5	NS	0.5	0.06	0.2
Variety p	0.056	0.007	0.330	0.008	0.019	0.001

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Appendix 4.C-Table 24. Analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Red Springs, NC during 2006 (date of evaluation 8/8/06).

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	25.8	15.9	4.1	11.8	1.63	5.0
COT67B(-)	22.8	15.6	4.1	11.5	1.46	5.1
Coker 312	25.4	16.0	4.1	11.9	1.59	4.8
LSD ($p \leq 0.05$)	NS[1]	NS	NS	NS	NS	NS
Variety p	0.214	0.894	0.970	0.900	0.115	0.696

[1] NS = Not significant

Appendix 4.C-Table 25. Analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at Haskell, TX during 2006 (date of evaluation 8/8/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	22.2	15.5	4.4ab	11.2	1.44	n/a
COT67B(-)	22.1	15.1	4.1b	11.0	1.47	n/a
Coker 312	23.6	15.6	4.5a	11.1	1.51	n/a
LSD (p≤0.05)	NS[2]	NS	0.3	NS	NS	n/a
Varirty p	0.312	0.580	0.043	0.899	0.174	n/a

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 26. Analysis of late bloom plant monitoring information for COT67B, COT67B(-) and Coker 312 evaluated at College Station, TX during 2006 (date of evaluation 8/9/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Nodes Above White Flower
COT67B	33.2	16.4	4.4b	12.0	2.03	2.2
COT67B(-)	34.7	15.9	4.8b	11.1	2.18	1.5
Coker 312	36.1	16.7	6.1a	10.7	2.16	2.4
LSD (p≤0.05)	NS[2]	NS	0.9	NS	NS	NS
Variety p	0.575	0.235	0.012	0.121	0.557	0.215

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 27. Final plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at Winterville, MS during 2006 (date of evaluation 9/21/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Vegetative Bolls/ Plant	Date of 50% Open Boll
COT67B	32.2	19.3	n/a	15.2	1.67b	15.7	4.5	30-Sep
COT67B(-)	28.5	19.2	n/a	14.9	1.50c	8.9	4.3	26-Sep
Coker 312	33.6	18.5	n/a	14.0	1.81a	14.6	12.1	24-Sep
LSD (p≤0.05)	NS[2]	NS	n/a	NS	0.11	NS	NS	NS
Variety p	0.118	0.809	n/a	0.673	0.019	0.297	0.300	0.366

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 28. Final plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at Estill, SC during 2006 (date of evaluation 9/6/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Vegetative Bolls/ Plant	Date of 50% Open Boll
COT67B	27.1	17.9	4.1	13.8	1.51b	14.6a	9.6	12-Sep
COT67B(-)	25.8	19.0	4.1	14.9	1.36c	9.2b	9.3	12-Sep
Coker 312	26.4	16.2	4.0	12.2	1.63a	11.4ab	12.9	11-Sep
LSD (p≤0.05)	NS[2]	NS	NS	NS	0.11	3.4	NS	NS
Variety p	0.697	0.143	0.296	0.157	0.009	0.050	0.500	0.385

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 29. Final plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at Red Springs, NC during 2006 (date of evaluation 9/29/06).

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Vegetative Bolls/ Plant	Date of 50% Open Boll
COT67B	27.8	17.7	4.6	13.1	1.57	8.7	1.0	4-Oct
COT67B(-)	24.1	16.5	4.7	11.9	1.46	6.9	1.1	6-Oct
Coker 312	24.9	16.5	4.4	12.2	1.52	7.5	0.0	5-Oct
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	NS	NS
Variety p	0.277	0.271	0.483	0.257	0.682	0.407	0.668	0.800

[1] NS = Not significant

Appendix 4.C-Table 30. Final plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at Haskell, TX during 2006 (date of evaluation 9/28/06)[1].

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Vegetative Bolls/ Plant	Date of 50% Open Boll
COT67B	21.7	18.5	5.0	13.5	1.17	8.5	11.8ab	28a-Sep
COT67B(-)	23.9	18.8	4.7	14.1	1.27	6.4	3.2b	25b-Sep
Coker 312	23.6	18.5	4.6	13.9	1.28	10.1	18.8a	23b-Sep
LSD (p≤0.05)	NS[2]	NS	NS	NS	NS	NS	11.0	2
Variety p	0.072	0.858	0.055	0.464	0.086	0.071	0.036	0.001

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 31. Final plant monitoring summary for COT67B, COT67B(-) and Coker 312 evaluated at Tifton, GA during 2006 (date of evaluation 10/4/06).

Genotype	Height (inches)	Total Nodes	Vegetative Nodes	Fruiting Nodes	Height to Node Ratio	Bolls/ Plant	Vegetative Bolls/ Plant	Date of 50% Open Boll
COT67B	39.1	17.9	5.0	12.9	2.18	8.5	7.4	16-Oct
COT67B(-)	39.0	17.9	4.6	13.3	2.19	9.5	7.2	13-Oct
Coker 312	41.8	18.2	5.1	13.1	2.30	10.8	18.6	11-Oct
LSD (p≤0.05)	NS[1]	NS	NS	NS	NS	NS	NS	NS
Variety p	0.154	0.749	0.485	0.695	0.305	0.093	0.243	0.089

[1] NS = Not significant

Appendix 4.C-Table 32. Across location analysis of COT67B, COT67B(-) and Coker 312 yield and seed productivity parameters evaluated at nine locations during the summer of 2006[1,2].

Location	Variety	Seed Cotton/ Acre (lbs)	% Lint Turnout	Lint/ Acre (lbs)	Plants/ RF	Seed Index	Seed/ Plant-g	Seed/ Plant
Alexandria, LA	COT67B	1397	30.7	432	3.47	9,43	7.15	75.58
	COT67B(-)	2295	31.8	731	3.76	8.86	10.70	120.88
	Coker 312	2172	31.4	680	3.17	9.34	12.48	133.64
LSD (p≤0.05)		514.25	NS	178.08	NS	0.18	2.95	31.45
Variety p		0.0177	0.5243	0.0223	0.1149	0.0010	0.0200	0.0159
Belle Mina, AL	COT67B	1295	37.0	478	3.47	8.99	6.63	73.60
	COT67B(-)	1426	36.2	516	3.54	8.90	7.03	78.91
	Coker 312	1387	34.7	482	2.83	9.13	8.62	94.53
LSD (p≤0.05)		NS	0.78	NS	NS	NS	NS	NS
Variety p		0.2805	0.0052	0.4007	0.0995	0.1804	0.0648	0.0641
Tifton, GA	COT67B	2355	35.2	830	4.85	8.62	7.84	91.05
	COT67B(-)	2945	34.6	1018	4.70	8.33	10.12	121.64
	Coker 312	2879	34.7	998	4.38	8.88	10.63	119.63
LSD (p≤0.05)		266.20	NS	103.83	NS	0.19	NS	NS
Variety p		0.0055	0.4990	0.0140	0.7274	0.0020	0.1275	0.1192
College Station, TX	COT67B	1629	28.9	472	4.20	7.54	7.18	95.63
	COT67B(-)	2072	28.8	600	5.00	7.45	7.61	102.29
	Coker 312	1958	28.6	562	3.65	7.25	10.35	142.21
LSD (p≤0.05)		NS[3]	NS	NS	0.89	NS	NS	NS
Variety p		0.0844	0.6351	0.1233	0.0441	0.2391	0.1209	0.0916
Estill, SC	COT67B	1748	33.6	586	2.79	10.01	10.95	109.48
	COT67B(-)	1595	32.5	521	3.25	9.88	8.65	87.89
	Coker 312	1833	31.2	572	3.10	9.96	10.90	108.96
LSD (p≤0.05)		NS	NS	NS	NS	NS	NS	NS
Variety p		0.2845	0.1970	0.4459	0.6081	0.9202	0.4342	0.4772
Hartsville, SC	COT67B	2120	30.8	656	3.76	8.96	9.96	111.17

Location	Variety	Seed Cotton/ Acre (lbs)	% Lint Turnout	Lint/ Acre (lbs)	Plants/ RF	Seed Index	Seed/ Plant-g	Seed/ Plant
	COT67B(-)	1790	29.1	535	4.24	8.97	7.74	85.92
	Coker 312	2105	29.7	628	2.95	9.36	12.69	136.84
LSD (p≤0.05)		NS	NS	NS	0.35	NS	NS	NS
Variety p		0.7526	0.2839	0.7304	0.0006	0.1302	0.1491	0.1628
Red Springs, NC	COT67B	1805	35.2	635	2.80	9.04	10.83	119.86
	COT67B(-)	1720	35.1	596	3.09	8.51	9.26	108.50
	Coker 312	2300	33.1	760	2.93	8.74	13.66	156.15
LSD (p≤0.05)		NS	NS	NS	NS	NS	2.85	29.26
Variety p		0.1127	0.3350	0.1513	0.6595	0.1573	0.0397	0.0281
Verona, MS	COT67B	1690	34.8	586	3.15	8.78	9.05	103.33
	COT67B(-)	2066	34.0	703	3.50	7.64	10.40	136.20
	Coker 312	1964	32.9	646	3.08	8.40	11.10	132.36
LSD (p≤0.05)		NS	NS	NS	NS	0.46	NS	NS
Variety p		0.1374	0.1066	0.2358	0.4286	0.0046	0.2689	0.1981
Winterville, MS	COT67B	2682	30.6	821	3.33	9.75	14.81	152.34
	COT67B(-)	3648	31.5	1141	3.18	9.15	20.09	219.62
	Coker 312	3060	29.5	903	2.91	9.55	19.53	205.14
LSD (p≤0.05)		NS	1.26	NS	NS	0.26	NS	40.61
Variety p		0.1487	0.0378	0.0869	0.3890	0.0058	0.0517	0.0253
Average	COT67B	1858 b	32.8 a	611 b	3.53 b	9.01 a	10.15 a	111 a
	COT67B(-)	2173 a	32.6 a	707 a	3.81 a	8.65 b	8.47 b	97 b
	Coker 312	2184 a	31.7 b	692 a	3.22 c	8.95 a	10.96 a	121 a
Variety p		0.0006	0.003	0.0028	≤0.0001	0.0023	≤0.0001	≤0.0001
LSD		175	0.58	56	0.24	0.12	1.0	11.19
Variety x Location p		0.0542	0.3058	0.0220	0.3685	0.0023	0.0525	0.0158
LSD		NS	NS	169	NS	0.36	NS	33.57

[1]See Figure 1 for formulae to calculate seed per plant

[2] Means followed by different letters are significantly different at p≤0.05

[3] NS = Not significant

Appendix 4.C-Table 33. Seed germination, viability and dormancy characteristics for COT67B, COT67B(-) and Coker 312 seed collected from multiple locations during 2006[1]

Location	Variety	4-Day Germ-Std[1]	9-Day Germ-Std.[2]	Cool[3]	Abnormal[4]	Cool Abnormal[5]	Vigor Index[6]	Hard Seed
Belle Mina, AL	COT67B	86.4	92.3	81.2	6.5	10.6	178.7	0.4
	COT67B(-)	86.4	89.5	81.1	8.6	12.3	175.9	2.9
	Coker 312	91.9	93.3	83.5	6.0	9.3	185.1	0.8
Tifton, GA	COT67B	75.0	80.8	58.5	8.6	15.5	155.8	7.6
	COT67B(-)	75.1	79.5	64.0	11.0	14.1	154.6	6.8
	Coker 312	76.9	83.9	60.1	9.9	19.6	160.8	10.4
College Station, TX	COT67B	81.4	84.8	73.6	8.3	11.0	166.1	3.1
	COT67B(-)	82.00	85.6	67.1	8.3	16.9	167.6	9.3
	Coker 312	81.6	85.8	68.3	6.1	9.8	167.4	5.0
Estill, SC	COT67B	74.0	78.3	61.6	12.0	23.5	152.3	3.9
	COT67B(-)	74.0	76.6	63.0	11.1	22.1	150.6	0.8
	Coker 312	76.4	79.4	71.1	13.4	13.9	155.8	0.8
Hartsville, SC	COT67B	68.6	72.3	51.1	11.1	17.0	140.9	9.8
	COT67B(-)	64.9	66.3	51.9	11.9	15.1	131.1	4.9
	Coker 312	73.1	77.4	55.0	8.5	15.8	150.5	6.4
Red Springs, NC	COT67B	77.0	81.4	65.5	8.5	14.0	158.4	9.8
	COT67B(-)	77.25	80.3	63.4	10.1	14.0	157.5	6.9
	Coker 312	77.9	82.7	61.0	9.9	14.3	160.5	10.4
Verona, MS	COT67B	86.1	88.9	75.1	8.9	14.6	175.0	1.1
	COT67B(-)	80.8	83.6	66.1	11.5	17.8	164.4	1.3
	Coker 312	83.3	85.8	68.3	9.6	18.6	169.0	2.1
Winterville, MS	COT67B	84.9	88.1	79.5	6.1	9.9	173.0	1.3
	COT67B(-)	84.6	86.8	75.4	8.9	11.9	171.4	2.1
	Coker 312	83.4	85.4	78.1	9.1	10.0	168.8	1.5
LSD (p≤0.05)		NS[9]	NS	NS	NS	NS	NS	NS
Loc x Var p		0.7902	0.5616	0.0621	0.9238	0.0815	0.7091	0.0563
LSMEAN Across Locations	COT67B	79.2	83.3 a	68.3	8.8	14.5	162.5	4.6
	COT67B(-)	78.1	81.0 b	66.5	10.2	15.5	159.1	4.4
	Coker 312	80.6	84.2 a	68.2	9.1	13.9	164.7	4.7
LSD (p≤0.05)		NS	2.2	NS	NS	NS	NS	NS
Variety p		0.1294	0.0185	0.3035	0.2338	0.3040	0.0525	0.9005

Appendix 4.C-Table 33. Continued (captions)

[1] Means followed by different letters are significantly different at p≤0.05

[2] 4-day count on standard germination test conducted at alternating temperatures of 20°C/30°C; 68°F/86°F; all seedlings longer than 1.5 inches counted.

[3] 7/9 day scoring of the standard germination test, all seedlings longer than 1.5 inches counted.

[4] Stressed germination at 18°C (64.4°F); 7-day reading of germination; all seedlings longer than 1.5 inches counted.

[5] Number of malformed seedlings per 200 from the standard germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[6] Number of malformed seedlings per 200 from the cool germination test. Typical abnormalities include swollen hypocotyl; blunt radicle; missing cotyledon(s).

[7] Vigor Index as calculated by 4 day warm germination + cool germ = seed index.

[8] Hard seed have not imbibed water and are hard to cut with a razor

[9] NS = Not significant

Appendix 4.C-Table 34. Across location analysis of fiber quality characteristics for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1].

Across Locations[1]						
Genotype	**Maturity**	**Micronaire**	**Length (inches)**	**Strength (g/tex)**	**Length Uniformity Index**	**Percent Elongation**
COT67B	86.9b	4.43b	1.15b	31.48b	83.20b	10.75a
COT67B(-)	87.1b	4.41b	1.16b	31.54b	83.23b	10.45b
Coker 312	87.6a	4.53a	1.18a	32.30a	83.94a	10.16c
LSD (p≤0.05)	0.28	0.08	0.013	0.64	0.20	0.19
Variety p	<0.0001	0.0070	<0.0001	0.0212	0.0006	0.0031

[1]Means followed by different letters are significantly different at p≤0.05

Appendix 4.C-Table 35. Relative fiber maturity for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

	Locations									
Genotype	**Alexandria, LA**	**Belle Mina, AL**	**Tifton, GA**	**College Station, TX**	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Verona, MS**	**Winterville, MS**	**Across Locations**
COT67B	88.3	89.2	86.0	83.8	88.0	85.8	87.5	87.8	86.0	86.9 b
COT67B(-)	88.8	89.3	86.5	84.3	88.0	87.0	87.5	86.5	86.0	87.1 b
Coker 312	89.5	89.5	86.5	84.3	89.8	86.8	88.3	87.3	86.5	87.6 a
LSD ($p \leq 0.05$)	0.57	NS[2]	NS	NS	0.85	NS	NS	NS	NS	0.28
Variety p	0.0090	0.4540	0.5787	0.1250	0.0062	0.2441	0.2441	0.0670	0.1250	≤0.0001

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Appendix 4.C-Table 36. Fiber micronaire for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

	Locations									
Genotype	**Alexandria, LA**	**Belle Mina, AL**	**Tifton, GA**	**College Station, TX**	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Verona, MS**	**Winterville, MS**	**Across Locations**
COT67B	4.78	5.25	4.18	3.18	4.83	4.25b	4.70	4.53a	4.20b	4.43b
COT67B(-)	4.78	5.30	4.25	3.28	4.78	4.60a	4.53	3.98c	4.20b	4.41b
Coker 312	4.88	5.40	4.25	3.20	5.08	4.63a	4.70	4.23b	4.43a	4.53a
LSD ($p \leq 0.05$)	NS[2]	NS	NS	NS	NS	0.24	NS	0.24	0.15	0.08
Variety p	0.3644	0.1393	0.8842	0.5502	0.2243	0.0261	0.4219	0.0074	0.0275	0.0070

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Appendix 4.C-Table 37. Fiber length for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

NO CBI

Locations										
Genotype	**Alexandria, LA**	**Belle Mina, AL**	**Tifton, GA**	**College Station, TX**	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Verona, MS**	**Winterville, MS**	**Across Locations**
COT67B	1.13	1.09	1.20b	1.11	1.18	1.19	1.13b	1.10	1.18	1.15b
COT67B(-)	1.16	1.13	1.22b	1.09	1.19	1.17	1.18a	1.15	1.19	1.16b
Coker 312	1.16	1.12	1.26a	1.14	1.30	1.22	1.17a	1.15	1.22	1.18a
LSD ($p \leq 0.05$)	NS[2]	NS	0.03	NS	NS	NS	0.03	NS	NS	0.013
Variety p	0.0723	0.4493	0.0153	0.1673	0.6792	0.0994	0.0300	0.0591	0.0727	≤0.0001

[1]Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

Appendix 4.C-Table 38. Fiber strength (g/tex) for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

Locations										
Genotype	**Alexandria, LA**	**Belle Mina, AL**	**Tifton, GA**	**College Station, TX**	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Verona, MS**	**Winterville, MS**	**Across Locations**
COT67B	30.13	33.31	33.30	30.38	34.00	30.30	30.55	31.20	30.15	31.48b
COT67B(-)	31.38	33.06	32.60	30.45	33.53	30.23	32.03	30.30	30.30	31.54b
Coker 312	32.08	32.60	33.33	31.63	35.28	31.58	32.03	31.98	30.33	32.30a
LSD ($p \leq 0.05$)	NS[2]	NS	NS	NS	NS	NS	NS	NS	NS	0.64
Variety p	0.2513	0.7098	0.7836	0.1596	0.4686	0.4272	0.0576	0.3050	0.9560	0.0212

[1] Means followed by different letters are significantly different at $p \leq 0.05$

[2] NS = Not significant

NO CBI

Appendix 4.C-Table 39. Fiber length uniformity index for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

	Locations									
Genotype	**Alexandria, LA**	**Belle Mina, AL**	**Tifton, GA**	**College Station, TX**	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Verona, MS**	**Winterville, MS**	**Across Locations**
COT67B	81.90	83.66b	85.03	81.03	83.95	83.60	82.77	83.20	83.60	83.20b
COT67B(-)	82.45	84.68a	84.43	81.15	83.65	83.70	83.38	81.95	83.73	83.23b
Coker 312	82.43	82.28c	84.70	82.08	84.43	84.70	84.13	84.13	83.60	83.94a
LSD (p≤0.05)	NS[2]	0.41	NS	NS	NS	NS	NS	NS	NS	0.20
Variety p	0.1706	0.0123	0.6811	0.3851	0.2525	0.1014	0.0599	0.1196	0.9713	0.0006

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

Appendix 4.C-Table 40. Percent fiber elongation for COT67B, COT67B(-) and Coker 312 evaluated at nine locations during 2006[1]

	Locations									
Genotype	**Alexandria, LA**	**Belle Mina, AL**	**Tifton, GA**	**College Station, TX**	**Estill, SC**	**Hartsville, SC**	**Red Springs, NC**	**Verona, MS**	**Winterville, MS**	**Across Locations**
COT67B	10.28a	10.53a	11.00	10.40	10.48	11.63	10.90a	10.00	11.28	10.75 a
COT67B(-)	9.15b	10.48b	10.65	10.65	10.03	11.43	10.10b	10.03	10.90	10.45 b
Coker 312	8.35c	10.13c	11.05	10.40	9.68	11.53	9.80b	9.65	10.80	10.16 c
LSD (p≤0.05)	0.55	0.22	NS[2]	NS	NS	NS	0.55	NS	NS	0.19
Variety p	0.0009	0.0225	0.5841	0.7721	0.1197	0.8101	0.0177	0.1863	0.0585	0.0031

[1] Means followed by different letters are significantly different at p≤0.05

[2] NS = Not significant

NO CBI

APPENDIX 5. THE ENVIRONMENTAL SAFETY OF COT67B

A. Introduction

The purpose of this appendix is to summarize in greater detail than in Chapter 7 the data on the environmental safety of COT67B and to draw conclusions about the likely environmental impact of its cultivation, and in particular whether it poses a plant pest risk. The environmental safety of commercial cultivation of COT67B cotton is considered in two parts: the likelihood that COT67B will harm nontarget organisms, including species beneficial to agriculture and endangered and threatened species; and the likelihood that COT67B will become a serious weed of agriculture or non-agricultural habitats. Some data are relevant to more than one of these categories of risk; for example, identification of the routes of exposure of nontarget organisms to FLCry1Ab in COT67B requires an evaluation of the potential of COT67B to become a weed.

B. Assumptions and Objectives

A fundamental assumption of this chapter is that the cultivation of nontransgenic cotton poses no currently unacceptable environmental risks, and hence if it can be shown that COT67B does not increase those risks significantly, COT67B can be regarded as environmentally safe and not a plant pest. Throughout the chapter, therefore, the environmental risks of COT67B are assessed relative to conventional cotton, rather than as absolute risks.

The objectives of the environmental assessment are two-fold: to show that COT67B is highly unlikely to be more harmful to nontarget organisms than is conventional cotton; and to demonstrate that COT67B is highly unlikely to be weedy in agricultural habitats, or to be more invasive of non-agricultural habitats than is conventional cotton. If these objectives are met, we demonstrate the protection of two assessment endpoints. The first is the diversity and abundance of nontarget organisms within and outside cotton fields. This endpoint includes, but is not limited to, natural enemies of pests of cotton; pollinators; animals that ingest or are otherwise exposed to cotton or its derivatives; and plants that grow in habitats that could be invaded by cotton. The second endpoint is the yield of crops in which cotton is a potential weed. Protection of these endpoints ensures that we meet the objectives of several environmental protection statutes, including the Plant Protection Act and the Endangered Species Act.

C. The Safety of COT67B to Nontarget Organisms

This section evaluates the likelihood that nontarget organisms will be exposed to harmful amounts of toxic substances that may be present in COT67B cotton. The potential for harm to nontarget organisms arising from increased weediness is considered in Section G.

The safety assessment for nontarget organisms first compares the composition of COT67B with that of near-isogenic, nontransgenic cotton, and with cotton in general. The aim is identify potentially toxic substances in COT67B that show changed concentration relative to nontransgenic cotton. The routes of exposure of nontarget

organisms to these substances are then identified, followed by an assessment of their hazard (toxicity). The hazard and exposure data are then combined to assess the risk, defined as the likelihood that COT67B will be associated with reduced abundance or diversity of nontarget organisms (see Section F).

C.1. The Composition of COT67B

Studies of composition and nutritional quality (Chapter 5) support the conclusion that COT67B is substantially similar to conventional cotton, apart from the presence of FLCry1Ab, and the DNA insert required for its production. Estimates of numerous compositional parameters in field-grown COT67B cotton and its null-isoline were compared, and most showed no statistically significant difference associated with the presence and absence of the *flcry1Ab* transgene. For analytes that differed significantly between COT67B and the null isoline, the concentration of the analyte in COT67B was within the range found in other cotton varieties. Overall, the hypothesis that no biologically significant differences in composition or nutritional value exist between COT67B and nontransgenic cotton was corroborated. The results of the compositional analysis demonstrate that any risk from toxicity of COT67B to nontarget organisms will arise from exposure to FLCry1Ab (exposure to transgene DNA or RNA poses no safety concerns; US FDA, 1992).

C.2. Routes of Exposure of Nontarget Organisms to FLCry1Ab

C.2.a. Exposure levels via crop tissue

Expression of FLCry1Ab in COT67B were measured in field trials in Arkansas, Georgia, Louisiana and Mississippi in 2004 (Chapter 4). Concentrations of FLCry1Ab in various tissues at several developmental stages were determined by enzyme-linked immunosorbent assay (ELISA; Tijssen, 1985), and the extraction efficiency of the proteins was estimated[32]. Table 5.1 summarizes the concentration of FLCry1Ab in COT67B, corrected for extraction efficiency.

Nontarget organisms within cotton fields may be exposed to FLCry1Ab by consuming COT67B tissues directly, or by consuming prey that has eaten COT67B tissue. COT67B pollen contains FLCry1Ab, but off-crop exposure is unlikely because cotton pollen is not readily dispersed by wind since it is large and sticky (*e.g.*, Llewelyn and Fitt, 1996). Emasculated cotton flowers rarely set seed if pollinators are excluded (Khan and Afzal, 1950; Sidhu and Singh, 1961), and very few pollen grains are captured in pollen traps suspended within the crop (Khan and Afzal, 1950; Thies, 1953; Sidhu and Singh, 1961). Insectivorous birds may be exposed to FLCry1Ab *via* pollen adhering to the bodies of

[32] The Cry1Ab in COT67B is referred to as 'FLCry1Ab' throughout this Chapter. It is recognized, however, that studies in which ELISA was used to measure the concentrations of Cry protein (using a polyclonal anti-Cry1Ab antibody) cannot distinguish between intact, full-length Cry1Ab and smaller immunoreactive derivatives. Therefore, the concentrations of 'FLCry1Ab' measured by ELISA and reported herein are not necessarily solely FLCry1Ab, but may include smaller Cry1Ab polypeptides.

pollinators; however, it is unlikely that this represents a significant route of exposure to birds. First, honeybees tend to pack cotton pollen inefficiently because of its spiny shape (Vaissière and Vinson, 1994). Secondly, birds are not abundant in intensively managed cotton in the United States (Cederbaum *et al*., 2004). Finally, birds near cotton fields are more likely to be exposed to FLCry1Ab directly *via* consumption of cotton tissue (*e.g.*, US EPA, 2002), or by eating cotton pests containing cotton tissue (*e.g.*, Bottrell and Adkisson, 1977; Bohmfalk *et al*., 1996). Therefore significant exposure of nontarget organisms to FLCry1Ab through plant tissue is likely to be restricted to cotton fields.

C.2.b. Exposure via residues in soil

Plants exude proteins from their roots (*e.g.*, Rengel, 2002) and hence it is possible that COT67B will exude FLCry1Ab into the soil during cultivation. FLCry1Ab may also enter soil in plant debris during and immediately after harvest of COT67B, and if COT67B is not harvested for any reason.

Most proteins do not persist or accumulate in the soil because they are inherently degradable in soils with healthy microbial activity (*e.g.*, Burns, 1982; Marx *et al*., 2005). Laboratory soil degradation studies on (truncated) Cry1Ab, Cry1Ac, Cry1F, mCry3A, Cry3Bb and Cry34/35 in field-collected soils indicate that these proteins are degraded rapidly. The period for protein concentration or bioactivity to fall to half its initial value (the DT_{50}) is typically between 2 and 22 days (US EPA, 2001a,b.; US EPA, 2003; US EPA, 2005b,c.; US EPA, 2007).

The short DT_{50}s of proteins in laboratory soil degradation studies suggested that Cry proteins were unlikely to accumulate in soil following cultivation of crops expressing these proteins. The hypothesis was tested by Head *et al*. (2002) using cotton expressing Cry1Ac, and by Dubelman *et al*. (2005) using maize expressing Cry1Ab. Both studies tested for the presence of Cry protein in soil in which the respective transgenic crop had been cultivated for at least three years. In neither study was the protein detectable by sensitive-insect bioassay, corroborating the hypothesis that inherent degradability of a protein in the laboratory is a good predictor of lack of accumulation of the protein in the field. The study of Cry1Ac cotton is particularly informative for predicting the behaviour of FLCry1Ab in soil because Cry1Ac and FLCry1Ab are similar molecules: they are full-length pro-toxins; they contain a 26 amino acid sequence called the Geiser motif towards their C-terminus; and they share sequence identity over more than 400 amino acids (*ca*. 70%) of their active regions (Chapter 4).

Appendix 5-Table 1. Expression levels of FLCry1Ab in COT67B grown in Arkansas, Georgia, Louisiana and Mississippi in 2004

		Mean µg FLCry1Ab/g fresh weight – corrected for extraction efficiency				
Tissue		**Squaring**	**1st White Bloom**	**Peak Bloom**	**1st Open Boll**	**Pre-harvest**
Young Leaves	Highest site Mean all sites	142.01 91.78	60.48† 	44.56 38.20	26.85 21.07	NA
Old leaves	Highest site Mean all sites	60.50 47.95	62.25 55.36	69.00 54.52	68.31 45.45	NA
Roots	Highest site Mean all sites	14.80 11.18	9.59 9.12	12.31 8.95	6.33 4.97	NA
Flowers	Highest site	---	---	25.68‡	---	---
Pollen	Highest site	---	---	5.45‡	---	---
Bolls	Highest site Mean all sites	---	---	NA	10.75 8.93	NA
Seeds	Highest site Mean all sites	---	---	---	---	27.57 20.11
Whole Plants	Highest site Mean all sites	NA	NA	NA	NA	22.59 15.34

NA = Not Analyzed

‡ Tissues collected from Louisiana only

† Tissue collected from Arkansas only

--- Tissue not produced at this stage

The degradability of FLCry1Ab in soil was evaluated by applying FLCRY1AB-0103, a 77.3% pure preparation of microbially produced FLCry1Ab, to a sandy loam soil collected from a cotton-growing area of North Carolina. The test substance was dissolved in an aqueous buffer and applied at a concentration equivalent to 80 µg FLCry1Ab/g dry weight soil, which is approximately 440X the concentration of FLCry1Ab following complete incorporation of pre-harvest plants of COT67B into soil to a depth of 6 inches (15.25 cm) (see D.1.b. below). After incubation of the treated soil at 25^{o}C for a specified interval (0, 1, 3, 7, 14, 30, 62, 94 or 120 days), samples of soil were removed and stored in a freezer until analyzed.

The degradation of FLCry1Ab was measured with a bioassay of European corn borer (ECB). First instars were exposed to 10% weight by volume of treated soil in a commercial diet for rearing Lepidoptera. Mortality of the larvae was assessed after 5 days exposure to the soil. The percentage mortality of the larvae declined with the incubation time of the soil; the decline appeared to be exponential with first-order kinetics. The estimated DT_{50} for insecticidal activity of FLCry1Ab under the conditions of the test was 17 days. The results indicate that FLCry1Ab is inherently degradable. Hence, the persistence of FLCry1Ab in soil following cultivation of COT67B will be brief; FLCry1Ab is unlikely to accumulate in soil, and the spread of FLCry1Ab outside cotton fields *via* soil is likely to be minimal.

Another theoretical route of entry to the soil is horizontal gene flow of *flcry1Ab* leading to expression of FLCry1Ab in soil micro-organisms. Recent reviews (US EPA, 2001a,b.; Connor *et al*., 2003) conclude that there is minimal likelihood of horizontal gene transfer between transgenic plants and soil micro-organisms. Should *flcry1Ab* from COT67B be integrated into a plasmid or chromosome of a bacterium, FLCry1Ab is extremely unlikely to be produced because its plant-derived promoter is unlikely to function in bacteria. In addition, codon use in *flcry1Ab* is optimized for expression in plants, not bacteria. Therefore, FLCry1Ab is extremely unlikely to be produced in soil *via* horizontal gene transfer.

C.2.c. Exposure via volunteer weeds

Volunteer cotton can occur through the persistence of perennial plants after harvest or the germination of spilled seed (Hennebury *et al*, 2003; Stewart *et al*., 2003). Cotton volunteers are most common where a failed cotton crop is replanted to soybeans; the volunteers do not reduce yield, but can act as reservoirs for insect pests of cotton, and therefore control is recommended (Stewart *et al*., 2003). Successful control of cotton volunteers, including herbicide resistant varieties, is possible using various combinations of herbicides (Stewart *et al*., 2003; Miller *et al*., 2004). There is no reason to suppose that COT67B will be more difficult to control with herbicides than the nontransgenic, null-isolene lines from which they are derived. Exposure of nontarget organisms *via* volunteers is likely to be minimal compared with exposure *via* the crop.

C.2.d. Exposure via feral populations

Seeds of cotton can be dispersed during transport after harvest, but plants rarely establish and form self-sustaining populations in the USA. There is no reason to suppose that expression of FLCry1Ab will increase the ability of cotton to form persistent populations outside cultivation because poor dispersal, rather than insect damage, prevents the establishment of feral populations of cotton (US EPA, 2001a,b,c.). Phenotypic characters that may be associated with weediness are not significantly different in COT67B and null-isoline plants (Chapter 5). Nontarget organisms outside cotton fields are very unlikely to be exposed to FLCry1Ab through the establishment of feral populations of upland cotton. This conclusion applies to varieties derived from COT67B, and to adventitious presence of one or both events in other varieties that may arise from cross-fertilization.

C.2.e. Exposure via gene flow

The genus *Gossypium* comprises about 50 species, with eight diploid genome lineages, designated A-G and K. There is one lineage of tetraploid species, with AD genomes. This lineage includes upland cotton, *G. hirsutum*, along with *G. tomentosum* (Hawaiian cotton), *G. barbadense* (Pima cotton), *G. darwinii* and *G. mustelinum* (Cronn and Wendel, 2003). *G. hirsutum* is interfertile with the other AD genome species "to some degree" (US EPA, 2001), but differences in chromosome number and incompatibility barriers render *G. hirsutum* incompatible with diploid *Gossypium* species (*e.g.*, Mehetre *et al*., 2003).

There are four species of cotton in the United States and its Territories and Possessions: *G. hirsutum*, *G. barbadense*, *G. tomentosum* and *G. thurberi* (desert cotton). *G. thurberi* is a diploid, D genome species, which grows at 2,500 to 5,000 feet in the mountains of New Mexico and Arizona. In the unlikely event of *G. hirsutum* pollinating *G. thurberi*, fertile progeny are extremely unlikely to be produced (US EPA, 2001a,b,c.) because hybrids between *G. hirsutum* and diploid species are almost always entirely sterile (*e.g.*, Mehetre *et al*., 2003, and references therein). There is anecdotal evidence that genes from *G. thurberi* have contributed to improvement of cultivated tetraploid cottons (*e.g.*, Jiang *et al*., 1998), but Cronn and Wendel (2003) record no evidence of introgression of genes into *G. thurberi* from other *Gossypium* species, although it is not clear that introgression has been searched for in *G. thurberi*.

The other three US cotton species are tetraploid and, in theory at least, it is possible for *flcry1Ab* genes to introgress into these species and, as a consequence, for FLCry1Ab to be expressed in these species. Upland cotton is self fertile, but does outcross where suitable pollinators, usually bees, are present; therefore, gene flow from COT67B to other cultivated upland cotton varieties is possible where the varieties are grown within a few hundred feet of each other and flower synchronously (*e.g.*, Van Deynze, 2005; Zhang *et al.*, 2005). Nontarget organisms in cotton fields may be exposed to FLCry1Ab *via* gene flow from COT67B to cultivated cotton, but exposure will be minimal compared with exposure through fields of COT67B cotton.

Feral populations of *G. hirsutum* occur in southern Florida, the US Virgin Islands, and possibly in Puerto Rico (US EPA, 2001). Feral populations of *G. barbadense* also occur in the Caribbean, including the Virgin Islands, where there may have been introgression between *G. hirsutum* and *G. barbadense* (US EPA, 2001a.). The other tetraploid cotton in the USA, *G. tomentosum*, grows wild in Hawaii.

The most likely areas where *flcry1Ab* could spread from cultivated cotton to feral or wild cotton populations are southern Florida, Hawaii, Puerto Rico and the Virgin Islands. There is a very low probability of *flcry1Ab* establishing in *G. thurberi* populations in Arizona and New Mexico. Elsewhere in the USA, there is a minimal probability of establishment of *flcry1Ab* outside cotton fields because of the absence of wild relatives or feral populations of commercial cotton varieties that have escaped from cultivation. Hence, if there is no large-scale commercial cultivation of COT67B in southern Florida, Hawaii, Puerto Rico and the Virgin Islands, the likelihood of exposure of nontarget organisms to FLCry1Ab *via* introgression of *flcry1Ab* into non-agricultural populations of *Gossypium* will be minimal.

C.2.f. Summary – nontarget organisms exposed to FLCry1Ab

Nontarget organisms will be exposed to FLCry1Ab mainly during cultivation of COT67B. Exposure to the highest concentrations of FLCry1Ab will occur *via* consumption of COT67B tissue. Another route of exposure to FLCry1AB is contact with or ingestion of soil in fields of COT67B during and immediately after harvest. Concentrations of FLCry1Ab are expected to be low and transient as the protein is likely to be degraded by soil proteases during decomposition of plant residues, and the probability of expression of FLCry1Ab in soil bacteria following horizontal gene transfer of *flcry1Ab* is minimal. Exposure *via* volunteers or feral plants will be insignificant compared with the crop, and exposure through hybridization of COT67B with wild relatives of cotton is unlikely.

D.1. Expected Environmental Concentrations

The following sections estimate the expected environmental concentrations (EECs) of FLCry1Ab to which nontarget organisms may be exposed as a result of cultivation of COT67B. In each case, a worst-case and a more realistic estimate are made: the worst-case EEC represents exposure *via* a diet of 100% of the relevant plant tissue; the more realistic EECs are refinements of that exposure to represent dilution of the protein through prey, in soil, or by other means. Realistic EECs assume that all individuals are present in or adjacent to fields in which COT67B is cultivated, and therefore are conservative values for estimating risks to local or regional populations of nontarget organisms.

D.1.a. Above ground nontarget arthropods

The highest mean concentration of FLCry1Ab in the above-ground parts of COT67B plants is 142.01 µg/g fresh weight leaves (Table 5.1). This can be regarded as the worst-case EEC for above-ground nontarget arthropods.

Nontarget arthropods rarely, if ever, eat leaves of cotton. The more likely route of exposure to transgenic proteins is consumption of prey that have fed on cotton, or consumption of pollen (*e.g.*, Torres *et al*., 2006; Coll and Guershon, 2002; US EPA, 2005b). The concentration of FLCry1Ab in COT67B pollen is 5.45 µg/g (Table 5.1). The concentration of FLCry1Ab in the prey of nontarget arthropods will vary depending on the prey species, its developmental stage, and the concentration of FLCry1Ab in plant parts on which they are feeding. Several studies have examined the concentration of Cry proteins in herbivores relative to the concentration of plants on which they are feeding; most tested the concentration of Cry1Ab in herbivores feeding on Bt maize (Head *et al*., 2001; Raps *et al*., 2001; Dutton *et al*., 2002; Obrist *et al*., 2005; Obrist *et al*., 2006a; b), and recently studies have been published of herbivores feeding on cotton and oilseed rape expressing Cry1Ac (Torres *et al*., 2006; Howald *et al*., 2003).

In general, the results show that herbivores contain lower concentrations of Bt toxin than the plants on which they are feeding. Sucking insects, such as aphids, contain only trace amounts of Cry1Ab when feeding on Bt maize (Head *et al*., 2001; Raps *et al*., 2001; Dutton *et al*., 2002; Obrist *et al*., 2006a). Lepidopteran larvae contain between 0.1X and 0.25X the concentration of Cry1Ab in Bt maize on which they are feeding (Raps *et al*., 2001; Dutton *et al*., 2002; Obrist *et al*., 2006b), and similar results were obtained by Torres *et al*. (2006) with *Spodoptera exigua* feeding on cotton expressing Cry1Ac. Thrips (*Frankliniella tenuicornis*) contain up to 0.35X the concentration of Cry1Ab in Bt maize, although this concentration is transitory; adults contain about half this amount and pupae less than 1/40th the concentration in larvae (Obrist *et al*., 2005). The herbivores with the highest concentrations of Cry protein are spider mites (*Tetranychus urticae*); they have been found to contain between 0.7 and *ca.* 3.0 X the concentration of Cry1Ab in Bt maize (Dutton *et al*., 2002; Obrist *et al*., 2006a; b).

Most predators in cotton fields are generalist feeders that do not depend on a single pest species for food (Bohmfalk *et al*., 1996). All of the pests discussed above are found in cotton, and therefore nontarget arthropods may be exposed to FLCry1Ab through consumption of these pests. Nontarget arthropods are particularly important as predators of eggs and small larvae of bollworms and tobacco budworms, but will switch to other prey, or to pollen and nectar, if Lepidoptera are scarce (Bohmfalk *et al*., 1996).

A precise realistic EEC is difficult to set given the variety of food that nontarget arthropods are likely to consume. Setting the EEC at 1/5th of the overall mean leaf concentration at the highest expressing developmental stage seems reasonably conservative as pollen and many lepidopteran larvae contain less than this amount, and aphids and lepidopteran eggs contain considerably less. However, spider mites may contain higher concentrations of FLCry1Ab than leaf tissue, but serious infestations of spider mites are uncommon in cotton except after insecticide treatments that kill their natural enemies (Bohmfalk *et al*., 1996). Therefore, spider mites are unlikely to form the sole food source for nontarget arthropods, and 0.2X the leaf concentration seems a reasonable balance between realism and conservatism. The average expression in leaf tissue across all sites at squaring (the stage with highest expression) is 91.78 µg FLCry1Ab/g fresh weight (Table 5.1). Therefore, the realistic EEC for above-ground nontarget arthropods is 91.78 x 0.2 = 18.35 µg FLCry1Ab/g fresh weight diet.

D.1.b. Soil dwellers

The highest mean concentration of FLCry1Ab in COT67B roots is 14.80 µg/g fresh weight (Table 1). This can be regarded as the worst-case EEC for soil-dwelling nontarget arthropods.

A realistic EEC can be calculated as the concentration of FLCry1Ab in soil following incorporation of cotton plants into soil post-harvest. The best estimate of the concentration of FLCry1Ab in plants post-harvest is the pre-harvest concentration. The whole plant concentration of FLCry1Ab at pre-harvest is 15.34 µg/g fresh weight, and the average fresh weight of a cotton plant at pre-harvest is 184 g; therefore, on average, COT67B plants at pre-harvest contain 2822.56 µg FLCry1Ab. Cotton is grown at about 148,200 plants/ha (US EPA, 2001), thus the mean amount of FLCry1Ab in fields of COT102 cotton is 148,200 x 2822.56 x 10^{-6} = 418.30 g/ha.

If the plants are incorporated into soil to a depth of 6 inches (15.25 cm), 418.30 g of FLCry1Ab will be incorporated into 10,000 x 10,000 x 15.25 = 1.525 x 10^{9} cm^3 soil. At an average density of 1.5 g/cm^3, 1.525 x 10^{9} cm^3 contains 2.2875 x 10^{9} g soil. Therefore the concentration of FLCry1Ab in soil will be

418.30/2.2875 x 10^{9} g = 2.29 x 10^{-8} g FLCry1Ab/g = 0.183 µg FLCry1Ab/g soil.

This is the realistic EEC of FLCry1Ab for soil-dwelling arthropods.

D.1.c. Pollinators

Honeybees collect pollen from various plants and feed it to larval brood, either intact or processed (Winston, 1991). Vaissière and Vinson (1994) suggested that honeybees do not extensively forage for cotton pollen because its spiny shape makes it difficult for honeybees to pack it in their pollen baskets. Pollen deposition on honeybees, and the ability of honeybees to pollinate cotton, is a consequence of foraging for nectar. Other pollinators, such as bumblebees, may have greater potential for dietary exposure to cotton pollen. Bumblebee larvae eat most of the pollen supply brought back to the nest; adult bees eat relatively little (Free and Butler, 1959). However, the extent to which cotton pollen contributes to the total pollen diet of bumblebees and other bee species is uncertain. Given this uncertainty, it is conservative to assume that cotton pollen may form the complete diet of some pollinators. Therefore, the EEC for pollinators can be set as 5.45 µg FLCryAb/g pollen (Table 5.1).

D.1.d. Wild birds

The US EPA (2002) determined that the main route of exposure of wild birds to transgenic proteins in cotton is though consumption of cotton seed and soil invertebrates. Worst-case exposure to FLCry1Ab through COT67B seed will be much higher than though soil invertebrates: seeds have higher concentrations of FLCry1Ab than roots, and dilution of FLCry1Ab will occur in the bodies of soil invertebrates that eat roots or other plant material. Therefore, the worst-case exposure of birds to FLCry1Ab would be consumption of a diet of 100% COT67B seeds.

The highest mean concentration of FLCry1Ab in COT67B seeds is 27.57 µg/g fresh weight (Table 1), which represents the worst-case EEC for birds. Exposure to birds may be expressed more suitably as a daily dietary dose (DDD), which is given by a simple formula:

$$DDD = \frac{FIR}{bw} \times C \quad \text{(Crocker et al., 2002)}$$

where FIR = food intake rate; bw = body weight; C = concentration of FLCry1Ab in food.

No figures are available for food intake rates or body weights of wild birds eating cotton seeds. Crocker *et al.*, 2002, give values for the linnet (*Carduelis cannabina*) eating seeds of oilseed rape (*Brassica napus*), which, being an oilseed crop, is a reasonable surrogate for cotton seed. The body weight of the linnet is 15.3 g and its food intake rate on oilseed rape seed is 4.9 g/day. Therefore, the worst-case DDD for birds feeding on cotton seeds can be estimated as

(4.9/15.3) x 27.57 = 8.83 µg FLCry1Ab/g body weight (≡ mg/kg bw).

Birds are highly unlikely to feed on a diet of 100% cotton seeds. First, birds are not common in intensively managed cotton (Cederbaum *et al.*, 2004), and secondly cotton seeds contain gossypol, which is toxic to birds. Gossypol is toxic to broiler chickens at concentrations of 800 mg/kg feed (*i.e.*, 0.08%) (Henry *et al.*, 2001). COT67B seeds contain about 0.64% gossypol, about 8X the gossypol concentration that is toxic to birds. Therefore a realistic EEC for birds should be based on consumption of no more than 12.5% cotton seed; higher concentrations would lead to toxic effects from gossypol. Also, more realism is introduced by using the mean expression across all sites, not the highest mean expression at any site. The mean expression across all sites for COT67B seeds is 20.11 µg FLCry1Ab/g fresh weight, and therefore the realistic EEC is 20.11 x 0.125 = 2.51 µg FLCry1Ab/g fresh weight seeds. The realistic daily dietary dose should also be based on a maximum proportion of 12.5% COT67B seeds in the diet and the average expression in seeds across all sites; therefore the realistic DDD is (4.9/15.3) x 20.11 x 0.125 = 0.81 mg FLCry1Ab/kg body weight.

D.1.e. Wild mammals

Rodents are pests of cotton in India where they cause serious damage to bolls; they eat the seeds and use the fibre for nests (Parshad, 1999). It is possible, therefore, that rodents in the USA may be exposed to FLCry1Ab through eating COT67B seeds. A worst-case EEC for wild mammals is therefore 27.57 µg/g fresh weight seeds (Table 1). A daily dietary dose can also be calculated from the formula used for birds. The best match for rodents eating cottonseed in Crocker *et al*. (2002) is the harvest mouse (*Micromys minutus*) eating cereal seeds: its body weight is 7.0 g and its food intake rate is 2.3 g/day. Therefore, the worst-case DDD for rodents is estimated as

(2.3/7.0) x 27.57 = 9.06 mg FLCry1Ab/kg bodyweight.

Gossypol is highly toxic to rodents. The LD_{50} is between 280 – 3,340 mg/kg body weight, and effects on reproduction are seen at 5.0 mg/kg body weight (Randel *et al*., 1992). The concentration of gossypol in COT67B seeds is 6.4 mg/g and therefore a diet of 100% COT67B seeds would give a dietary dose of

(2.3/7.0) x 6400 = *ca*. 2100 µg gossypol/g body weight (≡ mg/kg bw).

This DDD is roughly 60% of the LD_{50} for the least sensitive rodent tested and is greatly in excess of the no observable effect concentration (NOEC). It seems unlikely that rodents would eat even $1/10^{th}$ of the DDD, but this can be taken as a conservative estimate of realistic consumption of cotton seeds. Using the average concentration across all sites of 20.11 µg FLCry1Ab/g fresh weight, the realistic EEC for wild mammals is 2.01 µg FLCry1Ab/g fresh weight seeds and the realistic DDD is (2.3/7.0) x 20.11 x 0.1 = 0.66 mg FLCry1Ab/kg body weight.

D.1.f. Aquatic organisms

The main route of exposure of aquatic organisms to transgenic proteins in plants is through pollen. As discussed in Section C2.i., there is minimal movement of wind-borne cotton pollen. Therefore, there will be negligible exposure of aquatic organisms to FLCry1Ab *via* COT67B pollen.

Solvent-extracted cottonseed meal is used as a source of protein in commercial fish feeds; it is deficient in lysine and therefore the maximum proportion of cottonseed meal is 30% unless the feed is supplemented with lysine. Cottonseed meal generally comprises 10 to 15% of fish feed (Robinson and Li, 1996).

Worst-case exposures of farmed fish to transgenic proteins *via* cotton can be calculated as 30% of the concentration of the protein in solvent-extracted cottonseed meal; this is a conservative estimate because the manufacture of fish feed involves steam pelleting and extrusion, which are likely to destroy bioactive protein. Realistic exposures can be calculated as 15% of the concentration of the protein in solvent-extracted cottonseed meal. Again, there is a high amount of conservatism in such estimates: there is likely to be loss of bioactivity during manufacture; cottonseed meal may be toasted before incorporation into feed; and it is unlikely that a batch of fish feed will be manufactured solely from COT67B seed meal.

The concentration of FLCry1Ab in solvent-extracted COT67B seed meal is 47.50 ug/g, corrected for 69.2% extraction efficiency (Chapter 4). The worst-case EEC of Cry1Ab in fish feed is therefore 47.50 x 0.3 = 14.25 µg/g diet. The realistic EEC of Cry1Ab in fish feed is 47.50 x 0.15 = 7.13 µg/g diet.

E.1. Hazard Assessment of FLCry1Ab to Nontarget Organisms

E.1.a. Problem formulation

The insecticidal region of FLCry1Ab is similar to that of various truncated Cry1Ab molecules expressed in transgenic maize. The insecticidal regions of Cry1Ab and

Cry1Ac share sequence identity over more than 400 amino acids (*ca*. 70%) of their active regions. In addition, Cry1Ac is a full-length protein that contains the Geiser motif found in the C terminal region of FLCry1Ab; therefore, the effects of truncated Cry1Ab and of Cry1Ac are considered predictive of the effects of FLCry1Ab to nontarget organisms.

The mode of action of Cry1Ab, and laboratory and field studies of Cry1Ab-expressing maize and Cry1Ac-expressing cotton (*e.g.*, US EPA, 2001a,b.; Naranjo *et al*., 2005; Romeis *et al*., 2006; Cattaneo *et al*., 2006; Torres and Ruberson, 2007) provide a large weight of evidence that at concentrations found in transgenic plants, these proteins are toxic to Lepidoptera only, and therefore it is expected that toxicity of FLCry1Ab in COT67B will be limited to Lepidoptera. As exposure of nontarget organisms to FLCry1Ab is expected to be limited to COT67B fields, and as Lepidoptera that feed on cotton are regarded as pests, the exposure of nontarget organisms to FLCry1Ab is expected to be below concentrations necessary to induce toxic effects. In other words, the EEC is expected to be below the NOEC. If the risk hypothesis that EEC/NOEC ≤ 1 is tested rigorously and not falsified, COT67B can be regarded as safe to nontarget organisms (Raybould, 2007).

E.1.b. Hypothesis testing

To test the risk hypothesis, the toxicity (hazard) of FLCry1Ab to nontarget organisms was assessed in the laboratory. It was not possible to test all species for which the EEC of FLCry1Ab is greater than zero; therefore suitable representative indicator species were tested to act as surrogates for species not tested. Confidence in the risk assessment is strengthened by increasing the rigour with which the risk hypothesis is tested, and therefore the best representative indicators are those species most likely to reveal an effect. These species could be taxa closely related to the target pest, and hence likely to have lower NOECs than most of the species for which they are surrogates, or species that have high exposures, and hence likely to have higher EECs than most of the species for which they are surrogates.

As discussed above, there are no nontarget organisms related to the target pest for which the EEC is likely to be greater than zero. Therefore, indicator species were chosen on the basis of high exposure rather than the likelihood of high sensitivity to FLCry1Ab. Each of the categories of organism discussed in Section D. was represented by at least one test species (Table 5.2).

Most studies exposed test species to a microbial test substance, FLCRY1AB-0103, which contains purified FLCry1Ab from *Escherichia coli* expression system; FLCry1Ab in FLCRY1AB-0103 has been shown to be a suitable surrogate for FLCry1Ab produced in COT67B. Studies that exposed representative indicator species to test substances containing truncated Cry1Ab are also considered. The close similarity of the insecticidal regions of these molecules enables these studies to be used to make a broader risk assessment for certain categories of nontarget organism. The test substances used in the hazard assessments are summarised in Table 5.3.

Appendix 5-Table 2. Species used to assess the toxicity of Cry1Ab to nontarget organisms

Test species	Common name	Order: family	NTO group
Coleomegilla maculata	Spotted ladybird beetle	Coleoptera: Coccinellidae	Above-ground nontarget arthropod
Orius insidiosus	Insidious flower bug	Hemiptera: Anthocoridae	Above-ground nontarget arthropod
Aleochara bilineata	Rove beetle	Coleoptera: Staphylinidae	Soil-dweller
Folsomia candida	Springtail	Collembola: Isotomidae	Soil-dweller
Apis mellifera	Honeybee	Hymenoptera: Apidae	Pollinator
Colinus virginianus	Bobwhite quail	Galliformes: Phasianidae	Wild bird
Mus musculus	Mouse	Rodentia: Muridae	Wild mammals
Daphnia magna	Water flea	Cladocera: Daphniidae	Aquatic invertebrate
Ictalurus punctatus	Channel catfish	Silurifomes: Ictaluridae	Farmed fish

Appendix 5-Table 3. Test substances used to assess the toxicity of Cry1Ab

Test substance	Type	Concentration of active ingredient
FLCRY1AB-0103	Purified FLCry1Ab from *Escherichia coli* expression system	*ca.* 800 mg FLCry1Ab/g
LP176-0194	Maize leaf protein enriched with Cry1Ab	*ca.* 700 µg trCry1Ab/g
LLBt-0100	Lyophilized maize leaves	34.20 µg trCry1Ab/g
PHO176-0194	Maize pollen	12.36 µg trCry1Ab/g

NO CBI

Laboratory studies with test substances containing high concentrations of the active ingredient offer more rigorous tests of the risk hypothesis (EEC/NOEC ≤ 1) than field studies with transgenic plants because they are more likely to reveal effects. Laboratory studies can expose organisms to concentrations greatly in excess of the EEC and virtually eliminate uncontrolled confounding variables that will be present in field studies. If the risk hypothesis is not falsified under laboratory conditions, field studies should not be necessary as they do not provide a more rigorous test, and therefore do not increase certainty in the risk assessment. Field studies with COT67B were not undertaken as it was expected that nontarget organisms would be insensitive to FLCry1Ab, and hence the risk hypothesis would be corroborated.

E.1.c. Design of studies

All species tested have been used in safety evaluations for pesticides and other chemicals, and therefore protocols and testing guidelines setting out samples sizes, statistical power, validity criteria and endpoints were available. The springtail, honeybee, bobwhite quail, mouse, water flea and fish studies were carried out with minimal modification to the pesticide testing guidelines for these species. The ladybird beetle, flower bug and rove beetle studies were modified substantially from the pesticide protocols. First, these species had to be exposed to the test substance orally rather than topically; this necessitated the use of artificial diets that maintain bioactivity of FLCry1Ab while permitting normal development of the species. Secondly, exposure times were longer than the pesticide tests. In most studies, fresh diet was supplied daily to ensure exposure to bioactive protein throughout the study. Diets, exposure times and endpoints are summarized in Tables 5.4 and 5.5.

In all studies, a negative control group was exposed to a diet identical to that of the treatment group except that the test substance was omitted. For the study to be valid, the mortality in the negative control group had to be less than a certain value: 10 – 30% depending on the species. In the studies of insects, the test species were exposed to diets containing an insect growth regulator or potassium arsenate as a toxic reference substance; for the study to be valid, mortality in these positive control groups had to be above 50%. The sensitivity of the earthworms was assessed by determining the LC_{50} of 2-chloracetamide. All studies were carried out under international codes of Good Laboratory Practice (GLP).

In studies that exposed test species to FLCry1Ab *via* incorporation of FLCRY1AB-0103 in an artificial diet, the concentration of FLCry1Ab in diet was measured by ELISA, and the bioactivity and intactness of FLCry1Ab were tested by bioassays on ECB and western blots, respectively. In the springtail, honeybee and water flea studies exposure was taken to be the nominal concentration of FLCry1Ab after incorporation into the diet. Bobwhite quail and mouse were exposed through a single dose of test substance by gavage.

Appendix 5-Table 4. Summary of test methods used to assess the toxicity of Cry1Ab to nontarget organisms

Test organism	Life stage	Route of exposure to Cry1Ab	Duration	Guideline or protocol[†]
Ladybird beetle	2nd instar	FLCRY1AB-0103 in 50% bee pollen + 50% moth egg diet	21 days	US EPA OPPTS 885.4340
Flower bug	Nymph	FLCRY1AB-0103 in liver-based diet	12 days	Bakker et al. 2000
Rove beetle	Adult	FLCRY1AB-0103 in minced beef diet	11 weeks	Grimm et al. 2000
Springtail	Juvenile	LLBt11-0100 with yeast diet	28 days	ISO # 11267
Honeybee	Larvae	FLCRY1AB-0103 in sucrose solution	26 day	Oomen et al. 1992
Bobwhite quail	Juvenile	Single dose of LP176-0194 by gavage	14 days	US EPA OPPTS 850.2100
Mouse	Young adult	Single dose of FLCRY1AB-0103 by gavage	14 days	US EPA OPPTS 885.3050
Water flea	Neonate	PHO176-0194 in water	2 days	OECD # 202
Catfish	Juvenile	FLCRY1AB-0103 in fish feed	28 days	ASTM E 729-88

Abbreviations:

US EPA OPP = United States Environmental Protection Agency Office of Pesticide Programs

ISO = International Organization for Standardization

OECD = Organisation for Economic Co-operation and Development

[†] Nontarget arthropod tests following protocols in the literature also followed the US EPA Microbial Pesticide Test Guidelines where relevant

E.1.d. Results of toxicity studies

With one exception, there were no statistically significant differences between the FLCry1Ab treatment and the negative control groups in any study. The exception was the adult weight endpoint in the ladybeetle study. The mean weight of adults in the FLCry1Ab-treatment group was significantly lower than that of the negative control group; however, the difference was only 14%, and was not considered an adverse effect for the purposes of risk assessment. The positive and negative control validity criteria were met in all studies.

Diet analyses revealed intact, bioactive FLCry1Ab in all treatment diets. The concentrations of FLCry1Ab detected by ELISA were therefore taken to be the minimum value for the NOEC or no observable adverse effect concentration (NOAEC) of FLCry1Ab for the species tested. The NOEC for the honeybee study was taken as the nominal concentration of 76.98 µg FLCry1Ab/g diet. The NOECs for the studies using truncated Cry1Ab test substances were also taken as the nominal concentrations, and the no observable adverse effect levels (NOEL) in the quail and mouse studies were the amount of active ingredient in the dose of test substance.

Re-characterization of FLCRY1AB-0103 shortly after the completion of the experimental phase of the ladybird beetle, flower bug, rove beetle and catfish studies showed a statistically significant loss of bioactivity against first-instar ECB compared with the initial characterization of the test substance: the initial LC_{50} was 3.7 ng FLCry1Ab/cm^2 diet surface, and the LC_{50} at re-characterization was 16.5 ng FLCry1Ab/cm^2 diet surface. A conservative interpretation of these data is that for risk assessment the measured NOEC or NOAEC should be reduced by a factor of 4.5 to reflect the loss of bioactivity in the test substance. The adjusted NOECs are indicated as $NOEC_{ba}$. The results of the toxicity studies and diet analyses are summarised in Table 5.5

F.1. Risk Assessment of COT67B to Nontarget Organisms

The risk hypothesis under test is that the ratio of the EEC to the NOEC (the hazard quotient; HQ) is less than or equal to 1. The HQs for the representative indicator species used to test the risk hypothesis are presented in Table 5.6 and most are less than 1. It should be remembered that in all studies the NOEC or NOEL was the single concentration or dose used in the study; therefore the HQs are maxima.

F.1.a. Nontarget organisms in cotton fields

No adverse effects were seen in studies of the effects of full-length and truncated Cry1Ab on indicator species representative of NTOs found in cotton fields. Comparison of the NOECs (or NOAECs) or no observable effect levels (NOELs) with EECs or estimated DDDs indicated that in most studies, organisms were exposed to amounts of FLCry1Ab far greater than they are likely to be exposed to *via* cultivation of COT67B. Therefore the lack of adverse effects in the

studies indicates low risk to NTOs with high confidence, and studies with more realism are not required.

Appendix 5-Table 5. Summary of the results of ecotoxicology studies on Cry1Ab

Test organism	Endpoint	Concentration or dose	Study result
Ladybird beetle	Pre-imaginal mortality; development time	1000 µg FLCry1Ab/g diet	NOAEC ≥ 1000 µg FLCry1Ab/g diet†
			$NOAEC_{ba}$ ≥ 222.2 µg FLCry1Ab/g diet
Flower bug	Pre-imaginal mortality	1000 µg FLCry1Ab/g diet	NOEC ≥ 1000 µg mCry3A/g diet
			$NOEC_{ba}$ ≥ 222.2 µg FLCry1Ab/g diet
Rove beetle	Reproduction	1000 µg FLCry1Ab/g diet	NOEC ≥ 1000 µg mCry3A/g diet
			$NOEC_{ba}$ ≥ 222.2 µg FLCry1Ab/g diet
Springtail	Reproduction	17.1 µg Cry1Ab/g diet	NOEC ≥ 17.1 µg Cry1Ab/g diet
Honeybee	Brood development	76.98 µg FLCry1Ab/g diet	NOEC ≥ 76.98 µg FLCry1Ab/g diet
Bobwhite quail	Mortality and feeding	140 mg Cry1Ab/ kg bw	NOEL ≥ 140 mg Cry1Ab/ kg bw
Mouse	Mortality and various histological variables	1830 mg FLCry1Ab/ kg bw	NOEL ≥ 1830 mg FLCry1Ab/ kg bw
Water flea	Immobilization	1.8540 µg Cry1Ab/L	NOEC ≥1.8540 µg Cry1Ab/L
Catfish	Mortality and growth rate	8.13 µg Cry1Ab/g feed	NOAEC ≥ 8.13 µg Cry1Ab/g feed
			$NOAEC_{ba}$ ≥ 1.81 µg Cry1Ab/g feed

Abbreviations:

bw – body weight

NOEC – no observable effect concentration

$NOEC_{ba}$ – no observable effect concentration adjusted for bioactivity

NOAEC – no observable adverse effect concentration

$NOAEC_{ba}$ – no observable adverse effect concentration adjusted for bioactivity

NOEL – no observable effect level

† Statistically significant difference in mean weight of adults. Not considered an adverse effect – see text for details

F.1.b. Nontarget organisms outside cotton fields

The discussion of exposure in Section 5.C.2 indicates that significant exposure to FLCry1Ab is highly likely to be limited to cotton fields. Therefore, FLCry1Ab in COT67B presents low risk to NTOs outside cotton fields due to lack of exposure (if the EEC = 0, the HQ = 0). The exception is farmed fish that may be exposed to FLCry1Ab *via* fish feed manufactured from cottonseed meal. The maximum HQs for FLCry1Ab and fish are approximately 1 or greater. However, the HQs represent very conservative EECs, rather than high risk of FLCry1Ab. When more realistic exposures are considered, the risk to farmed fish is low.

F.1.c. Threatened and endangered species

The hazard data summarized above, and studies of the pest spectrum and mode of action of FLCry1Ab (*e.g.*, Gill *et al.*, 1992; Schnepf *et al.*, 1998), indicate that FLCry1Ab is highly unlikely to be toxic to non-Lepidoptera at concentrations produced by COT67B. A recent assessment by the US EPA (2005b) indicated that the only endangered or threatened Lepidoptera known to occur in a cotton-growing county in the United States is the Kern primrose sphinx moth (*Euproserpinus euterpe*). This species does not feed on cotton, nor do its food plants, the contorted suncup (*Camissonia contorta*) and red-stem filaree (*Erodium cicutarium*), occur near cotton fields. Therefore, FLCry1Ab in COT67B is predicted to have no adverse effects on endangered or threatened species in the United States.

Appendix 5-Table 6. Hazard quotients for NTOs exposed to FLCry1Ab *via* COT67B

Species	NTO Group Represented	Worst-case EEC	Realistic EEC
Ladybeetle	Above-ground arthropods	HQ ≤ 0.1420	HQ ≤ 0.0184
		HQ_{ba} ≤ 0.6391	HQ_{ba} ≤ 0.0826
Flower bug	Above-ground arthropods	HQ ≤ 0.1414	HQ ≤ 0.0183
		HQ_{ba} ≤ 0.6365	HQ_{ba} ≤ 0.0823
Rove beetle	Soil dwellers	HQ ≤ 0.0141	HQ ≤ 0.0002
		HQ_{ba} ≤ 0.0634	HQ_{ba} ≤ 0.0008
Springtail	Soil dwellers	HQ ≤ 0.8244	HQ ≤ 0.0099
Honeybee	Pollinators	HQ ≤ 0.0708	HQ ≤ 0.0708
Bobwhite quail	Wild birds	HQ ≤ 0.0630	HQ ≤ 0.0058
Mouse	Wild mammals	HQ ≤ 0.0050	HQ ≤ 0.0004
Water flea	Aquatic organisms	No exposure	No exposure
Catfish	Farmed fish	HQ ≤ 1.84	HQ ≤ 0.92
		HQ_{ba} ≤ 8.28	HQ_{ba} ≤ 4.14

HQ = EEC/NOEC. See Section ***D.1.*** for EECs; Table 5 for NOECs

F.1.d. Cumulative effects of transgenic cotton on nontarget organisms

Transgenic cotton expressing genes for control of lepidopteran pests comprised 57% of US cotton acreage in 2006 (USDA ERS, 2006). The diversity and abundance of nontarget organisms in lepidopteran-resistant cotton is at least as high as in conventional cotton, and in many studies higher biodiversity is associated with transgenic cotton (e.g., Head *et* al., 2005; Naranjo, 2005a;b; Torres and Ruberson, 2005; Whitehouse *et al*., 2005; Cattaneo *et al*., 2006; Torres and Ruberson, 2007). Control of lepidopteran pests by COT67B is highly unlikely to have direct toxic effects on nontarget organisms and it likely to be neutral or beneficial to biodiversity compared with conventionally managed cotton. Therefore, the likelihood of adverse cumulative effects on nontarget organisms following the introduction of COT67B is minimal.

G. Risk of Increased Weediness Potential of COT67B

This risk assessment seeks to demonstrate the protection of two assessment endpoints: the diversity and abundance of nontarget organisms within and outside cotton fields, and crop yield (Section F). Section E.1.d. showed minimal risk to nontarget organisms through toxic effects of

COT67B: the composition and nutritional quality of COT67B cotton are similar to conventional cotton, and FLCry1Ab has no observable toxicity at exposures likely to result from cultivation of COT67B.

Wild plants can also be regarded as nontarget organisms, and they could be harmed if COT67B has greater potential for weediness than conventional cotton. The abundance and diversity of wild plants could be reduced if feral populations of COT67B, or hybrids of COT67B with wild species, establish and spread into semi-natural or natural habitats; organisms that rely on these wild plants for food or shelter could also be harmed (*e.g.*, Raybould and Wilkinson, 2005). If COT67B is more likely to be a volunteer weed than conventional cotton, the yield of other crops may be affected. Volunteers reduce crop yield directly through competition, and indirectly by acting as "green bridges" for pests and pathogens, and are therefore regarded as plant pests (Froud-Williams *et al*., 1993; Raybould, 2005).

The risks to wildlife and agricultural productivity from weedy cotton populations are low. As described in Section C.2.c., volunteer cotton can occur through the persistence of perennial plants after harvest or the germination of spilled seed (Hennebury *et al*, 2003; Stewart *et al*., 2003). Cotton volunteers are most common where a failed cotton crop is replanted to soybeans; the volunteers do not reduce yield, but can act as reservoirs for insect pests of cotton, and therefore control is recommended (Stewart *et al*., 2003). Successful control of cotton volunteers, including herbicide resistant varieties, is possible using various combinations of herbicides (Stewart *et al*., 2003; Miller *et al*., 2004). If there is no significant difference between COT67B and nontransgenic cotton in characters associated with weediness, it is highly unlikely that COT67B will pose significantly greater risks to agricultural productivity or to semi-natural and natural habitats than nontransgenic cotton.

The potential for increased weediness is considered in four parts. First, the possibility that the intended increase in insect resistance could result in increased weediness potential of COT67B. Secondly, the phenotype of the COT67B is compared with near-isogenic, nontransgenic cotton to test the hypothesis of no unintended changes in phenotype have occurred during production of COT67B that may increase its weediness potential. Next, the possibility of increased weediness of wild relatives of cotton due to the introgression of *flcry1Ab* from COT67B is assessed. Finally, changes in agronomic practices that may result in increased weediness of COT67B are discussed.

G.1. Changes in Weediness Potential from the Intended Effects of FLCry1Ab

Feral populations of cotton are rare in the United States (Section *C.2.d.*, above) because of poor dispersal, competition from other plants and lack of water (US EPA, 2001a,b.). When cotton is damaged by insects, its canopy structure often changes to increase light capture and produce compensatory growth *(e.g.*, Sadras, 1996); therefore protection from insect damage may have little effect on the competitive ability of cotton. Expression of FLCry1Ab does not increase the

dispersal ability of COT67B (Chapter 4), nor is it likely that it increases water use efficiency; therefore COT67B is no more likely than conventional cotton to establish feral populations.

Cotton can be a volunteer weed (Section C.2.c., above), but is readily controlled by herbicides. Expression of FLCry1Ab is unlikely to confer broad-spectrum herbicide tolerance, and therefore COT67B is no more likely than conventional cotton to establish volunteer weed populations.

G.2. Changes in Weediness Potential Due to Unintended Effects of Transformation

A simple way to test for unintended phenotypic changes that may lead to increased weediness is to compare the growth of the transgenic crop with suitable nontransgenic counterparts in agronomic trials (White, 2002; Raybould, 2005). COT67B and the near-isogenic, nontransgenic line COT67B(-) cotton, derived from null segregants of the T_1 ancestor of COT67B, were grown in 43 sites throughout the U.S. in 2003 – 2006. The progenitor variety of COT67B and COT67B(-), Coker 312 was also grown. Plants were evaluated for various phenotypic characters that may act as indicators of changes in weediness (White, 2002).

G.2.a. Life span, dormancy and ability to overwinter

Although cotton is cultivated as an annual, its progenitors were perennial shrubs. Modern cotton cultivars retain the perennial habit, although it is less pronounced than in wild cotton species (De Souza and Da Silva, 1987). Carbohydrate is not exhausted by reproduction (Wells, 2002), and although defoliants are often applied to aid harvest, some regrowth of cotton can occur after harvest until the stubble is ploughed into the soil or otherwise removed.

Increased perenniality of COT67B could be regarded as indicating greater weediness potential. In the agronomic trials, there were no indications of greater allocation to vegetative growth from sexual reproduction or changes in plant morphology suggestive of increased perenniality of COT67B.

Cotton possesses primary and secondary seed dormancy. Primary dormancy is particularly associated with "hard seed" that have a seed coat that restricts uptake of water (Christiansen *et al*., 1960); secondary dormancy is induced by cold or salinity (IBPGR, 1985). Despite possessing dormancy, cotton does not appear to produce a persistent seed bank because the proportion of hard seed is low in modern cultivars and most seeds lose viability rapidly when moistened (*e.g.*, Furbeck *et al*., 1993).

Seed germination assays revealed no significant differences in germination between COT67B and nontransgenic cotton measured under standard conditions (alternating 20°C and 30°C) or cool conditions (constant 18°C). In agronomic trials there were no differences in the proportion of hard seed produced by COT67B and the nontransgenic comparators (Chapter 5). These data indicate no enhanced weediness potential of COT67B through increased dormancy.

G.2.b. Vegetative vigour

Vegetative vigour is a measure of competitive ability. There were no significant differences in seedling vigour or growth habit that suggest COT67B is more competitive than nontransgenic cotton (Chapter 5); therefore COT67B is expected to have no greater potential to be invasive of non-agricultural habitats.

G.2.c. Reproductive characteristics

Possible changes in reproduction associated with COT67B were assessed by measurement of several variables in field trials, including time to flowering, number of bolls per plant and number of seed per plant. No variable showed a trend indicating greater fertility or reproduction of COT67B compared with nontransgenic cotton. Greenhouse studies found no significant differences in the shape, weight or viability between COT67B pollen and pollen of Coker 312 (Chapter 5). These data predict no greater potential for weediness of COT67B due to increased fecundity or outcrossing.

G.2.d. Changes in interactions with pests and diseases

Apart from the intended effect of control of lepidopteran pests, no significant differences between COT67B and nontransgenic cotton in the incidence or severity of attack by pests and diseases were observed in field trials. Therefore, it is highly unlikely that COT67B will show increased weediness potential because of protection from a pest or disease that normally prevents cotton from becoming weedy.

G.3. Increases in Weediness Through Gene Flow

The risk from the production of weedy plants through hybridization and introgression of *flcry1Ab* from COT67B into wild relatives is negligible. The weediness hazard posed by the *flcry1Ab* is also minimal: no intended or unintended effects of COT67B are associated with increased weediness potential (Section C.2.e.).

G.4. Changes in Agronomic Practices

It is anticipated that COT67B will be grown in the same areas as current commercial cotton varieties: expression of FLCry1Ab is not intended to confer phenotypes that increase the range of habitats in which cotton can be grown. Apart from anticipated reductions in use of insecticides, cultivation of COT67B does not require different fertilizer or pesticide application, tillage, planting or harvesting from existing commercial cotton varieties (Chapter 5). Therefore, the likelihood of increased weediness potential due to indirect effects of cultivation of COT67B is negligible.

H. Summary Environmental Risk Assessment

In Section B, two objectives to demonstrate minimal environmental risk of cultivating COT67B were described: to show that COT767B cotton is highly unlikely to be more harmful to nontarget organisms than is conventional cotton; and to show that COT67B is highly unlikely to be a worse weed of agriculture, or be more invasive of non-agricultural habitats, than is conventional cotton. If these objectives were met, the protection of two assessment endpoints would be demonstrated: the diversity and abundance of nontarget organisms within and outside cotton fields, and crop yield. Protection of these assessment endpoints would meet objectives of several environmental protection statutes, including the Federal Plant Protection Act, the Endangered Species Act and the National Environmental Policy Act.

Nontarget organisms are highly unlikely to suffer toxicity from exposure to COT67B. The composition of COT67B is not significantly different from nontransgenic cotton, apart from the presence of FLCry1Ab. Laboratory studies of FLCry1Ab and truncated Cry1Ab indicate that these proteins are not toxic to wildlife at concentrations of FLCry1Ab expressed in COT67B. Hence, risks to the abundance and diversity of nontarget organisms from exposure to toxic substances in COT67B are minimal.

Conventional cotton rarely forms self-sustaining populations outside cultivation. Expression of FLCry1Ab is unlikely to increase the potential of cotton to become weedy, and field trials indicate no consistent effect of the presence of *flcry1Ab* or unintended effects of transformation on agronomic characters that are likely to determine weediness. In addition, the likelihood of weediness evolving in populations of feral cotton or wild relatives, as the result of gene flow from COT67B, is low because such populations do not occur near areas of cotton cultivation. Hence, the risks to wild plants from invasions of feral or wild populations derived from COT67B, and the risks to yields of crops potentially affected by volunteer cotton, are minimal.

In summary, no significant risks to the diversity and abundance of nontarget organisms within and outside cotton fields, or to crop yield, from the proposed cultivation of COT67B have been identified.

APPENDIX 6. PUBLIC INTEREST DOCUMENT IN SUPPORT OF REGISTRATION OF THE PLANT-INCORPORATED PROTECTANT PROTEINS VIP3A AND FLCRY1AB AS EXPRESSED IN THE COMBINED TRAIT COT102 X COT67B COTTON (VIPCOT™ COTTON)

EXECUTIVE SUMMARY

Cotton plant incorporated protectants based on *Bacillus thuringiensis* (Bt) Cry insecticidal proteins have been widely adopted and were planted either alone or in conjunction with plant incorporated herbicide tolerance on 95.5% of cotton acres in the US in 2006 (USDA AMS 2006). Three Bt crystalline insecticidal protein–based cotton products are currently registered by the US Environmental Protection Agency (EPA or the Agency) as Plant Incorporated Protectants (PIPs). These products are Bollgard®, Bollgard II® and WideStrike®. Bollgard II® and WideStrike® are combinations of two Bt Cry proteins, Cry1Ac plus Cry2Ab and Cry1Ac plus Cry1F, respectively, whereas Bollgard® produces only the Cry1Ac protein. Of these three products, Bollgard® and Bollgard II® represent more than 99% of the insect resistant cotton varieties sold in the US in 2006. This situation limits grower choice, but has provided significant grower, human health and environmental benefits resulting from the movement from conventional chemical cotton insecticides to Bt cotton.

Syngenta's VipCot™ cotton (VipCot™) product expresses both the Bt subsp. *kurstaki* full-length Cry1Ab insecticidal protein, FLCry1Ab, and also a novel vegetative insecticidal protein, Vip3Aa19 (Vip3A). VipCot™, provides high dose protection against the major Lepidopteran cotton pests (*Helicoverpa zea, Heliothis virescens, and Pectinophora gossypiella*; that is, cotton bollworm, tobacco budworm and pink bollworm). The Vip3A protein has a unique mode of action; thus, VipCot™ has the potential to extend the useful life of Bt Cry insecticidal protein-based technology generally. VipCot™ will provide growers with a legitimate alternative to current cotton PIP products; particularly, Bollgard® and Bollgard II® varieties that dominate the cotton market. Additional choice will also influence grower cost and help maintain or slightly expand the cotton acres planted to Bt-based PIPs.

The registration and market introduction of Syngenta's VipCot™ will result in agronomic, economic, human health, environmental, and resistance management benefits that are discussed in later sections of this document. These benefits provide strong support for the public interest finding needed for a conditional registration.

As discussed in the confidential marketing plan (Supplement 21), Syngenta will partner with a major cotton seed producer. This alliance ensures that the VipCot™ technology will be incorporated in elite germplasm. The strong VipCot™ product line will be marketed by two companies well-versed in the needs of cotton growers. Both Syngenta and the seed company

have large field forces to effectively provide needed support for growers. Combined with an aggressive marketing plan, this alliance ensures that VipCot™ will be a strong competitor in the Bt cotton market. Market share will be achieved, and additional grower choice will ensure that grower, environmental, and resistance management benefits will continue.

In summary, VipCot™ will be a welcome addition to the arsenal of products available to cotton growers. Benefits will accrue; the public interest will be served. This public interest document provides the support needed for these conclusions.

PUBLIC INTEREST FINDING

In order for EPA to issue a conditional registration for a new active ingredient, the Agency must determine that the registration is in the public interest. See §3(c)(7)(C) of the Federal Insecticide, Fungicide, and Insecticide Act (FIFRA). In a 1986 Federal Register Notice (51 FR 7628; March 5, 1986), EPA stated that it considered a variety of factors when deciding to grant a conditional registration for a new active ingredient. The Agency explained that it considered need, usage, performance, risk, and economic factors in reaching its public interest determinations.

Syngenta believes that registration of the active ingredients in VipCot™ meets the criteria for a conditional registration. The registration is clearly in the public interest. Registration and market introduction of Syngenta's VipCot™ product will result in agronomic, economic, human health, environmental, and resistance management benefits that are highlighted here, and discussed in greater detail in later sections of this document.

Syngenta's VipCot™ expresses high levels of the proteins, Vip3A and FLCry1Ab, through the combination of transgenic cotton events, COT102 and COT67B, respectively. The Vip3A protein is characterized by a range of properties that clearly distinguish it from the FLCry1Ab protein and the Cry proteins expressed by the Bt cotton varieties currently available to growers. The combination of the Vip3A and FLCry1Ab proteins offers effective protection from the principal Lepidopteran pests of cotton (cotton bollworm, tobacco budworm, and pink bollworm). As discussed in the confidential marketing plan (supplement 21), VipCot™ will eventually include a herbicide-resistant trait.

Syngenta efficacy trials show effective control of cotton bollworm, tobacco budworm, and pink bollworm. Preliminary yield data demonstrate no negative agronomic factors that will impact a variety development program. Strong efficacy and yield potential combined with Syngenta marketing and field expertise will result in varieties that are very competitive with those varieties now on the market. Current Bt-based PIPs offer agronomic and economic benefits compared to the use of chemical pesticides. The introduction of VipCot™ will continue and enhance the

agronomic and economic benefit stream by offering growers a new choice in germplasm, technology and terms of use.

In addition, a comparison of human health and environmental factors discussed in a later section of the report clearly demonstrates both the same low risk potential for VipCot™ as the current Bt PIPs, and the strong reduced risk potential of VipCot™ cotton compared to the use of alternative conventional chemical pesticides. While VipCot™ will primarily replace other Bt products as it gains market share, its presence in the marketplace will extend the useful life of Bt-based cotton technology generally, and thus, contribute to the continued human health and environmental benefits resulting from the use of Bt cotton compared to chemical alternatives.

Finally, VipCot™ will introduce the Vip3A vegetative insecticidal protein, that offers little chance for cross-resistance to its companion protein, FLCry1Ab, or the other Bt Cry insecticidal proteins currently marketed. Since VipCot™ expresses a protein with a unique mode of action, its combination with a viable competitive market presence will offer strong resistance management potential. As discussed in a later section of this report, risk assessment modeling of VipCot™ cotton confirms the low likelihood of cross-resistance and the potential to extend the useful life of Bt cotton technology generally.

GROWER BENEFITS

A. Agronomic Benefits

1. Expert Reports

The introduction of Bt cotton has transformed cotton production in the United States. The dramatic shift from conventional chemical insecticides to Bt cotton during the last 10 years has occurred because of the strongly positive agronomic and economic factors associated with Bt cotton technology. VipCot™ will continue in this tradition.

Syngenta asked three cotton experts to comment on the agronomics associated with Bt cotton generally and on VipCot™ specifically. Professor J.R. Bradley, North Carolina State University (Southeast); Professor B. R. Leonard, Louisiana State University (Mid-South); and Professor Don R. Rummel, Texas A & M University (Texas) each prepared a report that discusses the history of cotton production in their regions, the problems with chemical insecticides, the advantages of Bt cotton, and the potential for VipCot™ to contribute to the legacy of Bt cotton. In particular, the authors noted the anticipated effectiveness of VipCot™, and its anticipated usefulness in sustaining the longevity of Bt cotton technology. The professors' reports are included in this document as Report Numbers 6.1, 6.2 and 6.3.

2. Efficacy

In a study report provided to US EPA, Syngenta discusses evaluations of efficacy data developed during 2005 and 2006 (Supplement 20). The two years of field trials provide strong evidence that VipCot™ provides good resistance to both cotton bollworm and tobacco budworm. Work at multiple locations during the two years involving both artificial and natural infestations consistently demonstrated the strong superiority of COT 67B, COT102 and VipCot™ compared to non-transgenic Coker 312 cotton. Characteristics evaluated included average % square damage, average % boll damage, average % fruiting structure damage, and average % flower damage.

3. Yield and Fiber Quality

As discussed in the confidential marketing plan, Syngenta will partner with a major presence in the cotton seed industry which will provide the potential for VipCot™ to be incorporated into superior cotton seed variety lines. Preliminary yield and fiber quality data support the potential for developing strong VipCot™ varieties.

Insect efficacy is only one factor that must be considered when developing new transgenic technologies for use in commercial cotton. Yield potential and fiber quality traits must also be considered. In both cases, available data support the potential for viable VipCot™ varieties.

Concerning yield, Syngenta's marketing partner evaluated VipCot™ in a multiple location trial series representative of the US cotton growing region (Report 6.4). The results showed no significant differences in seed cotton yield between Coker 312 and VipCot™. The conclusion from this preliminary work is that VipCot™ demonstrates no negative agronomic characteristics that would impact a variety development program. Additional work is necessary, but initial results are very positive.

Concerning fiber quality, lint samples of the component events COT102 and COT67B (but not VipCot™) have been analyzed for fiber characteristics and quality cotton varieties. Such factors are important in the evaluation of possible unintended effects that could impact the agronomic suitability of VipCot™. In work conducted to date, there were no significant differences between events COT102, COT67B and non-transgenic genotypes for fiber micronaire, length or uniformity and thus, none are expected when these events are combined as VipCot™.

In summary, yield and fiber quality data all support the conclusion that VipCot™ technology can be incorporated into the varieties needed to give VipCot™ a strong market presence.

4. Conclusions

Agronomic, efficacy, yield and fiber quality data and information all support the strong potential for VipCot™ to become a quality competitive product in the Bt cotton market. These factors coupled with an aggressive marketing plan and strong market presence will provide growers with additional choice and generate the economic benefits discussed in the following section.

ECONOMIC BENEFITS

In its review and analysis of the public interest documents submitted to support current Bt cotton products, EPA determined that economic value would result from the sale and use of these products. Other studies have reached the same conclusions. For example, based on an analysis of biotechnology-derived crops planted in 2004, the National Center for Food and Agricultural Policy estimated that products providing protection against cotton bollworm, tobacco budworm, and pink bollworm increased cotton production by almost 600 million pounds, improved farm income by almost $300 million, and reduced chemical pesticide use by more than 1.6 million pounds (NCFAP, 2005). Numerous other studies have estimated the economic benefits from the adoption of transgenic cotton. Fernandez-Cornejo and Caswell, 2006, provide a summary of many of these studies that characterize effects on yield, pesticide use, and grower returns. In a summary table from this report, grower returns and yields consistently are reported as increased due to planting Bt cotton products.

Syngenta considers the major benefits resulting from the introduction of VipCot™ to be additional grower choice, increased competition and extended useful life of Bt cotton technology generally (resulting form the unique mode of action of the Vip3A protein expressed by VipCot™) rather than a major shift from chemical insecticide treated acres to new Bt planted acres. Syngenta projects additional economic benefits of $83 million will accrue from the regulatory approval and use of VipCot™. Thus, the technology will create significant economic value, strengthen the technology and maintain the dominance and overall benefits of Bt-PIP cotton compared to chemically treated acres.

Syngenta requested Dr. Eric Wailes, Department of Agricultural Economics and Agribusiness, University of Arkansas, to prepare an economic analysis of the introduction of VipCot™ into the marketplace. Dr. Wailes' report is attached as in Report 6.5. Dr. Wailes used a computational general equilibrium (CGE) model for his analysis. As discussed in his report, Dr. Wailes considers a CGE model superior to partial equilibrium models that require what he considers unrealistic assumptions in order to operate.

Reports 6.6 and 6.7 provide additional discussion and support for the use of a CGE model to analyze economic impacts. In his reference list, Dr. Wailes also provides a website link to a

chapter of a forthcoming book that discusses the Global Trade Analysis Project (GTAP) model he used for his work related to the potential impact resulting from the introduction of VipCot™ and the resulting additional choice that will be available to American cotton growers.

Dr Wailes based his analysis on the confidential Syngenta marketing plan; and utilized conservative estimates to predict the net present value of VipCot™ replacing inferior cotton varieties at $42 million over the first eight years of sales and use. He considered the additional value that VipCot™ will bring to the market in terms of added competition and grower choice. He conservatively estimated the net present value of this component at $41 million. Thus, the total net present value resulting from the first eight years of VipCot™ sales is estimated at $83 million. Finally, Dr Wailes estimated the regional distribution of these benefits based on existing adoption levels of transgenic cotton varieties.

HUMAN HEALTH AND ENVIRONMENTAL BENEFITS

A. Introduction

EPA has consistently found that the registration of Bt PIPs is in the public interest. These findings have largely been based on a determination that Bt PIPs present less risk than conventional chemical pesticide alternatives. The Agency's view concerning Bt PIPs is well accepted and supported by the work of others. In an interesting analysis, Brookes and Barfoot (2005) presented findings that the global introduction of genetically modified crops resulted in the reduction in use of chemical pesticides by 172 million kilograms, and "reduced the environmental footprint associated with pesticide use by 14% during the period 1996 to 2004".

VipCot™ also presents a strong reduced risk profile. The registration of VipCot™ is not expected to significantly expand the number of Bt cotton acres; acreage is expected to remain relatively stable. However, VipCot™'s strong market presence and unique mode of action will contribute to resistance management and the long-term viability of Bt PIP cotton technology. The significant replacement of conventional chemical insecticides by Bt PIP cotton is well documented. The introduction of VipCot™ may result in a small replacement of cotton acres currently treated with chemical insecticides, but its major contributions will be to support the continuation of Bt PIP cotton acreage and continue the reduction in the use of chemical cotton insecticides already achieved.

B. VipCot™

The studies conducted by Syngenta to support the US EPA FIFRA Section 3 registration of VipCot™ are discussed in the reports provided to the Agency. A subset of those studies is provided separately from this Petition as CBI Supplements 1 through 22. There are no human

health issues associated with VipCot™. Neither the Vip3A nor the FLCry1Ab protein is likely to be a food allergen, and toxicity studies indicate no hazard concern. The same profile holds for the marker protein, hygromycin B phosphotransferase, which is exempt from the requirement for a tolerance. Syngenta studies also provide the same solid evidence for the environmental safety of the proteins expressed in VipCot™. Extensive testing shows no real risk concern.

C. Conventional Chemical Pesticide Risk Profile

The human health and environmental safety data presented in other studies clearly demonstrate reduced risk compared to the conventional chemical pesticides used to protect cotton against cotton bollworm, tobacco budworm, and pink bollworm. The following discussion briefly highlights human health and environmental risk concerns of these chemical insecticides.

Tables 6.1 through 7 provide market information regarding the pesticides used to control cotton bollworm, tobacco budworm and pink bollworm, including the pounds applied, acres treated and total grower expenditures. Table 1, shows that the pounds of active ingredients used to control these three pests has ranged from about 600,000 to almost 1,000,000 pounds of active ingredient during the 2002-2005 time period. While acres and grower costs have decreased, both figures are substantial in each year during this period.

Table 6.2, shows that a substantial portion of the increase in pounds applied came from increased use of organophosphates (OPs), which rose from approximately 264,000 pounds in 2002 to approximately 493,000 pounds in 2005. Table 3 presents use information on a percentage basis. OP, carbamate and pyrethroid products account for a substantial percentage of the insecticides used in each year including over 90% in 2003 and 2004.

Tables 6.4 through 7 present the top ten compounds by pounds of active ingredient used on the target pests from 2002 through 2005. With limited exceptions, the top ten insecticides in each year are OPs, carbamates and pyrethroids.

These three classes of pesticides require numerous safety warnings and extensive use restrictions as described in Tables 6.8 through 11. The products clearly present greater potential risks than VipCot™ and Bt PIP cotton products. The grower movement from chemical insecticides has clearly and dramatically reduced risk to human health and the environment.

TABLE 6.1

USAGE INFORMATION FOR PESTICIDES USED TO TREAT COTTON BOLLWORM, TOBACCO BUDWORM, AND PINK BOLLWORM

Year	Pounds	Acres Treated	Expenditure
2002	724,737	9,626,617	$56,113,616
2003	592,575	5,277,667	$26,675,567
2004	596,876	6,090,454	$31,966,188
2005	966,611	4,456,605	$24,667,171

TABLE 6.2

USE BY CHEMICAL CLASS FOR PESTICIDES USED TO TREAT COTTON BOLLWORM, TOBACCO BUDWORM, AND PINK BOLLWORM

Chemical Class	2002		2003		2004		2005	
	Pounds AI	**Acres Treated**	Pounds AI	**Acres Treated**	Pounds AI	**Acres Treated**	Pounds AI	**Acres Treated**
OP	264,021	511,685	394,775	742,145	285,866	723,093	493,513	816,657
Carbamate	98,892	199,121	37,586	136,959	95,445	180,273	30,768	86,644
Pyrethroid	241,174	6,924,048	135,302	3,962,964	180,726	4,445,635	106,999	2,870,133
Other	120,650	1,991,763	24,912	435,599	34,839	741,453	335,331	683,171
Grand Total	**724,737**	**9,626,617**	**592,575**	**5,277,667**	**596,876**	**6,090,454**	**966,611**	**4,456,605**

TABLE 6.3

PERCENT OF USE BY CHEMICAL CLASS FOR PESTICIDES USED TO TREAT BOLLWORM, BUDWORM, AND PINK BOLLWORM

Chemical Class	2002		2003		2004		2005	
	Pounds AI	Acres Treated	Pounds AI	Acres Treated	Pounds AI	Acres Treated	Pounds AI	Acres Treated
OP	36%	5%	67%	14%	48%	12%	51%	18%
Carbamate	14%	2%	6%	3%	16%	3%	3%	2%
Pyrethroid	33%	72%	23%	75%	30%	73%	11%	64%
Other	17%	21%	4%	8%	6%	12%	35%	15%
Grand Total	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**

TABLE 6.4

MAJOR COTTON BOLLWORM, TOBACCO BUDWORM, AND PINK BOLLWORM PESTICIDES BY POUNDS OF ACTIVE INGREDIENT – 2002

Compound	Chemical Class	Pounds	Acres Treated	Expenditures
Spinosyn	Other	97,659	1,693,144	$16,500,447
Acephate	OP	91,305	209,811	$998,332
Profenofos	OP	67,051	109,321	$948,109
Cyfluthrin	Pyrethroid	59,525	1,828,023	$9,731,280
Zeta-cypermethrin	Pyrethroid	55,005	1,440,416	$5,946,676
Cyhalothrin-lambda	Pyrethroid	52,726	1,687,917	$8,505,838
Aldicarb	Carbamate	41,805	52,933	$840,543
Disulfoton	OP	35,612	59,354	$265,907
Phorate	OP	28,250	19,425	$296,622
Cypermethrin	Pyrethroid	27,664	476,407	$1,748,985
Total		**556,602**	**7,576,751**	**45,782,739**

TABLE 6.5

MAJOR COTTON BOLLWORM, TOBACCO BUDWORM, AND PINK BOLLWORM PESTICIDES BY POUNDS OF ACTIVE INGREDIENT – 2003

Compound	Chemical Class	Pounds	Acres Treated	Expenditures
Acephate	OP	142,993	346,118	$1,441,128
Profenofos	OP	121,691	137,277	$1,305,772
Cyhalothrin-lambda	Pyrethroid	44,845	1,494,299	$7,282,063
Methyl parathion	OP	44,377	57,101	$248,292
Chlorpyrifos	OP	34,482	41,523	$445,435
Azinphos-methyl	OP	31,246	98,687	$488,836
Cyfluthrin	Pyrethroid	30,793	926,580	$4,779,941
Cypermethrin	Pyrethroid	24,560	478,520	$1,369,898
Zeta-cypermethrin	Pyrethroid	21,885	633,855	$2,957,471
Oxamyl	Carbamate	17,587	92,394	$331,095
Total		**514,459**	**4,306,354**	**20,649,931**

TABLE 6.6

MAJOR COTTON BOLLWORM, TOBACCO BUDWORM, AND PINK BOLLWORM PESTICIDES BY POUNDS OF ACTIVE INGREDIENT – 2004

Compound	Chemical Class	Pounds	Acres Treated	Expenditures
Dicrotophos	OP	148,436	361,654	$1,550,591
Acephate	OP	109,598	289,974	$1,287,601
Cypermethrin	Pyrethroid	82,301	1,072,143	$4,678,681
Aldicarb	Carbamate	59,635	72,413	$1,169,880
Cyfluthrin	Pyrethroid	40,042	1,229,031	$6,534,719
Cyhalothrin-lambda	Pyrethroid	38,803	1,253,028	$6,100,081
Oxamyl	Carbamate	21,524	69,237	$409,896
Zeta-cypermethrin	Pyrethroid	12,147	533,481	$2,268,945
Thiodicarb	Carbamate	11,357	28,699	$141,963
Profenofos	OP	10,451	33,515	$107,518
Total		**534,294**	**4,943,175**	**24,249,875**

TABLE 6.7

MAJOR COTTON BOLLWORM, TOBACCO BUDWORM, AND PINK BOLLWORM PESTICIDES BY POUNDS OF ACTIVE INGREDIENT – 2005

Compound	Chemical Class	Pounds	Acres Treated	Expenditures
Acephate	OP	298,338	443,523	$3,654,391
Dichloropropene	Other	250,749	10,156	$255,715
Malathion	OP	91,955	74,663	$255,973
Dicrotophos	OP	62,333	209,482	$692,486
Chloropicrin	Other	53,014	10,156	$79,429
Cypermethrin	Pyrethroid	41,712	653,834	$2,804,910
Aldicarb	Carbamate	27,937	63,161	$564,487
Cyhalothrin-lambda	Pyrethroid	23,894	793,507	$3,877,601
Cyfluthrin	Pyrethroid	21,369	611,304	$3,100,233
Methyl parathion	OP	15,234	30,468	$104,732
Total		**886,535**	**2,900,254**	**15,389,957**

Tables 6.8 through 11 provide summary risk information for the top ten competitive compounds from 2005 as listed in Table 7.

Table 6.8 provides the signal word and EPA toxicity category information for the major competitive compounds. Many of these products are signal word Danger or Warning (EPA Toxicity Category I or II).

Table 6.9 presents a summary of the Personal Protective Equipment (PPE) requirements for each of the top ten competitive products. Many of the products have extensive PPE requirements including respirators.

Table 6.10 identifies ecological concerns noted on the labels of the competitive products. Eight of the ten competitive products list toxicity to fish and other aquatic organisms, birds, bees, and other wildlife in their environmental hazard statements or restricted use statements. Two of the ten products have groundwater warnings.

Table 6.11 summarizes the restricted use classification for the top ten alternative compounds. This table shows that eight of the ten alternatives are classified as restricted use. Four of these compounds are restricted use for ecological reasons. Four of the compounds are restricted use due to human toxicity, including carcinogenicity. One product is restricted use due to concerns regarding groundwater contamination.

In summary, a review of labels for the major cotton insecticides demonstrate the much greater risk potential resulting from the use of these products compared to Bt products. Signal words, statements of hazard, PPE requirements, ecological concerns, and restricted use determinations document the increased risk that exists when these insecticide products are used rather than Bt cotton products.

TABLE 6.8

SIGNAL WORD AND TOXICITY CATEGORY INFORMATION

Active Ingredient	End Use Product Name	Signal Word	Toxicity Category	Reason
Acephate	Bracket 90	Caution	III	Harmful if swallowed. Causes eye irritation. Avoid contact with eyes, skin and clothing. Avoid breathing dust or spray mist. Wash hands thoroughly after handling. Do not allow children or pets to come into contact with treated areas until sprays have dried.
	Orthene® 75 S			
Dichloro-propene	Telone C-17	Danger	I	Hazardous liquid and vapor. Do not swallow any of this product. May be fatal if swallowed. Do not get in eyes. Corrosive. Causes irreversible eye damage. Do not get on skin. May be fatal if absorbed through the skin. Causes skin burns. May cause allergic skin reaction. Do not breathe vapor. May be fatal if inhaled. May cause lung, liver, and kidney damage and respiratory system irritation upon prolonged contact. Product contains 1,3-dichloropropene, which has been determined to cause tumors in laboratory animals. This fumigant has the capacity to cause marked irritation to the upper respiratory tract.
Malathion	Fyfanon®	Warning	II	May be harmful if swallowed, inhaled, or absorbed through skin. Causes substantial but temporary eye injury. Do not take internally. Avoid contact with eyes, skin and clothing. Avoid breathing vapors, dust, or spray mist. Flush contaminated eyes with plenty of water and get medical attention if irritation persists.
	Gowan Malathion 8	Caution	III	Harmful if swallowed. Avoid contact with skin. Avoid breathing of spray mist.
	Malathion 5 EC	Warning	II	Harmful if swallowed. Causes substantial but temporary eye injury. Do not get in eyes or on skin or clothing. Avoid contact with skin. Wash thoroughly after using. Avoid breathing of spray mist.
Dicroto-phos	Bidrin	Danger	I	Fatal if swallowed. May be fatal if absorbed through skin. Harmful if inhaled. Rapidly absorbed through skin. Inhalation or skin contact may, without symptoms, progressively increase susceptibility to BIDRIN insecticide poisoning. This product has a strong skin sensitizing potential. Do not swallow or get in eyes, on skin, or on clothing. Do not breathe vapors.

TABLE 6.8 (CONTD.)

Active Ingredient	End Use Product Name	Signal Word	Toxicity Category	Reason
Chloropicrin	Telone C	Danger	I	See Dichloropropene listing above
Cypermethrin	Ammo 2.5 EC Battery 2.5 EC Up-Cyde 2.5 EC	Caution	III	Harmful if absorbed through skin, inhaled, or swallowed. Causes eye irritation. Avoid breathing vapor or spray mist. Avoid contact with skin, may cause sensitization reaction in some individuals. Avoid contact with eyes or clothing.
Aldicarb	G (Lock 'n Load®, Aldicarb Pesticide, CP Aldicarb Pesticide)	Danger	I	Fatal if swallowed. Causes cholinesterase inhibition. May be fatal or harmful by skin or eye contact or by breathing dust. Rapidly absorbed through skin or eyes. Do not get on skin or in eyes. Do not breathe dust. Keep away from domestic animals. Always stand up-wind from hopper when loading.
Cyhalothrin-lambda	Karate® Insecticide	Danger	I	Corrosive. Causes skin burns. May be fatal if swallowed or inhaled. Causes substantial but temporary eye injury. Do not get in eyes on skin or clothing. Do not breathe vapor or spray mist. Harmful if absorbed through skin. Wear protective clothing, gloves, protective eyewear (goggles, face shield, or safety glasses) and respirator as indicated under Personal Protective Equipment. Prolonged or frequently repeated skin contact may cause allergic reactions in some individuals.
	Karate® Insecticide with Zeon Technology Warrior® with Zeon Technology	Warning	II	May be fatal if swallowed. Causes moderate eye irritation. Harmful if absorbed through skin. Avoid contact with eyes, skin or clothing. Prolonged or frequently repeated skin contact may cause allergic reaction in some individuals.

NO CBI

TABLE 6.8 (CONTD.)

Active Ingredient	End Use Product Name	Signal Word	Toxicity Category	Reason
Cyfluthrin	Baythroid® 2 Emulsifiable Pyrethroid Insecticide	Danger	I	Corrosive. Causes irreversible eye damage. Do not get in eyes or on clothing. Wear protective eyewear (goggles or face shield). Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash before reuse. May be fatal if inhaled. Do not breathe vapors or spray mist. Harmful if swallowed or absorbed through skin. Prolonged or frequently repeated skin contact may cause allergic skin reactions in some individuals.
	Baythroid® XL	Warning	II	Causes substantial but temporary eye injury. Do not get in eyes or on clothing. Wear protective eyewear (goggles or face shield). Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash before reuse. May be fatal if swallowed. Harmful if inhaled or absorbed through skin. Do not breathe vapors or spray mist. Prolonged or frequently repeated skin contact may cause allergic skin reactions in some individuals.
Methyl parathion	Methyl 4EC	Danger	I	Can kill you if swallowed: This product can kill you if swallowed even in small amounts; spray mist may be fatal if swallowed. Can kill you by skin contact: This product can kill you if touched by hands or spilled or splashed on skin, in eyes or on clothing (liquid goes through clothes). Can kill you if breathed: This product can kill you if vapor or spray mist are breathed.

NO CBI

TABLE 6.9

REQUIRED PERSONAL PROTECTIVE EQUIPMENT

Active Ingredient	End Use Product Name	Worker Protection Standard – Personal Protective Equipment
Acephate	Bracket 90 Orthene 75 S	Applicators and other handlers and mixers and loaders must wear long-sleeved shirt and long pants, waterproof gloves, shoes plus socks and chemical-resistant headgear for overhead exposure. Requirements for early entry: coveralls, waterproof gloves, shoes plus socks and chemical-resistant headgear for overhead exposure.
Dichloropropene	Telone C-17	Handlers performing tasks with liquid contact potential must wear at minimum: Coveralls over short-sleeved shirt and short pants, chemical-resistant gloves, such as barrier laminate (EVAL) or viton, chemical-resistant footwear plus socks, chemical resistant headgear for overhead exposure, chemical resistant apron, face shield or safety glasses with brow and temple shields (do not wear chemical goggles), half-face respirator or full-face respirator. Handlers performing tasks with no liquid contact potential or handlers in treated area 1 to 5 days after application must wear at minimum: Loose fitting or well ventilated long-sleeved shirt and long pants, shoes and socks, face shield or safety glasses with brow and temple shields (do not wear chemical goggles), half-face respirator or full-face respirator. Handlers exposed to high concentrations must wear at minimum chemical resistant suit, chemical resistant gloves, such as barrier laminate (EVAL) or viton, chemical resistant footwear plus socks, chemical resistant headgear, supplied air respirator.
Malathion	Fyfanon Malathion 5 EC	Applicators and other handlers must wear long-sleeved shirt and long pants, chemical-resistant gloves, such as barrier laminate, butyl rubber, nitrile rubber, or viton, shoes plus socks, chemical-resistant headgear for overhead exposure, and protective eyewear. Requirements for early entry: coveralls, chemical-resistant gloves, such as barrier laminate, butyl rubber, nitrile rubber or viton, shoes plus socks, chemical-resistant headgear for overhead exposure, and protective eyewear.
	Gowan Malathion 8	Applicators and other handlers must wear long-sleeved shirt and long pants, chemical-resistant gloves, such as barrier laminate, butyl rubber, nitrile rubber, or viton, and shoes plus socks. Requirements for early entry: coveralls, chemical-resistant gloves, such as barrier laminate, butyl rubber, nitrile rubber or viton, and shoes plus socks.

NO CBI

TABLE 6.9 (CONTD.)

Active Ingredient	End Use Product Name	Worker Protection Standard – Personal Protective Equipment
Dicrotophos	Bidrin 8	Applicators and other handlers must wear coveralls over short-sleeve shirt and short pants, chemical-resistant gloves, such as barrier laminate or butyl rubber or nitrile rubber or neoprene rubber or polyvinyl chloride (PVC) or viton, chemical-resistant footwear plus socks, protective eyewear, chemical-resistant headgear for overhead exposure, chemical-resistant apron when cleaning equipment, mixing or loading. Respirator for enclosed areas. Requirements for early entry: coveralls over short-sleeved shirt and short pants, chemical-resistant gloves, such as barrier laminate, butyl rubber, neoprene rubber, poly-vinyl chloride (PVC), viton or nitrile rubber, chemical-resistant footwear plus socks, protective eyewear, and chemical-resistant headgear for overhead exposure.
Chloropicrin	Telone C-17	See Dichloropropene listing above
Cypermethrin	Ammo 2.5 EC Battery 2.5 EC Up-Cyde 2.5 EC	Handlers who may be exposed through application, or mixing or loading must wear coveralls over short-sleeved shirt and short pants, chemical-resistant gloves, such as barrier laminate or nitrile rubber or neoprene rubber or viton, shoes plus socks, protective eyewear, and for those handling concentrate, chemical resistant apron when mixing or loading. Requirements for early entry: coveralls over short-sleeved shirt and short pants, chemical-resistant gloves, such as barrier laminate or nitrile rubber or neoprene rubber or viton, shoes plus socks, and protective eyewear.
Aldicarb	Temik® brand 15 G (Lock ‘n Load®, Aldicarb Pesticide, CP Aldicarb Pesticide)	Applicators and other handlers must wear coveralls over short-sleeved shirts and short pants, waterproof gloves, chemical-resistant footwear plus socks, chemical-resistant headgear for overhead exposure, protective eye wear, chemical-resistant apron when cleaning equipment, mixing or loading and a dust/mist filtering respirator or a NIOSH approved respirator. Requirements for early entry: coveralls over short-sleeved shirt and short pants, waterproof gloves, chemical-resistant footwear plus socks, protective eyewear and chemical-resistant headgear for overhead exposure.

NO CBI

TABLE 6.9 (CONTD.)

Active Ingredient	End Use Product Name	Worker Protection Standard – Personal Protective Equipment
Cyhalothrin-lambda	Karate	Applicators and other handlers must wear coveralls over long-sleeved shirt and long pants, chemical resistant gloves, Category E, such as barrier laminate, nitrile rubber, neoprene rubber or viton, chemical –resistant footwear plus socks, protective eyewear, chemical-resistant headgear for overhead exposure, chemical-resistant apron when cleaning equipment and mixing or loading. For exposures in enclosed areas, use a NIOSH approved respirator with an organic vapor (OV) cartridge or canister. For exposures outdoors, use a NIOSH approved respirator with any R, P or HE filter. Requirements for early entry: coveralls over long-sleeved shirt and long pants, chemical-resistant gloves, Category E, such as barrier laminate, nitrile rubber, neoprene rubber or viton, chemical-resistant footwear plus socks, protective eyewear, chemical-resistant headgear for overhead exposure.
	Karate with Zeon Technology	Applicators and other handlers must wear long-sleeved shirt and long pants, chemical-resistant gloves, Category G, such as a barrier laminate or viton, shoes plus socks, and protective eyewear. Requirements for early entry: coveralls, chemical-resistant gloves, Category G, such as barrier laminate or viton, and shoes plus socks.
	Warrior with Zeon Technology	Applicators and other handlers must wear long-sleeved shirt and long pants, chemical-resistant gloves, Category F, such as barrier laminate, butyl rubber, nitrile rubber, or viton, shoes plus socks, protective eyewear. Requirements for early entry: coveralls, chemical-resistant gloves, Category F, such as barrier laminate, butyl rubber, nitrile rubber or viton, shoes plus socks
Cyfluthrin	Baythroid® 2 Emulsifiable Pyrethroid Insecticide, Baythroid® XL	Applicators and other handlers must wear long-sleeved shirt and long pants, chemical-resistant gloves, such as barrier laminate or viton, shoes plus socks, and protective eyewear. Requirements for early entry: coveralls, chemical-resistant gloves, shoes plus socks, and protective eyewear.

NO CBI

TABLE 6.9 (CONTD.)

Active Ingredient	End Use Product Name	Worker Protection Standard – Personal Protective Equipment
Methyl parathion	Methyl 4 EC	Applicators and other handlers must wear: Coveralls over long-sleeved shirt and long pants, chemical-resistant gloves, such as barrier laminate or Viton ≥ 14 mils, chemical-resistant footwear plus socks, protective eyewear, chemical-resistant headgear for overhead exposure; chemical-resistant apron when cleaning equipment, mixing or loading. For exposures in enclosed areas, a respirator with either an organic vapor-removing cartridge with a prefilter approved for pesticides, or a canister approved for pesticides, or a NIOSH approved respirator with an organic vapor cartridge or canister with any R P, or HE prefilter. For exposures outdoors, a dust/mist filtering respirator. Discard clothing and other absorbent materials that have been drenched or heavily contaminated with this products' concentrate. Do not reuse them. Follow manufacturers instructions for cleaning/maintaining PPE. If no such instructions for washables, use detergent and hot water. Keep and wash PPE separately from other laundry. Requirements for early reentry: Coveralls over long-sleeved shirt and long pants, chemical-resistant gloves, such as Barrier Laminate or Viton ≥ 14 mils, chemical-resistant footwear plus socks, protective eyewear, chemical-resistant headgear for overhead exposure.

NO CBI

TABLE 6.10

ECOLOGICAL/ENVIRONMENTAL CONCERNS

Chemical	End-Use Product	Ecological Concerns
Acephate	Bracket 90	This pesticide is toxic to birds. This pesticide is highly toxic to bees exposed to direct treatment or residues on blooming crops or weeds. Exposed treated seed may be hazardous to birds and other wildlife.
	Orthene 75S	This pesticide is toxic to birds. This pesticide is highly toxic to bees exposed to direct treatment or residues on blooming crops or weeds.
Dichloropropene	Telone C-17	1,3-dichloropropene is known to move through soil and under certain conditions has the potential to reach groundwater as a result of agricultural use.
Malathion	Fyfanon, Gowan Malathion 8, Malathion 5 EC	This pesticide is toxic to fish, aquatic invertebrates, and aquatic life stages of amphibians. This pesticide is highly toxic to bees exposed to direct treatment on blooming crops or weeds.
Dicrotophos	Bidrin 8	Extreme toxicity to fish, birds, and other wildlife. Birds feeding on treated area may be killed. This pesticide is highly toxic to bees exposed to direct treatment on blooming crops or weeds.
Chloropicrin	Telone C-17	NA
Cypermethrin	Ammo 2.5 EC Battery 2.5 EC Up-Cyde 2.5 EC	Restricted use product due to extreme toxicity to fish and aquatic invertebrates. This pesticide is highly toxic to bees exposed to treatment on blooming crops or weeds.

NO CBI

TABLE 6.10 (CONTD.)

Chemical	End-Use Product	Ecological Concerns
Aldicarb	Temik® brand 15 G (Lock 'n Load®, Aldicarb Pesticide, CP Aldicarb Pesticide)	Restricted use product due to ground water contamination potential. Aldicarb residues may move into shallow ground water under certain conditions. Toxic to fish, birds, aquatic invertebrates and wildlife Birds feeding on exposed granules may be killed.
Cyhalothrin-lambda	Karate® Insecticide, Karate with Zeon Technology, Warrior with Zeon Technology	Extreme toxicity to fish and aquatic organisms, and toxic to wildlife. This pesticide is highly toxic to bees exposed to direct treatment on blooming crops or weeds.
Cyfluthrin	Baythroid® 2 Emulsifiable Pyrethroid Insecticide, Baythroid® XL	Extremely toxic to fish and aquatic invertebrates. This pesticide is highly toxic to bees exposed to direct treatment on blooming crops or weeds.
Methyl parathion	Methyl 4 EC	This pesticide is highly toxic to aquatic invertebrates and wildlife. Birds in treated areas may be killed. Shrimp and other aquatic organisms may be killed at recommended application rates. This product is highly toxic to bees exposed to direct treatment or residues on blooming crops or weeds.

NO CBI

TABLE 6.11

RESTRICTED USE CLASSIFICATION

Active Ingredient	End Use Product Name	Restricted Use	Restricted Use Rationale
Acephate	Bracket™ 90	No	N/A
	Orthene® 75 S		
Dicloropropene	Telone C-17	Yes	High acute inhalation toxicity and carcinogenicity
Malathion	Fyfanon®	No	N/A
	Gowan Malathion 8		
	Malathion 5 EC		
Dicrotophos	Bidrin	Yes	Rationale not given
Chloropicrin	Telone C-17	Yes	High acute inhalation toxicity and carcinogenicity
Cypermethrin	Ammo 2.5 EC	Yes	Toxicity to fish and aquatic organisms.
	Battery 2.5 EC		
	Up-Cyde 2.5 EC		
Aldicarb	Temik® brand 15 G (Lock 'n Load®, Aldicarb Pesticide, CP Aldicarb Pesticide)	Yes	Acute toxicity and ground water contamination.
Cyhalothrin-lambda	Karate® Insecticide, Karate with Zeon Technology, Warrior with Zeon Technology	Yes	Toxicity to fish and aquatic organisms
Cyfluthrin	Baythroid® 2 Emulsifiable Pyrethroid Insecticide	Yes	Toxicity to fish and aquatic organisms.
	Baythroid® XL		
Methyl parathion	Methyl parathion	Yes	Very high acute toxicity to humans and birds.

NO CBI

D. RESISTANCE MANAGEMENT BENEFITS

The insect resistance management benefits offered by the introduction of VipCot™ are substantial. Not only is cross-resistance between the Vip3A and FLCry1A components unlikely, so is the potential for cross-resistance between the Vip3A and Cry proteins in currently marketed Bt cotton PIPs. Thus, the registration and marketing of VipCot™ has the potential to extend the useful life of Bt cotton technology generally.

As discussed in Syngenta's resistance management volume (Supplement 22), VipCot™ provides a high dose for cotton bollworm, tobacco budworm, and pink bollworm. Supporting information and study results demonstrate the lack of cross-resistance between Vip3A and other Bt Cry proteins, including not only the FLCry1Ab protein in VipCot™, but also the Cry proteins in currently sold Bt cotton PIPs. Resistance risk assessment modeling by Dr. Michael Caprio, Mississippi State University, predicts that the risk of resistance developing to VipCot™ is very low, and further, modeling predicts that the use of VipCot™ can delay the development of resistance to cotton varieties expressing Cry toxins.

Syngenta plans to link an aggressive marketing program designed to achieve substantial market share with a strong product stewardship program with the highest possible standards to maintain the longevity of both VipCot™ and Bt cotton technology, generally. Extending the viability of Bt cotton PIPs will enable growers and society to continue to accrue the human health, environmental, and economic benefits that have resulted from the replacement of conventional chemical pesticides with Bt products.

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REPORT 6.1

COTTON IN THE SOUTHEASTERN UNITED STATES AND THE EXPECTED BENEFITS OF SYNGENTA'S COT 102/67B STACK

NO CBI

COTTON IN THE SOUTHEASTERN UNITED STATES AND THE EXPECTED BENEFITS OF SYNGENTA'S COT 102/67B STACK

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ABSTRACT

The southeast is unique among U.S. cotton growing regions with respect to agro-ecosystem diversity and historical problems with insect pests. No other region has witnessed the collapse of an industry due to an insect pest, the boll weevil; nor has any other region had a comparable industry resurgence following eradication of that insect pest. The other enabling event behind resurgence of the southeastern cotton industry was Bt cotton.

Bt cotton varieties now dominate cotton acreage in the southeast and have resulted in economic and environmental benefits to the farmer and to society in general. Thus, the southeastern cotton field may now be characterized as a "low-spray" environment that hopefully, will persist well into the future. The planting of transgenic cottons that produce a novel Bt protein, Vip3A, may be the key to the sustained effectiveness of Bt technology and a low insecticide input system.

BACKGROUND

Agriculture in North Carolina and other Southeastern states consists of highly diverse agro ecosystems characterized by a mosaic of forests interspersed with numerous crops including cotton, corn, soybeans, peanuts and tobacco.

The history of cotton production in North Carolina is characterized by dramatic changes in acres planted. Cotton was the major cash crop grown in North Carolina from the late 1700s until shortly after the boll weevil invaded the state in the 1920s. Cotton production in the state peaked at 1.8 million acres in 1926; rapidly declined to 1 million acres by 1935 and reached the modern era low of less than 50 thousand acres by the late 1970s. The demise of the North Carolina cotton industry during the second quarter of the 20th century is attributed largely to insect pests and problems associated with their management (Dickerson et al. 2001).

The reversal of fortune for the North Carolina cotton industry began in the 1980s in response to eradication of the boll weevil. The cotton industry infrastructure was rapidly rebuilt and cotton

acreage resurged. By the early 1990s cotton was planted to more than 500 thousand acres in North Carolina and for the past decade cotton has been planted to greater than 800 thousand acres annually (NASS 2005).

The entire Southeast has experienced a recent rebirth of the cotton industry in response to the elimination of the boll weevil and advances in technology for caterpillar control, mainly the advent of Bt cotton. The continued effectiveness of Bt technology is an important factor in maintaining cotton as a major crop in the southeastern United States. A strong resistance management program is essential to ensure the long-term success of Bt cotton (Bates et al. 2005).

PEST MANAGEMENT PRACTICES

Prior to the boll weevil entering North Carolina (and other Southeast states), arthropod pests were of relatively minor importance to cotton production. There were no key pests, and only the bollworm and cotton aphid were occasional pests. The region consisted of a patchwork of small farms with multi-crop production systems that provided alternate hosts for caterpillars that were more preferred than cotton. Cotton was not a preferred food source or oviposition host for insect pests because the naturally infertile soils of the Southeast produced a smaller cotton plant that "cut-out" early, and was a poor late-season host (Bradley 1993).

The emergence of the boll weevil resulted in dramatic changes in cotton production. During the years (1930-1946) following entrenchment of the boll weevil, but prior to the organic insecticide era, North Carolina cotton acreage steadily declined, and yield expectations were generally low. During this time, farmers had little concern for pests other than boll weevil.

Farmer attitudes toward cotton changed when organic insecticides became available at the end of World War II. For the first time, it was thought that control of cotton insect pests, including boll weevil, could be guaranteed. In response, there was wholesale adoption of yield maximization strategies with little concern for the impacts upon arthropod pests. Cultural controls that had been employed successfully to lower the mean level of abundance of many pests, particularly caterpillars, were largely abandoned in quest of increased yields. A protective blanket of effective, inexpensive insecticides allowed cotton breeders to develop high-yielding varieties that would produce fruit as long as the season allowed and would positively respond to irrigation and increased amounts of fertilizers. This surge of new technologies produced the desired result; long-season, maximum input production systems that allowed the cotton plant to more completely realize genetic yield potential and to produce longer-stable lint that was highly sought by textile mills. This period of optimism lasted less than a decade with the elevation of secondary and potential pests to major pest status. A great diversity of arthropod pests thrived in these long-season cottons that remained lush for the entire summer. Among the species that began to exploit cotton on a regular basis were cotton aphids, spider mites, thrips, whiteflies,

armyworms, loopers, bug pests, bollworm, and tobacco budworm. The situation was further exacerbated through the emergence of insect strains that were resistant to many insecticides (Bradley 1980).

North Carolina cotton farmers discovered that they were losing the battle with insect pests. By the late 1970s cotton acreage in North Carolina had fallen to less than 50 thousand acres. A rapid resurgence of the cotton industry occurred in the 1980s following boll weevil eradication. The boll weevil was the key pest and its elimination was the enabling factor that allowed successful management of other pests by cultural means, selective insecticides, and genetically engineered plants that produced insecticidal proteins (Bradley 1994).
First and foremost of current insect management tactics employed by North Carolina farmers is the preventive use of insecticides at planting as either seed treatments or as a granule applied in-furrow to control thrips and aphids on the seedling plant. Control of these insect pests is practiced on essentially 100% of the cotton acreage because yield reductions as high as 70% have been documented in tests where they were not controlled (Faircloth et al. 1999).

The second most prevalent insect management practice employed by North Carolina farmers is the planting of Bt cotton for control of caterpillar pests. In 2005, approximately 94% of the cotton acreage in North Carolina was planted to a Bt variety (J. S. Bacheler, per comm.).

Hemipteran pests are thought to be increasing in North Carolina with the reduced use of broad-spectrum insecticides following boll weevil eradication and the wholesale planting of Bt cotton. The current, low-spray environment and use of selective insecticides for control of cotton aphid and spider mites should allow hemipterans to reach their pest potential. Thus, “bug” pests have become the current focus of pest managers in Bt cotton (Bacheler and Mott 2005).

Early season monitoring for plant bug, *Lygus lineolaris*, begins with retention counts of small squares. If square retention counts drop below a threshold value and plant bug presence is confirmed through sweep net sampling, an insecticide application is recommended. Once flowering has commenced, square retention is an unreliable indicator of plant bug feeding because of square shed from weather related factors. Thus, late season plant bug damage is assessed in North Carolina by monitoring for “dirty” blooms and internal damage to small bolls, as well as for the presence of live plant bugs. Adult and nymphal stages are monitored by sweep net, drop cloth, or by visual inspection (Bacheler 2005).

Stink bugs are pests of cotton only after bolls are present; thus, their management is a mid-to-late season phenomenon. Extensive feeding may completely destroy small bolls, causing them to abort. When stink bugs feed on larger bolls, they not only destroy individual seeds, but they also often introduce boll-rot pathogens resulting in partially or entirely destroyed locks, hard-lock condition, and a lower grade for the harvested fiber. Scouting for stink bugs commences 1-2

weeks after first flower and continues through the boll development period. Stink bug damage levels are determined by cutting open quarter-size bolls and assessing internal damage to include callous growth, or warts on the internal surfaces of the carpel walls or stained or spotted lint. At present, stink bug management consists of insecticide application when a 10% boll damage threshold is met or exceeded.

There are several other arthropods that are occasional pests of cotton in North Carolina; these include cotton aphids, two-spotted spider mites, and cutworms. Populations of these pests are monitored whenever they are observed, thresholds are considered, and insecticide applications are made when warranted. With few exceptions, these arthropod pests may be controlled through the application of selective insecticides with minimal adverse environmental impacts. For example, cotton aphid control may be achieved with any of several members of the chloronicotinyl family.

Highly effective technologies are available for control of arthropod pests of cotton with the exception of hemipteran pests. Bt cotton now includes two-gene products- Bollgard II® and WideStrike® that effectively control all caterpillar pests with the exception of cutworms. Furthermore, VipCot™ cottons will soon be registered that are equally effective against caterpillar pests. Thrips and cotton aphids may be controlled with highly selective insecticides in the chloronicotinyl family with minimal adverse environmental impacts. Also, effective miticides are available for control of two-spotted spider mites.

The major shortcoming of current management practices available to North Carolina farmers concerns hemipteran pests. Adequate control of "bug" pests may be achieved, but only through the application of broad-spectrum insecticides that may lead to excessive costs and potential environmental problems. Furthermore, monitoring procedures are often too time consuming and costly to be fully implemented; thus insecticide application is often not based on confirmed need.

IMPACT OF Bt COTTON

Cotton plants genetically modified to express insecticidal proteins from *Bacillus thuringiensis* have revolutionized insect control and in so doing have had a profoundly positive impact on agriculture. The rapid acceptance of these Bt cottons by US cotton producers is a testament to their benefits at the farm level. Perhaps no other technology has received such a rousing endorsement in the initial years of commercialization. Generally, these crops have shown substantial economic benefits to growers and reduced the use of other insecticides (Shelton et al. 2002).

Specifically, yields of Bt cottons averaged over years and locations exceeded that of conventional cotton. The mean profit advantage ranged from about $16 to almost $173 per acre, including the costs of the technology fee (Marra et al. 2002). Bacheler and Mott (2003) reported

that over the period of 1996-2002, Bollgard® cotton in North Carolina was sprayed with insecticides only 0.79 times annually, whereas non-Bt cotton required 2.75 foliar insecticide applications over the same period.

Introduction of Bt cotton technology has definitely had positive environmental benefits through reduced insecticide usage. The U.S. EPA using data from NASS noted that reductions in insecticide use on cotton, from 1.5 to 3 applications, occurred in all regions with high adoption of Bt cotton (US EPA). For 1999 alone, EPA estimated that there was a 7.5-million-acre reduction in insecticide use on cotton due to the extensive planting of Bt varieties. The environmental benefits are obvious, but the extended benefits in fuel savings and reduced labor expenditures must also be considered. In North Carolina, Bt cottons are now planted where cotton could not be planted in the past because of concern for insecticide drift onto housing developments, schools, parks and other areas of frequent human activities.

Transgenic cottons engineered to produce a novel Bt protein, Vip3A, have recently received a non-regulated status for planting in the US. The Vip3A protein is unique in that it is active in the vegetative phase, as well as during the sporulation phase; whereas, Cry1 and Cry2 protein activities are restricted to the sporulation phase (Estruch et al. 1996).

Bradley et al. (2004) presented preliminary field data from North Carolina suggesting that cottons producing the Vip3A protein, COT202 and COT203, exhibited levels of cotton bollworm control equal to that of Bollgard II® and superior to that of WideStrike®. A 2006 field test also located in North Carolina and confirmed that Syngenta's COT 102/67B stacked cottons may be expected to provide efficacy against caterpillar pests equal to that of Bollgard II® and superior to that of WideStrike®. Under extremely high bollworm larval populations the stacked cottons provided exceptional control of a bollworm population that decimated the non-Bt cotton genotype. These tests confirm that the technology will provide cotton producers with a viable alternative to currently registered Bt cottons. Not only will the COT 102/67B cottons provide variety choices with no loss in insect control, but they offer a mechanism of resistance management as well. Jackson et al. (2006) reported that Cry1Ac-resistant strains of tobacco budworm were susceptible to Vip3A toxins and Vip3A producing plants in laboratory and greenhouse experiments. These experiments demonstrated that cross-resistance is non-existent between Cry1Ac and Vip3A in tobacco budworm. Thus, the planting of Vip3A-expressing cotton varieties to a substantial portion of cotton acreage should delay Cry1Ac resistance evolution in heliothines and increase the sustainability of all Bt technologies.

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REPORT 6.2

AN EXAMPLE OF COTTON PRODUCTION IN THE "MID-SOUTH" REGION OF THE UNITED STATES AND THE EXPECTED BENEFITS OF SYNGENTA'S COT 102/COT67B STACK

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AN EXAMPLE OF COTTON PRODUCTION IN THE 'MID-SOUTH' REGION OF THE UNITED STATES AND THE EXPECTED BENEFITS OF SYNGENTA'S COT 102/67B STACK

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ABSTRACT

Arthropod pest control in cotton fields across the Mid-South states of Arkansas, Louisiana, Mississippi, and Tennessee represents a source of significant annual variable production costs. Pest management strategies continue to evolve with the changing status of several pest problems. These changes in pest status are likely the consequence of target-selective insecticides, Bt cotton, and boll weevil eradication programs. Adopting these technologies have decreased the application frequency of broad-spectrum insecticides and allowed changes in pest status. This shifting spectrum of potential pests across the Mid-South requires producers to incorporate a broad range of tactics into a holistic integrated management system to be economically successful.

A critical component of Mid-South cotton IPM relies upon the effective use of *Bacillus thuringiensis* (Bt) cotton technologies. Bollgard®, Bollgard II®, and WideStrike® are currently registered for use and provide effective control of several species of caterpillar pests. Syngenta is currently developing a novel transgenic product with activity against a broad spectrum of Lepidopteran pests. Syngenta's VipCot™ cotton lines express two insect control proteins, Vip3A and Cry1Ab. The Vip3A protein is different in structure and mode of action compared to that of that of the Cry proteins expressed by other transgenic Bt cottons. The Cry1Ab protein in combination with Vip3A further enhances the efficacy and the spectrum of Lepidopteran pest activity compared to that observed for single and selected combinations of Cry proteins expressed in cotton plants. VipCot™ also will also provide another option to the Bt resistance management alternative. Based upon the data generated in Louisiana and from other state and federal scientists, the VipCot™ technology will reduce, and in some instances, eliminate supplemental control of caterpillar pests with foliar applications of insecticides in cotton. Additional options for Bt resistance management could prolong the efficacy of transgenic control strategies not only in cotton, but also in other crops that utilize similar Cry proteins and are attacked by target pests that overlap in cotton fields. Providing producers with another approved IRM tactic also could improve and sustain Bt resistance management compliance levels by offering acceptable alternatives that do not create operational and short-term economic problems.

BACKGROUND

The lands of the Lower Mississippi River Valley represent one of the most productive agricultural regions of the United States. The states of Arkansas, Louisiana, Mississippi, and Tennessee border the Mississippi River and compose an area of the country termed the 'Mid-South'. Many of the agronomic practices and pest management strategies are similar across the Mid-South production systems. The primary agricultural commodities in these states include forests, livestock and pastures, as well as intensively managed row crops. The most common fiber and grain crops are cotton, field corn, soybean, rice, and grain sorghum. Other noteworthy crops of substantial acreage in this region include sugarcane and sweet potatoes.

Cultivation of cotton was reported in the 1700's in the Mid-South States, but fiber use was limited to household spinning. Cotton became a cash crop in 1793 with Eli Whitney's development of the cotton gin. Commercial cotton production spread to the Mid-South region from the Atlantic coast states during the early 1800's (May and Lege 1999). Cotton acreage and production increased steadily until the outbreak of the Civil War. Historically, cotton, field corn, soybean, and rice have represented the greatest acreage of row crop commodities harvested annually across this region. In the last five years, cotton acreage in the Mid-South States of Arkansas, Louisiana, Mississippi, and Tennessee have ranged from 910,000 to 1.17million acres, 500,000 to 630,000 acres, 1.11 million to 1.22 million acres, and 530,000 to 700,000 acres, respectively. In addition, annual lint yields during 2002-2006 generally were among the highest historical levels produced in these states (NASS 2006).

Prior to the 1950's, the lack of available production input resources limited the average cotton producer in the Mid-South to only about 20 acres. With mechanization and improved pesticides during the 1960's and 1970's, cotton farm size increased over 10-fold in size. During the last twenty years, major advances improving cotton productivity and reducing on-farm risk generally have been associated with changes in pest management practices. Boll weevil eradication, genetically engineered caterpillar resistant varieties, herbicide tolerant cultivars, and producers in Louisiana, as well as in the other Mid-South states have rapidly adopted seed treatments for insect, disease, and nematode management. Adoption rates of these technologies have exceeded 90% in some areas and clearly demonstrate the value of these tools to cotton producers (USDA-AMS 2006, Williams 2006).

Regardless of the aforementioned technologies, it is the responsibility of the entire cotton industry to develop and implement strategies to maintain their sustainability. The development of scientific information to support temporal and/or spatial use patterns is essential to maintain efficacy levels and create opportunities for additional crop production/protection technologies.

STATUS OF ARTHROPOD PEST MANAGEMENT

The agricultural landscape has gone through some dramatic changes during the last decade. Recent advances in pest management for cotton in the Mid-South have resulted in less reliance on foliar applications of synthetic pesticides. Notably, efforts to eradicate the boll weevil have significantly reduced the total numbers of insecticide applications to cotton. Prior to the boll weevil eradication program, the total annual insecticide applications ranged from 12.4 to 19.9 per acre in the Mid-South (King et al. 1987). During the 2002 growing season, after boll weevil eradication had been initiated or completed in each of these states, the total numbers of insecticide applications declined, ranging from 2.1 to 8.0 per acre (Williams 2003). These figures directly reflect the numbers of insecticide applications used against boll weevils as well as applications targeting other pests (primarily Lepidopteran larvae). These pests were released from natural control by biological agents that were destroyed by broad spectrum insecticides used against boll weevils. Concurrent with implementation of the eradication program was the registration of the Bollgard® technology used against tobacco budworm and bollworm. Bollgard® has reduced insecticide application frequency against lepidopteran pest targets over 50% compared to previous insecticide use patterns (Williams 1996; 2006). However, the Cry1Ac protein in Bollgard® cottons only provides satisfactory control of a limited range of Lepidopteran pest species. For example, supplemental insecticide oversprays have been required to prevent economic injury from bollworm infestations that persist for several days in many mid-South and Southeastern Bollgard® fields (Bacheler and Mott 1997, Layton et al. 1998, Leonard et al. 1998, Roof and DuRant 1997, Smith 1997).

In the Mid-South, cotton is a long season crop, which is attacked by a diverse group of pest species throughout its development and maturity (Johnson et al. 1996, Leonard et al. 1999). In Louisiana, this extended list of arthropod pests includes one or more species that attacks cotton during nearly every stage of crop development. The most common pest problems until recently have been cutworms, *Agrotis spp.*; thrips, Thysanoptera; cotton aphid, *Aphis gossypii* Glover; tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois); cotton fleahoppers, *Pseudatomoscelis seriatus* (Reuter); stink bugs, Pentatomidae; boll weevil, *Anthonomus grandis grandis* Boheman; spider mites, Acari; bollworm, *Helicoverpa zea* (Boddie); tobacco budworm, *Heliothis virescens* (F.); fall armyworm, *Spodoptera frugiperda* (J. E. Smith); beet armyworm *Spodoptera exigua* (Hübner); and soybean looper, *Pseudoplusia includens* (Walker). Indirect losses from cotton insect pests such as quality reductions, harvest efficiency decreases, and delayed maturity are difficult to quantify, but can be extensive in some years.

The number of pests and requirement for multiple control measures cause arthropod pest management to be a significant annual variable production cost for cotton. During 2005, the total economic loss in Louisiana from cotton arthropod pests was $15.63 million. Cotton producers had average expenditures of $99.45 per acre for arthropod pest control that year

(Williams 2006). During periods of prolonged and intense pest infestations, control costs can exceed $150/acre.

The most injurious pests during the decade of the 1990's were a complex of the previously listed Lepidopteran pests and the boll weevil. However, beginning in the late-1990's, the status of cotton pests shifted to more consistent problems from a complex of Heteropteran pests that currently exists today. These pests have included tarnished plant bug, brown stink bug, *Euschistus servus* (Say), southern green stink bug, *Nezara viridula* (L.), and green stink bug, *Acrosternum hilare* (Say). This change in pest status was likely the consequence of an overall decrease in the application frequency of broad-spectrum insecticides that are applied against cotton pests (Roberts 1999, Leonard and Emfinger 2002). Those treatments formerly directed toward boll weevil and other Lepidopteran pests coincidently controlled infestations of tarnished plant bugs and stink bugs. In 2005, tarnished plant bug and stink bugs were responsible for nearly 50% of the yield losses attributed to arthropod pests in cotton (Williams 2006). In Louisiana, tarnished plant bug and stink bug pests infested 96.7% and 53.2%, respectively, of cotton acreage during 2005. Stink bugs were not included in cotton loss estimates for each state until 1993, because they occurred infrequently. During 1995, Louisiana reported <1.0% stink bug infested acreage (Williams 1996). Stink bugs have been recognized as a potential but uncommon pest of cotton for many years, but within the last decade they have been elevated to the status of a major cotton pest. In addition, the frequency of insecticide applications targeting tarnished plant bugs and stink bugs have increased in response to these changes in pest management practices. During 1995, the numbers of insecticide applications targeting tarnished plant bugs were 1.1 applications per acre. By 2004, 5.4 treatments were applied per acre for this pest (Williams 1996, 2005). Tarnished plant bug and stink bugs now represent significant pest problems in conventional non-Bt and transgenic Bt cotton fields in the absence of the boll weevil.

The cotton industry has consistently relied heavily on insecticide use strategies to manage arthropod pests (Leonard et al. 1999). However, the overall availability of insecticides has decreased due to difficulties in the discovery of new chemistry, the exorbitant cost of registration and re-registration, cancellation of uses, and the development of insect resistance to insecticides. The lack of effective products against specific pests has caused producers to increase the doses and application frequency of available insecticides to obtain satisfactory control. Frequently, the use of products in such a manner induces economic outbreaks of other cotton insect pests by disrupting native beneficial arthropod populations that limit pest populations.

2006 RECOMMENDED ARTHROPOD PEST MANAGEMENT STRATEGIES

The current strategy to manage insects is multi-faceted, but still requires the timely application of insecticides. The majority of the cotton acreage is scouted once or twice weekly by a professional agricultural consultant who dictates pest management strategies to the producer.

Insecticide selection and application timing is determined by specific pest problems that are detected in each field or farm (Bagwell et al. 2006).

The abbreviated summary of recommended practices listed below includes cultural, chemical, biological, and host plant resistance (transgenic *Bacillus thuringiensis* var *kurstaki* [Bt] plants). During the winter months, field selection is based upon proximity to alternate crops, CRP/WRP land, crop rotation patterns, and Bt cotton refuge areas. This information is used by the consultant to estimate areas of initial field infestations from emigrating populations and prepare sampling plans that consider these areas.

Variety selection focuses on greater than 80% of the acreage planted to a Bt cotton cultivar (Bollgard®, Bollgard II®, or WideStrike®). VipCot™ varieties are equally effective against Lepidopteran pests, and once they are registered and commercially available, will be recommended in a similar manner. In 2005, 83.8% was planted to varieties expressing the Bt cotton trait. Herbicide-tolerant cottons (Round-up Ready, Round-up Flex, or Liberty Link) are stacked in the Bt varieties, but the conventional non-Bt acreage is also planted with herbicide-tolerant cottons for ease of weed management in Bt cotton IRM refuges. The herbicide traits are not necessarily used to manage arthropods, but must be included in this discussion due to fact that herbicide tolerance is an important consideration in varietal selection. Herbicide-tolerant cotton varieties were planted on over 95% of the cotton acreage in the Mid-South states in 2006 (USDA-AMS 2006).

Cultural practices include herbicide use strategies that terminate all winter and spring vegetation to ensure a weed-free seedbed at least four weeks in advance of planting. The absence of living vegetation in the field reduces the probability of insect pests being present at the time cotton seedlings emerge.

Seed treated with insecticides and/or soil applied products are used at the time of planting as a preventative treatment against thrips and cotton aphids. From all estimates, nearly 100% of the cotton acreage in the Mid-South region is treated with these products. An insecticide treatment, usually a pyrethroid, is applied post-planting, but pre-emergence to control cutworms and preserve plant stand densities. During seedling development a single insecticide application may or may not be used between 7 and 21 days after emergence to control thrips after the residual efficacy of the seed or soil insecticide has decayed to ineffective levels (Anonymous 2006, Bagwell, et al. 2006).

Beginning at the pin-head flower bud (square) stage of plant development and continuing until the majority of the plants are flowering, fields are sampled by monitoring square retention and infestation levels of tarnished plant bugs and cotton fleahoppers. Reactive control strategies with insecticides are used only if pests are present, and square retention falls below an action threshold.

Cotton aphids may develop to detectable levels during this same period. However, the entomopathogen, *Neozygites fresenii* Nowakowski, typically reduces cotton aphid densities after they have reached a certain level and environmental conditions are favorable for an epizootic event. In some instances, an insecticide treatment is recommended when excessive numbers of cotton aphids are present, beneficial are low, and plants are experiencing environmental stresses (Chappell et al. 2005, Bagwell et al. 2006).

During the flowering and boll maturation stages, a complex of Heteropteran pests, including tarnished plant bugs and stink bugs, can be yield limiting pests (Greene et al. 2006, Williams 2006). Fields are sampled by visual observation of damaged fruiting forms (internal injury to squares or bolls), insect samples with sweep nets and shake sheets, or a combination of these methods. In addition, a group of Lepidopteran (caterpillar) pests can reach damaging levels at several periods during the flowering and post-flowering stages. These pests may include bollworm, tobacco budworm (except on Bt cotton), fall armyworm, beet armyworm, and soybean looper. All caterpillar pests except tobacco budworm (which is eliminated) are reduced in Bt cotton to levels that may or may not need insecticidal oversprays. Action thresholds are designed to initiate treatments for all of these pests. At the end of the production season, many consultants use boll maturity (based upon heat unit accumulation after the last effective boll population has developed on the plant) to terminate arthropod pest management strategies (O'Leary et al. 1996, Leonard et al. 2007).

Cotton producers in Louisiana and across the Mid-South are fortunate to have a number of highly effective technologies to control most arthropod pests of cotton. The primary concern during 2006 and in the foreseeable future is a lack of available options to control the tarnished plant bug. Populations of this pest have exhibited resistance to most classes of currently available insecticides. The few alternative products demonstrate lower levels of efficacy and are more expensive than the standard organophosphates and carbamates.

IMPLEMENTATION OF Bt COTTON INTO MID-SOUTH COTTON IPM

In 1996, the world's first transgenic cotton cultivars expressing insecticidal activity were commercially planted on 1.8 million acres in the United States (Gould 1998). Those initial cotton cultivars expressing the Bt gene (trademarked Bollgard®) provided exceptional control of two important target pests, tobacco budworm and pink bollworm. The main target of Bollgard® cotton was and is still populations of tobacco budworm in the Mid-South. Widespread resistance to conventional insecticides made this insect difficult to control during the early to mid 1990's (Sparks et al. 1993, Elzen et al. 1994). The excessive cost of controlling caterpillar pests with foliar insecticides on non-Bollgard® cotton and the risk of economical yield losses has allowed Bollgard® to become widely accepted by producers. Consequently, Bollgard® cultivar acreage rapidly increased across the Mid-South and essentially eliminated the yield limiting effects of tobacco budworm.

The efficacy of Cry1Ac in Bollgard® against several other lepidopteran pests is not acceptable and foliar applications of conventional insecticides are required to manage these pests in many fields (Macintosh et al. 1990, Stewart and Knighten 2000). The most consistent common pest after the tobacco budworm is the bollworm. The bollworm is significantly less susceptible to the insecticidal protein in Bollgard® cotton than tobacco budworm (Gore et al. 2001). Despite less than adequate control of bollworms with Bollgard® cotton, the numbers of insecticide applications for the bollworm/tobacco budworm complex have declined since the broad adoption of Bollgard® cotton. From 1990 to 1995, cotton producers in Louisiana experienced an average loss of 4.1 percent attributable to the bollworm and tobacco budworm complex. From 1996 to 2002, losses dropped to 2.1 percent for both Bt and non-Bt varieties. Since 1999, Bt varieties have averaged less than 1% yield loss from this insect compared to yield losses in the range of 2-5% for non-Bt varieties (Williams 1996, 2001, 2003, 2004, 2005, 2006).

Recent advances in genetic engineering (GE) technologies have produced a second generation of caterpillar-resistant cotton. Field and laboratory studies have been conducted during the past several years with Bollgard II®, WideStrike® and VipCot™ products in Louisiana. Bollgard II® and WideStrike® express multiple insecticidal proteins and typically exhibit a much greater spectrum of caterpillar control compared to that for Bollgard. Similar data across the Mid-South demonstrates enhanced efficacy with VipCot™ against bollworm and satisfactory control of a wider spectrum of Lepidopteran pests including fall armyworm, beet armyworm, soybean looper, cotton leaf perforator (*Bucculatrix thurberiella*), and black cutworm (*Agrotis ipsilon*) compared to that for other single Cry protein technologies (Mascarenhas et al. 2003, Shotkoski et al. 2003, Cook et al. 2004, Mascarenhas 2004, Leonard et al. 2005). Numbers of insecticide applications targeting Lepidoptera are expected to further decline as improved transgenic cottons (Bollgard II®, WideStrike®, VipCot™) are released into commercial production (Leonard et al. 2003).

Syngenta's experimental VipCot™ cotton line expresses two Bt proteins, the Vip3A or vegetative insecticidal protein, and the Cry1Ab protein. The Vip3A is different from proteins expressed in other Bt cottons in structure and mode of action (Estruch et al. 1996). These proteins were combined or "stacked" through the conventional breeding of COT102 (Vip3A) with COT67B (Cry1Ab). Cotton entomologists expect the performance of the COT102/COT67B or VipCot™ stacked cottons to provide satisfactory control against caterpillar pests equal to or better than that of currently registered alternative Bt cottons. In addition, this is the first transgenic introduction into cotton varieties that uses something other than a Cry protein. Therefore, the COT102/COT67B insecticidal proteins could have considerable value as a Bt resistance management tool. In laboratory populations of tobacco budworm, no cross-resistance between Cry1Ac and Vip3A proteins of Bt were detected (Jackson et al. 2006). Given the adoption rates of Bt cottons expressing Cry proteins, the availability of VipCot™ cotton varieties containing Vip3A offers an acceptable alternative to the other Bt technologies. Additional Greater choice for growers in Bt cotton technology will reduce the chance of insects developing resistance to

any one technology. It is likely that the cotton industry in the Mid-South would greatly suffer and perhaps cease to exist in the absence of Bt technologies.

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Report 6.3

TEXAS COTTON AND THE EXPECTED BENEFITS OF NEW TRANSGENIC VARIETIES

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TEXAS COTTON AND THE EXPECTED BENEFITS OF NEW TRANSGENIC VARIETIES

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ABSTRACT

Texas is the largest cotton producing state in the U.S. with almost six million acres harvested in 2005. Because of its vast size, there are several distinct cotton production regions in Texas with varying production and variety requirements. With the introduction of boll weevil eradication and Bt cottons, Texas has entered a new era of cotton production with increased yields and greatly reduced insecticide use. New transgenic cotton varieties containing the Bt protein Vip3A offer a means of sustaining the current successful insect management system.

BACKGROUND – THE TEXAS EXPERIENCE

Texas, due to its size and extreme variation in environmental conditions, includes 10 distinct vegetational areas. These areas range from pine forests in the east, across blackland prairies, gulf prairies and marshes, post oak savannas, and rolling and high plains, to mountains and desert valleys and plateaus in the west. Rainfall varies from annual averages of more than 55 inches in extreme east Texas to less than 8 inches in the desert areas of the far western portion of the state (Gould 1975).

Within the various vegetational areas lie many millions of acres of cultivated land devoted to the production of cotton, wheat, grain, sorghum, corn, rice, vegetables, and citrus. Cotton however, has long been the major cash crop with the first commercial plantings made in the Brazos river flood plain in the 1822. Cotton planting increased following the Civil War with nearly 7 million acres being harvested in 1900. This figure had increased by 1926 to near 18 million acres (Walker 1983).

Throughout the world, cotton has proven vulnerable to the attack of many insect species. If there is a single explanation for this, it is found in the protracted fruiting period the plant expresses.

Ironically, this same fruiting habit contributes to the level of lint production that producers seek (Walker 1983).

Insect damage was apparently not a major factor in the early days of cotton production in Texas. The cotton leafworm, a tropical species, usually migrated northward each season into cotton growing areas of Texas. However, infestations of this foliage feeding insect often did not occur until late in the season after the crop was satisfactorily mature. Early reports refer to occasional "ravages" of the bollworm, which destroyed up to 50% of the cotton in some countries. Damaging bollworm infestations were reported to occur mainly in late planted cotton and in cotton delayed in maturity by excessive rainfall. Likewise, the ranker growing cotton of the fertile river bottoms was more susceptible to damage than the less vigorous cotton grown on upland soils (Quaintance and Brues 1905). It is likely that the tobacco budworm also was involved in these infestations but was not identified because of the similar appearance of the two species (Walker et al. 1978, Rummel et al. 1978).

There were other insects species recognized as enemies of cotton: the cotton fleahopper, several species of lygus, and the cotton aphid. These insects usually infested cotton in a spotted, haphazard manner and often did not occur in numbers sufficient to cause significant damage (Walker 1983).

This favorable situation changed with the invasion of the boll weevil in 1894. Boll weevil infestations determined how and where cotton would be grown in Texas. Gradually, acreages were eliminated in the eastern 40-48 inch rain zone as growers discovered that the 18-25 inch annual rainfall in the western part of the state permitted effective production. At the end of the 1920's growers farmed near 18 million areas of cotton with over 5 million grown in the western half of the state where boll weevils were not a problem (Walker 1983). Cultural controls offered some relief from weevil damage, but there were no truly effective insecticides available. Calcium arsenate provided marginal control of the boll weevil but damaging outbreaks of bollworms an aphids often followed treatment with this material.

The problem of major insect damage in Texas cotton seemed answered with the introduction of the synthetic organic insecticides following World War II. Inexpensive, highly effective, and formulated as easy-to-use sprays, these materials were quickly adopted into various styles of automatic application programs.

Cultural controls, which had evolved over the years were replaced by multiple treatment insecticide programs. Producers, seeking maximum yields, adopted long season production systems with increasing levels of irrigation, nitrogen fertilizers, and insecticides. Secondary pest outbreaks involving numerous species became common, but these also could be controlled with

the new insecticides. Difficult to control bollworm and tobacco budworm infestations were addressed with multiple treatments of mixtures of two, three, and even four different insecticides.

The "glory" days of producing cotton under a blanket of synthetic organic insecticides was short lived. By 1960, it had become difficult to control bollworms with organochlorines and entomologist soon documented resistance in both the bollworm and the tobacco budworm. Insect damage and the costs of control greatly reduced profits. By the late 1960's cotton production in the Texas Gulf Coast, the Texas Lower Rio Grande Valley and the Texas Trans-Pecos seemed to be on the verge of extinction. Entomologists and producers finally realized that insects could not be dominated in cotton production by repeated applications of insecticides (Walker et al. 1978).

Another important problem, largely unanticipated by producers was the growing public concern over the annual use of millions of pounds of toxic chemicals in cotton production. In the late 1960's the concept of integrated pest management was introduced to Texas cotton producers. Economic injury levels and treatment thresholds were developed for major pests and producers began to depend increasingly on cotton scouting to determine insect infestations levels (Walker et al. 1978). Farmers were encouraged to take advantage of natural insect control factors and apply insecticide only when treatment thresholds were reached. Insecticide treatments were still needed but producers began to realize that sometimes insecticide usage resulted in more problems than it solved.

Walker (1983) concluded that if there was a single lesson learned from these experiences it was that the cotton insect fauna, always a delicate balance of interactions, become a dangerous affair for producer interests when the crop was encouraged through plant breeding, irrigation, or fertilizer to prolong the fruiting habit. Cotton producers turned to earlier maturing, short season cottons to reduce boll weevil and Heliothine pest damage (Walker et al. 1978). Rather than seeking maximum yields, farmers accepted lower yields and increased profits through minimum inputs. This system was far from perfect but it was something most producers could live with.

Cotton producers also benefited from reduced public concern over the use of insecticides.
Over 19 million pounds of insecticide were applied to Texas cotton in 1964. By 1976, this figure was reduced to less than 3 million pounds. By 1980, less than 10% of Texas cotton was being grown under the multiple insecticide treatment programs that once were commonly followed (Walker 1983).

Encouraged by the success of boll weevil eradication in southeastern states, producers made plans to begin eradication programs in Texas by the mid-1990's (Stavinoha and Woodward 2001). While boll weevil eradication was expected to effect a major improvement in Texas cotton production, there was a missing element. Producers still desired longer season production

systems to maximize yields, but this type system was known to encourage infestation by Heliothine pests. This problem was addressed by the introduction of Bt cottons in the late 1990's. The initial Bollgard® offering provided excellent control of tobacco budworm and usually satisfactory control of bollworms (Rummel et al.1994). Even pests not specifically targeted by this Bt cotton such as the beet armyworm were often held to non damaging levels. The introduction of Bollgard II® cotton proved even more effective.

Under the cover of boll weevil eradication and Bt cottons, Texas has entered a new era of cotton production. Growers are seeking improved, adapted varieties, which offer longer season production to better realize the genetic potential of the plant. Per acre, yields and lint quality have increased. Cotton planting has rebounded in some older production areas and has spread into previously non-cotton areas of the Texas Panhandle.

Hemipterian pests, which became a problem following boll weevil eradication in southeastern states, have so far not presented a significant problem in Texas. The cotton aphid, which reached major pest status during the 1980 to early 1990's period when pyrethroid insecticides were widely used, (Kidd et al. 1996), is now only an occasional pest. The cotton fleahopper while not an annual pest, sometimes reaches damaging levels. However, this pest can be controlled with limited treatments of available, selective insecticides.

The use of Bt cotton in dryland production in low rainfall areas such as the Texas High Plains, and Texas Rolling Plains is generally not feasible due to the high technology fees. However, the planting of Bt cottons in the higher rainfall area of the Texas Gulf Coast is increasing (R.D. Parker, per. comm.). Bt cotton varieties are now widely used in irrigated cotton plantings throughout Texas.

NEW TRANSGENIC COTTONS IN TEXAS

Due to the vast geographical area covered by Texas, cotton production requirements vary greatly across the state. Arguably, at least seven distinct production regions can be identified: 1) Texas High Plains; 2) Texas Rolling Plains; 3) Texas Trans-Pecos; 4) Texas Blackland Prairies; 5) Texas Central River Valley; 6) Texas Gulf Coast; and 7) Texas Lower Rio Grande Valley (Rummel et al. 1986). Rainfall, soil type, length of growing season, availability of irrigation, and production techniques vary greatly among the regions. Cotton varieties, which provide optimum performance in one region, may exhibit only marginal performance in another. Thus, Texas growers require access to numerous insect resistant cotton varieties to meet the requirements of the various production regions.

Fortunately, new transgenic cottons are entering the market. The registration of the recently developed VipCot™ cotton is now under consideration. The commercial product will contain the genes COT102 (Vip3A component) and COT67B (Cry1Ab component). This technology will primarily target Heliothine and Pectinophora pests with good activity on a broad spectrum of other lepidopterian species (Mascarenhas et al. 2003, Cloud et al. 2004).

Tests conducted across the cottonbelt have demonstrated the effectiveness of the VipCot™ System (Bradley et al. 2004, Bacheler et al. 2004, Cook et al. 2004, Parker and Livingston 2005). It is expected that VipCot™ cotton varieties will provide control of Lepidopteran pests at least equal to that of Bollgard II®.

VipCot™ employs a recently discovered protein (Vip3A) with a novel mode of action (Estruch et al. 1996). Shotkoski et al. (2003) showed that the mode of action of Vip3A differed in several respects compared to Cry1Ab. These authors concluded that incorporation of Vip3A into insect control programs would serve to address Bt resistance concerns in addition to its own value as a control agent. Research conducted in North Carolina with Cry1Ac resistant bollworms showed no cross-resistance between Cry1Ac and Vip3A (Marcus et al. 2005). Also, there was no cross-resistance between Cry 1Ac and Vip3A for resistance tobacco budworms. Because of the low risk of cross-resistance to Cry toxins the introduction of Vip3A is expected to enhance the sustainability of Transgenic Lepidopteran-resistance cotton technology (Negrotto and Martin 2005).

The introduction of the VipCot™ technology in high yielding, high quality varieties would be a welcome addition to Texas cotton production. VipCot™ varieties would help growers meet the different varietal requirements in the various production regions of Texas. The competition resulting from greater choice for producers would also tend toward lower technology fees. The potential for using VipCot™ varieties in insect resistant management programs also is an important consideration in Texas.

Production systems utilizing Bt cottons offer the greatest promise yet seen in the almost two hundred year history of Texas cotton. This system not only benefits farmer but society as a whole. The amount of insecticide released into the environment each year by cotton production practices in now only a tiny fraction of what it once was. The success of the Texas cotton industry depends on the sustainability and improvement of this system.

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Report 6.4

VipCot™ Cotton Seed Yields - Preliminary Report

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VipCot™ Cotton Seed Yields - Preliminary Report

Delta and Pine Land Company

December 1, 2006

BACKGROUND

In the process of developing new transgenic technologies for use in commercial cotton production, many factors must be evaluated in addition to insect efficacy. Among those factors are plant growth characteristics, yield potential and fiber quality traits. The attached data is a preliminary report of yield potential as measured in seed cotton harvested per acre.

MATERIALS AND METHODS

During the 2006 cropping season, D&PL endeavored to evaluate the agronomic characteristics and yield potential of the leading candidate stacked product for commercialization. As part of a larger research project, the breeding stack of events COT 102 x COT67B cotton (VipCot™ cotton) was planted in a multiple location trial series.

Prior to planting, all seed were treated with commercial seed treatments containing both fungicides and insecticides. Fungicidal treatments consisted of Dynasty™ CST providing the following active ingredients (rates): azozystrobin (8.82g ai/CWT), fludioxynil (1.40g ai/CWT), and mefenoxam (4.34 g ai/CWT.). Insecticidal treatments for thrips and aphid control consisted of thiomethoxam (Cruiser®@ 0.34 mg/kernel), which were applied to all seeds before planting.

All plots were planted under USDA notification number 06-039-16n between May 18 and June 14, 2006 using cone planting equipment. Specific planting dates by location were as follows: Bell Mina, AL (05-18-06), College Station, TX (05/18/06), Estill, SC(05-18-06), Hartsville, SC (05-23-06),Haskell, TX (05/19/06), Portageville, MO (05/18/06), Red Springs, NC (06-14-05), Tifton, GA (06/08/06), and Winterville, MS(06-18-05).

Seeds were packaged for planting before shipment to the various locations. Two hundred seeds per packet were packaged, which enabled stands of 3.5 to 4 plants per row foot to be obtained in all trials. Plots consisted of two rows per plot, four replicates per variety, and finished plots were 35-43 feet in length. Trials were designed as randomized complete blocks to allow for proper statistical analysis.

All plots maintained reproductive isolation via the use of at least sixteen 38-inch row spacing border rows. Border rows were planted using DP 565 at 4.0 seeds per row foot allowing establishment of finished stands of 3.5 plants per row foot.

Typical management inputs including preplant applications of nitrogen fertilizers at rates ranging from 70-110 pounds per acre, preplant incorporation of residual herbicides for grass and weed control, layby applications of various residual herbicides and timely in-season applications of growth regulators (mepiquat chloride) were used as needed in this trial series. Some local modifications were made to management depending on location, trial type, and/or local custom as needed. Weed management consisted of a comprehensive program of preplant burndown herbicides and both pre and postemergence in-season herbicide applications in combination with hand weeding, as needed, throughout the season.

Since agronomic evaluation was the primary goal of these plots, they were protected from insects in a manner similar to conventional cotton. Lepidopteran pests consisting primarily of *Helicoverpa zea* (Boddie) and *Spodoptera frugiperda* (J.E. Smith) were controlled at all sites using university extension thresholds and applications of pyrethroid insecticides (ex. Karate - lambda cyhalothrin), Sucking pests were effectively excluded in all locations via the use of selective sprays. Lygus/sucking pest control was achieved by using standard scouting procedures followed by timely applications of acephate, acetamiprid, and/or dicrotophos.

At the appropriate timing, all plots were chemically defoliated for harvest. Defoliant selection varied across the locations depending on the localized climatic conditions. After successful defoliation plots were machine picked for yield estimations.

Data from the lines of interest were placed in subsets and analyzed in statistically appropriate manner using JMP software. Replicates were preserved within locations allowing separation of replicate effect, line effect, location effect, and the line x location effect. The threshold for significant difference was set at $p \leq 0.05$ for all parameters.

RESULTS AND DISCUSSION

Trial results are listed in Table 1. Data represented here are shown as seed cotton per acre. When analyzed as randomized complete block trials across locations, several preliminary conclusions can be drawn from the 2006 data including:

- No significant Location x Variety interaction (p=.1031) occurred in the 2006 testing series.

- No significant differences (p=0.7729) in seed cotton yield between Coker 312 and the COT 102 x 67B stacked materials were measured.

CONCLUSIONS

From the 2006 data, we preliminarily conclude that the VipCot™ cotton exhibits no negative characteristic in seed cotton yield, which could present difficulties in a variety development program. Several other characteristics will be measured throughout the ginning process. Further interpretation of in season plant mapping and fiber quality measurements should provide further confidence in the agronomic performance of VipCot™ cotton.

Table 9-1. LSMEAN analysis with appropriate statistics from D&PL VipCot™ evaluations conducted during 2006[1].

Location	**Line**	
	Coker 312	**COT 102 X 67B**
Belle Mina, AL	1387	1330
College Station, TX	1958	2036
Estill, SC	1833	1713
Hartsville, SC	2467	2222
Haskell, TX	2254	1999
Portageville, MO	2014	2306
Red Springs, NC	2300	2072
Tifton, GA	2879	3303
Winterville, MS	3060	3339
Variety x Location (p<0.05)	0.1031-NS	
	Line	
Variety	**Coker 312**	**COT 102 X 67B**
	2239	2257
Variety (p<0.05)	0.7729-NS	

[1]All values represent estimates for seed cotton generated per acre. Values are listed for both an across location analysis (upper, by location table) and the line mean (lower, by line table) for the tested entries.

Report 6.5

ECONOMIC ANALYSIS OF VipCot™/HERBICIDE STACKED BIOTECH COTTON

ECONOMIC ANALYSIS OF VIPCOT™/HERBICIDE STACKED BIOTECH COTTON

Eric Wailes[33]

ABSTRACT

The introduction of VipCot™/Herbicide stacked biotech cotton will provide producers expanded choice in selecting varieties and increase competitiveness in the cottonseed industry. The unique protein combination of Vip3A and Cry1Ab is expected to delay the onset of resistance to cotton varieties expressing Cry toxins (Kurtz et al. 2006). Economic benefits will result from lower pesticide applications, lower pesticide costs, lower technology fees, and higher cotton yields than without the introduction of VipCot™/herbicide stacked cotton varieties. Because existing stacked varieties in cotton exist (Bollgard II®/Roundup Ready® and BollgardII®/Roundup Ready Flex®, Liberty Link®/Bollgard II®), this study focuses on the incremental value of introducing an additional stacked event, which expands producer choice and delays resistance. Without this event, cotton yields will be lower, pesticide use will be higher, and returns to the industry will be lower. The study estimates the value of VipCot™ technology replacing existing varieties that become inferior at a net present value over the first eight years at $42 million. There is an additional value estimated from the increased competition that VipCot™ varieties bring to the market in terms of choice for farmers in pesticides, seeds and business services. The net present value of this component over the first eight years is estimated to be $41 million. The total net present value of the VipCot™ technology is then estimated at $83 million for the first eight years of the market plan.

INTRODUCTION

Genetically modified cotton varieties have been widely adopted in the U.S. (Figure 1). Transgenic varieties have accounted for 90% or more of the acreage in the Southeastern and Mid-South cotton regions since 2003, and by 2006 for 70% in the Southwest and over 50% in the West (Appendix Table 1).

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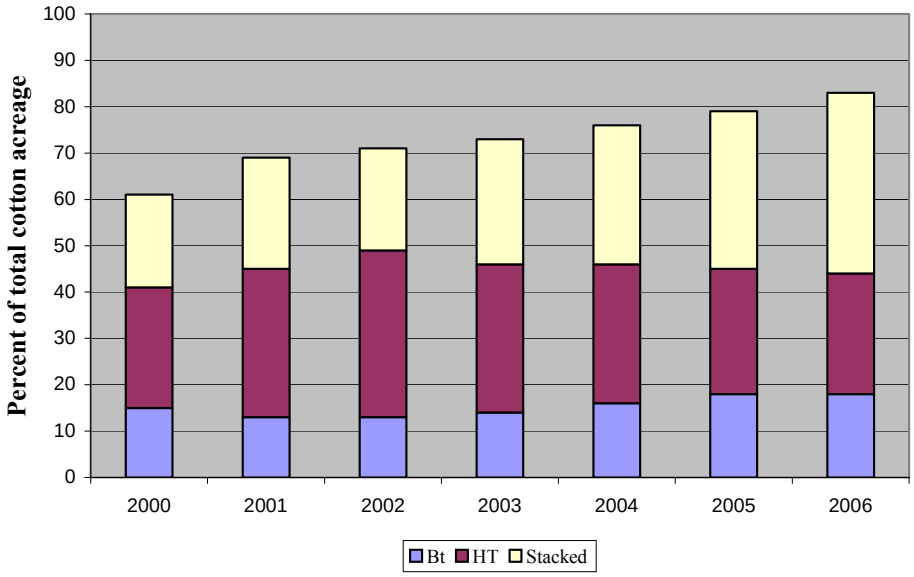
Figure 1. U.S. Biotech Cotton Acreage, 2000-06



Source: U.S. Department of Agriculture, National Agricultural Statistics Service (NASS), *Acreage.* 2001, 2002, 2003, 2004, 2005, 2006.

Numerous studies have provided estimates of economic and environmental benefits from the adoption of transgenic cotton varieties (Bryant et al. 2003, 2004; Cattaneo et al. 2006; Fernandez-Cornejo and Caswell, 2006; Fernandez-Cornejo and McBride, 2002; Edge et al., 2001; Frisvold and Pochat, 2004; Frisvold and Tronstad, 2004; Qaim and Zilberman, 2003; and Wossink and Denaux, 2006; Frisvold et al., 2006). Fernandez-Cornejo and Caswell, 2006 provide summary of studies that characterize the effects on yields, pesticide use, and producer returns. Their summary is provided in Appendix Table 2 of this report. Other studies have also examined why farmers adopt transgenic cotton varieties (Fernandez-Cornejo and McBride, 2002; Gianessi and Carpenter, 1999; Carlson et al., 1998; Kalaitzandonakes and Suntornpithug, 2001; and Klotz-Ingram et al., 1999.)

METHOD OF ANALYSIS

The objective of this study is to provide an *ex ante* economic assessment of the introduction of Syngenta's VipCot™ / Herbicide stacked varieties. The demand by farmers for transgenic varieties to be marketed by Syngenta can be expressed in a two-stage decision process, where producers at the first level determine their demand to plant

a transgenic versus a conventional variety. Their second level decision is then to select which particular transgenic variety to purchase.

At the first level, the demand might be expressed as:

Demand for transgenic varieties:

$D_{Tt} = f(ER_{Tt} / ER_{Ct})$
Where, ER_{Tt} = expected returns from Tt (Transgenic varieties in time period t)
ER_{Ct} = expected returns from Ct (Conventional varieties in time period t)

And $ER_{it} = (EP_{it} * EY_{it}) - EC_{it}$

Where, EP_{it} = expected price of cotton varieties (i=T or C) in time period t.
EY_{it} = expected yield per acre of cotton varieties (i=T or C) in time period t.
EC_{it} = expected costs per acre of cotton varieties (i=T or C) in time period t.

Where expected costs include expected input prices and input rates (including the technology fee for transgenics, prices of pesticides, fuel, labor, etc as well as expectations of pest infestation).

Competition in the cottonseed market then is influenced by the producer's expectations with regard to the price received as affected by staple quality and marketing assistance, by the field yield, and the associated expenditures required to produce the crop (Stewart, 2004).

The second level demand decision essentially follows from the first level where once the choice to plant a transgenic is made then the same expected relative returns framework among alternative transgenic varieties is made. Therefore, success of the marketing plan and market penetration of the Syngenta VipCot™ varieties will ultimately depend upon the expected value of these varieties in terms of staple quality, marketing assistance, field yield, input use and prices, and technology fees charged relative to other transgenic varieties. Unfortunately, publicly available estimates of the demand parameters of the above equations do not exist. Nor is there data publicly available to estimate these parameters. Further, data on expected yields of proposed Syngenta varieties are not available. Therefore, this study relies on assumptions in order to estimate economic impacts regarding the adoption and market penetration of Syngenta varieties, based on the proposed marketing plan.

As Appendix Table 1 shows, the level of demand for transgenics varies considerably among cotton production regions. The literature cited above identifies various reasons for this differential in terms of pest levels, types of pests, etc. In the Southeast and Mid-South, adoption rates of transgenics are over 90%, implying that expected returns relative

to conventional varieties are higher. Therefore, the demand for Syngenta VipCot™ varieties will be more dependent upon second level demand factors such as marketing assistance and technology fees relative to other transgenics.

In this analysis, we assume that the expansion in Syngenta's market share is determined by two competitive elements of Syngenta VipCot™ varieties. The first element is increased productivity based on higher yields relative to conventional cotton and potentially to other transgenic varieties. The second is increased productivity based on cost efficiencies available through more competitive technology fees and services to be provided by Syngenta relative to other transgenic varieties.

Differences in adoption rates by region are important and will be an important dimension of the regional distribution of benefits associated with VipCot™ market development. Cotton yields in the Southeast U.S. were found to increase by 2.1 percent for every 10-percent increase in Bt cotton acreage. For every 10-percent increase in HT cotton acreage, yields have increased by 1.7 percent (Fernandez-Cornejo and McBride, 2002). This study also found that for every 10-percent increase in Bt cotton acreage, net returns increase by 2.2 percent and for every 10-percent increase in HT cotton acreage, net returns increased by 1.8 percent.

The impact on pesticide use as reported in the literature is mixed, with studies showing either a decrease or the same as compared with conventional varieties. A recent study by Wossink and Denaux 2006 found, however, that for a sample of cotton farms in North Carolina that adoption of stacked gene cotton improved environmental efficiency by reducing the leaching potential of chemical pesticide use, but had no impact on cost savings due to technology fees.

The cotton biotechnology events have evolved in two significant dimensions. First, events for both herbicide tolerance and Bt insect management have required innovations that address the onset of pest resistance. Bollgard II® and Roundup Ready Flex® reflect advances that have already been commercialized. VipCot™/herbicide tolerant stacked varieties will enhance the existing set of biotech cotton events by providing varieties that are unique novel proteins. Thus, the primary economic benefit will be an expansion in the choice of transgenic varieties from which producers can select. Further, the VipCot™/herbicide tolerant varieties will provide the benefit of delaying pest resistance in other transgenic varieties compared to what would have occurred without them. This benefit, which will accrue to non- VipCot™ transgenic varieties however is not captured in the analysis which follows.

ANALYTICAL FRAMEWORK

In order to evaluate the incremental benefit of this biotechnology event for the U.S. cotton sector, a computable general equilibrium (CGE) model of the U.S. and global economy is used. The standard and well-received Global Trade Analysis Project (GTAP) model of the global economy is used to provide insights into the effects of enhancing the

biotechnology choice available to U.S. cotton producers. A global model is relevant because exports of cotton are an essential aspect of the U.S. cotton market[34]. Hertel (1997, 1999) provides a comprehensive documentation of the GTAP model, which is a neo-classical, multi-regional, static, applied general equilibrium model that assumes perfect competition, constant returns to scale and unchanging aggregate employment of all factors of production. The choice to use a CGE model in this analysis is based on the fact that the GM transgenic technology has multi-market effects. It has direct impacts on the cotton product market but also on the input markets to produce cotton including seeds, machinery, chemicals and business services. Further, the CGE model is global and therefore captures the technology impact on global cotton trade.

The use of CGE models to evaluate technology has been made more accessible through the development of the GTAP framework. A CGE framework is superior to the partial equilibrium model from an analytical perspective since it provides the ability to link the product and factor markets simultaneously. This is an important feature when the technology innovation is linked to both the cotton product market and several factor input markets as in this case. Partial equilibrium analysis requires the assumption that all other markets are held constant, which is a strong assumption when the transgenic technology package proposed with the introduction of VipCot™ is being evaluated.

We use the latest Version 6.05 of the GTAP database (see Dimaranan, 2006), which draws on global economic structure policies and trade flows of 2001. The GTAP model has been aggregated for this study to depict the global economy as having 6 sectors (cotton, seeds, fuel, machinery, chemicals, business services, and all other sectors) and 2 regions (U.S. and the rest-of-the world). The GTAP model does not measure environmental or human health externalities and therefore welfare effects of any such externalities are not included in the welfare measures. To the extent that transgenic cotton may result in the use of fewer chemicals, it is likely more environmentally friendly than conventional cotton, and therefore estimates presented in this study are likely to understate the benefit.

MODEL SIMULATION SCENARIOS

To simulate the economic effects of introducing the VipCot™ novel biotech cotton varieties we assume that factor productivity will increase in correspondence with the introduction and market penetration of such cotton varieties (Mascarenhas, 2004). More specifically, we follow the proposed marketing plan as proposed by Syngenta and assume three alternative adoption rates as reflected in Table 1. In the first two years,

[34] For a recent application of the GTAP model to GM cotton see Anderson et al. "Recent and Prospective Adoption of Genetically Modified Cotton: A Global CGE Analysis of Economic Impacts." World Bank Policy Research Working Paper 3917, May 2006. Another recent study on transgenic cotton adoption by Frisvold et al. 2006 also employs a CGE framework.

commercialization will only include the VipCot™ component. Stacked VipCot™/herbicide tolerant varieties will not be commercialized until 2010.

TABLE 1

ALTERNATIVE ADOPTION LEVELS OF VipCot™/HERBICIDE TOLERANT VARIETIES IN THE U.S.

Time Period	Adoption Levels (Per cent of U.S. cotton acreage)		
	Low	Medium	High
2008*		0.035	
2009*		0.143	
2010	3.6	10.7	14.3
2011	10.7	17.9	21.4
2012	10.7	25.0	35.7
2013	10.7	32.1	39.3
2014	10.7	32.1	39.3
2015	10.7	32.1	39.3

* VipCot™ only, no herbicide tolerant varieties

To estimate the incremental impact of VipCot™/herbicide tolerant varieties on value-added productivity improvement (land, labor and capital) we assume that the productivity impact would be incremental over time since without this technology pest resistance would increase (Flanders and White, 2003). The assumed incremental impact on productivity is given in Table 2. These percent increases are assumed since field level data is not yet available. They are, however, conservative relative to productivity estimates experienced and reported in the literature (Brookes and Barfoot, 2005; Fernandez-Cornejo and Caswell, 2006; Marra et al., 2002; Qaim and Zilberman, 2003; Huang et al., 2004).

TABLE 2

TIME PATH OF YIELD PRODUCTIVITY IMPROVEMENT WITH VipCot™/HT VARIETIES ABOVE BASELINE YIELD

Time Period	Productivity Improvement in Value Added Inputs
2008	0.5%
2009	0.5%
2010	0.5%
2011	1.0%
2012	1.5%
2013	2.0%
2014	2.5%
2015	3.0%

To measure the value-added effect in the GTAP model, we simulate the model relative to a baseline of no adoption/introduction of VipCot™ varieties for eight years and for the six years with stacked varieties at low, medium, and high levels of adoption. We specify the productivity improvement only on the percent of cotton area with VipCot™ adoption. This again is most likely a conservative estimate because the introduction of VipCot™ will also extend the value and life of competing transgenic technologies if choice is introduced and pest resistance is delayed in other Bt varieties (Matten and Reynolds, 2003).

RESULTS

The results of this simulation exercise are reported in Table 3. These results reflect the impact on producer's net income of VipCot™ acres replacing acres otherwise planted to competing transgenic varieties, again as reflected in the marketing plan.

TABLE 3

PRODUCER NET INCOME MEASURES OF VipCot™ TECHNOLOGY AT ALTERNATIVE ADOPTION LEVELS (MILLION USD)

Time Period	Adoption Levels		
	Low	Medium	High
	Million US dollars		
2008		$ 0.004	
2009		$ 0.017	
2010	$ 0.410	$ 1.250	$ 1.640
2011	$ 2.470	$ 4.129	$ 4.934
2012	$ 3.715	$ 8.633	$ 12.320
2013	$ 4.934	$ 14.763	$18.020
2014	$ 6.176	$ 18.430	$ 22.470
2015	$ 7.393	$ 22.062	$ 26.925

Finally, to approximate the potential net income gains from increasing competitiveness in the cottonseed market, the model is simulated to estimate the impact of a 1% increase in the productivity of the chemical, seed and business services sectors. This simulation is conducted relative to the medium level adoption of VipCot™ in order to estimate the incremental value of more competition in the stacked cottonseed sector. To reflect that the first two years are based on an assumption of commercialization of VipCot™ varieties without herbicide tolerance, the estimated impact of productivity increase is assumed to be only 0.5%. This again is a conservative estimate based on previous literature cited above. The results of this exercise are given in Table 4.

In Table 4, the first column is based on numbers for the economic value to farmers' net income as reported in Table 3 at the medium level of adoption. The GTAP model is then re-simulated, incorporating improvements in efficiency in factor markets due to competition, to estimate a total value, as shown in the second column. This estimate includes not only the value on cotton output but on productivity through competitive

pricing and efficiency in the input markets of chemicals, seeds, and business services. The net effect of increased competition in the input industries is derived from subtracting the first column from the second to obtain the value of input market productivity gains. The estimate as given in the third column again reflects increases in producer net returns.

TABLE 4

IMPACT OF COST EFFICIENCY IN THE SEED, CHEMICAL AND BUSINESS SERVICES (SCBS) SECTORS FROM VipCot™. (MILLIONS USD)

Time Period	Middle Adoption Level (Million US dollars)		
	No SCBS gain	1% SCBS gain	Net gain
2008	$ 0.004	$ 4.312	$ 4.308
2009	$ 0.017	$ 4.325	$ 4.308
2010	$ 1.250	$ 9.820	$ 8.570
2011	$ 4.129	$ 12.708	$ 8.579
2012	$ 8.633	$ 17.217	$ 8.584
2013	$ 14.763	$ 23.351	$ 8.588
2014	$ 18.430	$ 27.024	$ 8.594
2015	$ 22.062	$ 30.657	$ 8.595

REGIONAL DISTRIBUTION OF BENEFITS

The regional distribution of benefits of VipCot™ cotton varieties can be suggested by existing adoption levels of transgenic cotton varieties. In 2006, the share of Bt only transgenic by regional shares was 22.3% Southeast, 28.9% Mid-South, 46.9% Southwest, and 1.8% West. The regional shares of the stacked varieties were 36.1% Southeast, 41.7% Mid-South, 21.5% Southwest, and 0.7% West. The total value of the mid-level adoption rate with both yield and factor cost efficiencies as given in the third column of Table 4 are allocated by regions in Table 5 based on 2006 regional adoption rates.

TABLE 5

REGIONAL ALLOCATION OF NET PRODUCER RETURNS OF VipCot™ TRAIT TECHNOLOGY

Year	Southeast	Mid-South	Southwest	West
	Million U.S. dollars			
2008	0.962	1.247	2.024	0.078
2009	0.965	1.251	2.030	0.079
2010	3.547	4.091	2.109	0.073
2011	4.590	5.294	2.730	0.094
2012	6.218	7.173	3.698	0.127
2013	8.434	9.729	5.016	0.172
2014	9.761	11.259	5.805	0.200
2015	11.073	12.772	6.585	0.226

SUMMARY AND CONCLUSIONS

The economic analysis of VipCot™/Herbicide Stacked biotech cotton is presented in this study. The framework used is a computable general equilibrium (CGE) model that simulates both product and factor markets simultaneously in the global economy. The GTAP model is used to simulate the value of introducing the new technology over an eight-year period, with the first two years based only on VipCot™ and the remaining six years including the stacked herbicide trait. The model is simulated based on reasonable assumptions of productivity gains as field data does not yet exist. However, the assumed values are conservative relative to estimates reported in the literature for existing transgenic cotton varieties.

The results of this study estimate the value of introducing VipCot™ technology based on 1) productivity gains resulting from replacement of varieties that become inferior due to

pest resistance and 2) productivity gains resulting from increased competition as a result of providing producers a choice of alternative transgenic varieties. The results suggest that based on a discount rate of 8% that the discounted net present value in the cotton product market over the eight-year period of analysis ranges from a low of $15 million to a high of $52 million, with the medium adoption level yielding a net present value of $42 million. This value reflects the replacement of transgenic varieties that become less valuable as pests become resistant. The analysis also estimates the additional value of increased competition in the chemical, seed, and business services sector based on the competition that VipCot™ technology provides by expanding the choices available to producers and therefore improvements in pricing efficiency. This is estimated to add a net present value of $41 million. The total net present value in the first eight years of market introduction and penetration of VipCot™ technology is estimated to be $83 million.

Appendix Table 1. Transgenic cotton acre shares by region, 2000-06.
Source: USDA, NASS, Crop Acreage

Region	Year	Bt	HT	Stacked	Total GM
Southeast	2000	0.15	0.31	0.34	0.80
	2001	0.11	0.41	0.32	0.85
	2002	0.10	0.44	0.36	0.90
	2003	0.15	0.31	0.47	0.93
	2004	0.15	0.24	0.54	0.93
	2005	0.21	0.19	0.54	0.94
	2006	0.17	0.17	0.62	0.97
Mid-South	2000	0.32	0.16	0.27	0.76
	2001	0.17	0.19	0.47	0.83
	2002	0.22	0.24	0.38	0.84
	2003	0.21	0.19	0.50	0.90
	2004	0.24	0.18	0.51	0.93
	2005	0.23	0.20	0.53	0.96
	2006	0.18	0.20	0.57	0.95
Southwest	2000	0.07	0.33	0.06	0.46
	2001	0.07	0.35	0.06	0.48
	2002	0.07	0.40	0.04	0.51
	2003	0.08	0.39	0.06	0.53
	2004	0.10	0.40	0.08	0.58
	2005	0.14	0.35	0.14	0.63
	2006	0.18	0.34	0.18	0.70
West	2000	0.03	0.17	0.04	0.24
	2001	0.11	0.27	0.02	0.40
	2002	0.06	0.26	0.01	0.33
	2003	0.09	0.27	0.03	0.39
	2004	0.06	0.39	0.07	0.52
	2005	0.08	0.40	0.05	0.53

Southeast: Alabama, Florida, Georgia, Kentucky, North Carolina, South Carolina, and Virginia

Mid-South: Arkansas, Illinois, Louisiana, Mississippi, Missouri, and Tennessee

Southwest: Kansas, New Mexico, Oklahoma, and Texas

West: Arizona, California, and Nevada

APPENDIX TABLE 2. SUMMARY OF PRIMARY STUDIES ON EFFECT OF BIOTECH COTTON ON YIELDS, PESTICIDE USE AND RETURNS.

Crop/researcher/date	Data source	Effects on		
		Yield	Pesticide Use	Returns
Herbicide tolerant cotton				
Vencill, 1996	Experiments	Same	na	na
Keeling et al., 1996	Experiments	Same	na	na
Goldman et al., 1998	Experiments	Same	na	na
Culpepper & York, 1998	Experiments	Same	na	Same
Fernandez-Cornejo et al., 2000	Survey	Increase	Same	Increase
Bt cotton				
Stark, 1997	Survey	Increase	Decrease	Increase
Gibson et al., 1997	Survey	Increase	na	Increase
ReJesus et al., 1997	Experiments	Same	na	Increase
Bryant et al., 1998	Experiments	Increase	na	Increase
Marra et al., 1998	Survey	Increase	Decrease	Increase
Fernandez-Cornejo et al., 2000	Survey	Increase	Decrease	Increase

Source: Fernandez-Cornejo and Caswell, 2006.

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REPORT 6.6

RECENT AND PROSPECTIVE ADOPTION OF GENETICLLY MODIFIED COTTON: A GLOBAL CGE ANALYSIS OF ECONOMIC IMPACTS

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Recent and Prospective Adoption of Genetically Modified Cotton: A Global CGE Analysis of Economic Impacts

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Abstract

This paper provides estimates of the economic impact of initial adoption of genetically modified (GM) cotton and of its potential impacts beyond the few countries where it is currently common. Use is made of the latest version of the GTAP database and model. Our results suggest that by following the lead of China and South Africa, adoption of GM cotton varieties by other developing countries – especially in Sub-Saharan Africa – could provide even larger proportionate gains to farmer and national welfare than in those first-adopting countries. Furthermore, those estimated gains are shown to exceed those from a successful campaign under the WTO's Doha Development Agenda to reduce/remove cotton subsidies and import tariffs globally.

JEL codes: D58, F17, Q16, Q17

Key words: GMOs, cotton biotechnology, computable general equilibrium modeling, economic welfare, subsidy and tariff reform

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Recent and Prospective Adoption of Genetically Modified Cotton: A Global CGE Analysis of Economic Impacts

1. Introduction

Cotton is important for many developing countries, either as a cash crop and/or as an input into their textile industry. It is receiving more attention of late for two reasons. One is because, thanks to genetic modification using modern biotechnology, new insect-resistant and herbicide-tolerant cotton varieties are emerging that are proving to be more productive than traditional varieties of cotton. Over the decade following their first release, genetically modified (GM) cotton rose to account for 28 percent of all land sown to cotton globally in 2005 and to one-ninth of the world's total area of GM crops. But the United States and China account for almost all of that, where the proportion of plantings that are GM are already more than four-fifths and two-thirds, respectively (Table 1).[1] The only other countries with high GM adoption rates by 2005 are Australia and South Africa, both with slightly more than four-fifths of their cotton areas under GM varieties. Apart from India and Mexico, where legal adoption began to take off only in 2003-04,

[1] China's adoption share is lower because insect infestations are low in the Western part of China where much of the crop is grown, so the gains from switching to current varieties of GM cotton are not yet sufficient to make the change. The drop in China's GM cotton acreage in 2005 (see Table 1) paralleled a drop in its non-GM cotton acreage as farmers moved away from land-intensive to labour-intensive crops.

and an unknown extent of (possibly illegal) plantings in Argentina, no other developing countries have widespread adoption yet of this new technology.[2]

The other reason cotton is in the news is because four poor cotton-exporting West African countries (Benin, Burkina Faso, Chad and Mali) have demanded that cotton subsidy and import tariff removal be part of the World Trade Organization's Doha Development Agenda. However, cotton subsidies are mostly provided by governments in high-income countries, and those governments have yet to be persuaded by other cotton-exporting countries to abandon them – notwithstanding the fact that part of the US cotton subsidy program has been ruled illegal following a WTO dispute settlement case brought by Brazil.

What is at stake here in terms of economic welfare in various developing countries? Specifically, how much are developing countries foregoing by procrastinating in their approval of GM cotton production? How does that compare with the effects on developing country and global welfare of removing cotton subsidies and import tariffs? And how much greater would be the gains to cotton-producing developing countries from GM cotton adoption if global cotton markets were not distorted by subsidies and tariffs?

After presenting a brief background to the world's cotton market in Section 2, this paper seeks to address these questions by using a well-received model of global economy known as GTAP (developed by Purdue University's Global Trade Analysis Project) and the latest version of its related trade and protection database, described in Section 3. Empirical simulation results are presented in Section 4. These are followed by a

[2] Experimental work has begun in numerous other developing countries though, including in countries as poor as Burkina Faso. For a thorough review of such developments, see FAO (2004, Ch. 4).

discussion of caveats in Section 5. The concluding section summarizes the findings and draws policy implications for developing countries.

2. The global cotton market

Cotton production is highly concentrated in several respects. One is that most production is in a few countries: as of 2005/06, nearly half is produced by just China and the United States, and that rises to more than two-thirds when India and Pakistan are added and to more than three-quarters when Brazil and Ukbekistan are included. Also highly concentrated are exports of cotton lint, with the US, Australia, Uzbekistan and Brazil accounting for almost two-thirds of the world's exports, while the cotton-four in West Africa and the other four countries in Central Asia bring that total to almost four-fifths (Table 2).

Cotton usage, on the other hand, is distributed across countries roughly in proportion to their volumes of textile production. Because of high domestic usage by exporters of textiles and clothing in developing Asian countries (and Mexico because of its preferential access to the US and Canadian markets under NAFTA), even large cotton producers such as China, Pakistan and India export only a small fraction of their crop, in contrast to Sub-Saharan Africa and Central Asia where textile production is relatively minor. This explains the pattern of net exports of cotton and textiles across regions (columns 3 and 4 of Table 3), an understanding of which is helpful in explaining the signs of the welfare effects of some of the technology and policy shocks considered below.

3. The GTAP model and database

The standard Global Trade Analysis Project (GTAP) model of the global economy is used to provide insights into the effects of governments allowing GM technology adoption in some countries without and then with cotton trade and subsidy policy reform globally. See Hertel (1997) for comprehensive documentation of the GTAP model, which is a neo-classical multi-regional, static, applied general equilibrium model that assumes perfect competition, constant returns to scale and unchanging aggregate employment of all factors of production. We use the latest Version 6.05 of the GTAP database (see Dimaranan and McDougall, 2005), which draws on global economic structures, policies and trade flows of 2001. The GTAP model has been aggregated to depict the global economy as having 27 sectors and 38 regions (to highlight the main participants in the world's cotton markets, two of which are newly disaggregated countries: Nigeria and Pakistan). Trade is modeled using a nested Armington structure in which aggregate import demand for each sector's product is the outcome of allocating domestic absorption between domestic goods and aggregate imports, and then aggregate import demand is allocated across source countries to determine the bilateral trade flows.

This economy-wide GTAP model does not include environmental or human health externalities, so the welfare consequences of any such externalities are not measured. This unfortunate situation is a result of the uncertainty surrounding the relationships among various economic and environmental variables. What can be said, though, is that the net environmental effects of producing GM crops could be positive or

negative – just as they could be for producing non-GM crops, which also are not captured in our model. On the one hand, many GM crop varieties have some attributes that are more environmentally friendly than their conventional non-GM counterparts. They also are less dangerous to farmers and the soil where they require reduced applications of pesticides. On the other hand, there is concern that some long-term and possibly irreversible negative environmental effects might occur in the future, although we are not aware of significant scientific evidence of such adverse effects.[3]

4. Model simulations and results of GM cotton adoption

To simulate the economic effect of adoption of GM cotton, we assume total factor productivity (TFP) in cotton production would rise by 5 percent in most adopting countries, net of any higher cost of GM seed.[4] This output-augmenting, Hicks-neutral TFP shock is a conservative estimate of the gain to farmers, according to experience to date (FAO 2004, Table 7; Marra, Pardey and Alston 2002; Qaim and Zilberman 2003; Huang et al. 2004) and bearing in mind that typically, in a small number of years after GM cotton adoption is allowed, more than four-fifths of production moves to GM varieties. In India and Sub-Saharan Africa other than South Africa, however, we assume a TFP shock of 15 percent. Even that higher value is conservative for those countries, according to Qaim and Zilberman (2003), because those countries' yields per hectare with conventional varieties are less than half the yields in the rest of the world (see last

[3] Federoff and Brown (2004) give reasons why that null finding is not surprising from the viewpoint of a molecular biologist.

[4] In the GTAP database, cotton is part of a sector called 'plant-based fibers' but it represents well over 90 percent of the value of that sector. The only country for which this is likely to be of any significance is Bangladesh, which is still a large flax producer.

column of Table 2) and the GM field trials in India have been boosting yields by as much as 60 percent. More-recent commercial planting data suggest yield per hectare gains in India of more than one-third from adopting GM cotton varieties, and higher net profits despite the GM seed costing three times as much as non-GM seed (Qaim et al. 2006, Bennett et al. 2006).[5]

Three GM cotton adoption simulations are presented below. The first one aims to measure the market and welfare effects of adoption that had already taken place by 2001 in the United States, China, Australia, and South Africa. In China's case it was only about halfway through its adoption process as of 2001, so only a 2.5 percent TFP shock is applied in this case. The simulation is a negative one, in the sense that we examine how the world would have been had that 5 percent shock (2.5 percent in China's case) not taken place.

That first simulation is then compared with two other shocks: one in which all other countries except the rest of Sub-Saharan Africa adopt GM cotton (and China completes its adoption process), and the other in which Sub-Saharan Africa also adopts. The reason it is worth examining separately the impact of adoption by the rest of Sub-Saharan Africa is that the region has a history of very slow adoption of new agricultural technologies in the 1970s and 1980s, and during the 1990s its investments in agricultural R&D grew only 1 percent per year and spending actually fell in about half the countries for which data exist (Science Council 2005). To reiterate, the TFP shock in these latter two simulations is also 5 percent except for India and Sub-Saharan Africa (excluding South Africa) where it is 15 percent and for China where it is 2.5 percent. The potential

[5] There are also benefits from insect-resistant Bt cotton in terms of improved health for farmers (see Hossain et al. 2004), and also less pesticide damage to soil and water, but these benefits are ignored in what follows.

net effect of this new biotechnology as of 2001 is thus the sum of effects from the first simulation (what had already taken place by 2001) and those from the third simulation (what still remained to be embraced after 2001).

First simulation (what had already taken place by 2001)

Results from the first simulation, presented in the final three columns of Table 3, suggest that world cotton output had hardly changed up to 2001. This is because the output gains in the first four GM-adopting countries were offset by output losses in the non-adopting countries, which were driven by the downward pressure on the average price of cotton in international markets (which fell by 2.5 percent as a result of this initial adoption, according to our model).[6] Globally, both value added by cotton farmers and the value of cotton exports were reduced by about 1 percent, and by more than that in most non-adopting regions. Note in particular that the largest changes in net income to cotton farmers are in Sub-Saharan Africa, with a rise in South Africa of 3.5 percent and a fall in the rest of Sub-Saharan Africa of 4.4 percent. Note also that among the GM cotton adopters, net incomes from cotton farming were lowered in both the United States and China, in part because of the decline in export prices. This is not to say individual farmers in those countries were irrational in adopting GM cotton, because had they not they would have still suffered from the product price fall, following adoption by other farmers, but would not have had a productivity improvement to partly offset it. For China, its small volume of cotton exports also was lowered, as most output is used by its domestic textile industry which expanded in response to the lower price of raw cotton.

[6] That estimated price fall would have been somewhat less had we also included GM corn and soybean adoption at the same time, since that would have reduced the extent of diversion of resources to cotton.

The net economic welfare effects of this initial adoption of GM cotton are summarized in Table 4. For all four adopting countries this was positive despite the loss due to their terms of trade deterioration and, in all but Australia's case, a small loss from domestic resource reallocation to the cotton sector (the latter because resources are attracted from sectors that were less assisted by government policies than cotton). But notice also that welfare improves in all non-adopting regions but one. This is because they are net importers of cotton and so enjoy a terms of trade improvement. The exceptional non-adopting region is Sub-Saharan Africa (excluding South Africa) which as a net exporter of cotton faces lower cotton export prices and also has resources move to sectors in which it had a lesser comparative advantage. Globally, annual economic welfare was enhanced by more than $0.7 billion from this technology's adoption as of 2001, plus whatever net profits accrued to the biotech and seed firms.

Second and third simulations (technology catch-up)

If all other countries then adopt GM cotton, cotton output in the early-adopting countries falls in response to the output expansion in newly adopting regions. If Sub-Saharan Africa continues to procrastinate, its cotton output, value added and exports would fall even further; but if it also were to embrace this technology, its cotton industry would expand more than any other region's and would more than make up its losses to 2001 from adoption by the first four adopters (compare the final three columns of Tables 3 and 5). Note too that the value of global exports shrinks more in these two simulations than in the first one, indicating that more cotton would be grown in the regions where it is consumed the more developing countries adopt this technology.

Global welfare could be boosted very much more with greater adoption by developing countries. Even without Sub-Saharan Africa adopting, it would jump to $2.0 billion per year, even though that would lower slightly Sub-Saharan Africa's (and Australia's) welfare (Table 6). But adoption by the rest of Africa would raise that global benefit to $2.3 billion, with two-thirds of that extra $0.3 billion being enjoyed by Africa (more than offsetting its loss shown in Table 4 because of adoption by others up to 2001), and the rest by cotton-importing regions. Asia's developing countries that are net importers of cotton gain even if they grow little or no cotton, because the international price of that crucial input into their textile industry would be lowered further, by an average of 2.4 percent in this scenario (and as much as 4.1 percent when Sub-Saharan Africa also adopts, as compared with 2.5 percent from GM adoption by just the first four adopting countries). Note though that Australia's earlier gain would be erased by the fall in its cotton export price in this scenario. With complete catch-up as in this third scenario, the gains to Central Asia, Sub-Saharan Africa and South Asia are ten, thirteen and twenty-three times greater than the global gains when expressed as a percentage of regional GDP (Table 6b and Figure 1). South Asia's are especially large because it is a large producer of both cotton and textiles (Table 1).

Clearly, there are large benefits being foregone by developing countries that are procrastinating in their release of GM cotton varieties. It is gratifying to see that the governments of India and Mexico are now allowing growers access to them (see Table 1), and hopefully other governments will soon follow suit.

What if cotton subsidies and tariffs were removed?

How do the above prospective gains from adopting GM cotton compare with the effects of eliminating all cotton subsidies and tariffs, as called for by several African cotton-exporting countries as part of the WTO's Doha Development Agenda? And how much greater would be the developing countries' gains from GM cotton adoption if the world was free of cotton subsidies and tariffs?[7]

The extent of subsidies to cotton production and exports, and of tariffs on cotton imports, is non-trivial (see Anderson and Valenzuela 2006, Appendix Table A3). Large though some of the interventions are, the estimated global welfare gain from removing them ($283 million per year) is only one-eighth the above estimate of the gain from completing the adoption of GM cotton technology ($2.3 billion).[8] Furthermore, most of that protection cost is felt by the countries imposing those distortions. Indeed many developing countries – as net importers of cotton (see Table 3) – benefit from those subsidies and tariffs because they lower prices for cotton in international markets.

What is striking about the distribution of the welfare effects that would result from removing those distortions, however, is the relatively large benefit it would bestow on Sub-Saharan Africa. Indeed that potential gain of $147 million per year is almost as large as the region's estimated gain from joining with the rest of the world in embracing GM cotton technology. Such reform would boost the international price of cotton by an

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[7] The juxtaposing of gains from trade reform with gains from new technology adoption is uncommon among CGE modelers, but an early exception in the case of Africa is Hertel, Masters and Elbehri (1998).

[8] Of course if textile and clothing tariffs also were removed, global welfare would increase far more: by an extra $6.8 billion per year, according to our model's results.

average of 12.9 percent,[9] and lead to an estimated increase in Sub-Saharan African cotton output and value added of nearly one-third. The real value of cotton exports from Sub-Saharan Africa would increase by more than 50 percent, while cotton output and exports would fall by one-quarter in the United States and would halve in the EU (Table 7). That would raise Sub-Saharan Africa's share of global cotton exports from 12 to 17 percent, and the share of all developing countries from 52 to 72 percent.

Also striking is a comparison of the welfare result from cotton reform with that from removing *all* merchandise tariffs and agricultural subsidies. While the latter gain is nearly 300 times as great as the former globally, for Sub-Saharan Africa cotton reform is crucial: its potential contribution to the region's welfare of $147 million per year is one-fifth of the estimated $733 million gain for the region from the freeing of *all* goods markets globally.

If those distortions to cotton markets were removed, how different would be the estimated effects of further GM cotton adoption beyond that achieved by 2001? Globally it would be virtually no different, for reasons explained in Alston, Edwards and Freebairn (1988) and Anderson and Nielsen (2004). But the gains to developing countries in the absence of distortionary cotton policies would be slightly greater (12 percent so in the case of Sub-Saharan Africa), while those to high-income countries would be less (middle columns of Table 6).

Were these two reforms (GM catch-up and subsidy removal) to occur simultaneously, they would reinforce each other in Sub-Saharan Africa as each expands the region's cotton production and exports and so makes the gain from the other change

[9] This is close to the 10 percent estimated by Sumner (2006, p. 282), which is also the simple average of the studies surveyed by Baffes (2005, p. 122).

larger. This is evident in the final column of Table 8, which shows that the gain to Sub-Saharan Africa would then be ($223m + $147m =) $370m. This is equivalent to $199m + $172m, the former appearing in column 1 of Table 8 and the latter being the gain to Sub-Saharan Africa from global removal of cotton subsidies and tariffs had GM catch-up occurred before that reform. With these two reforms the average price of cotton in international markets would be 7.4 percent above the baseline, instead of 4.1 percent below as in the case of just GM catch-up alone. That is why the loss shown in Table 7 for South Asia following subsidy removal becomes a gain in the final column of Table 8 when that reform is accompanied by GM cotton adoption. Clearly this is an example of complementarity between the trade and development components of the Doha Cotton Initiative.

5. Caveats

We have ignored the owners of intellectual property in GM varieties, and simply assumed the productivity advantage of GM varieties is net of the higher cost of GM seeds. If that intellectual property is held by a firm in a country other than the GM-adopting country, then the gain from adoption is overstated in the adopting country and understated for the home countries of the relevant multinational biotech companies.

Also, we do not have enough knowledge of the potential positive and negative effects of GM varieties on the environment to incorporate them into our simulation model. As with food safety concerns, it would in any case not be sufficient to include them only for GM varieties; they would also need to be included for non-GM varieties to

ensure even-handedness in the analysis. It happens that, prior to GM varieties, cotton farming in all but low-income countries has involved one of the most chemical-intensive forms of agricultural production. By switching to GM cotton, farmers have been able to lower substantially their applications of insecticides, thereby reducing soil, water and air pollution and improving the health of farmers and their neighbors. For cotton farmers in low-income countries (including much of India and Sub-Saharan Africa – see final column of Table 2), who have not yet had access to insecticides and other farm chemical and hence have relatively low yields and profits, GM cotton varieties offer an opportunity to leapfrog the chemical-intensive technology and provide a win-win-win for farm profits, human health, and the environment.

The technology shocks in our simulations assume a uniform increase in productivity of all factors and inputs used in GM cotton production. We use that assumption because it is simpler to describe, and it turns out there is little difference to the welfare results when we allow some factors to be saved more than others or some intermediate inputs such as pesticides to be needed less by GM crop varieties.

6. Conclusions

Adaptation and adoption of new genetically modified (GM) cotton varieties are within the powers of developing countries themselves. Unlike the Cotton Initiative in the WTO's Doha Development Agenda, governments in Sub-Saharan Africa and elsewhere do not need to wait until that round concludes to boost the incomes of their cotton farmers. Indeed the above results suggest that developing country welfare could be

enhanced by more from allowing GM cotton adoption than by the removal of all cotton subsidies and tariffs.[10] Furthermore, our results support the notion that the gains to developing countries from the Doha Cotton Initiative will be even greater if GM cotton is adopted first, providing yet another reason not to delay approval of this new biotechnology.

Those developing countries with well-developed public agricultural research and extension systems (such as India) are well placed to benefit promptly from the new biotechnology by working in partnership or in parallel with private biotech and seed companies. Approving investments in those activities by the private sector – and the overall investment climate – will allow the process of adaptation and adoption to move forward. The experiences in China, India and South Africa all indicate that rapid and widespread adopt is then possible, including by small farmers. Many of Sub-Saharan Africa's low-income countries have poorly developed public agricultural research and extension public research agencies and unattractive investment climates though (Beintema and Stads 2004; Sithole-Niang, Cohen and Zambrano 2004; Cohen 2005). As those systems and associated intellectual property rights are improved, so the payoff from R&D spending to adapt appropriate local crop varieties will be enhanced. The potential benefits shown above from this new biotechnology should make that expenditure even more affordable now.

Moreover, the fear of adverse environmental or food safety issues have not been vindicated during the first decade of adoption by those countries and the US and Australia, not least because scientists and regulators have found ways to manage those

[10] There is no expectation that *all* cotton subsidies and tariffs will be removed as a result of the Doha round (see Sumner 2006 and Anderson and Valenzuela 2006), so the gains from GM adoption are even greater relative to prospective trade policy reform over the next decade.

risks. Indeed farmer, water and soil health have all improved thanks to the lesser pesticide needed with Bt varieties of GM cotton. Nor does GM cotton carry the stigma that GM food carries in high-income countries of Europe. If embracing GM cotton helps developing country governments to streamline also the process of approving the release of GM varieties of food crops (given the steady flow of scientific reports such as by King (2003) concluding that there is no evidence that GM foods are harmful either to the environment or to human or animal health), these economies would be able to multiply that $2 billion gain from GM cotton adoption by at least two, according to the numbers presented in Anderson and Jackson (2005) and Anderson, Jackson and Nielsen (2005).

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Table 1: Area of GM cotton and other GM crops, by country, 2002 to 2005

(million hectares)

(a) Total area

	2002	**2003**	**2004**	**2005**
United States	4.2	3.9	4.2	5.6
China	2.1	2.8	3.7	3.3
Australia	0.2	0.1	0.2	0.3
South Africa	0.0	0.0	0.02	0.03
India	0.0	0.1	0.5	1.3
Mexico	0.0	0.0	0.07	0.12
Total, cotton	**6.8**	**7.2**	**9.0**	**9.8**
TOTAL of all GM crops	**58.7**	**67.7**	**81.0**	**90.0**

(b) Area by product and variety, 2005

		Global GM area (m. ha)	Crop's share of global GM area (%)	Area under GM varieties as a % of crop's global area
Cotton:	Bt (insect resistant)	4.9	6	
	herbicide tolerant	1.3	2	
	Bt/herbicide tolerant	3.6	4	
	ALL COTTON	**9.8**	**11**	**28**
Soybean		54.4	60	60
Maize		21.2	24	14
Canola		4.6	5	18
TOTAL of four crops		**90.0**	**100**	**30**
TOTAL of all crops				**5**

Source: James (2005) and earlier issues.

Table 2: Volume of cotton[a] production, yield, trade and utilization, 2005-06

	Output (Kt)	Change in stocks (Kt)	Exports (Kt)	Imports (Kt)	Utilization (Kt)	Share of supply[b] exported (%)	National share of global output(%)	National share of global exports (%)	Yield per ha, % of global average
China	5819	9	10	2800	8600	0	23.5	0.1	163
United States	4735	408	3039	7	1296	70	19.1	37.5	122
India	4250	550	225	125	3600	6	17.1	2.8	63
Pakistan	2308	42	100	250	2415	4	9.3	1.2	103
Brazil	1191	-85	425	50	900	33	4.8	5.2	161
Uzbekistan	1100	14	837	1	250	77	4.4	10.3	110
Turkey	805	0	25	770	1550	3	3.2	0.3	181
Australia	496	-97	582		11	98	2.0	7.2	258
Greece	358	6	258	5	100	73	1.4	3.2	144
Syria	298	-9	150		158	49	1.2	1.9	192
Egypt	263	-8	125	75	220	46	1.1	1.5	137
Burkina Faso	254	-14	264		4	99	1.0	3.3	64
Mali	250	-1	247		4	98	1.0	3.0	68
Turkmenistan	219	6	114		100	54	0.9	1.4	52
Tajikistan	162	6	132		25	85	0.7	1.6	80
Argentina	155	-5	50	20	130	31	0.6	0.6	63
Mexico	152	-33	45	287	428	24	0.6	0.6	169
Kazakhstan	147	5	134	5	12	94	0.6	1.7	99
Benin	140	-49	186		3	98	0.6	2.3	67
Côte d'Ivoire	124	11	103		10	91	0.5	1.3	62
Iran	120	0	10	10	120	8	0.5	0.1	114
Cameroon	112	-78	57	1	132	30	0.5	0.7	69
Spain	110	0	63	15	62	57	0.4	0.8	178
Sudan	96	0	92		4	96	0.4	1.1	67
Tanzania	96	-24	104		16	87	0.4	1.3	31
Paraguay	90	42	43		5	90	0.4	0.5	49
Nigeria	87	2	30	15	70	35	0.4	0.4	33
Zambia	76	0	55		20	72	0.3	0.7	39
Chad	72	-5	77		1	100	0.3	0.9	33
Zimbabwe	72	-13	58		26	68	0.3	0.7	36
Peru	70	1	2	23	90	3	0.3	0.0	118
Togo	70	-9	79		0	100	0.3	1.0	54
Myanmar	59	0	11		47	19	0.2	0.1	29
Colombia	55	21		78	111	0	0.2	0.0	109
Azerbaijan	55	5	41		8	82	0.2	0.5	71
Kyrgyzstan	38	0	39	3	3	103	0.2	0.5	121
Uganda	37	-5	38		4	90	0.1	0.5	52
Mozambique	25	-3	26		2	93	0.1	0.3	16
Ethiopia	22	0	2		20	9	0.1	0.0	38
South Africa	21	0		39	60	0	0.1	0.0	73

Source: ICAC (2005).

[a] Cotton, refers to ginned lint or raw cotton. It does not include seed cotton, linters, cotton mill waste, or cotton fibers subjected to any processing other than separation of lint from seed by the gin. Annual data are for the cotton year beginning 1 August. [b] Supply is output plus change in stocks.

Table 3: Global market shares and net exports of cotton, and effects of GM cotton adoption as of 2001 on cotton output and exports, 2001

	Share (% by value) of global cotton:		*Net exports[a] ($b) of:*		% change from GM cotton adoption in:		
Adopters as of 2001:	*output*	*exports*	*cotton*	*textiles and clothing*	cotton output volume	value of cotton exports	value added in cotton prod'n
United States	*18*	*27*	*2.2*	*-60.7*	4.8	4.4	-0.1
China	*17*	*1*	*-0.1*	*41.9*	0.4	-4.3	-1.6
Australia	*3*	*13*	*1.1*	*-2.6*	7.2	4.3	2.1
South Africa	*0.1*	*0.3*	*-0.0*	*-0.2*	8.1	4.3	3.5
Non-adopters as of 2001:							
Other high-income countries	*5*	*13*	*-1.7*	*-28.4*	-3.5	-5.7	-3.2
Eastern Europe and Central Asia	*16*	*18*	*0.2*	*7.4*	-1.0	-4.5	-0.8
Southeast Asia	*1*	*1*	*-1.5*	*18.4*	-2.3	-8.4	-1.4
South Asia	*21*	*3*	*-1.0*	*24.5*	-1.0	-8.8	-0.6
Middle East and North Africa	*8*	*7*	*0.3*	*-3.3*	-1.6	-7.8	-1.5
Sub-Saharan Africa (excl S. Africa)	*5*	*13*	*1.1*	*-1.5*	-4.6	-7.5	-4.4
Latin America and Carib.	*6*	*4*	*-0.5*	*4.9*	-2.5	-8.9	-2.1
World	***100***	***100***	***0.0***	***0.0***	**0.2**	**-1.1**	**-1.0**

[a] Exports minus imports, both valued at f.o.b. prices as in the GTAP database 6.05

Source: Authors' GTAP model simulation results and (for columns 1 to 4) the GTAP database

Table 4: Effects of GM cotton adoption on national economic welfare as of 2001

(equivalent variation in income, 2001 US$m)

	Welfare changes due to effects of:			Total welfare change
Adopters as of 2001:	resource re-allocation	new technology	terms of trade change	
United States	-47	485	-114	324
China	-18	214	-34	162
Australia	2	63	-39	26
South Africa	-1	2	1	2
Non-adopters as of 2001:				
Other high-income countries	46	0	101	147
Eastern Europe and Central Asia	0	0	5	5
Southeast Asia	-15	0	51	36
South Asia	4	0	10	14
Middle East and North Africa	5	0	9	14
Sub-Saharan Africa (excl S. Africa)	-4	0	-13	-17
Latin America and Carib.	7	0	22	29
World	-22	764	0	742

Source: Authors' GTAP model simulation results

Table 5: Prospective effects of GM cotton adoption by non-adopters as of 2001 on cotton output and exports, without and with Sub-Saharan Africa participating

(percent change from baseline)

	Without Sub-Saharan Africa adopting, % change in:			*With* Sub-Saharan Africa adopting, % change in:		
First adopters as of 2001:	cotton output volume	value of cotton exports	value added in cotton prod'n	cotton output volume	value of cotton exports	value added in cotton prod'n
United States	-3.8	-9.5	-2.7	-5.4	-13.7	-3.9
China	0.2	-0.9	-1.7	-0.1	-8.4	-1.9
Australia	-6.1	-8.2	-5.6	-10.1	-13.5	-9.3
South Africa	-4.7	-7.5	-5.0	-13.7	-14.4	-14.7
New and prospective adopters:						
Other high-income countries	5.0	0.9	0.0	0.5	-5.9	-4.0
Eastern Europe and Central Asia	2.0	0.3	-2.3	0.6	-6.4	-3.1
Southeast Asia	0.4	-0.3	-1.6	0.0	-6.3	-1.9
South Asia	6.2	10.4	-2.9	5.6	3.1	-3.2
Middle East and North Africa	2.1	1.3	-2.7	0.2	-6.4	-4.5
Sub-Saharan Africa (ex S. Africa)	-7.4	-11.8	-7.2	26.7	22.2	10.0
Latin America and Carib.	3.0	2.0	-1.7	1.1	-6.4	-3.4
World	**1.0**	**-5.3**	**-2.7**	**1.0**	**-6.2**	**-2.9**

Source: Authors' GTAP model simulation results

Table 6: Prospective effects of GM cotton adoption by non-adopters as of 2001 on national economic welfare, without and with Sub-Saharan Africa participating
(equivalent variation in income, 2001 US$m)

(a) *Without* Sub-Saharan Africa adopting

	Welfare changes due to effects of:			Total welfare change	
First adopters as of 2001:	resource re-allocation	new technology	terms of trade change	in US$m	as % of GDP
United States	106	0	-45	61	0.001
China	-13	204	-78	113	0.010
Australia	1	0	-15	-14	-0.004
South Africa	1	0	4	5	0.004
New and prospective adopters:					
Other high-income countries	54	93	124	271	0.002
Eastern Europe and Central Asia	3	323	-1	325	0.049
Southeast Asia	-1	26	6	31	0.008
South Asia	75	880	9	964	0.157
Middle East and North Africa	10	133	14	157	0.018
Sub-Saharan Africa (ex S. Africa)	-4	0	-14	-18	-0.009
Latin America and Carib.	12	116	-4	124	0.006
World	**244**	**1775**	**0**	**2018**	**0.006**

(b) *With* Sub-Saharan Africa adopting

	Welfare changes due to effects of:			Total welfare change	
First adopters as of 2001:	resource re-allocation	new technology	terms of trade change	in US$m	as % of GDP
United States	139	0	-83	57	0.001
China	-14	204	-90	100	0.009
Australia	0	0	-28	-28	-0.008
South Africa	1	0	11	12	0.010
New and prospective adopters:					
Other high-income countries	82	91	165	337	0.003
Eastern Europe and Central Asia	0	321	-5	317	0.048
Southeast Asia	-11	25	49	63	0.009
South Asia	80	877	13	970	0.158
Middle East and North Africa	14	132	28	175	0.020
Sub-Saharan Africa (ex S. Africa)	36	221	-69	187	0.091
Latin America and Carib.	12	115	9	135	0.007
World	**338**	**1985**	**0**	**2323**	**0.007**

Source: Authors' GTAP model simulation results

Table 7: Impact of removing cotton subsidies and tariffs[a] on cotton output, exports and value added, and on national economic welfare

(percent and 2001 US$m)

	Change in cotton output volume (%)	Change in cotton value added (%)	Change in value of cotton exports (%)	Welfare changes ($m) due to effects of:		
				resource re-allocation	terms of trade change	TOTAL
All high-income countries	**-20.4**	**-15.4**	**-18.2**	**187**	**275**	**462**
Australia	25.0	22.2	38.1	12	125	137
United States	-24.6	-17.9	-29.0	-15	443	428
EU25	-54.0	-53.3	-48.8	124	-109	15
Japan	0.7	1.5	61.9	25	-49	-24
Korea-Taiwan	11.9	6.9	33.6	21	-84	-63
Other High income	**-36.1**	**-36.6**	**-41.7**	**190**	**-293**	**-103**
All developing countries	**5.7**	**4.3**	**46.3**	**96**	**-275**	**-179**
E. Europe and Central Asia	7.0	3.3	35.9	21	-36	-15
China	2.0	1.5	75.7	5	45	50
Other East Asia	8.7	5.1	65.3	39	-82	-33
India	-0.6	-0.4	31.1	-5	-79	-84
Other South Asia	6.0	3.5	59.8	9	-20	-11
Middle East & North Africa	6.2	6.1	37.4	-7	26	19
South Africa	19.4	20.6	46.5	2	-2	0
Other Sub-Saharan Africa	32.1	30.6	55.0	32	115	147
Argentina	13.6	10.7	66.1	1	6	7
Brazil	9.8	10.3	57.6	1	12	13
Mexico	13.0	10.5	42.3	11	-136	-125
Other Latin American & Car.	9.4	7.3	44.7	-13	-34	-47
World	**-0.8**	**-1.8**	**7.9**	**283**	**0**	**283**

[a] Removal of those distortions left after the eventual phase-out of the quotas under the Multifibre Agreement at the end of 2004.

Source: Authors' GTAP model simulation results

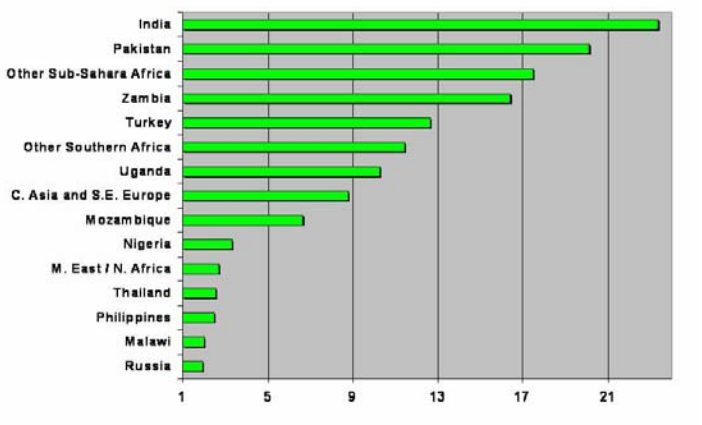
Table 8: Prospective effects of GM cotton adoption by non-adopters as of 2001 on national economic welfare, without and with cotton subsidies and tariffs removed first

(equivalent variation in income, 2001 US$m)

	Without subsidy and tariff reform	With cotton subsidies and tariffs first removed	With simultaneous cotton subsidy/tariff removal and GM catch-up
All high-income countries	366	279	744
Australia	-28	-58	80
United States	57	-25	404
EU25	269	281	295
Japan	36	37	14
Korea-Taiwan	-14	-6	-68
All developing countries	1957	2043	1866
E. Europe and Central Asia	317	317	303
China	100	94	144
Other Southeast Asia	63	83	-48
India	822	855	771
Other South Asia	148	151	140
Middle East & Nth Africa	175	211	194
Sub-Saharan Africa	199	223	370
Latin American & Carib.	135	146	-8
World	**2323**	**2322**	**2610**

Source: Authors' GTAP model simulation results

Figure 1: Welfare gain from GM cotton adoption as a percent of GDP, as a multiple of the percentage gain to the world as a whole



Source: Authors' GTAP model simulation results

REPORT 6.7

APPLIED GENERAL EQUILIBRIUM ANALYSIS OF AGRICULTURAL AND RESOURCE POLICIES

APPLIED GENERAL EQUILIBRIUM ANALYSIS OF AGRICULTURAL AND RESOURCE POLICIES*

by
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APPLIED GENERAL EQUILIBRIUM ANALYSIS OF AGRICULTURAL AND RESOURCE POLICIES*

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Abstract

This paper reviews the literature on applied general equilibrium analysis of agricultural and resource policies. It begins with an historical overview, followed by an assessment of the benefits of this methodology for examining sectoral policies. The chapter then turns to questions of disaggregation of commodities, households, regions and factors of production. Parameter specification and model closure are discussed, as well as problems of modeling policies which affect agriculture. There are also special sections on agriculture and the environment, product differentiation and imperfect competition and model validation. The paper closes with a discussion of future challenges to the field.

Chapter Outline

APPLIED GENERAL EQUILIBRIUM ANALYSIS OF AGRICULTURAL AND RESOURCE POLICIES*

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Introduction

Applied general equilibrium analysis as we know it today has intellectual origins in the debate over the feasibility of the centralized computation of a Pareto optimal allocation of resources within an economy (Whalley, 1986, pp. 30-34).[1] During the first half of this century, quantitative economists were preoccupied with the question of whether or not it was computationally feasible to solve the associated system of behavioral equations. Since that time, rapid developments in operations research have proven the optimists correct. It became possible to solve very large models representing national economies and indeed, the global economy. Initially these were solved as centralized planning problems, intended to deduce the optimal allocation of resources in the economy. With the demise of central planning, decentralized "computable" general equilibrium models have become dominant. While this has not brought an end to the debate over the operational relevance of general equilibrium theory, the increasing use of such models in policy analysis has served to sharpen the debate. It now focuses heavily on questions of model specification, parameter choice, and the appropriate representation of policies (Whalley, 1986). In this sense, many of the issues raised in this survey are no different from those which arise in other areas of applied economics. This is why I prefer to use the term "applied" general equilibrium (AGE), in place of the popular "computable" general equilibrium (CGE) label.

Leif Johansen (1960) developed the first operational AGE model in the late 1950's. Variants of this model are still used in Norway (Shreiner and Larsen). Since Johansen's pathbreaking contribution, AGE models have been applied to a very wide range of topics. John Shoven and John Whalley and their students spearheaded work in the analysis of tax issues (Shoven and Whalley, 1992) and Whalley (1985) led the way with multiregion AGE modeling of trade policy questions. The Australian school of AGE modeling, led by Peter Dixon, has been analyzing issues of protection in the Australian economy for more than twenty years (e.g., DPSV, 1982). Applied GE models have also been popular in the development economics literature (Dervis, de Melo and Robinson, 1982; Robinson, 1988).

This survey focuses on AGE modeling issues and applications related to agricultural policy analysis. In order to keep this task manageable, I have elected to limit the bulk of the discussion to issues arising in comparative static, AGE analysis of agricultural policies in national market economies, as well as globally. As noted above, there are a number of surveys of AGE analysis focusing specifically on developing economies. To this we might add the work of Sadoulet and de Janvry (1995), which has a strong developing economy orientation. While many of the issues are common, regardless of the level of economic development, there are some salient differences having to do with underdeveloped markets and other rigidities. I will not have much to say about these "structural" issues here, as they tend to be locationally and institutionally specific. Additionally, I

[1] Sections I and II draw heavily on material in Hertel (1990).

will not attempt to cover the specialized topics relating to AGE analysis under uncertainty or dynamics. Appropriate treatment of these topics would take me well beyond the page limits for this chapter.[2]

Another area of modeling which I will omit in this survey has to do with the incorporation of financial variables in the model. This work, aimed at a synthesis of micro- and macro-economics, is quite challenging. In his 1991 review of this subject, Sherman Robinson highlights the theoretical tension between the neoclassical paradigm and AGE models with financial behavior. "We are still far from a theoretical reconciliation between Walras and Keynes and empirical models cannot help but reflect the theoretical gap." (p. 1522). Nevertheless, the need for this type of synthesis remains. In their recent survey of issues arising in this area, Bevan and Adams (1997) point out that many of the structural adjustment packages presented to developing countries and economies in transition ignore the real sector consequences of their macroeconomic prescriptions. Analysis of monetary variables in an AGE model could help to fill this gap, and this clearly represents an important topic for research (see, for example, McKibbin and Sachs, 1991). However, as the Bevan and Adams survey indicates, no clear consensus exists and many difficult issues remain to be resolved. Consequently, all of the work reviewed below will relate only to *real* models, in which monetary variables, such as the money supply, price levels and nominal exchange rates have no role to play.

Why AGE Analysis of Agriculture?

Benefits of AGE Analysis

An important question to be raised at the outset of this survey bears on the relevance of AGE analysis for agriculture. With food and agriculture representing an ever shrinking share of GDP and consumer expenditure, why should we go to the trouble of constructing an economywide model to analyze policies in these sectors? There are several important advantages offered by this approach to policy analysis.

Household focus: Traditional agricultural economic analysis has tended to focus on commodities, and associated factor returns. In contrast, AGE models begin with households as the primitive concept. Households supply factors of production and consume goods and services. Welfare in the model is computed directly in terms of household utility and not some abstract summation of producer, consumer and taxpayer surplus. After all, most households embody a combination of all three of these attributes, namely, income generation, consumer expenditure and the payment of taxes or the receipt of subsidies. The focus also on people, services, resources and

[2] For a recent AGE application with uncertainty, which focuses on agriculture, see Boussard and Christensen (1997). Readers interested in intertemporal models are referred to Keuschnigg and Kohler (1997), McKibbin and Wang (1998), and Wilcoxen (1989). Recursive-dynamic applications are quite common in agriculture (Fisher *et al.*, 1988; Burniaux and van der Mensbrugghe, 1991; Wang, 1997), and Diao, Roe and Yeldan (1998) have an application drawing on endogenous growth theory.

the environment, instead of just commodities is increasingly important, as the share of farm household income generated outside of agriculture increases.[3]

Finite resources and accounting consistency: AGE models rely on social accounting matrices (SAMs) for their empirical structure (Pyatt and Round, 1979; Hanson and Robinson, 1988). These SAMs detail all the basic accounting identities which must hold for the economy to be in equilibrium. Those who work with AGE models quickly recognize that these identities are as important as the behavioral assumptions. The fact that households cannot spend more than they earn, or that the same unit of labor, land or capital cannot be simultaneously employed in two different places, serves to tightly circumscribe the range of possible GE outcomes.

A related issue has to do with the fiscal integrity of the analysis. Historically, agricultural economists have rarely posed the question: Who pays for farm subsidies? (Alston and Hurd, 1990). Yet it has been shown, using AGE methods, that the marginal excess burden of raising revenue in the United States is often very high (e.g., Ballard, *et al.*, 1985b). By incorporating an explicit budget constraint for the government, AGE models can capture the cost of higher levels of agricultural subsidies — or alternatively, the fiscal benefits of reducing expenditures on farm programs. Chambers (1995) takes the distortionary effect of taxation into account in his general equilibrium analysis of alternative forms of agricultural subsidies. He shows that traditional, partial equilibrium calculations of farm subsidy incidence misrepresent social losses and systematically overestimate the benefits agricultural producers derive from farm programs by ignoring the impact on government revenue requirements.

A final benefit resulting from the exhaustive accounting in AGE analysis derives from the applicability of Walras' Law. This "law" states that if: (a) all households are on their budget constraint (subject to explicitly defined inter-household transfers or borrowing), (b) all firms exhaust their revenues on factor payments, taxes, and transfers of excess profits to households, and (c) all markets are in equilibrium (i.e. supply = demand), then one of the equilibrium relationships in the model will be redundant and may be dropped. This provides an extremely powerful check on the consistency of the AGE model, since the redundant equilibrium condition may be checked — after the fact— to verify that there were no errors in data base management, model coding, or possibly in the theoretical structure. Indeed, most AGE modelers will admit to having discovered many errors via the use of this check.[4] Given the complexity of implementing a large-scale empirical model, this can be a very powerful tool indeed.

Second-best analysis: One of the distinguishing features of agricultural policy analysis is the high degree of public intervention in the farm and food sector. This includes programs which: (a) subsidize inputs such as credit, water, and fertilizer, (b) restrict acreage planted to certain crops, (c) intervene in output markets with subsidies or production quotas, (d) subsidize (or tax less) the

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[3] The latest information from the U.S. indicates that 88% of farm household income is derived from nonfarm sources (USDA, 1995).

[4]For example, if one decided to introduce imperfect competition in an existing AGE model, but forgot to distribute the excess profits to owners of the enterprise, Walras' Law would reveal this in the form of insufficient demand in the omitted market.

consumption of food relative to other goods and services, and (e) intervene at the border with export subsidies, import tariffs and quotas, etc. This complex web of policy interventions makes it very difficult to anticipate the efficiency consequences of a marginal perturbation in, or reform of, farm and food policies (Clarete and Roumasset, 1990). Chambers (1995) derives conditions under which, *at the margin*, land retirement may dominate decoupled transfers to producers due to their impact on the government budget and hence existing levels of distortionary taxation.

In an AGE application focusing on US agriculture in the mid-1980's, Hertel and Tsigas (1991) show that, *at the margin*, tradeable output quotas could have been welfare-enhancing. This stemmed from the fact that existing agricultural, food, and tax policies had retained excessive resources in agriculture and the quotas would provide a mechanism for moving some of these inputs out of the farm sector. However, those authors also show that the supply control approach which was preferred at the time, namely acreage restrictions, would have reduced efficiency in the economy. Finally, they demonstrate that the first-best alternative of removing all of the distortions would generate welfare gains an order of magnitude larger than the tradable quotas. In summary, AGE models provide an excellent vehicle for conducting welfare analysis in a second-best setting, and this makes them particularly well-suited for use in agricultural policy analysis.

Interindustry linkages: Often when one is conducting policy analysis in the farm sector, it is difficult to know where to draw the line between the commodities and sectors affected by a given policy and the rest of the economy. More generally, distinguishing agriculture from non-agriculture in the modern, industrialized economies has become quite difficult. Increasingly, large, commercial farms contract out some of their operations. The firms providing these services — ranging from pesticide applications to financial services — may not be exclusively tied to agriculture. Sayan and Demir (1998) assess the degree of interdependence between agriculture and non-agriculture industries in Turkey using techniques from input-output analysis. They find that when backward linkages from agriculture to non-agriculture are ignored, the agricultural multipliers are understated by about 20%. Linkages from the agriculture to non-farm sectors producing energy, fiber and other nonfood items are also important. When backward linkages from non-agriculture to agriculture are omitted from Sayan and Demir's analysis, the non-farm multipliers for Turkey are about 8% too low. A final, important reason for capturing the non-farm linkages has to do with the diversification of farm households' earnings. They often have significant financial or wage earning interests in other sectors, so that their welfare depends on much more than the changes in agricultural activity.

Economywide Perspective: AGE analysis also provides a valuable tool for putting things in an economywide perspective. Microeconomic theory emphasizes the importance of relative, as opposed to absolute, levels of economic variables. For example in the case of technological progress, it is not the absolute rate of TFP growth that matters for agricultural production and prices, but rather the rate of TFP growth *relative to* the non-farm sector (Simon, 1947; Gruen, 1961). Similarly, a tax reform which raises tax rates for agriculture, may not discourage farming activity if the non-agricultural tax rates rise by more. In an AGE analysis of the US tax system, Hertel and Tsigas (1988a) find that relatively low tax rates on capital, labor and output in agriculture, as well as relatively lower consumption taxes on food, have all conferred an implicit subsidy on the farm and food sector. Nowhere is the importance of relative vs. absolute comparisons more evident than in international trade. It is very common for agricultural economists to compare production costs in different regions, and, when they are lower in one country than another, to conclude that country is

more competitive. However, this ignores the most fundamental proposition of international trade, namely that countries will export the product in which they have a *comparative advantage*. Where do they go wrong? Any partial equilibrium comparison of costs invariably must make an assumption about the terms of trade. Yet, the terms of trade are fundamentally endogenous. They adjust to ensure external balance. In equilibrium, a given economy may be the most efficient producer of both agriculture and manufactures. But if its comparative advantage is in manufactures, it will import agricultural products.

In order to better understand where partial equilibrium analysis of competitiveness can lead one astray, it is useful to think about a specific example. Consider the case whereby the US embarks on an effort to become more competitive by investing in the skill-base of its workforce. *A priori* we might think that this should result in an increase in agricultural output, since a more highly skilled workforce will result in more productive farmers. However, once we take into account general equilibrium constraints, we will find that the opposite conclusion is more likely correct. The reasoning is as follows. First of all, more productive labor will tend to boost output across the board. Consequently, at constant prices, exports will increase and imports will be displaced by domestic production in all sectors. Furthermore, foreign investment is also likely to increase in response to the higher level of labor productivity. This leads to a violation of the general equilibrium condition for external balance:

$$(1) \quad S - I = X + R - M$$

where S = national savings, I = investment, X = exports, R = international transfers, and M = imports.

Without any GE adjustment, the left hand side of (1) becomes more negative (I↑) and the right hand side becomes more positive (X↑, M↓). Something clearly must adjust to ensure that (1) will hold. In general equilibrium, this is the real exchange rate. Goods produced in the US must become more expensive abroad, and imports must become relatively cheaper. As the system re-equilibrates, what will happen to farm output? Since agriculture is relatively more intensive in land, the availability of which is unchanged, and relatively less intensive in skilled labor, the supply of which has increased, we expect agricultural outputs (and exports) to *fall* in this instance. (This is the well-known Rybczynski theorem.) In summary, here we have a case where the economywide constraints are strong enough to actually reverse partial equilibrium intuition.

Hidden Challenges to AGE Analysis

Having made the argument that general equilibrium analysis is called for in some circumstances, the next question is: What type of AGE model is appropriate? In a paper titled "Hidden Challenges in Recent Applied General Equilibrium Exercises," John Whalley (1986) emphasizes the need to move from general to special-purpose models if AGE analysis is to become more policy relevant. He notes that the AGE models of the 1960s, 1970s and early 1980's were developed partially in order to "demonstrate the feasibility of constructing applied general equilibrium models ... showing they could handle much larger dimensions than theoretical models" (p. 37). Application of such models to particular policy issues often involved redesigning the basic model, while carrying along considerable excess baggage. With model construction and

computational cost now less burdensome, Whalley suggests that future efforts be directed at developing special purpose models, tailored to address specific issues. He notes that particular attention should be paid to parameter specification and the manner in which policies are modeled. The remainder of this survey may be viewed as an overview of recent attempts to meet some of these "hidden challenges", which have often limited the impact which general purpose AGE models have had on agricultural policy issues.

Most of the early AGE models of developed market economics (DMEs) treated agriculture (possibly along with forestry and fisheries) as a single, aggregate sector, producing one homogeneous product (e.g., Ballard, *et al.,* 1985a). This type of aggregation was essential in order to permit complete commodity coverage at a relatively uniform level of aggregation. Also, this is often the level of aggregation provided in published input-output tables. However, when it comes to analyzing farm policies, more detail is required. This is because intervention varies widely across farm commodities, with some receiving a great deal of support (1985 U.S. sugar prices were five hundred percent of the world price), while others (such as the U.S. poultry industry) are virtually free of intervention. By lumping all of these products into one single aggregate, little can be said that would carry any weight with agricultural policy makers. The question of appropriate disaggregation of AGE models for agricultural policy analysis will be addressed in section III of the chapter.

A second important feature of general purpose models which has limited their applicability to agricultural issues is their failure to distinguish land from other capital inputs. Yet the presence of farm land in the agricultural production function is critical. It is perhaps the most distinguishing feature of this sector of the economy. Furthermore, land can also be an important instrument of public policy. Historically, a significant aspect of intervention in U.S. agriculture involves the idling of productive acreage in order to raise commodity prices. The European Union and Japan have recently also directed more of their policies towards limiting land use. In addition, farm land prices are themselves often a policy target. With relatively limited alternative uses (outside of agriculture), the price of farmland not adjacent to cities tends to be determined predominantly by expected farm product prices. Therefore land prices are potentially quite volatile. Since land usually represents the major form of wealth holding for the farm population, the impact of public policy on farm prices and hence returns to landowners is of paramount importance to farmers and agricultural policymakers. There is simply no way around dealing with land markets if one wishes to appropriately model the agricultural sector, and so disaggregation of factors of production, including land, is also dealt with in section III.

A third critical limitation of the most common, general purpose AGE models of the last two decades is their tendency to devote too little attention to the specification of key behavioral parameters in the farm and food system. As a consequence, there is a wide gulf between the partial equilibrium models currently used in agricultural policy analysis, and the partial equilibrium behavior of their AGE counterparts. In some cases these discrepancies may be justified. However, in most instances the AGE models' parameters simply lack sufficient empirical justification. As a consequence, they often generate implausible results.

Generous federal and state funding, and close working relationships with other scientists and with industry have combined to result in an agricultural economic data base which is the envy of many applied economists. There is also more than half a century of applied econometric analysis of

supply and demand behavior in agricultural markets upon which to draw. To be effective, any AGE modeler who wishes to seriously tackle farm and food policy issues must be willing and able to capitalize on this wealth of data and behavioral information. In some cases this will require use of more general functional forms for representing preferences and technology in the AGE model. Section IV of this paper addresses the issue of parameter specification as well as the related questions of length-of-run and model closure.

Section V of this paper focuses on one of the specific hidden challenges identified by John Whalley - namely the need for explicit modeling of public policies. There are many cases in which simple ad *valorem* equivalent representations, common among general purpose models, give rise to inaccurate, or even misleading, conclusions. Of course, time spent at detailed modeling of individual policies must be balanced against the need to provide a comprehensive picture of distortions in the economy. For some purposes this extends to analyses of agriculture and the environment, which is the topic of Section VI.

Section VII addresses a few of the issues which arise in the context of product differentiation and imperfect competition. This can be very important when it comes to validation of AGE models, which is the subject of Section VIII. The chapter closes with some thoughts about future directions for AGE analysis of agricultural and resource policies.

Data and Aggregation Issues: How Detailed Should the Model Be?

Sectoral and Commodity Disaggregation

Obviously there are limits to the amount of detail which can be provided by an economywide model. The general purpose models have logically opted for a relatively balanced treatment of the entire economy, given the constraints imposed by national accounting conventions. For example, the U.S. tax model outlined in Ballard, Fullerton, Shoven, and Whalley has nineteen sectors. Sectoral gross output, as a percentage of the U.S. total, ranges from slightly less than one percent (mining) to a little more than ten percent (services). But most sectors fall in the 2-8% range (Ballard, *et al.*, 1985a, table 4.13).

A special purpose model focused on agricultural policy will necessarily be more lopsided in order to focus attention on particular issues. Perhaps the most extreme example of this is the world wheat model of Trela, Whalley, and Wigle (1987). In their framework, each country consumes two goods: wheat, and everything else. This permits them to focus on the global effects of wheat policies within a consistent AGE model. It also makes data and calibration particularly straightforward. Benchmark equilibrium wheat production and consumption data are readily obtained from (e.g.) the FAO and they may then obtain data on the other sector as a residual. Constant elasticity of substitution (CES) or transformation (CET) preferences and technology are calibrated to reproduce published supply and demand elasticities for wheat, and they are "off and running" with a model. The IIASA model was based on the same idea, only its authors disaggregated agricultural production into ten commodities, with one residual, "nonagriculture" commodity (Fisher, et al., 1988). The multiple commodity work of Horridge and Pearce (1988)—based on the Tyers and Anderson (1992) PE trade model)— as well as that of Peterson, Hertel and Stout (1994) and McDonald (1990) — both based on USDA's SWOPSIM model (Roningen *et al.*, 1991) —are similar in spirit.

Given the difficulty of constructing a benchmark equilibrium data base for an AGE-trade model, there are obvious advantages in a model specification which has a large "residual" sector. However, there are important drawbacks associated with this backdoor approach to arriving at a complete AGE model. The first of these is due to aggregation bias. Gehlhar and Frandsen (1998) illustrate how aggregation of agricultural sectors changes key qualitative findings with respect to APEC trade liberalization. This is due to the tendency to create false competition between countries producing fundamentally different products (e.g., rice and wheat). Excessive aggregation also can alter the welfare effects by smoothing out tariff peaks which may exist at a disaggregate level. Bach and Martin (1997) show how this problem can be overcome via the use of Anderson and Neary's Trade Restrictiveness Index in concert with an AGE model. They find that the welfare gains from tariff reform in China double when their analysis begins at the level of individual tariff lines, as opposed to simple aggregation to 10 sectors.

Another problem with excessive sectoral and commodity aggregation stems from the fact that the dividing line between the agricultural and nonagricultural economy is not at all clear. Furthermore, in the case of some agricultural policies, the "grey area" between these two groups of sectors is where the most interesting "action" is. Consider, for example, the U.S. sugar program. Support for U.S. sugar producers is achieved indirectly by administering an import quota on partially refined sugar, which is adjusted until the domestic price of sugar reaches a prespecified target. The greatest source of pressure on the U.S. sugar quota has come not from the farm sector's supply response, but rather from the manufacturers of substitute sweeteners - in particular high fructose corn sweeteners (HFCS). The HFCS industry is dominated by a handful of firms who have become a very effective lobby for the sugar program. They have also made a concerted attempt to mobilize corn producers in support of this import quota on sugar, arguing that the derived demand for corn generated by production of this sweetener substitute lends considerable support to the market price of corn. While it has already been partially processed, traded sugar must be further refined for use in the domestic market. As a consequence, successive tightening of the quota has seriously hurt domestic sugar refiners.

Rendleman and Hertel (1993) show how the sugar quota can be analyzed using a special purpose AGE model. They conclude that short-run losses to sugar producers and the manufacturers of substitute sweeteners are, to a great extent, offset by gains to the ailing sugar refiners when the quota is eliminated. They also conclude that corn producer support for the U.S. sugar program is likely misplaced, since the HFCS industry produces by-products, corn oils and gluten food, which competes with corn grain. Consequently analysis of the sugar program which ignored the livestock sector seriously overstated the impact on corn prices. The message here is that analysis of particular commodity programs often requires disaggregation of nonfarm, food manufacturing activity as well.

Applied GE models attempting to address the overall impact of farm and food programs need to disaggregate sufficiently to isolate distinct types of commodity market intervention. Hertel, Thompson, and Tsigas distinguish nine different farm products and about a dozen agri-processing sectors in their attempt to assess the impact of unilateral agricultural policy liberalization in the United States. In their work on U.S. agricultural policies, Robinson *et al.* began with a model in which 3 farm sectors were broken out (Robinson, Kilkenny, and Adelman, 1989). They subsequently found it desirable to disaggregate to 8 agricultural and 8 food processing sectors (e.g., Hanson,

Robinson and Tokarick, 1988; Kilkenny and Robinson, 1990) in order to capture the major differences among various farm and food policies.

The question of disaggregation becomes more difficult in those cases where the general equilibrium modeler wishes to deal explicitly with agricultural trade and related domestic policies, among a variety of countries. This is because multiple data sources must be used, making disaggregation more difficult. The OECD "Rural-Urban, North-South" RUNS model (Burniaux and van der Mensbrugghe, 1991), had 15 commodities, of which 8 pertain to farm and food products. More recently, the Australian Industry Commission developed a 16 region, 37 commodity model with 11 farm and food sectors, nicknamed SALTER (Dee, *et al.*,1992). Much of the data base underpinning SALTER was adopted by the Global Trade Analysis Project and built into the GTAP model (Hertel, 1997). The most recent version of the GTAP data base disaggregates 20 farm and food products and 30 non-food products. However, obtaining this degree of sectoral detail for many different countries necessarily involves some compromises. Also, even with that degree of sectoral detail, the breakdown may not be sufficient for a particular policy issue in a specific country.

Household Disaggregation

From the point of view of welfare analysis, disaggregation of *households* in the economy is probably even more important than sectoral disaggregation. Unfortunately, data on factor payments to households is difficult to obtain. For this reason many researchers choose to aggregate all private consumption into a single household. Some notable exceptions in the case of research on U.S. agriculture, include Boyd (1988), who distinguishes households by income class, and Kilkenny (1993), who distinguishes rural and urban households. Of course income distribution is often much more skewed in the case of developing countries and consequently there has been more of this sort of work done in that context (e.g., Brandao, *et al.*, 1994; de Janvry and Sadoulet , 1987; Robinson, *et al.*, 1993; Warr and Coxhead, 1993). If AGE analysis is to address the important *distributional* implications of farm and food policies, this type of household disaggregation must become standard practice. This will require additional data work on the part of the researcher.

Given the strong interest in income distributional consequences of public policies, some researchers have adopted a second-best approach to the problem. In particular, they first solve a household-aggregated model for a set of relative price changes for commodities and factors of production. They then engage in *ex post* calculations of the implied welfare changes for different household groups. This can make it feasible to examine the welfare implications for thousands of different household types. A recent example of this approach in the analysis of Vietnamese rice policies is provided by Minot and Goletti (1997). Provided the implied changes in income distribution have minimal implications for aggregate commodity demand, this is a very attractive approach to the problem of household disaggregation, since it permits the researcher to report results at a very high level of detail.

Regional Disaggregation

Just as it is often necessary to disaggregate sectors and households, so too is regional disaggregation frequently required to adequately capture the impact of agricultural and resource policies. Such disaggregation can take place at the sub-national level. For example, Kraybill *et al.* (1992) disaggregate the U.S. into the State of Virginia and the rest of the U.S. in order to analyze the regional incidence of national macroeconomic policy. A major challenge in such efforts at sub-national disaggregation arises from the scarcity of state-level social accounting matrices or input-output tables. Typically these must be "estimated" based on national accounts and selected sate level information (e.g., employment by sector and final demand). Another problem arises from the absence of observations on intra-national (inter-state) trade flows. As a consequence, multiregion AGE models are more common at the international level, where researchers can build on national accounts and international data sources on trade-flows.

International AGE models may be broken into two groups: those with a regional focus, and those with global coverage. In some cases, the issue being considered has a clear regional dimension which suggests analysis in the context of a two or three country model. The U.S. - Mexico component of the North American Free Trade Agreement (NAFTA) was successfully analyzed by Robinson *et al.* (1993) in a two region (US - Mexico) model wherein the rest of the world responses were simply captured with excess supply and demand equations. Harrison *et al.* (1989, 1991) develop a disaggregated data base for the European Union in order to analyze the welfare and distributional consequences of policies associated with the European Community.

Increasingly, however, many policy makers are seeking answers to global economic policy questions. In this case, global applied general equilibrium analysis is often the most appropriate tool. The drawn-out negotiations under the Uruguay Round of the GATT/WTO provided ample opportunity for quantitative analysis. The volume edited by Martin and Winters (1996) offers the most comprehensive analysis of the Uruguay Round Agreement. All five of the quantitative assessments contained therein are based on global AGE models. While these global models generally share the same basic structure as the national and regional models, there are some specific issues which arise in making the transition from one to the other. Hertel, Ianchovichina and McDonald (1997) provide an extensive discussion of these differences.

In some cases, global modeling is desired, not because the policy scenario under consideration is global in nature, but rather because the consequences of a regional shock are expected to be widespread. Thus Arndt *et al.* (1997) use a global AGE model to analyze which countries gain, and which lose, from rapid economic growth in China. Coyle, McKibbin and Wang (1998) use a global AGE model to analyze the impact of the Asian financial crisis on U.S. agriculture. One reason why the list of global AGE analyses has been growing so rapidly in recent years is the public availability of a global economic data base to support such studies. Nicknamed GTAP (Global Trade Analysis Project), this data base is now on its fourth release (Hertel, 1997)[5].

[5]For a comprehensive listing of references to global AGE studies based on the GTAP data set (150 at the time of this writing), the reader may visit the following web site: *www.agecon.purdue.edu/gtap/apps*

Agriculture as a Multiproduct Industry

The generic, general purpose AGE model is typically characterized by single commodity, constant returns to scale industries. However, agriculture departs significantly from this mold. Econometric tests for nonjointness in aggregate agricultural production are consistently rejected (e.g., Ball, 1988). There are numerous explanations for this apparent jointness in production including technological interdependence, the presence of lumpy/shared inputs, and the presence of an allocatable fixed input, namely land (Shumway, Pope, and Nash, 1984).

The problem posed by the presence of multiproduct sectors in an AGE model is that the addition of potential output-output, and input-output interactions vastly increases the number of parameters to be specified. One common solution is to impose input-output separability (e.g., Dixon, *et al.*,1982). The implication of this particular restriction is that the optimal output mix is invariant to changes in relative input prices. This is a strong assumption which violates one's intuition (e.g., the optimal mix of corn and soybeans is sensitive to the price of fertilizer). It also is persistently rejected by the data (e.g., Ball, 1988).

Another problem confronting the modeler seeking to treat agriculture as a multiple product sector is the presence of commodity-specific factor market interventions. For example, in order to qualify for corn output subsidies in the U.S., it was previously necessary to idle a certain percentage of one's established corn acreage. This in turn had a differential effect on the shadow price of land in corn vs. (e.g.) soybean production. Lee and Helmberger (1985) demonstrate how this can result in own-price effects which are "too small" relative to cross-price effects. As a result, nonconvexities can arise in a multiproduct profit function representation of the farm sector.

If one is willing to argue that jointness in agricultural production is solely due to the presence of an allocatable fixed input, then it is possible to revert to modeling commodity production as a set of single product activities -- bound together by the presence of a fixed amount of land. Indeed, attempts have been made to estimate agricultural technology under these assumptions (Just, Zilberman, and Hochman, 1983). It is also a common specification in agriculturally focused AGE models, and has the advantage of facilitating commodity-specific interventions in the land market (Hertel and Tsigas, 1991; Kilkenny, 1991).

Producer Heterogeneity

Another type of heterogeneity in the farm sector is that which arises due to differences in producers. This could arise due to differences in entrepreneurial capacity, as hypothesized by Friedman, 1976), or due to differences is risk preferences, or for other reasons. In any case, we observe a great deal of variation in farm size as well as production technology in the farm sector. One reason which such differences can persist in the face of market forces is the tendency for farmland to absorb any differences in profitability. As long as the farmer owns his/her own land, and as long as he/she is willing to take a sub-market return on this asset, then they can remain in farming in spite of lower levels of efficiency. This is particularly likely in the case of smaller, part-time operations in which farming is part of the household's lifestyle.

In the U.S. a relatively small group of commercial farms produces the majority of agricultural output. There are a great number of small farms, many of which are part-time operations. For example, in 1987, 52% of the farms had sales of less than $10,000 and consequently accounted for only 6% of gross farm income (Sumner, 1990). The inexorable downward slide of average costs leaves small producers with below average, sometimes negative, returns to their equity and own-labor. This process is driven by persistent technological change, and at any particular moment, the agriculture sector is in a state of disequilibrium with regard to the composition and size of farms. For example, in their econometric analysis of the period from 1947-74, Brown and Christensen (1981) show that, while family labor in agriculture dropped by two-thirds over this period, the estimated optimal level of this input also dropped dramatically. As a result, the ratio of observed to optimal family labor hardly changed.

While the issue of farm size is an important one, it is essential that AGE modelers with an interest in agriculture focus on aspects of the farm sector: (a) which are central to the questions they seek to answer, and (b) to which they can contribute some added insight. I would argue that neither of these applies (in most instances) to the farm size issue in developed market economies. Most production comes from a relatively small group of commercial farms. These operations dominate the data used to estimate price elasticities, and their behavior is more nearly consistent with the neoclassical paradigm prevalent in AGE analysis. Thus, in most cases, we should focus on modeling representative commercial farm operations. Modeling the evolution of the distribution of farms by size is an important policy issue, but not one in which AGE models have any comparative advantage.

Of course there are exceptional cases in which farm size becomes relevant for AGE analysis of agricultural policies. A good example is provided by the Canadian dairy program, whereby individual farms are assigned a production quota. Econometric evidence indicates that this has contributed to the presence of unexploited scale economies (Moschini, 1988). Thus it is important to build this inefficiency into the initial equilibrium. Robidoux, Smart, Lester, and Beausejour (1988) have done this (both for dairy and poultry) in their analysis of Canadian farm policies. They find that agricultural policy liberalization generates considerable "rationalization" in the dairy industry as some farms exit and the remaining operations move down their long-run average cost curve.[6]

Establishing an Appropriate Benchmark

The vagaries of weather, long gestation periods, price-inelastic demands, and heavy (but unpredictable) intervention by governments all contribute to greater volatility of agricultural assets, relative to their nonfarm counterparts (Irwin *et al.,* 1988). It is not uncommon to find enormous swings in the components of agricultural value-added reported in the national accounts. This volatility in observed "cost shares" can translate directly into volatile model results, as has been

[6]In addition to the differences in farm size, there are other observed types of heterogeneity which can have important policy implications. Using USDA survey data, Hertel, Stiegert and Vroomen (1996), show that Indiana corn producers exhibit strikingly different propensities to apply nitrogen fertilizer to their crops. Even after controlling for terrain, soil type, manure applications and crop rotation, those authors observe application rates ranging from 30 to more than 200 lbs./acre in 1989. In the face of a proposed tax on nitrogen fertilizer, the authors argue that this producer heterogeneity can give rise to an additional source of nitrogen-land substitution — namely a composition effect. As the price of fertilizer rises, it has a strong negative impact on profitability for the most profligate users of this input. This induces a shift of corn land from the high-intensity users to the low-intensity farm managers.

demonstrated for Australia by Adams and Higgs (1990), using the ORANI model. Since the share of fixed capital and land in the primary factor aggregate is a key parameter in the calibration of ORANI's agricultural supply response (see also Section IV below), variation in this share translates directly into variation in the supply elasticity. The authors show that such variation can even alter the predicted macroeconomic consequences of farm sector shocks. This led Adams and Higgs to the development of a "representative year" data base for Australian agriculture.

In a somewhat more ambitious undertaking, Harrison, Rutherford, and Wooten (1989) construct a sequence of SAMs for the European Community with which they proceed to analyze the same experiment (removal of the Common Agricultural Policy) over a period of 12 years. A logical extension of this effort would be to use this time series data to estimate a representative benchmark equilibrium for the entire economy. A more modest undertaking might involve the econometric estimation of cost shares for the agricultural sector alone.

Finally, there is a question of what benchmark should be used to assess the impact of policies which are due to be implemented over a relatively long period of time. A good example is provided by the Uruguay Round Agreement (UR) which was concluded in 1995 but which was due to be phased in over ten years. Furthermore, in the most contentious areas — agriculture and apparel — many of the reforms are "back-loaded" with the deepest cuts in protection scheduled for the later years. Yet most of the studies of this agreement employed data bases which described the global economy in the early 1990's. Bach *et al.* (1998) evaluate the difference between the welfare effects of the URA in 1992 vs. 2005, where the latter benchmark is constructed by projecting the global economy forward using World Bank estimates of endowment and productivity growth. Since the deepest UR cuts are in Asia, and since this region was projected to growth rather rapidly as well, the authors found that the global gains from the UR were larger in 2005. In addition, they projected that without the UR, the textile and apparel quotas would have become significantly more binding. This, too, serves to make the UR more valuable in 2005 than would have been foreseen in the context of the 1992 global economy.

Treatment of Land in AGE Models

The role of land in agricultural production is arguably one of its most distinguishing features in terms of AGE analysis. This section focuses on the treatment of farmland in these markets. Here there are two key issues which I will address. The first pertains to the sector-specificity of land. I.e., are there significant alternative nonfarm uses for this input which might contribute to determining its price in the long run? The second issue has to do with the heterogeneity of farm land and subsequent limitations on its mobility among uses within the ' agricultural sector.

Sector-Specificity of Farm Land: Unlike labor and capital, land is geographically immobile. As a result, it is common to assume that it is a sector-specific asset which ultimately bears all of the producer burden of a reduction in farm support. For example, Hertel, Thompson, and Tsigas (1989) estimate an 18% reduction in land rents following unilateral elimination of U.S. farm programs. Vincent (1989) estimates that Japanese farm land rents would fall by 68% following unilateral liberalization in that country's agricultural sector. Is there a chance that such price reductions might stimulate nonagricultural uses of farm land? If so, this type of quantity adjustment would serve to dampen the landowner losses (e.g., McDonald, 1990). The answer to this question will clearly vary

by region and by country. In the U.S., nonagricultural uses have been shown to play a role in determining the value of farmland in selected metropolitan areas (Lopez, *et al.,* 1988), but this has not proven to be an important determinant of aggregate agricultural land values. Furthermore, most of the commodities grown near urban areas are not the traditional program commodities which are most dramatically affected by U.S. farm policy. Thus the potential for nonfarm uses of agricultural land dampening the downward adjustment of rental rates following unilateral agricultural liberalization would seem quite limited.

The case of Japan is quite different. There, the capitalized value of farm program benefits represents a larger share of land's claim on agricultural output. Furthermore, the proximity of farm land to major population centers is much greater. Thus the demand for residential, recreational, and commercial land may be expected to place a significant floor under farm land values. Of course, the degree to which such adjustment can occur depends on accommodating changes in land use legislation. In Japan, "landowners must obtain the permission of the prefecture or of the Ministry of Agriculture, Forestry, and Fisheries in order to transfer farmland into other uses" (ABARE, 1988, p. 75). Extremely favorable property and inheritance taxation of farmland, coupled with high rates of capital gains taxation serve to further discourage movement of land into nonfarm uses. As a result, the percentage of land devoted to agricultural uses in the three major metropolitan areas in Japan (16%) exceeds the share of this land devoted to residential, commercial, and industrial plant uses (11.5%). It also exceeds the share of farmland in Japan's total land area (15%) (ABARE, 1988, p. 316). Despite these distortions in the land market, there is evidence that nonfarm demands support agricultural land values. For example, between 1979 and 1985 the relative price of rice to rice paddy land fell by about 20% (ABARE, 1988, p. 321).

Heterogeneity of Land: Abstracting from the question of how much land might move between farm and nonfarm uses, there are important modeling issues deriving from the heterogeneity of such land in agricultural production. The capacity of a given acre of land to produce a particular farm product varies with soil type, location in the watershed, and climatic conditions. These characteristics all combine to determine the yield, given a certain level of nonland inputs. To treat all farmland as homogeneous is to assert that one can grow oranges in Minnesota at the same cost as Florida (i.e. without greenhouses)! Models based on this structure will overstate supply response, since they don't take into account the agronomic and climatic constraints placed on the production of specific farm commodities. The trick for an AGE model is to capture the essence of such constraints without being forced to develop a full-blown model of agricultural production by locality and land type.

Perhaps the simplest method of constraining acreage response in a AGE model is that employed by Hertel and Tsigas (1988a). They specify a transformation function which takes aggregate farm land as an input and distributes it among various uses in response to relative rental rates. Given a finite elasticity of transformation, rental rates will differ across uses and acreage response may be calibrated to econometrically estimated values.

The next level of complexity in modeling the heterogeneous nature of agricultural land involves drawing a distinction between land types and land uses. In this framework, equilibrium in the land market involves the equalization of after-tax rates of return on any given type of land. However, provided these land types substitute imperfectly in the production of a given crop, there

may exist differential rental rates across land types. Robidoux, Smart, Lester, and Beausejour (1989) adopt this type of specification in their AGE model of Canada. They specify CES aggregator functions that combine three land types, each of which is used - to some degree -- in the production of six different farm products. An interesting wrinkle in their approach is the way in which they estimate benchmark equilibrium rental rates, by land type. These are obtained by regressing total land rents in each sector on the observed quantity of each land type used in that sector. In equilibrium, the land-specific rental rate (i.e., the coefficient on acreage) must be equal across uses.

The Robidoux, *et al.* approach deals with differences in land type, but not regional or climatic differences. Models designed to assess the effects of climate change, or the regional implications of policy shocks must disaggregate land endowments still further. Darwin *et al.* (1995), have taken a similar approach to their analysis of the economic impacts of climate change in a global AGE model focused on agriculture. They disaggregate land classes into six types, each of which is characterized by its length of growing season. These land classes are employed differentially across farming and forestry sectors, according to current patterns of production. In addition, the authors explicitly identify water as an input into the production function of each crop. The authors then turn to the results of the global climate simulation models in order to assess the impact of alternative climate change scenarios on the temperature and precipitation by region. This causes a shift in each region's land endowment across land classes and therefore causes patterns of agricultural production to change. Darwin *et al,* are then able to assess the consequences of climate change for patterns of trade, consumption and welfare.

The Role of Water

As can be seen from the previous example, it is often important to distinguish farm land by its access to water. Berck, Robinson and Goldman (1991) provide an overview of the use of AGE models to assess water policies. A key question is how to model water supply. Decaluwe, Petry and Savard (1997) have wrestled with this issue in the context of an AGE model of the Moroccan economy. In particular, they distinguish between groundwater and surface water collected by dams. Supply response is modeled via a Weibull distribution, and their analysis focuses on the economywide implication of water pricing policy in Morocco. In contrast, Robinson and Gehlhar (1995) develop an AGE model of Egypt in which land and water are combined in a linear fashion in the sectoral production function. As water scarcity becomes an increasingly important issue in the drier areas of the world, appropriate modeling of the water supply and demand in AGE models will become a pressing area for research

Parameter Specification and Model Closure

Specification of Preferences and Technology

Consumer Demand: The long history of applied econometric work in agricultural economics represents an asset which AGE modelers must capitalize on if their work is to have an impact on farm and food policy analysis. In the area of consumer demand, for example, there is a considerable body of work available which reports the results of disaggregated, complete demand systems for food and nonfood commodities (e.g., George and King, 1971; Huang and Haidacher, 1993). While there is a strong tendency for food products to be price- and income-inelastic, individual elasticity

values vary widely among food groups, with consumer demands for grains being quite unresponsive to price and income, while livestock products are more responsive. It is impossible to capture this diversity of price-responses with simple, explicitly additive demand systems such as the Constant Elasticity of Substitution (CES) on the Linear Expenditure System (LES). Some studies simplify even further, by assuming Cobb Douglas preferences (e.g., Robidoux, *et al.* (1989);Robinson, Kilkenny, and Adelman (1989)). In so doing, the authors risk overstating some uncompensated price elasticities by a full order of magnitude. This is particularly problematic when agricultural price policies are being examined, since consumer demand elasticities are critical in determining the incidence of changes in these policies. By overstating consumers' ability to respond to a price increase, such models will overstate the backward shifting of the effects of such a shock.

This naturally takes us into the problem of functional form, which lurks beneath the surface in any discussion of parameter specification for AGE models. Since the demand (and supply) relations in these models are the outcome of well-defined optimization problems, it is not possible to arbitrarily specify some elasticities and then plug into the model equations. Any elasticities must be compatible with the parameters of the underlying utility function. This part of the "calibration problem" can be quite challenging. Most of the work on functional forms has focused on "fully flexible" forms, i.e. those which do not arbitrarily restrict the matrix of N * (N-1)/2 partial substitution elasticities, where N is the number of commodities. Here the work of Diewert and Wales (1987); and Perroni and Rutherford (1995, 1997) on global well-behaved flexible forms is particularly important. However, for significant disaggregations of commodities and sectors, obtaining this much information is simply not possible. Therefore some intermediate ground is often needed.

In a somewhat overlooked 1975 article, Hanoch proposed a class of implicitly additive functional forms which are associated with N independent substitution parameters. He made precisely the argument alluded to above - namely that there may be cases where a generalization of the CES which falls short of being "fully flexible" might be useful. Furthermore, under implicit additivity, N is precisely the number of free parameters required to match up with a vector of N own-price elasticities of supply (demand). In addition, unlike explicitly additive functions, implicit additivity does not rule out complementary relations. The implicit additivity restriction was first employed empirically in order to represent production possibilities in Australian agriculture, within the context of the ORANI model (Vincent, Dixon, and Powell, 1977). These authors used the CRETH (Constant Ratio Elasticity of Transformation Homothetic) system, which is a primal specification. The Constant Difference Elasticity (CDE) functional form is a dual (potentially non-homothetic) specification. It has been employed to estimate demand relationships in agriculture (Hjort, 1988; Surry, 1989; Herrard *et al.*, 1997). Recently, it has been used in AGE analysis to calibrate consumer demand to a vector of own-price and income elasticities of demand (Hertel *et al.*, 1991).

Most of the literature on functional forms has focused on flexibility in price space. This is generally the most relevant dimension for comparative static analysis of agricultural policies with highly aggregated households, since the impact of these policies on aggregate income is generally quite small, compared to the impact on relative prices. However, when the AGE analysis involves accumulation of factors of production, as in a dynamic AGE model, or exogenous shocks to endowments in a comparative static AGE model (e.g., Anderson *et al.*, 1997), then the income

elasticities of demand can play a very important role in the results. In such cases it will be important to not only capture variation in income elasticities of demand across commodities, but also the tendency for the income elasticity of demand for food products to fall over time. The need for this type of "Engel-flexibility" has been emphasized by Rimmer and Powell (1994), based on non-parametric analysis. This precipitated development of a new functional form, nicknamed AIDADS, which restricts the price space via implicit additivity, but which provides third-order Engel flexibility (Rimmer and Powell, 1996). AIDADS can capture the change in the income elasticities of demand for food over time, as per capita incomes rise (Cranfield *et al.*, 1998).

Producer Technology: The predominance in AGE models of Leontief (fixed coefficient) technology with CES substitution in value-added has its origins in the computational advantages which once flowed from this specification. By assuming fixed intermediate input coefficients, the entire equilibrium problem can be reduced to one of finding a fixed point in factor price space (Ballard, *et al.,* 1985a). This vastly reduces the computational cost of AGE analysis, which was an important consideration prior to the development of more efficient algorithms and more powerful computers. However, intermediate input substitution plays an important role in the farm and food system.

Wohlgenant (1987) shows that substitution of agricultural products for marketing-inputs plays a key role in determining farm-level demand elasticities. The potential incidence of farm programs is also closely circumscribed by the ability of livestock producers and food processors to substitute among raw agricultural products. As noted above, high fructose corn syrup has been widely substituted for sugar in the U.S. food and beverage sectors, as a consequence of the sugar import quota. In the EU, the gains from price support programs for grains have been shared with non-grains producers in the EU and overseas. Peeters and Surry (1997) review the literature on price - responsiveness of feed demand in the EU, where this issue has received a great deal of attention due to the constraints it has placed on the Common Agricultural Policy. They distinguish between three approaches: linear programming, the synthetic modeling approach and econometric approaches. One of the more innovative is offered by Folmer *et al.* (1990) who incorporate a detailed treatment of feed demand into the European Community Agricultural Model (ECAM) using the Linear Expenditure System.

Substitution among intermediate inputs and between intermediate and primary inputs also plays an important role at the farm level. Empirical evidence from U.S. agriculture (e.g., Hertel, Ball, Huang, and Tsigas, 1989) indicates greater potential for such substitution, than for substitution within the primary factor aggregate (land, labor, and capital). Warr (1995) also finds significant substitution possibilities between fertilizer and some primary factors. Because many important farm policies represent interventions in the primary factor markets (e.g., acreage reduction programs and subsidized investment), proper assessment of their impact on target variables such as employment and land rents hinges crucially on the specification of farm technology.

Trade Elasticities: Since cross-price effects play an important role in the domestic farm and food economy, it is no surprise that they also show up in the rest of the world's response to domestic price movements, and hence in the trade elasticities facing food exporters (Carter and Gardiner, 1988). Unfortunately such cross-price export demand elasticities are notably difficult to estimate (Gardiner and Dixit, 1986). Thus, single region models are forced to rely on simulation results from

global trade models to measure them. Based on Seeley's (1985) work with the IIASA model, these cross-price effects are empirically quite important. For example, while he estimates a four-year own-price elasticity of export demand for U.S. wheat of -2.15, he finds that the *total elasticity* (when all grain and oilseed prices move together) is only -0.54. Since most farm sector interventions affect these commodities simultaneously, cross-price elasticities of export demand can be expected to play an important role in any policy simulation. Yet most one-country, general purpose AGE models abstract from cross-price effects in export demand.

One of the special features about agricultural trade — particularly in grains— is that it is controlled by state marketing agencies in many regions. This has led Abbott, Patterson and Young (1997) to conclude that the appropriate model for analysis of grains trade does not treat the individual agents in the economy as the decision makers for imports, but rather focuses on the problem faced by the individuals managing the state trading agencies. The resulting "plans and adjustment" model, which these authors propose, appears to fit the data quite well. Given the emerging importance of state-trading as a topic in multilateral trade negotiations, it may be worthwhile for AGE modelers to work on ways of incorporating this type of behavior as an explicit policy regime into their analysis of grains trade.

Implications for Policy Analysis: There will always be limitations in the way one is able to represent the basic structure of an economy in an AGE model, and so the critical question becomes: Are these limitations sufficient to warrant the extra effort involved in remedying them? In order to investigate this issue I have chosen to focus on one of the most inefficient farm policy tools - namely the idling of productive acreage in order to boost farm prices. Results are based on a special purpose AGE model outlined in Hertel, Ball, Huang, and Tsigas (1989), which utilizes a flexible representation of consumer preferences and producer technology. I then ask the question: What is the cost of successively restricting preferences and technology along the lines suggested by some of the general purpose models?

The results from the unrestricted experiment are summarized in Hertel and Tsigas (1991). Results for the restricted cases are reported in an appendix which is available on request from the author.[7] They indicate that a generic, general purpose AGE model which oversimplifies consumer preferences (Cobb Douglas case) and producer technology (no intermediate input substitution), and which omits cross-price effects in export demand, will overstate the welfare costs of acreage controls. In particular, the welfare cost of incremental acreage controls designed to raise program crop prices by 10% is overstated by 60% ($4.2 billion vs. $2.6 billion in the unrestricted model). This follows from two basic flaws in the general purpose models. First of all, they tend to overstate the farm level demand elasticity for these crops. Secondly, they tend to overstate the ability of farmers to substitute away from the land input. It should be noted, however, that the direction of bias is ambiguous. For example, when taken alone, the assumption of no substitutability in intermediate uses leads to an understatement of these welfare costs. Of course none of these parameters can be specified without some reference to the time frame for the simulation, and this is the subject to which we now turn.

[7]Readers can access this appendix on the worldwide web at *www.agecon.purdue.edu/gtap/wkpapr.*

Short, Medium. or Long-run?

Commodity Stocks: The time frame chosen for a AGE simulation has important implications for a variety of features which are critical to the outcome of the experiment. In the very short run, crop production has little scope for adjustment and, in the absence of stocks, supply shocks cause wide swings in commodity prices. As a result, there are substantial incentives for stockholding - either private or public - in the case of nonperishable crop commodities. In the longer run, the importance of stocks is diminished, since continued stock accumulation or decumulation quickly becomes infeasible in the context of a global agricultural economy.

Since the majority of AGE analyses focus on deterministic, comparative static analysis with respect to the medium run (which I take to be 3-4 years), it is common to abstract from commodity stockpiling - assuming that the associated price effects will only be transitory. However, any annual agricultural data set will include this type of "inventory demand" (or supply). One solution is to purge such demands from the benchmark equilibrium data set, in the process of constructing a representative year data set (Adams and Higgs, 1990 ; James and McDougall, 1993).

An alternative approach is to explicitly incorporate the stockpiling of commodities into the AGE analysis. Harrison, Rutherford, and Wooten (1989) develop a model of the European Community's Common Agricultural Policy in which excess market supplies are purchased, and either stored or unloaded onto world markets (with the help of an export subsidy). Stored commodities "are 'eaten' by EC government agents" (presumably they are stored until they spoil). Thus, they do not return to the marketplace, and hence do not generate future utility for private agents in this model.

Factor Mobility: As the time horizon for an AGE model lengthens, there is increased potential for production to adjust in response to a policy shock. In the limit, if all factors were perfectly mobile and the farm sector were relatively small, supply response would be perfectly elastic. However, some farm factors of production are probably never perfectly mobile. As noted above, farm land, in particular, often has few alternative uses and thus experiences more of a price adjustment than other factors in the long run. Also, family labor, farm structures, and some types of capital are relatively immobile in the short- to medium-run (Vasavada and Chambers, 1986).

To highlight the importance of factor mobility assumptions in determining the incidence of farm programs, consider the following evidence taken from Hertel, Thompson, and Tsigas (1989). (See also Kilkenny and Robinson, 1990, for further analysis of factor mobility). They analyze the impact of unilateral elimination of U.S. agricultural support policies in both the short run and the long run. The short run is characterized as the period over which both U.S. and foreign farm labor and capital are unable to adjust to this major shock. Thus short-run export demand elasticities are used, and U.S. farm labor, crop and livestock capital are all assumed immobile out of agriculture. The estimated short-run loss to these factors (in 1987 dollars) is $12.8 billion. The distribution of these losses is determined by the estimated elasticities of substitution in the farm sector. In this case the losses are distributed as follows: labor 37.3%, land 36.5%, livestock capital 18.2%, and crop capital 8.0%. In the medium run, the effect of mobile labor and capital on the elasticity of farm supply dominates the impact on farm level demand of larger export demand elasticities. As a result, the total producer burden falls to $5.7 billion. However, now all of this is borne by the sector-specific

factor -- land. Thus the pattern of factor incidence can vary considerably, depending on assumptions about factor mobility.

Exactly how "long" is the medium run in models assuming over which perfect mobility of labor and capital is realized? This depends in part on the size of the shock. In the above experiments, the adjustments to attain a new equilibrium include a 5.5% reduction in the agricultural labor force, and a 14% decline in the stock of farm capital. Are these adjustments large? Not when compared to other forces at work in the farm sector. For example, Hertel and Tsigas (1988b) estimate that the average *annual* decline in the derived demand for farm labor as a consequence of technological change during the post WWII period was 4.3%. The needed capital stock adjustment is also not too large when compared to average annual rates of economic depreciation for farm machinery, which range from about 10% to 25% depending on the equipment in question. Of course, these relatively modest adjustments likely mask more dramatic regional and farm-specific effects. Also, if yours is the farm that goes under as a result of the new policies, the adjustment is hardly marginal! Nevertheless, in view of the fact that: (a) rigidity is greatest for downward price movements, and (b) this policy experiment is the most dramatic one that could be inflicted on U.S. agriculture (policies are *completely and unilaterally* eliminated), it seems reasonable to expect that the period of adjustment required to obtain a new equilibrium is not more than the 3-4 year time horizon usually assumed.

It would be inappropriate to conclude this section without mentioning the increasing importance of *international* factor mobility. Given the relatively small share of the total national capital stock employed in agriculture, international capital mobility is probably not an area of central concern. However, concerns about international migration of labor have placed that issue at the center of the debate over possible effects of a North American Free Trade Agreement (NAFTA). Advocates of NAFTA cited the need to stem the tide of migration from Mexico into the United States. In their AGE analysis of this issue, Burfisher, Robinson, and Thierfelder (1992, 1994) conclude that such an agreement would likely *increase* migration from Mexico to the U.S., largely due to its negative impact on the demand for agricultural labor in Mexico. This reversal of conventional wisdom is an important reminder of the need for careful empirical analysis of agricultural and trade policy questions.

Supply Response

Assumptions about factor mobility and technology combine to determine the supply elasticities for agricultural commodities in an AGE model. In order to highlight this interaction, it is useful to consider a simple CES production function which combines two groups of inputs with a constant elasticity of substitution (σ). The first group of (variable) inputs is assumed to be in perfectly elastic supply and comprises a share of costs equal to C_v. The second group is in fixed supply with cost share C_F. In this case, the sector of supply elasticity may be computed as: $\eta_z = \sigma(C_v/C_F)$. Calibration of this model may proceed by one of two routes. The first is to take some estimate, $\hat{\eta}_z$ such as that from the cross-section study of Peterson (1988), and combine this with the benchmark equilibrium values for C_v and C_F and to obtain $\hat{\sigma}$. The problem with this approach is that η_z varies as a function of relative prices, (provided $\sigma \neq 1$). In the developed market economies in Peterson's sample, where purchased inputs are cheap relative to the opportunity cost of family labor,

we observe a large value of C_v relative to C_F. In this case, Peterson's cross-section estimation of η_s will understate supply response. In the poorer economies the opposite will be true.

The second approach to calibration of supply response in an AGE model involves estimating σ directly and inferring something about η_s based on alternative factor mobility assumptions. Problems with conventional estimates of supply response led Griliches (1960) to this type of indirect approach. Using factor demand relationships he estimated a long run supply elasticity for U.S. agriculture to be between 1.2 and 1.3. (This is quite close to the cross-section estimate by Peterson (1988) of 1.19.)

Hertel (1989) generalized the indirect approach to estimation of supply response to the case of multiple, quasi-fixed factor, and a fully flexible production technology. He combines an estimated matrix of Allen partial elasticities of substitution with two alternative factor mobility assumptions. In the first case, land and capital are assumed fixed and aggregate farm labor is partially mobile with a factor supply elasticity of 0.5. This generates a commodity supply elasticity of 0.84. In the second case, with labor and capital perfectly mobile, the aggregate supply elasticity is simply equal to the absolute value of the own-Allen partial elasticity of substitution for land, which is estimated to be 3.2.

These indirect estimates of supply response are all considerably larger than those obtained using single equation models fitted to time series data. Such studies have generally yielded aggregate agricultural supply elasticities in the range of 0.1 to 0.4 (Peterson, 1988). In such an environment output subsidies look a lot like lump sum transfers! One problem with such studies is that multicollinearity often precludes inclusion of a complete set of disaggregate prices (or quantities). Consequently, it is unclear what is being assumed about particular decision variables facing the farm firm. Are they fixed or variable? To overcome such problems a preferred approach to the direct estimation of supply response from time series data involves specification of a restricted profit function which, in turn, gives rise to a complete system of supply and demand equations in which the treatment of decision variables is explicit. Use of symmetry, homogeneity, and curvature restrictions help to overcome the problem of collinearity in such a system.

One example of the profit function approach is provided by Ball, who estimates a 5 output, 6 input system for U.S. agriculture. It is restricted on an exogenously determined quantity of own-labor (i.e., self-employed farmers). He obtains individual commodity supply elasticities ranging from 0.43 to 1.11. Furthermore, his outputs all exhibit gross complementarity (the so-called "normal case" (Sakai, 1974)). Thus aggregate supply response is larger than individual commodity response. Indeed, revenue share-weighted row sums of the output price submatrix sum to an aggregate supply elasticity of 3.6, which is again much larger than traditional estimates. If this is correct, then agricultural price support policies are much more distorting than is indicated by the agricultural sector models based on conventional time series estimates of supply response.

Model Closure

Economists using the comparative static, AGE framework face a fundamental problem in closing their models. This is because any SAM will have an activity related to investment, yet there is no intertemporal mechanism for determining the level of this activity in a static model. Sen (1963) defined this as a problem of *macroeconomic closure*. Following Dewatripont and Michel (1987), four popular solutions to this problem may be identified. The first three are non-neoclassical closures in which investment is simply fixed and another source of adjustment is permitted. In the fourth closure, investment adjusts endogenously to accommodate any change in savings. This, neoclassical closure, is the most common one in comparative static AGE models.

In addition to adopting a closure rule with respect to investment, it is necessary to come to grips with potential changes in the current account. (Recall from equation (1) from Section II above, that the difference between national savings and investment must equal exports plus international transfers less imports.) How much of the investment will be financed by domestic savings and how much by foreign savings? This question is difficult to address in the context of a single region, comparative static model. Therefore, it is common to fix the trade balance exogenously, in which case any change in investment must be financed out of national savings. In opting to exogenize this balance, the modeler is acknowledging that it is largely a macroeconomic phenomenon. To a great extent, the causality in equation (1) runs from left to right. That is, changes in global capital markets dictate what will happen on the current account. This approach also facilitates analysis by forcing all adjustment onto the current account. In addition, if savings does not enter households' utility function, then fixing the trade balance is the right approach for welfare analysis, since it prevents an arbitrary shift away from savings towards current consumption from being confused with a welfare improvement.

Finally, there is the question of labor market closure. The most common alternatives involve either assuming flexible wages and full employment on the one hand, or fixed real wages and unemployment on the other. In their review of alternative modeling approaches and the implications for the incidence of agricultural policy in India, de Janvry and Sadoulet (1987) explore the implications of these two extreme specifications, as well as an intermediate case in which wages are partially indexed to the cost of living. They find that the labor market closure plays a significant role in determining the incidence of techological change in agriculture on the rural population — particularly the landless poor.

Equilibrium Demand Elasticities

One very useful way of summarizing the combined effect of all of the assumptions about preference, technology, factor mobility and model closure is via a matrix of equilibrium demand elasticities. Each column in this matrix captures the change in demand for all products in the model, when the market price of one particular product, say corn, is raised by one percent and all other markets in the model are permitted to clear. Brandow (1961) was the first to use this technique for summarizing his multi-market, farm-to-retail model of US agriculture. Hertel, Ball, Huang and Tsigas (1989) updated Brandow's work in a general equilibrium setting. They find, for example, that feedgrains and foodgrains are GE, farm-level substitutes, while feedgrains and livestock products are complements.

Systematic Sensitivity Analysis

We cannot conclude this section on parameters in AGE models without a discussion of systematic sensitivity analysis. Anyone who has been involved in quantitative economic analysis is familiar with the concept of sensitivity analysis. It is also common in AGE modeling to vary key assumptions and parameters. However, given the large number of parameters involved in any economywide model, some sort of *systematic* sensitivity analysis (SSA) is advisable (Harrison *et al.*, 1987). Unfortunately, since most realistic AGE models require more than a few seconds to solve, standard Monte Carlo analysis (typically involving thousands of solutions) is generally infeasible. Pagan and Shannon (1987) proposed an approach based on a local, Taylor series approximation of the model results, expressed as a function of the model parameters. Harrison and Vinod (1992) have proposed an approach based on a numerical integration procedure, whereby they sample from a discrete approximation to the true distribution of parameters.

Recently a new approach to SSA has been proposed by DeVuyst and Preckel (1997). Like Harrison and Vinod, their approach is based on numerical integration techniques. They use multivariate Gaussian Quadrature, which draws a sample and associated weights in order to satisfy a set of conditions equating the moments of the approximating distribution to the moments of the true parameter distribution up to some finite order of moments (usually 3 to 5). This has proven to be a very powerful tool. For example, in their SSA of the Whalley-Wigle carbon tax model, DeVuyst and Preckel find that a Gaussian Quadrature requiring only 12 model evaluations vastly dominates both the Pagan and Shannon approach using 25 evaluations and Harrison and Vinod approach using 64 model evaluations. Indeed, the *error* from the true mean of the carbon tax required (to obtain a prespecified reduction in omissions) is only one-tenth of that with Pagan and Shannon's method and one-hundredth of that with Harrison and Vinod's method. The good news for AGE modelers is that the Gaussian Quadrature approach to SSA has now been automated for the case of symmetric, independent distributions (Arndt, 1996; Arndt and Pearson, 1996).

Modeling Policies That Affect Agriculture

As noted in the introduction, one of the important areas for future work identified in Whalley's "Hidden Challenges" paper involves improved modeling of public policies. This is nowhere more important than in agriculture, where, for some commodities in certain countries, the value of policy transfers exceeds the gross domestic value of production (USDA, 1988). Such interventions are not only large, they are also diverse. For example, it is not uncommon for agricultural policies to send conflicting signals regarding resource allocation. Input subsidies frequently coexist with supply control measures. Furthermore, many agricultural policies are not easily amenable to "ad valorem equivalent" modeling (Gohin *et al.*, 1998; Kilkenny, 1991; Kilkenny and Robinson, 1988; McDonald, 1990; Veenendaal, 1998; Whalley and Wigle, 1990).

Modeling Voluntary Participation

One of the more vexing problems in agricultural policy modeling has involved the search for an appropriate framework with which to model voluntary farm programs. Voluntary participation has been a hallmark of the U.S. grains programs. Until recently, they required farmers to idle a certain proportion of their base acreage in order to qualify for a variety of program benefits including

payments on output. The fact that participation rates varied from year to year, indicated that producers are an economically heterogeneous group (see discussion of this topic above). The most common approach to modeling these programs was to derive an average "incentive price" which, when combined with the supply shift due to idled acreage, would have induced the observed market supply of the crop in question (Gardner, 1989). However, such efforts ignored the impact that changing program parameters on important components of the problem such as variable costs per acre, optimal yields, and the nature of the supply shift. In reality, this is a complex, highly nonlinear problem.

Whalley and Wigle (1990) propose an alternate approach to modeling participation in the U.S. grains programs. They specify an explicit distribution of farms that reflects differences in their underlying cost structure such that the incentive to participate varies across five broad classes of farms. As program parameters or market conditions change, the participation rate varies endogenously. Hertel, Tsigas and Preckel (1990) extended this framework to incorporate a continuous distribution of land capacities, which in turn provide the motivations for differential participation. Shoemaker (1992) incorporates the voluntary participation decision in a dynamic model examines steady-state effects of the farm programs. All of this work highlights the differential incidence of farm programs on participants, nonparticipants, and those who are roughly indifferent to participation.

Interventions in the Processed Product Markets

A large void in many AGE models with an agricultural policy focus rests in their treatment of the food manufacturing and marketing sectors. In the U.S., only about one-third of every dollar spent on food goes to the farmer. Value-added in food manufacturing, and in wholesale/retail activities, are each roughly equal to that of agriculture. Furthermore, in many cases, support for farm commodities is provided indirectly, by purchase of (or protection for) processed products. For example, the primary mechanism for supporting U.S. fluid milk prices involves purchases of cheese, butter, and skim milk powder by the Commodity Credit Corporation (CCC). This type of indirect approach to supporting the farm sector can have important implications for policy analysis, and hence for the appropriate structure of AGE models. For example, CCC purchases of dairy products have generated considerably more processing capacity in the industry than would otherwise be required. Any lowering of support prices translates into redundant capacity. As a consequence, dairy processors have moved into the forefront of the dairy lobby. Similarly, as noted above, the U.S. sugar quota generated a new set of advocates in the corn milling industry. These processing sector impacts, in addition to the change in returns to dairy and sugar farms, must be captured by any model choosing to focus on such policies.

Agricultural Policies in a Changing World Economy

In many cases agricultural policies are tied to particular targets. For example, the policy makers may be required to defend a given level of domestic price, to maintain farm incomes, or to ensure a given level of self-sufficiency. Also, it is not uncommon for those seeking reform to legislate constraints on budgetary outlays. In the case of the Uruguay Round Agreement on Agriculture, export subsidies were constrained both in terms of volume (21% reduction in the volume of subsidized exports) and value (36% reduction). Such policy targets introduce the potential

for endogenous changes in policy regimes once the constraint becomes binding. Of course whether, for example, the EU export subsidy commitments become binding will depend on conditions in the EU, as well as those in the world markets. Frandsen, Bach and Stephensen (1998) have explored this issue in the context of a global AGE model. Their analysis focuses on the eastward enlargement of the European Union to include a number of Central and Eastern European countries (CEECs). They consider projections from 1992 to 2005 with the Uruguay Round commitments, as well as the explicit specification of compensatory payments, set-aside requirements, base area restrictions and milk quotas. The authors conclude that the current specification of policies is likely to render EU enlargement infeasible. Some sort of reform of the Common Agricultural Policy will be necessary.

Political Economy of Policies: General Equilibrium Dimensions

The AGE framework can also provide valuable insights into the political economy of agricultural policies.[8] For example, there is a strong tendency for relative rates of protection to shift as countries grow wealthier. Poor countries tend to tax agriculture and subsidize industry. While wealthier countries tend to subsidize agriculture, relative to industry (Anderson and Hayami, 1986). Anderson (1995) has used a small AGE model to illustrate why this particular pattern of intervention is so compelling[9]. The model which he employs has three sectors: agriculture, industry and non-tradeables (services). Capital is sector-specific, and the welfare of farmers and industrialists is closely tied to the return to their respective capital stocks. Anderson then proceeds to analyze the relative impact of trade policies on farmer and industrialist returns in each of these two archetype economies. He concludes that farmers who successfully seek agricultural price supports in poor countries reap only one-sixth to one-ninth the percentage improvement in returns, as compared with their counterparts in the rich economy. This has to do with a variety of features of lower income economies, including: (a) the relatively large share of agriculture in GDP, (b) the large share of food in household consumption, and (c) the relatively lower dependence of farming on industrial inputs. By contrast, industrial protection in the lower income country yields ten times the benefits for manufacturing lobbyists, as compared to their counterparts in the industrialized economy. These findings lead Anderson to conclude that these *general equilibrium,* structural differences in rich and poor countries are a key force between observed differences in protection patterns.

AGE analysis also has an important role to play in the political economy of reforming agricultural and trade policies. The IMPACT project in Australia turned to AGE analysis in the early 1970's in an attempt to stem the tide of special interests in tariff deliberations (Powell and Snape, 1992). The goal of the AGE modeling work developed under this project was to explicitly identify the opportunity cost of pursuing protectionist policies. While any individual tariff hike might not cost the average consumer very much, when taken together the costs of protection were quite substantial. It is interesting to note that, in the wake of these studies, the position of Australian agriculture with respect to policy reform was eventually reversed. AGE analysis showed that the effects of trade

[8]Some authors have used AGE models in conjunction with game theory to examine the endogenous formation of agricultural and trade policy (Rutstrom, 1995; Rutstrom and Redmond, 1997).

[9]de Janvry and Sadoulet (1992) also seek to explain the differences in observed policies affecting agriculture and the rural sector in India and Ecuador using an applied general equilibrium framework which captures linkages between rural and urban activity. They develop an index of political feasibility which permits them to take six different determinants of political power into account.

liberalization in Australia was to leave agriculture *better off* after removal of support - provided similar measures are taken in the industrial sectors (Higgs, 1989). The insight that relatively higher support for the Australian manufacturing sector amounted to an implicit tax on agriculture was an important revelation which could not have been communicated without AGE analysis. Similarly, in those economies where agriculture is relatively heavily protected, one of the best hopes for reform involves enlistment of export-oriented manufacturers who stand to benefit from a more competitive economy.

In sum, appropriate modeling of agricultural policies is an important, but difficult task. There is much to be gained by focusing on a particular policy and doing a good job of modeling it. However, in some circumstances it will be essential to incorporate a relatively complete set of economywide distortions in order to capture the consequences of potentially second-best interventions. This tradeoff between breadth of coverage and depth of analysis is evident in most areas of AGE analysis. There is no simple answer as to which approach is correct. Indeed, in many cases, both will be needed.

Agriculture and the Environment

Increasingly agricultural policy is being driven by environmental considerations (Gardner, 1993). Therefore, demand for analyses of the impact of agricultural and trade policies on the environment has been rapidly increasing (Bredahl *et al.*, 1996). Many environmental issues are very location-specific. This might lead one to conclude that there is little role for AGE analysis. However, Shively (1997) shows that GE interactions can also be important at the level of an individual watershed. He examines the case where deforestation and erosion from an upland region lowers productivity in lowland agriculture. In addition to being linked through erosion, the two regions are also linked through the labor market and diminished productivity in lowland agriculture puts downward pressure on wages, thereby reducing off-farm income opportunities for upland farmers. This leads to more deforestation and a downward spiral. Technological change aimed at increasing employment opportunities for upland farmers in the lowland region can have the opposite effect, by relieving pressure on the upland forest, thereby improving downstream productivity and wages.

In the context of national-level, environmental applications, it is most common for AGE modelers for focus on the economywide costs of restricting pollution. Rendleman (1993) analyzed the impact of chemical restrictions on US agriculture. Komen and Peerlings (1995) used an AGE model to calculate the costs of manure restrictions in the Netherlands as well as to assess the impact of environmentally motivated energy taxation on agriculture (Komen and Peerlings, 1998). However, ultimately the policy problem is one of weighing the costs of abatement against the benefits of a cleaner environment. Perroni and Wigle (1994) argue that, despite the conceptual and empirical pitfalls, it is essential to build the benefits of environmental clean-up into AGE models. They do so by specifying an initial endowment of environmental quality, some of which gets consumed by pollution activities. Firms can abate pollution by substituting commercial inputs (e.g., new machinery) for emissions. Households value the environment as a consumption good, and the marginal valuation rises with per capita income. They use this model to explore the interactions between trade policy and environmental policy.

Tsigas, Gray and Krissoff (1997) have built upon the approach proposed by Perroni and Wigle with an application which focuses on agriculture in the Western Hemisphere. In particular, they incorporate estimates of soil erosion, pesticide toxic releases, and nitrogen releases from agriculture, in addition to industrial pollution. Like Perroni and Wigle, they are forced to extrapolate from the US, where relatively good emissions data are available, to other regions in their analysis (Canada, Mexico, Brazil and Argentina). The authors use this AGE model to analyze the impact of Western Hemisphere free trade on environmental quality in the region. They find that environmental damages in Mexico, Brazil and Argentina are likely to increase under free trade, unless trade liberalization is combined with more stringent environmental policies. When the two are undertaken in concert, the welfare gain to these three countries is considerably enhanced.

However, agriculture not only generates pollution, it also provides environmental amenities (Legg and Portugal, 1997). There is increasing interest in linking farm payments to the level of such amenities provided. The OECD's Joint Working Party between the Committee for Agriculture and the Environment Policy Committee is currently developing a set of agri-environmental indicators to support policy analysis in this area (OECD, 1998). The initial set of indicators will cover the areas of farm management and financial resources, agricultural land conservation, soil and water quality, nutrient balance, pesticide use, greenhouse gases, biodiversity and wildlife habitat, landscape and the agricultural use of water resources.

Product Differentiation and Imperfect Competition

The theme of product differentiation has come to play an increasingly important role in analysis of agricultural trade policies (Carter, McCalla, and Sharples, 1990). A computational motivation for product differentiation is the specialization problem in small open economies facing exogenous world prices (de Melo and Robinson, 1989). By differentiating home and foreign goods, the elasticity of world price transmission into the domestic economy is dampened and drastic swings in the sectoral composition of output are avoided. This also opens the possibility of intra-industry trade, which is a commonly observed phenomenon. The oldest tradition in this area is the so called Armington approach in which products are *exogenously* differentiated by origin. This seems most appropriate in the case of those agricultural products for which agronomic and climatic considerations limit the scope for production of particular types of commodities [e.g., wheat (by class) or fruits and vegetables (by season)]. The market share rigidity provided by the Armington specification also serves as a proxy for non-price considerations which often play an important role in agricultural trade (Hjort, 1988). This specification may also be modified so that the law of one price applies in the long run (Gielen and van Leeuwen, 1998). However, in light of the increased importance of trade in processed food products, and the globalization of the food manufacturing industry, the Armington approach seems increasingly irrelevant for many sectors. Consumers pay less and less attention to the origin of the products which they consume.[10]

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[10]Another criticism of the Armington approach has to do with functional form. Winters (1984) and Alston *et al.,* (1990) argue that the CES representation is too restrictive and that the non-homothetic, AIDS specification is preferable. Robinson *et al.*(1993) have used this functional form in their AGE analysis of the North American Free Trade Agreement (NAFTA). As with the specification of consumer and producer behavior, more flexibility is better than less, provided sound estimates and calibration procedures can be provided. The main problem with a non-homothetic specification for import aggregation is the absence of a well-defined price index for the resulting composite commodity, since unit expenditure now depends on the level of utility. This eliminates the scope multi-stage budgeting which is the

While consumers are growing less concerned with the country of origin, they appear to be growing more aware of brand names. The fact that firms have become important actors in the field of product differentiation fundamentally changes the appropriate modeling approach, since this differentiation is now *endogenous*. That is, firms invest fixed costs in research and development and marketing activities in order to establishing a market niche, which then permits them to markup price over marginal cost. This type of formulation was originally introduced by Dixit and Stiglitz (1979) in order to investigate the trade-off between fixed costs and the benefits which accrue to consumers as additional varieties are provided. It has since provided a foundation for much of the work on international trade under imperfect competition. This approach seems highly relevant for large parts of the farm and food complex. Food manufacturers are among the most important sources of advertising expenditures, accounting for 32% of all manufacturer outlays but only 12% of total sales (Connor, *et al.*, 1985).[11] In these circumstances, product differentiation is quite clearly endogenous, and supported by firms pricing above marginal cost. Lanclos and Hertel (1995) demonstrate that this alternative approach to product differentiation tends to magnify the impact of trade liberalization on the US food manufacturing industries. Philippidis and Hubbard (1998) find similar magnification effects in their analysis of the European Union's Common Agricultural Policy.

The number of AGE analyses of trade policy incorporating imperfect competition has mushroomed since the pathbreaking work on Harris (1984). Many alternative approaches have been identified and the key constraint seems to be availability of high quality data to support the calibration of markups, excess profits and scale economies. Francois and Roland-Holst (1997) offer a comprehensive survey of this topic. They distinguish between the cases in which products are homogeneous and the market power is derived from barriers to entry, and those in which products are differentiated in the manner discussed above. They also distinguish between the so-called "small group" and "large group" cases. In the former instance, markups are endogenous and vary with the nature of inter-firm rivalry, relative prices and the number of firms in the industry. This is often difficult to implement in AGE models, since industries tend to be highly aggregated. In the small group case, firms ignore potential interactions with other firms and markups are dictated by the degree of product differentiation.[12]

Model Validation

One question which consumers of AGE model results often ask is: "Has the model been validated?" This is a reasonable question to expect from an analyst seeking advice on a policy reform which may end up shifting hundreds of millions of dollars around the economy. How can we be assured that the model bear any relationship to reality? The typical answer is that the AGE model,

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foundation of most disaggregated AGE models of consumer and producer behavior.

[11]Peterson (1989) provides some of the first attempts to incorporate imperfect competition in food manufacturing into an AGE model.

[12]One important feature of the Francois and Roland-Holst chapter is their approach to handling endogenous product differentiation. By cleverly re-scaling output to obtain "variety-scaled output" they are able to introduce this additional complexity into a standard AGE model at relatively low cost. Anyone thinking about introducing imperfect competition into an existing AGE model should definitely take a look at this before proceeding.

like any simulation model, has not been econometrically estimated and therefore cannot be subjected to the usual forecasting tests. To the extent that (a) the individual components of the system are based on plausible, perhaps even econometrically estimated, relationships, (b) the underlying social accounting matrix is accurate and reflects the best economy-wide data available, and (c) the equilibrium assumptions and macro-closure are plausible, then the assertion is that the results will indeed shed relevant light on what might actually happen if the proposed reforms were implemented.

However, if AGE modelers are successful in obtaining a higher policy profile for their results, more will be demanded in the way of model validation. Several relatively ambitious validation efforts have been undertaken in recent years. Kehoe, Polo and Sanchez (1991) conducted an *ex post* analysis of the impact of tax reform in the Spanish economy. They conclude that, with some adjustments, their AGE model is able to predict the broad pattern of resource reallocations precipitated by the change in tax policy. Fox (1998) has conducted a similar, *ex post* analysis of the predictions made by Brown and Stern (1989) using the Michigan model to evaluate the U.S.- Canada Free Trade Agreement. He finds that the model performs better for Canada than for the U.S. This is likely due to the fact that this agreement was of much greater significance to the Canadian economy. In contrast, its role in redirecting the sectoral allocation of resources in the U.S. was much more modest, and therefore difficult to detect.

Gehlhar (1997) attempted a somewhat different validation exercise, whereby endowments and productivity are shocked instead of policies. In this "backcasting" exercise with the multiregion GTAP model in which he attempts to predict 1982 East Asian export shares based on a model calibrated to 1992 data. Unlike the usual econometric models which have hundreds of exogenous variables, he uses only exogenous shocks to primary factor endowments and technology. Once he incorporates a proxy for human capital, he finds that the model performs reasonably well as regards prediction of changes in export shares. Coyle *et al.* (1998) attempt something similar, but more narrowly focused than Gehlhar. They seek to explain the dramatic change in composition of world food trade which occurred between 1980 and 1995. They employ a modified version of the GTAP model incorporating an econometrically estimated demand system. Coyle *et al.* (1998) are able to explain about half of the observed shift from bulk to non-bulk food trade over this period.

Realistically, any such "validation" effort will inevitably involve a certain amount of tinkering with the model in order to improve its performance. In this sense, such exercises are really a more elaborate method of calibration (but something short of formal econometrics) in which the model is fitted to multiple data points.[13] In this sense they do not constitute proof that the model will perform well in future simulations. However, such efforts to compare model performance to economic history will go a long way to addressing the criticism that AGE models bear little or no relationship to reality. As such, this type of work should be a high priority for future research.

Conclusions

As noted in Section II, this paper may be viewed as a survey of agriculturally-related attempts to meet some of the "hidden challenges" outlined by John Whalley in the mid-1980's. I am happy to

[13]Arndt and Robinson (1998) have recently used a 5 year time series of data on the Mocambique economy to formally adjust their AGE model parameters based on the maximum entropy approach.

report that considerable progress has been made. Many of the AGE-based studies reported in this survey represent excellent applied economic research with important policy insights and implications. In fact, there is a clear parallel with developments in other areas of applied economic research. Most of the hidden challenges which Whalley identifies — appropriate disaggregation, parameter specification, modeling of strategic behavior and treatment of policies — are universal challenges facing applied economists. Of course, GE modelers face some special challenges. In particular, the constraints imposed by the requirement for an economy-wide, micro-consistent data set have precluded systemwide econometric estimation. Nor do AGE modelers have the luxury of specifying reduced form elasticities. Agricultural supply response must be the outcome of producers' constrained optimization decisions subject an explicitly specified technology, and conditioned by clear assumptions about factor mobility. Nevertheless, there are fewer differences between AGE analysis and other areas of applied economics than many would suggest.

Indeed, I believe that one of the main avenues for improvement in AGE analyses of agricultural policies over the coming decade will be through increased collaboration with economists working on partial equilibrium studies. As highlighted in Section II of this chapter, the AGE approach has many important benefits in the context of policy analysis. However, in order to be fully effective, those working in this field must learn from economists with detailed knowledge of the sector, industry, households, or policies being analyzed. In order for this collaboration to blossom, AGE modelers will have to extend themselves in a number of ways.

The first area in which improvements need to be made involves the communication of key assumptions and parameters in a form which others can interpret and evaluate. Very few AGE analyses of agricultural policy report their assumed supply and demand elasticities for key products. Yet we all know that these are key parameters in determining the economic incidence of any price intervention. Why this paucity of information? The main problem is that AGE models are not typically specified in terms of supply and demand elasticities. Rather they involve the specification of explicit production and utility functions. Deriving the supply and demand elasticities involves some further computations. In addition, there is no longer one simple "supply elasticity". What is to be assumed about factor market adjustment? Are non-agricultural prices and incomes assumed constant? Similar problems exist with the specification of demand elasticities. However, this multiplicity of options is also a strength.

The researcher can report elasticities under a range of assumptions, showing how they are altered as one moves from partial to general equilibrium.[14] In so doing, they will assist the partial equilibrium analyst who is trying to grasp the differences between the two approaches.

A second step which will help to facilitate communication between AGE modelers and other economists involves a more widespread use of systematic sensitivity analysis (SSA). Economists accustomed to dealing with models with only a few behavioral parameters are often quite skeptical of models in which there are dozens of elasticities of substitution. Given the difficulty we have of obtaining robust estimates of such parameters, how can we have any confidence whatsoever in the results from such a model? This is a legitimate question, and it can only be addressed by the use of

[14]11For a discussion of partial and general equilibrium elasticities of demand in a multiregion AGE model, see Hertel, Lanclos, Pearson and Swaminathan (1997).

parametric SSA. In the past, authors of prospective journal articles could plead that their model was so big that it would take several months of computing to implement a complete Monte Carlo analysis. However, as pointed out in Section IV above, recent developments in this field have rendered SSA eminently practicable. In some cases, researchers have been pleasantly surprised with the robustness of AGE results to parametric uncertainty. This is because the data base and equilibrium assumptions also play key roles in determining the range of possible outcomes. Furthermore, as more of the AGE-based work draws on high quality, published data bases, the data dependence of these studies will be viewed as a strength of the approach.

Once non-AGE economists have been convinced that the findings are based on reasonable assumptions and that they are robust, they will want to know more about what is driving the results. This is where experienced AGE modelers and novices have parted company in the past. While some results are easy to explain (e.g., why output falls in a sector when a subsidy is removed), the welfare impact of a marginal change in policies in the context of a heavily distorted economy can be very difficult to interpret. AGE modelers interested in policy analysis need to invest much more time and energy in techniques of analysis which permit them to understand, and explain to others, the basic mechanisms driving their results. One illustration of this is the welfare decomposition technique derived by Keller.[15] He fully decomposes the change in economywide welfare into the efficiency consequences for each market captured by the model. Thus one can make statements such as: "25% of the welfare gain was due to improved allocation of labor in the economy". Or: "the welfare loss came about because the partial tax reform lured resources into the relatively protected agricultural sector." Without recourse to such explanations, backed up by detailed tables of data and results, the consumer of AGE model results is left with a black-box which they must either accept or reject as a matter of faith.

In my experience, once an AGE modeler has convinced the audience that the analysis is not only robust, but also sheds light on an important issue, s/he will very likely face requests by others to replicate the study. Replication is standard practice in other sciences, but it has been slow to penetrate the economics profession (Dewald, Thursby and Anderson, 1986). However, given the availability of a number of relatively easy to use software packages for AGE modeling (Harrison and Pearson, 1996; Rutherford, 1997), it is now within reach for most studies. In fact, I would like to see journal editors require that all AGE-based articles be submitted along with those files needed for replication. Ideally, reviewers would also have the opportunity to vary key assumptions such as model closure and parameter settings. This would greatly enhance the credibility of work in the area. It would also aid those seeking to build on previously published work, thereby facilitating more rapid scientific progress.

One reason why AGE modelers have been reluctant to make their models easier to use is the fear that they will be mis-used. This fear is well-founded. There is no doubt that as construction and implementation of an AGE model becomes routine and accessible to those outside the close-knit fraternity of modelers, foolish applications will abound. However, this is no different from any other branch of quantitative economics. The only distinguishing feature of AGE analysis is that, due to the

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[15]Keller's technique provides a local approximation to this decomposition. for a small, open economy. Huff and Hertel (1996) have adapted Keller's approach to the case of large changes, and apply this in the context of the multiregion GTAP model.

size of many of these models, one can generate foolish numbers at an extremely rapid rate! Ultimately it will be up to the process of professional peer review to sort the wheat from the chaff. It will no longer be the case that when one gets an AGE application to review, you can assume that the individual writing the paper has assembled the data and built the model themselves. This is a drawback. They may be ill-informed, simply offering a mechanistic set of model runs. However, an experienced reviewer can quickly identify such a paper. Furthermore, since model construction is no longer such an onerous task, one can now reasonably expect much more from the author in the way of analysis and exposition of results.

Indeed, I believe that successful AGE applications related to agricultural and resource policies in the future will increasingly exhibit six key features:

(1) Relevant institutional and behavioral aspects of the sector in question are taken into account.
(2) Key policies are modeled explicitly. Voluntary program participation, quantitative restrictions, price ceilings and floors, as well as state trading, are all common types of farm sector interventions which lend themselves to explicit treatment in an AGE framework.
(3) Key behavioral parameters are reported and related to econometric work in the literature.
(4) Results are reported in terms of means and standard deviations generated by SSA procedures which take parametric uncertainty into account.
(5) Central findings are exhaustively decomposed and explained.
(6) Finally, results can be easily replicated, and key assumptions altered, by the reviewer.

Regardless of how forthcoming the partial, and general equilibrium analysts are in their dialogue, one cannot avoid the fact that there is an inevitable tension between the mandate for AGE studies to be comprehensive and the need to delve into the specifics of the industries / households directly affected by specific policies. By definition, compromises are required, and the most distinguishing feature of high quality AGE policy applications is that they make the *right* compromises. In particular, they preserve key features of the sector in question. For this, a dialogue with industry experts is essential. Such dialogue is often cumbersome and, at times tedious. However, it is the only way applied general equilibrium studies can avoid falling prey to Robert Solow's (1973) criticism of Jay Forrester's early work on global modeling. In this debate, Forrester asserted that rather than "go to the bottom of a particular problem ... what we want to look at are the problems caused by the interactions." To this Solow (p. 157) responds:

> I don't know what you call people who believe they can be wrong about everything in particular, but expect to be lucky enough to get it right on the interactions. They may be descendants of the famous merchant Lapidus who said that he lost money on every item sold, but made it up on the volume.

In summary, after several decades of rapid development and application to many different areas of economic analysis, AGE models are maturing. They must be subjected to the same scrutiny and skepticism, and validation efforts as other models. Ultimately their usefulness in delivering policy insights and guidance will determine whether or not this field of endeavor has been a success.

Some striking examples of AGE-based impact in the policy sphere exist.[16] However, the ratio of policy-oriented, AGE applications to effective policy input is still quite low. If this situation is to be rectified, it is essential that the use of AGE analysis extend beyond the narrow modeling community to a broader group of policy economists. It is my hope that this survey will encourage such cross-fertilization.

[16] See Powell and Snape (1993) on the Australian experience. Francois and Shiells (1994) describe the importance of AGE analysis in the NAFTA debate.

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APPENDIX 7. DATA SUMMARY FOR SUPPLEMENTAL REPORTS

Appendix 7.-Table 1.

Supp. No.	MRID No.	Title	Summary
1	**468859-01**	Harper, B (2006), Molecular characterization of Event COT76B cotton. Report No. SSB-125-06.	Data from Southern analyses and DNA sequencing demonstrated that a single copy of the full-length *cry1Ab* gene (*flcry1Ab*) was present in the T-DNA insert in Syngenta's Event COT67B cotton. COT67B does not contain the selectable marker gene, hygromycin B phosphotransferase (*aph4*), from the transformation plasmid pNOV1914. Additionally, COT67B cotton does not contain any of the backbone sequences from the transformation plasmids pNOV4641 or pNOV1914. Sequence analysis of the entire T-DNA insert present in COT67B cotton confirms the overall integrity of the insert and that contiguousness of the functional elements has been maintained. Statistical analysis of segregation patterns over several generations of COT67B confirms the expected Mendelian inheritance ratio for *flcry1Ab* demonstrating that the transgenic locus in COT67B is inherited across generations in an expected manner.
2	**468859-02**	Hill, K. (2006), Quantification of Cry1Ab protein in Event COT67B cotton tissues and whole plants. Syngenta Seeds Biotechnology Report No. SSB-022-06.	To characterize the range of expression of Cry1Ab protein in Event COT67B cotton plants, the concentration of Cry1Ab was determined by ELISA in several plant tissues and whole plants at five growth stages (squaring, 1st white bloom, peak bloom, 1st open boll and pre-harvest) of plants grown at four locations. The concentration of Cry1Ab in pre-harvest stage whole plants was estimated on a per-acre and a per-hectare basis. Corresponding near-isogenic, nontransgenic control cotton plants were analyzed in parallel.
3	**47017604**	Graser, G. (2005). Characterization of Cry1Ab test substance FLCRY1AB-0103 and Certificate of Analysis. Syngenta Seeds Biotechnology Report No. SSB-001-05.	Re-analysis of FLCRY1AB-0103 *ca*. five months after its initial characterization demonstrated that the test substance was substantially stable when stored desiccated at -20C.

Appendix 7.-Table 1. Continued

4	**47017605**	Kramer, C. (2006). Re-characterization of Cry1Ab test substance FLCRY1AB-0103. Syngenta Seeds Biotechnology Report No. SSB-015-06.	Test substance retained a substantial concentration of intact, bioactive Cry1Ab protein during storage for *ca*. 26 months.
5	**47017608**	Graser, G. and X. Li. (2006). Characterization of the Cry1Ab protein produced in Event COT67B-derived cotton plants and comparison with Cry1Ab protein produced in recombinant *Escherichia coli*. Syngenta Seeds Biotechnology Report No. SSB-016-06.	FLCry1Ab from COT67B cotton plants (LPCOT67B-0106, IAPCOT67B-0106) and from microbially produced test substance FLCRY1AB-0103 were shown to have a molecular weight consistent with the predicted molecular weight of *ca*. 133.5 kDa and immunologically cross-reacted with anti-Cry1Ab antibodies, as shown by Western analysis. Both test substances were highly active in *Ostrinia nubilalis* bioassays. No evidence of post-translational glycosylation was found. Results indicate that test substance FLCRY1AB-0103 is a suitable surrogate for Cry1Ab expressed in COT67B cotton. Peptide mass mapping analysis provided additional and strong evidence of identity of the insecticidal proteins expressed in COT67B cotton and in test substance FLCRY1AB-0103.
6	**47017609**	Pence, K. (2006). Stability of Cry1Ab protein expression across multiple generations of Event COT67B cotton. Syngenta Seeds Biotechnology Report No. SSB-048-06	Levels of Cry1Ab protein expression at open boll stage for three conventionally bred generations of COT67B cotton plants were comparable, indicating a consistent expression profile for the Cry1Ab protein in COT67B cotton plants at the open boll stage of development.
7	**47017611**	De Fontes, J. and K. Hill. (2006). Analysis for the presence of Cry1Ab protein in linters, toasted cottonseed meal and once-refined cottonseed oil from processed seed of Event COT67B cotton expressing a full-length Cry1Ab protein. Syngenta Seeds Biotechnology Report No. SSB-027-06.	A quantifiable level of Cry1Ab was found in the fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B.

Appendix 7.-Table 1. Continued

8	**47017614**	Barnes, E. (2005). FLCRY1AB-0103: Single dose oral toxicity study in the mouse. Central Toxicology Laboratory Study No. AM7516, Macclesfield, Cheshire, UK.	The oral administration of 1830 mg Cry1Ab protein/kg bodyweight (2127.9 mg FLYCRY1Ab-0103 test substance/kg bodyweight) as a single dose resulted in no treatment related effects. The data support the prediction that the full-length Cry1AB protein will be non-toxic to humans.
9	**47017615**	Graser, G. (2006). *In vitro* digestibility of full-length Cry1Ab protein (test substances FLCRY1AB-0103 and IAPCOT67B-0106) under simulated mammalian gastric conditions. Syngenta Seeds Biotechnology Report No. SSB-026-06.	FLCry1Ab from two sources, Event COT67B transgenic cotton and recombinant *Escherichia coli*, was readily degraded in SGF. No intact FLCry1Ab (molecular weight ca. 133.5 kDa) from either source was detected following incubation in SGF for one minute, as assessed by Western blot analysis. The data support the conclusion that FLCry1Ab expressed in transgenic COT67B cotton plants will be readily digested under typical mammalian gastric conditions.
10	**47017616**	Graser, G. and G. Mims. (2006). Effect of temperature on the stability of full-length Cry1Ab protein. Syngenta Seeds Biotechnology Report No. SSB-035-06.	At 25 and 37ºC there was no significant effect on FLCRY1AB-0103 bioactivity compared to that observed at 4ºC, as shown by the estimated LC_{50}s of 5.2, 6.0 and 7.8 ng FLCry1Ab/cm^2, respectively. FLCry1Ab inactivation occurred at temperatures of 65ºC and 95ºC; LC_{50}s and 95% confidence intervals could not be calculated due to low mortality at all concentrations. Very weak activity was retained in the 65ºC sample at the two highest concentrations (i.e., 500 and 1250 ng FLCry1Ab/cm^2), while the FLCry1Ab sample incubated at 95ºC was completely inactive at all concentrations in the *O. nubilalis* bioassay.
11	**47017619**	Harper, B. (2006). Full-length Cry1Ab as expressed in Event COT67B cotton: Assessment of amino acid sequence homology with known allergens. Syngenta Seeds Biotechnology Report No. SSB-137-06.	Two different searches were performed comparing the FLCry1Ab sequence to the sequences in the SBI Allergen Database to determine if the amino acid sequence of the FLCry1Ab protein as expressed in Event COT67B cotton has any significant homology to known allergens. FLCry1Ab showed no significant amino acid sequence homology to known or putative allergenic proteins.

Appendix 7.-Table 1. Continued

12	**47017620**	Raybould, A. (2006). Environmental safety assessment of COT102 x COT67B cotton expressing the insecticidal proteins Vip3Aa19 and FLCry1Ab.	The purpose of this volume was to evaluate the environmental safety of VipCot™ cotton to non-target organisms (NTOs). Safety is demonstrated if there is high certainty that the assessment endpoints of abundance and diversity of NTOs will not be harmed by cultivation of VipCot™ cotton. Harm would be significant loss of an ecological function such as pollination or biological control. The safety of VipCot™ cotton is assessed by comparison with non-transgenic cotton, which is considered to have no unacceptable effects on NTOs. If the effects of VipCot™ cotton are not significantly different from those of non-transgenic cotton, COT102 x COT67B cotton can be deemed safe to the environment. No adverse effects were seen in 23 laboratory or semi-field studies of the effects of Vip3Aa19 or FLCry1Ab indicator species representative of NTOs found in cotton fields. Therefore the lack of adverse effects in the studies indicates negligible risk to NTOs from COT102 or COT67B cotton with high certainty.
13	**47017625**	Hoberg, J. (2006). Channel catfish (*Ictalurus punctatus*) feeding study comparing a fish food containing FLCry1Ab protein to a standard diet. Springborn Smithers Laboratories Study No. 1781.6659, Wareham, MA, USA.	No mortality or abnormal behavior was observed throughout the 28-day study. Of the biological endpoints monitored, day 28 mean total biomass and mean weight gain of fish fed the treated diet were found to be significantly reduced relative to the control. Although significantly reduced, day 28 mean total biomass and mean weight gain in the treatment were reduced by only 12 and 18%, respectively, relative to the control. Feed consumption and feed:gain ratios were similar between the treatment and control fish.
14	**47017629**	Jeker, L. (2006). FL Cry1Ab: A study to evaluate the effects of full-length Cry1Ab protein on brood development of the honeybee, *Apis mellifera* L. (Hymenoptera: Apidae). Syngenta, Jealott's Hill International Research Centre, UK Study No. 2033098.	The consumption of FLCRY1AB-0103 by bee colonies at a rate of 107.82 mg test item /L sucrose solution, representing a 10X mean concentration of Cry1Ab in COT67B leaf tissue, (10X EEC), had no adverse effects on the colony conditions and survival of honeybee life stages (eggs, young larvae and old larvae) developing in brood cells within the hives. Also, the test item had no apparent adverse effects on survival of exposed worker bees. These results suggest that incidental ingestion of Cry1Ab proteins would not adversely affect the hive condition, survival of larvae in brood cells, or exposed worker bees.

Appendix 7.-Table 1. Continued

15	**47017631**	Kramer, C. (2006). Laboratory soil degradation of full-length Cry1Ab protein. Syngenta Seeds Biotechnology Report No. SSB-044-06.	The degradation of Cry1Ab in a sandy loam soil was rapid. The DT_{50} and DT_{90} of Cry1Ab based on a simple first-order kinetic model were 17 and 52 days, respectively. The results indicate that Cry1Ab that enters the soil will be rapidly degraded.
16	**47017626**	Patnaude, M. (2006). Fl Cry1Ab: A laboratory study to determine the effects of full-length Cry1Ab protein and the combined effects of Cry1Ab and Vip3A on the predatory beetle *Coleomegilla maculata*. Springborn Smithers Laboratories Study No. 1781.6661, Wareham, MA, USA.	There were no detrimental effects on larval, pupal, or adult survival, days to pupation, or days to adulthood when *Coleomegilla maculata,* a generalist predator, were exposed to a diet of bee pollen and *Ephestia* eggs containing 10X mean concentration of Cry1Ab in COT67B leaf tissue (10X EEC). However, *C. maculata* adults exposed to both treatments weighed approximately 13% less than their assay control counterparts; the difference was statistically significant in both treatments. ELISA and Western analyses of test diets confirmed quantifiable levels of Cry1Ab protein was present in the diet and that the protein was intact and stable throughout the exposure phase of the toxicity study. Results of the sensitive insect bioassays at test initiation and termination confirmed the biological activity of the test substances.
17	**47017627**	Stacey, D. (2006). FL Cry1Ab: A laboratory study to determine effects of full-length Cry1Ab protein on the rove beetle *Aleochara bilineata* (Coleoptera: Staphylinidae). Syngenta, Jealott's Hill International Research Centre, UK Study No.T004347-05.	When *Aleochara bilineata* parasitic larvae were exposed to test substance FLCRY1AB-0103 at a dose representing a 10X mean concentration of Cry1Ab in COT67B leaf tissue (10X EEC) there were no statistically significant differences between the numbers of beetles that emerged from the test item treatment compared to the control. Therefore, it can be concluded that the microbially expressed full-length Cry1Ab protein had no effects on the reproduction of the ground-dwelling beetle, *Aleochara bilineata*, when exposed orally *via* a treated meat-based diet.

Appendix 7.-Table 1. Continued

18	**47017628**	Raybould, A. (2006). The effects of Vip3Aa19 and FLCry1Ab on *Orius* species and implications for the environmental safety of COT102 x COT67B cotton.	Several laboratory studies of the predatory bugs *Orius insidiosus* and *O. laevigatus* (Heteroptera: Anthocoridae) were completed for the safety assessment. The studies exposed the bugs to Vip3Aa19 and FLCry1Ab alone and in combination. The purpose of this summary volume is to present the rationale for the studies and discuss the implications of the results for the environmental safety of COT102 x COT67B cotton. Laboratory studies of *Orius* species exposed to microbial test substances VIP3A-0104 or FLCRY1AB-0103 showed no observable effects on pre-imaginal mortality and indicate minimal risk to *Orius* species from the cultivation of COT102 and COT67B cotton. Sporadic increases in mortality were observed in *O. laevigatus* exposed to a combination of Vip3Aa19 + FLCry1Ab at approximately 10X the concentration of these proteins in leaves of COT102 x COT67B cotton. The apparent synergistic effect was unexpected as no synergism between Vip3Aa19 and FLCry1Ab was detected in bioassays with TBW and CBW, which are sensitive to both proteins; and because synergism between *Bt* proteins at or below their NOECs has not be observed previously. It is possible that increased mortality in *O. laevigatus* exposed to Vip3Aa19 and FLCry1Ab resulted from interactions between components of the microbial test substances and the liver-based diets that had an anti-feedant effect on the *O. laevigatus* nymphs. If this is the case, there is minimal risk to *Orius* species from cultivation of COT102x COT67B cotton
19	**47017632**	Raybould, A. (2006). The environmental fate of Vip3Aa19 and FLCry1Ab in COT102 x COT67B cotton.	This volume is summarizes the environmental fate of Vip3Aa19 and FLCry1Ab during and following cultivation of VipCot™ cotton. The purpose is to predict the exposure of nontarget organisms (NTOs) to these proteins. Two types of EEC's are calculated for NTO's: 1) worst-case EECs for NTOs potentially exposed in cotton fields calculated from concentrations of the transgenic proteins in plant tissue, and 2) realistic EECs based on estimates of dilution of the transgenic proteins in the diet of the NTOs. The EECs are summarized in this volume and in a separate volume (Raybould, 2006, vol. 21), these EECs are compared with estimates of hazard to assess the risks of COT102 x COT67B cotton to NTOs.

Appendix 7.-Table 1. Continued

20	**47017633**	Dickerson, D., Negrotto, D., O'Reilly, D., Martin, S., Minton, B. (2006). Field efficacy evaluation of component Events COT102 and COT67B and stacked COT102 x COT67B cotton ((VipCot™ cotton) Syngenta Seeds Biotechnology Report No. SSB-175-06.	Side by side efficacy trials against TBW and CBW over two successive growing seasons comparing single gene events with stacked VipCot™ cotton demonstrated that the single gene events and double hemizygous stacked cotton provided excellent control of both pests while double homozygous VipCot™ cotton significantly outperformed the single gene events.
21	**47034701**	Stone, T., T. Martin and R. Tinsworth (2006). Public interest document in support of registration of the plant incorporated protectant proteins Vip3A and FLCry1Ab as expressed in the combined trait COT102 x COT67B cotton (VipCot™ cotton) Syngenta Seeds Biotechnology Report No. SSB-171-06.	VipCot™ provides high dose protection against the major Lepidopteran cotton pests (*Helicoverpa zea, Heliothis virescens, and Pectinophora gossypiella*; that is, cotton bollworm, tobacco budworm and pink bollworm). The Vip3A protein has a unique mode of action; thus, VipCot™ has the potential to extend the useful life of Bt Cry insecticidal protein-based technology generally. VipCot™ will provide growers with a legitimate alternative to current cotton PIP products; particularly, Bollgard® and Bollgard II® varieties that dominate the cotton market. Additional choice will also influence grower cost and help maintain or slightly expand the cotton acres planted to Bt-based PIPs. The registration and market introduction of Syngenta's VipCot™ will result in agronomic, economic, human health, environmental, and resistance management benefits that are discussed in sections of this document. These benefits provide strong support for the public interest finding needed for a conditional registration. In summary, VipCot™ will be a welcome addition to the arsenal of products available to cotton growers. Benefits will accrue; the public interest will be served. This public interest document provides the support needed for these conclusions.

Appendix 7.-Table 1. Continued

22	**47017634**	Kurtz, R., A. McCaffery, D. O'Reilly and T. Stone. (2006). Insect resistance management considerations for VipCot™ cotton. Syngenta Seeds Biotechnology Report No. SSB-170-06.	The current volume provides details of the studies performed to demonstrate several features of VipCot™ cotton, which include: 1) the efficacy of VipCot™ cotton on target pests, 2) study results demonstrating VipCot™ cotton provides a high dose for *H. zea*, *H. virescens* and *P. gossypiella*, 3) supporting information and study results performed to demonstrate the lack of cross resistance between Vip3A and Bt Cry proteins; and 4) risk assessment simulation modelling to understand the potential for target pest resistance to VipCot™ cotton. In addition, this volume contains details of the implementation of Syngenta's IRM strategy; including the planting of refugia, a rigorous program of grower education and plans for insect resistance monitoring.
23	**46885903**	Harper, B. (2006). Full-length Cry1Ab: Assessment of amino acid sequence homology with known toxins. Report No. SSB-124-06.	Using conservative search criteria, it was concluded that the FLCRY1Ab query sequence showed no significant sequence homology to any proteins identified as, or known to be, toxins other than delta-endotoxins, including other Cry proteins.

Supplemental Section. Copies of Reports Submitted to the EPA [CBI-Deleted]

Copies of the following reports submitted to the EPA (Supplements 1-23), which are claimed as Confidential Business Information (CBI), are provided as a separate enclosure with this petition.

EPA

The following reports were submitted to the EPA BPPD on Dec 15, 2006 in support of FIFRA Section. 3 Registration of the Vip3Aa19 and full-length Cry1Ab plant-incorporated protectants in COT102 x COT67B cotton (VipCot™ cotton)

Supp. 1. Harper, B (2006), Molecular characterization of Event COT76B cotton. Report No. SSB-125-06. (41 pages)

Supp. 2. Hill, K. (2006), Quantification of Cry1Ab protein in Event COT67B tissues and whole plants. Report No. SSB-022-06. (28 pages)

Supp. 3. Graser, G. (2005). Characterization of Cry1Ab test substance FLCRY1AB-0103 and Certificate of Analysis. Syngenta Seeds Biotechnology Report No. SSB-001-05. (18 pages)

Supp. 4. Kramer, C. (2006). Re-characterization of Cry1Ab test substance FLCRY1AB-0103. Syngenta Seeds Biotechnology Report No. SSB-015-06. (16 pages)

Supp. 5. Graser, G. and X. Li. (2006). Characterization of the Cry1Ab protein produced in Event COT67B-derived cotton plants and comparison with Cry1Ab protein produced in recombinant *Escherichia coli*. Syngenta Seeds Biotechnology Report No. SSB-016-06. (23 pages)

Supp. 6. Pence, K. (2006). Stability of Cry1Ab protein expression across multiple generations of Event COT67B cotton. Syngenta Seeds Biotechnology Report No. SSB-048-06 (14 pages)

Supp. 7. De Fontes, J. and K. Hill. (2006). Analysis for the presence of Cry1Ab protein in linters, toasted cottonseed meal and once-refined cottonseed oil from processed seed of Event COT67B expressing a full-length Cry1Ab protein. Syngenta Seeds Biotechnology Report No. SSB-027-06. (23 pages)

Supp. 8. Barnes, E. (2005). FLCRY1AB-0103: Single dose oral toxicity study in the mouse. Central Toxicology Laboratory Study No. AM7516, Macclesfield, Cheshire, UK. (200 pages)

Supp. 9. Graser, G. (2006). *In vitro* digestibility of full-length Cry1Ab protein (test substances FLCRY1AB-0103 and IAPCOT67B-0106) under simulated mammalian gastric conditions. Syngenta Seeds Biotechnology Report No. SSB-026-06. (16 pages)

Supp. 10. Graser, G. and G. Mims. (2006). Effect of temperature on the stability of full-length Cry1Ab protein. Syngenta Seeds Biotechnology Report No. SSB-035-06. (13 pages)

Supp. 11. Harper, B. (2006). Full-length Cry1Ab as expressed in Event COT67B: Assessment of amino acid sequence homology with known allergens. Syngenta Seeds Biotechnology Report No. SSB-137-06. (61 pages)

Supp. 12. Raybould, A. (2006). Environmental safety assessment of COT102 x COT67B expressing the insecticidal proteins Vip3Aa19 and FLCry1Ab. (42 pages)

Supp. 13. Hoberg, J. (2006). Channel catfish (*Ictalurus punctatus*) feeding study comparing a fish food containing FLCry1Ab protein to a standard diet. Springborn Smithers Laboratories Study No. 1781.6659, Wareham, MA, USA. (57 pages)

Supp. 14. Jeker, L. (2006). FLCry1Ab: A study to evaluate the effects of full-length Cry1Ab protein on brood development of the honeybee, *Apis mellifera* L. (Hymenoptera: Apidae). Syngenta, Jealott's Hill International Research Centre, UK Study No. 2033098. (131 pages)

Supp. 15. Kramer, C. (2006). Laboratory soil degradation of full-length Cry1Ab protein. Syngenta Seeds Biotechnology Report No. SSB-044-06. (30 pages)

Supp. 16. Patnaude, M. (2006). Fl Cry1Ab: A laboratory study to determine the effects of full-length Cry1Ab protein and the combined effects of Cry1Ab and Vip3A on the predatory beetle *Coleomegilla maculata*. Springborn Smithers Laboratories Study No. 1781.6661, Wareham, MA, USA. (67 pages)

Supp. 17. Stacey, D. (2006). FL Cry1Ab: A laboratory study to determine effects of full-length Cry1Ab protein on the rove beetle *Aleochara bilineata* (Coleoptera: Staphylinidae). Syngenta, Jealott's Hill International Research Centre, UK Study No.T004347-05. (61 pages)

Supp. 18. Raybould, A. (2006). The effects of Vip3Aa19 and FLCry1Ab on *Orius* species and implications for the environmental safety of COT102 x COT67B. (130 pages)

Supp. 19. Raybould, A. (2006). The environmental fate of Vip3Aa19 and FLCry1Ab in COT102 x COT67B. (34 pages)

Supp. 20. Dickerson, D., Negrotto, D., O'Reilly, D., Martin, S., Minton, B. (2006). Field efficacy evaluation of component Events COT102 and COT67B and stacked COT102 x COT67B ((VipCot™ cotton) Syngenta Seeds Biotechnology Report No. SSB-175-06. (19 pages)

Supp. 21. Stone, T., T. Martin and R. Tinsworth (2006). VipCot™ cotton marketing plan. (15 pages)

Supp. 22. Kurtz, R., A. McCaffery, D. O'Reilly and T. Stone. (2006). Insect resistance management considerations for VipCot™ cotton. Syngenta Seeds Biotechnology Report No. SSB-170-06. (190 pages)

Supp. 23. Harper, B. (2006). Full-length Cry1Ab: Assessment of amino acid sequence homology with known toxins. Report No. SSB-124-06.

[Supplements 1-23 are CBI-deleted]

Janet Reed
Regulatory Affairs Manager
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3054 East Cornwallis Road
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July 20, 2007

Ms. Margaret Jones
USDA-APHIS-Biotechnology Regulatory Services
4700 River Road, Unit 147
Riverdale, MD 20737

Re: Review for Technical Completeness of Petition Numbered 07-108-01p for a Determination of Non-Regulated Status for COT67B

Dear Ms. Jones:

This letter is in response to APHIS/BRS letter dated June 19, 2007 outlining deficiencies noted in Petition No. 07-108-01p, application for determination of non-regulated status for COT67B. Syngenta's responses are incorporated directly into the Agency letter followed by a confidential attachment comprised of the requested field trial reports. A CD of this letter and Syngenta's responses accompany the hard copy sent by overnight courier July 20, 2007.

If there are additional questions regarding the responses or the Petition please do not hesitate to contact me.

Sincerely,

Janet N. Reed
Regulatory Affairs Manager
Syngenta Seeds, Inc.
Tel. (919) 765-5076; Fax: (919) 541-8535; janet.reed@syngenta.com

cc: Terry B. Stone, Manager, Regulatory Affairs, Syngenta Seeds, Inc.
Lawrence W. Zeph, Regulatory Manager, NAFTA, Syngenta Seeds, Inc.
Lisa W. Zannoni, Head, Global Biotechnology Regulatory Affairs, Syngenta Seeds, Inc.

June 19, 2007

United States Department of Agriculture

Animal and Plant Health Inspection Service

Marketing & Regulatory Programs Business Services

4700 River Road
Riverdale, MD 20737

Janet Reed
3054 East Cornwallis Road
Research Triangle Park, NC 27709

Subject: Review for Technical Completeness of Your Petition Numbered 07-108-01p for a Determination of Non-Regulated Status for COT67B

Dear Ms. Reed:

This letter is in reference to the Petition for Determination of Non-Regulated Status for COT67B lepidopteran resistant cotton submitted to the USDA, Animal and Plant Health Inspection Service/Biotechnology Regulatory Services (APHIS/BRS) on April 18, 2007. APHIS/BRS has assigned this petition the number 07-108-01p. APHIS has reviewed this petition and has identified the following deficiencies:

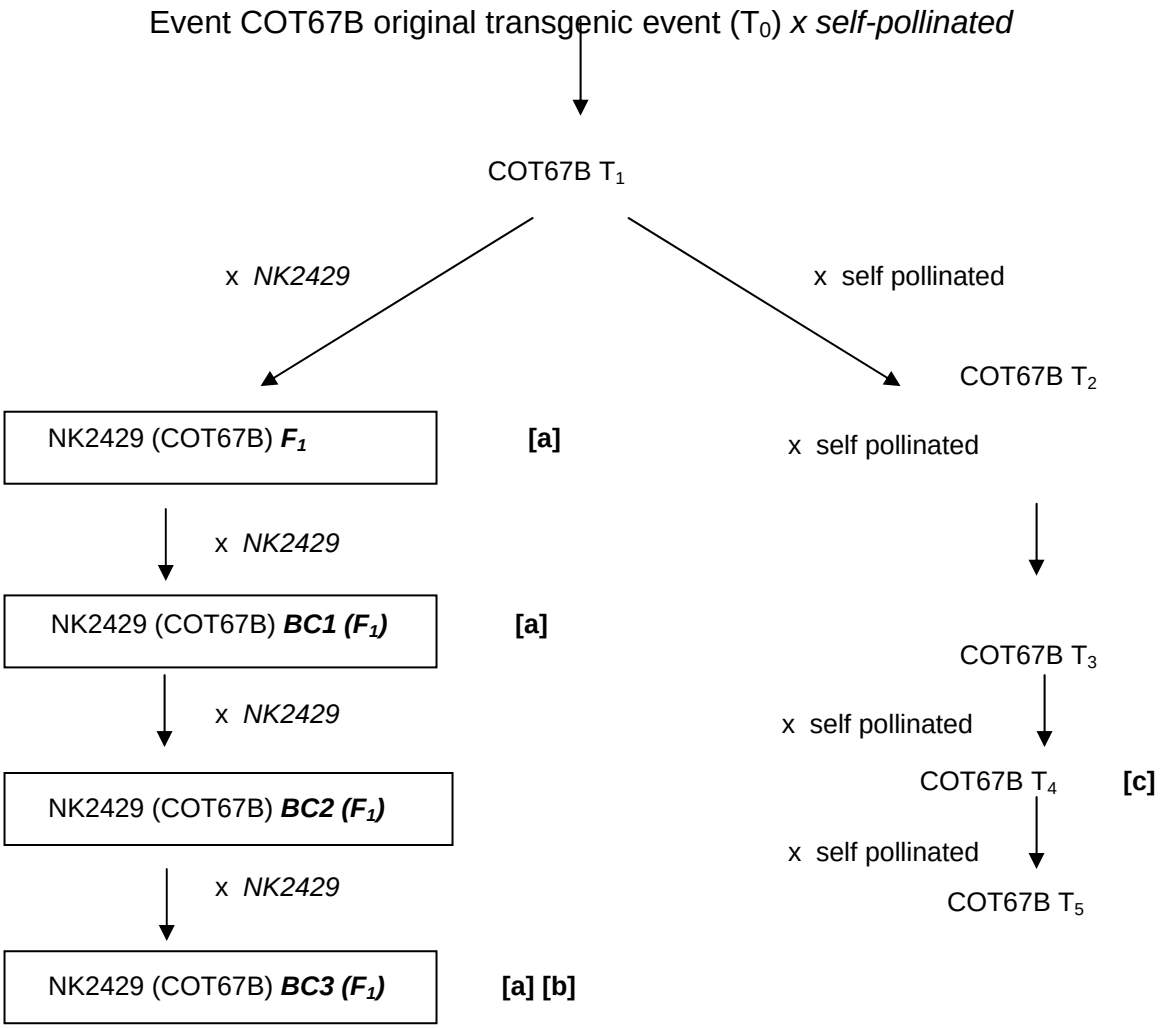
1. **p 28** Please submit all field test reports for the notifications referenced in the petition, including those listed as "pending".

   Syngenta response:
   - COT67B field trial reports from the following notifications are provided as a confidential attachment to this document: 03-098-08n, 03-268-04n, 04-041-01n, 04-064-05n, 04-079-01n, 05-034-02n, 05-102-01n, 05-266-01n, 05-339-04n, 06-039-16n, and 06-060-04n.
   - CBI and CBI deleted copies of Notification No. 06-039-16n (SYN2006-108) are also provided.
   - Notification Nos. 06-223-109n and 06-223-110n refer to counter season trials currently underway in Hawaii. Field trial reports for these trials will be submitted immediately upon termination of the trials.

2. **p 28** The submitted field test reports include lines designated as: 43-67B, CE43-67B, 67B, 43-67. Provide a flowchart that describes the pedigree of these lines and COT67B. Indicate in the flowchart which lines were used for which studies submitted in the petition. Provide a table that indicates which constructs are contained in each line.

   Syngenta response:
   The lines above are one and the same, COT67B. The different designations come from nomenclature associated with various phases of research and development. Constructs pNOV4641 and pNOV1914 were used to create COT67B. A pedigree

chart showing the source material for all studies is provided below. A similar flowchart is provided on page 42 of the Petition.



[a] Materials used for Mendelian inheritance and generational stability Southern analyses
[b] Materials used for all Southern analysis and sequencing
[c] Material used for compositional analysis, processing study, protein analysis (including IAPCOT67B-0106)

3. **p 28 and p 230-280** The petition (p 230) indicates that line COT67B contains only the *flcry1A(b*) gene, and does not contain the *aph4* gene. However, according to the data submitted with the notifications, all the lines planted under the notifications, referenced in the petition (p 28), and used in the agronomic studies (p 230-280) contain both the *flcry1Ab* and the *aph4* gene. Were COT67B lines that only contained the *flcry1Ab* gene (and did not contain the *aph4* gene)

used in any of the field studies referenced in the petition? If not provide justification for use of lines containing the *aph4* gene.

Syngenta response:
COT67B does not contain the aph4 gene. The USDA notifications include the constructs used to generate the initial transformation event. Subsequent breeding activities to remove any transgenic elements would not be captured in the notifications. As indicated on page 230 and demonstrated in Chapter 3 of the Petition (molecular characterization), the two T-DNA system of transformation was employed specifically to enable the identification of transformation events in which the selectable marker gene had segregated away from the insecticide gene. Such was the case for event COT67B.

4. **p 37 & 41** The petition indicates that the *aph4* promoter (in pNOV1914) includes the ubiquitin 3 intron, however the pNOV1914 construct in notifications 03-098-08n, 03-268-04n and 04-079-01n contains the pep-carboxylase intron #9 from maize, and in notification 06-233-110n contains no intron. Explain the discrepancy in the descriptions of the constructs and how this would or would not affect the submitted field test data.

Syngenta response:
pNOV1914 contains the aph4 gene and the ubiquitin 3 promoter and intron. In cases where it indicates otherwise, it is incorrect. The pep-carboxyylase discrepancy was recognized and corrected in the 2005 and 2006 notifications. Notification 06-233-110n should have had ubiquitin3 promoter and intron listed. The discrepancy was noted and is reflected in subsequent notifications. These inadvertent errors have no impact on the submitted field trial data.

5. **p 40-41** Indicate the organism donor of all the plasmid and inserted sequences to the species level.
   Syngenta response: (next page)

Table 3-1 Continued (page 40 of Petition)

Genetic Element	Location in pNOV4641 (bp)	Size (bp)	Function
virG	8903 - 9628	726	VirGN54D (*Agrobacterium tumefaciens*) from pAD1289 (similar to Entrez Accession Number AF242881 (NCBI, 2007)). The N54D substitution results in a constitutive virG phenotype. virG is part of the two-component regulatory system for the vir regulon in *Agrobacterium tumefaciens* (Hansen *et al.*, 1994).
repA	7800 - 8873	1074	pVS1 replication protein from *Pseudomonas aeruginosa*, which is a part of the minimal pVS1 replicon that is functional in gram-negative plant associated bacteria (Entrez Accession Number AF133831 NCBI, 2007) (Heeb et al., 2000).
VS1ori	7353 - 7757	405	Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas aeruginosa* (similar to Entrez Accession Number U10487 (NCBI, 2007)). Serves as origin of replication in *Agrobacterium tumefaciens* host (Itoh et al., 1984).
ColE1ori	5869 - 6675	807	Origin of replication that permits replication of plasmid in *Escherichia coli*. (similar to Entrez Accession Number V00268 (NCBI, 2007)) (Itoh and Tomizawa, 1978).
RB (right border)	5732 - 5756	25	Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01826 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang et al., 1984).

Table 3-2. Selectable marker cassette and plasmid backbone components of plasmid pNOV1914 (Page 41 of the Petition)

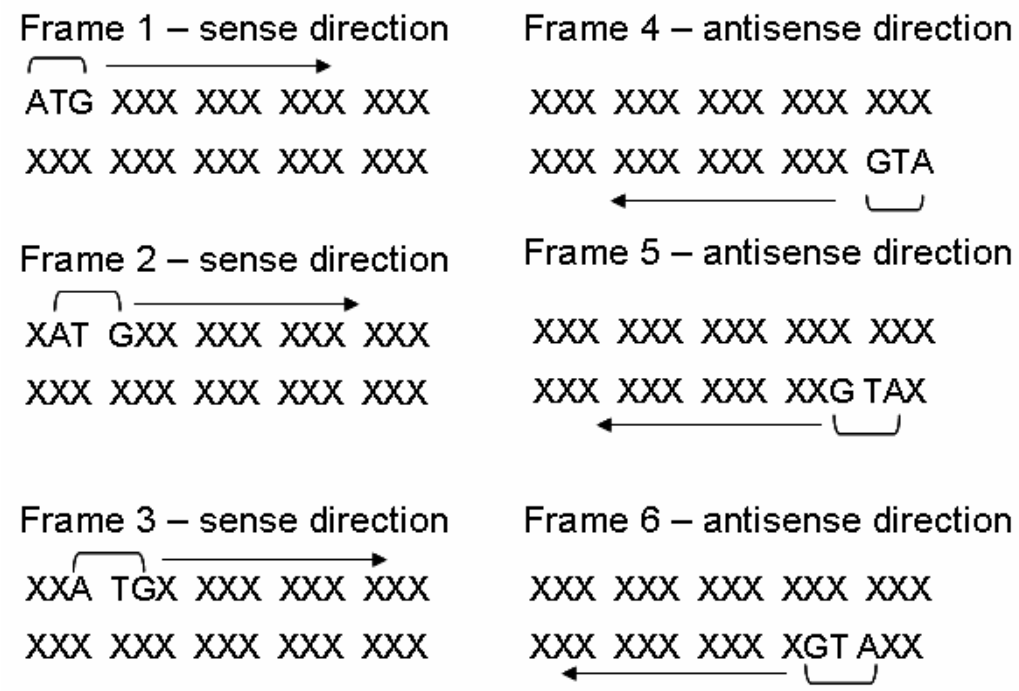
Genetic Element	Location in pNOV1914 (bp)	Size (bp)	Function
SELECTABLE MARKER CASSETTE			
Ubq3	245 - 1965	1721	Promoter region plus the first intron from the ubiquitin 3 (ubi3) of *Arabidopsis thaliana* (Norris *et al.*, 1993).
aph4	1997 - 3022	1026	The *aph4* gene encodes a synthetic phosphotransferase enzyme (hygromycin B phosphotransferase; an aminocyclitol phosphotransferase from E*scherichia coli*) that catalyzes the phosphorylation of hygromycin and some related aminoglycosides (Waldron, 1997). The *aph4* gene, when transformed into some plant cells, enables the transformed cells to grow in the presence of the selection agent hygromycin.
NOS	3056 - 3308	253	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Enterz Accession Number V00087 (NCBI, 2007)). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).
PLASMID BACKBONE COMPONENTS			
LB (left border)	71 - 95	25	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01825, (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).
trfA	10262 - 11410	1149	Encodes the replication initiation protein (*Pseudomonas aeruginosa*) essential for plasmid replication (Smith and Thomas, 1984).
npt2	9169 - 9963	795	5' region from the *Escherichia coli* gene encoding the 3'5'-aminoglycoside phosphotransferase type III conferring kanamycin resistance (Trieu-Cuot and Courvalin, 1983).
oRK2	6880 – 7590	711	Region covering the origin of replication oriV of plasmid RK2 from *Escherichia coli* (Stalker *et al*., 1981)
*tra*J	6378 – 6749	372	Encodes the relaxosome protein (*Escherichia coli*) for plasmid replication (Guiney and Yakobsen, 1983).
virG	4543 - 5268	726	VirGN54D (*Agrobacterium tumefaciens*) from pAD1289 (similar to Entrez Accession Number AF242881 (NCBI, 2007)). The N54D substitution results in a constitutive virG phenotype. virG is part of the two-component regulatory system for the vir regulon in *Agrobacterium* (Hansen *et al*., 1994).
RB (right border)	3390 - 3414	25	Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01826 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*., 1984).

6. **p 42** What is the cotton variety of the NK2429 backcross line?

Syngenta response:
NK2429 (*Gossypium hirsutum)*, also called D2429, PVP Certification # 200400100 issued August 27, 2004. The experimental number of NK2429 is B429-NX2429

7. **p 46** Second paragraph. What are the six ORFs referred to in the text?

Syngenta response:
This refers to the six possible frames of an ORF in sense and antisense directions that may be found using ATG as the start codon and TAA, TAG, or TGA as the stop codon. Novel putative ORFs that were at least 150 base pairs long were searched for in the sense and antisense directions at the 3' and 5' regions of the COT67B insert. Specifically the six ORF frames are:



8. **p.82** What database(s) were searched and what search criteria were used to determine lack of homology to known toxins and allergens? Was a national or international standard used as guidance for the searches? If so, what guidance?

Syngenta response:
To demonstrate lack of homology to allergens two different searches were performed comparing the query sequence to the sequences of entries in the SBI Allergen Database. The homology searches follow the guidance of the Codex Alimentarius Commission (2003) that recommends using a bioinformatics search using a FASTA or a BLASTP algorithm for greater than 35% identity over 80 or

more amino acids. The second guidance from the Codex Alimentarius Commission recommends a search for short contiguous amino acid identities between the query protein and an allergen. It is also recommended that the size of the contiguous amino acid search be based on scientifically justified rationale in order to minimize negative or false positive results. The searches were carried out for FLCry1Ab used 80 amino acid windows (1-80, 2-81…) of the query sequence for greater than 35% identity and a search for 8 or more consecutive identical amino acids based on Hileman *et al*. 2002.

The SBI Allergen Database used is updated annually by searching the latest updates of the public protein databases as well as the SWISS-PROT and FARRP Allergen databases, and additional entries identified in the scientific literature as putative allergens, but which are not found in the public databases. The latest version of the SBI Allergen Database (version 5.0) contains a total of 1,735 nonredundant entries and was last updated in July 2006.

To show lack of homology to toxins the NCBI Entrez protein database was searched with the BLASTP program using the query sequence. This database is a large public database containing over 4.7 million sequences and thus represents a good source to identify any potential toxin homologies. No international standard was used for this search however the method provides a robust way to identify potential toxin homologies using conservative search criteria.

The criteria for evaluating BLASTP results were based on a generated E-value threshold. Since meaningful analysis of database "hits" requires that a threshold is established below which similarity to the query sequence is considered statistically significant and not the result of random amino acid similarity, shuffled versions of the query sequence were created. These were also searched with the BLASTP program to retrieve E-values, the lowest of which was used to set a threshold for which query sequence search hits would be evaluated. E-values for matches to the query sequence below this threshold were considered to represent proteins with statistically significant amino acid homology and these sequences were evaluated for biological significance and homology to toxins.

References:

Codex Alimentarius Commission (2003). Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy 30 June-5 July, 2003.

Hileman, R. E., A. Silvanovich, R. E. Goodman, E. A. Rice, G. Holleschak, J. D. Astwood and S. L. Hefle (2002). Bioinformatic methods of allergenicity assessment using a comprehensive allergen database. *Int. Arch. Allergy Immunol.* 128: 280-291.

9. **p 83** Provide updates of all relevant submissions to EPA & FDA involving FLCry1Ab. All studies submitted to EPA and FDA do not necessarily need to be provided to APHIS.

   Syngenta response:
   A Section 3 commercial registration application for the breeding stack COT102 x COT67B was submitted to EPA in late 2006. A Safety and Nutritional Assessment of COT67B was submitted to FDA in 2007.

10. **p 89** Third paragraph, fourth line should read: "poses no more of a plant pest risk"

    Syngenta response:
    The corrected sentence reads:
    Consequently, COT67B poses no more of a plant pest risk either directly to the cotton ecosystem, indirectly to agriculture as a whole, or cumulatively, taking into consideration incremental past, present and reasonably foreseeable future impacts resulting from the deregulation and unconfined planting of COT67B compared to cotton varieties currently cultivated.

11. **p 130** Describe the truncated trCry1Ab. Justify why the truncated protein was used. Is the truncated protein equally effective as the full length protein? Justify by literature citations or by providing data.

    Syngenta response:
    Truncated Cry1Ab protein results from either trypsinized FLCry1Ab or from enzyme cleavage in the midgut of the target insect. The truncated and FLCry1Ab proteins have very similar insecticidal regions. Corn Events Bt11 and Bt176 contain truncated Cry1Ab. Because of the similarity of the insecticidal regions, studies supporting Bt11 and Bt176 were included in the weight of evidence to support the safety of FLCry1Ab. See Ch. 4, Section A.2, Figure 4-1, page 74 of Petition and question 21 this document for additional information.

12. **p 134** The statement at the end of the third paragraph on this page suggests 95.5% of the cotton varieties grown in the US are Bt. Based on refuge requirements, it is not possible for there to be 95.5% Bt grown in the U.S. annually. Clarify this statement.

    Syngenta response:
    The statement refers to 95.5% of the *varieties* sold – not 95.5% of the *acreage*

13. **p 194** Provide a pedigree for IAPCOT67B-0106.

    Syngenta response:
    The plants used to generate IAPCOT67B-0106 were the T4 generation as shown in the pedigree chart provided in response to question 2. A similar chart can be found on page 42 of the petition

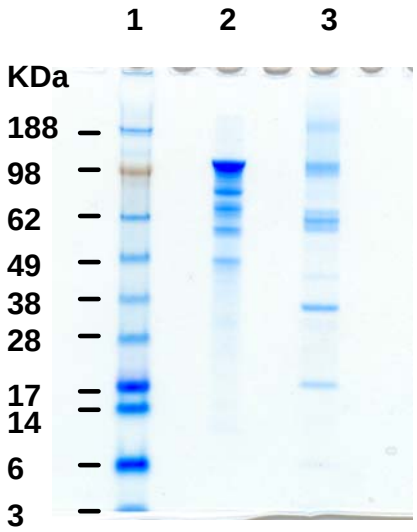
14. **p 199** First paragraph in the text refers to Figure 3.B.3 (not 3.B.2 as stated in the text)

    Syngenta response:
    The text should read Figure 3.B.3.

15. **p 201**
    - Provide a protein gel used for this Western, if available

    Syngenta response:
    It is technically impossible to provide a protein gel for this particular Western because it was used to transfer the proteins onto the PVDF membrane (followed by detection with antibodies). However, a protein gel containing Cry1Ab proteins from test substances FLCRY1AB-0103 and IAPCOT67B-0106 (see figure below) prepared for peptide mass mapping analysis is provided. This particular gel does not include the crude leaf extract of LPCOT67B-0106, however, after Coomassie staining, this fraction would show all cotton crude extract proteins thereby making it impossible to identify FLCry1Ab protein.

Coomassie Blue Stained SDS 4-12% NuPage Gel



Lane 1: See Blue plus2 marker

Lane 2: FLCRY1AB-0103

Lane 3: IAPCOT67B-0106

- Discuss all major bands found in Lane 4 (immuno-purified protein).

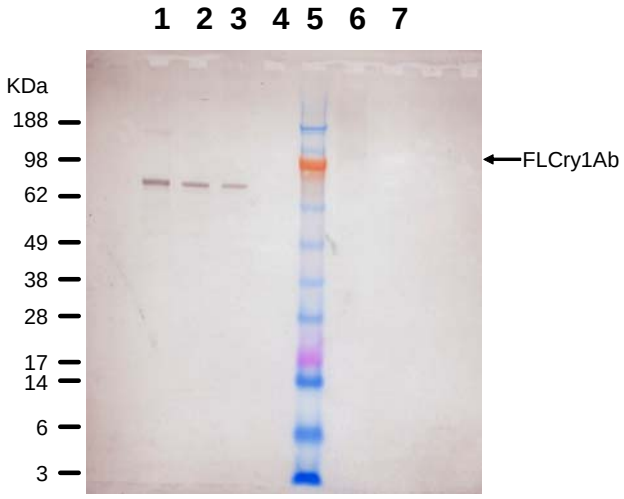
  Syngenta response:
  Lane 4 contains immuno-purified Cry1Ab proteins from cotton leaves. In addition to the intact FLCry1Ab band with molecular weight of 133.5 kDa, lower molecular weight protein fragment bands are visible, e.g. 67, 62, 58 and 36 kDa bands. These fragments most likely represent break-down products from the Cry1Ab protein purification process because they are not present in the crude extracts as shown in lane 3 of the same Western blot on page 201. Peptide mass mapping analysis identified all major protein bands as Cry1Ab related fragments.

- What is the significance in terms of functional activity (or lack of significance of the extra proteins found in lane 5 (*E. coli*).

  Syngenta response:
  The extra proteins most likely represent breakdown fragments of the Cry1Ab protein as evidenced by cross-reactivity with Cry1Ab-specific antibodies. Densitometry analysis showed that 90% of the protein in test substance FLCRY1AB-0103 is Cry1Ab, consequently, the functionality of these breakdown fragments was not investigated. Breakdown products are not likely to be biologically significant in terms of functionality.

16. **p 202** The gel photo in the submitted petition does not show a faint band as so indicated in the text on page 199. Submit a photo that shows the band.

Syngenta response:
As reported, this band is very, very faint. A new scan of the figure did not result in a better resolution of the described band.



Lanes 1, 2 and 3: 100, 50, 25 ng transferrin (positive control), respectively
Lane 4: 2 µg creatinase (negative control)
Lane 5: Molecular weight standard SeeBlue® Plus2
Lane 6: 2.2 µg Cry1Ab from IAPCOT67B-0106
Lane 7: 2.3 µg Cry1Ab from FLCRY1AB-0103

17. **p 205** What is the pedigree of the near isogenic comparator line?

Syngenta response:
The near isogenic comparator line is the COT67 null line (COT67b(-))in Coker 312 background. The derivation on the COT67B(-) is shown below along with a description on the origin and breeding history of Coker 312, the near isogenic comparator line used for the protein developmental expression study, is provided below:

13 A. EXHIBIT A, ORIGIN AND BREEDING HISTORY OF VARIETY - COKER 312

Stage	Year	Activity
1	1948	Cross; Coker 100 Staple X Deltapine 15
2	1950-1959	Line selection program thru successive generations produced the strain Coker 60-111.
3	1960-1966	Line selection in Coker 60-111 produced the strain Coker 66-115, later named Coker 310.
4	1966-1968	Line selection in Coker 66-115 produced the strain Coker 68-312, now named Coker 312.
5	1968-1971	Coker 68-312 evaluated in replicated yield trials and disease screening trials across the Cotton Belt. Concurrent seed increase was accomplished to produce a small volume of foundation seed during the 1970 season in South Carolina. Continued reselection within Coker 68-312 has produced maintenance strains which will be used to produce foundation seed in years ahead.
6	1971	Produced certified seed of Coker 312 under contract with Canyon Gin, Lubbock, Texas, for distribution to farmers for 1972 plantings in that area.

Variants: Occasional variants are to be found in any cotton variety due to frequency of natural cross pollination. However these are at a minimum in Coker 312 due to the long period of selection and reselection and relatively high degree of homozygosity.

18. **p 210** Paragraphs B.2. refers to Table 8, and B3 refers to Table 9 and these should be Table 7 and Table 8 respectively.

Syngenta response:
Paragraph B.2 refers to Table 7; it should be “Table 8”. Paragraph B.3. refers to Table 8; it should be “Table 9” The corrected text is included below:

B.2. Estimated Total FLCry1Ab Protein per-Acre and per-Hectare

Assuming a planting density of 50,000 plants/acre (123,500 plants/hectare), estimates of mean FLCry1Ab concentrations in the transgenic plants on a per-acre (and per-hectare) basis at pre-harvest ranged from *ca.* 46.49 g FLCry1Ab/acre (114.84 g FLCry1Ab/hectare) at WLA to *ca.* 182.59 g FLCry1Ab/acre (451.01 g FLCry1Ab/hectare) at LMS (Table 8). Values have been corrected for extraction efficiency.

B.3. FLCry1Ab Extraction Efficiency

The apparent extraction efficiency of FLCry1Ab across Event COT67B plant tissues ranged from 70.7% in whole plants to 77.0% in bolls (Table 9). Extraction efficiency of FLCry1Ab from pollen averaged 78.5%.

19. **p 250** Table 10 and Table 14 describe results from field tests located in Texas that are not summarized with the other 2005 locations on page 239. Why?

Syngenta response:

The data for Haskell, TX submitted with the Petition is the only data taken at that site in 2005. All data were not collected from all locations due to logistical reasons or adverse weather conditions.

20. **p284** Provide a non-CBI toxicity table of the non-target organism studies, as submitted in the CBI Supplement 12.

Syngenta response: (See also question 22)

Summary of ecotoxicology studies on FLCry1Ab and Cry1Ab

Species	Test substance	Endpoints	Result
Mouse	Microbial FLCry1Ab	14-d mortality and growth	NOEL ≥ 1830 mg FLCry1Ab/ kg bw
Bobwhite quail	Cry1Ab leaf protein	14-d mortality and growth	NOEL ≥ 140 mg Cry1Ab/ kg bw
Poultry		49-d mortality and growth	Not determined
Honeybee	Microbial FLCry1Ab	34-d larval survival	NOEC ≥ 76.98 µg FLCry1Ab/g diet
Ladybeetle[†]	Microbial FLCry1Ab	Larval and adult mortality	NOAEC ≥ 1000 µg FLCry1Ab/g diet $NOAEC_{ba}$ ≥ 223.1 µg FLCry1Ab/g diet
Orius	Microbial FLCry1Ab	Pre-imaginal survival	NOEC ≥ 1003.9 µg FLCry1Ab/g diet $NOEC_{ba}$ ≥ 223.1 µg FLCry1Ab/g diet
Rove beetle	Microbial FLCry1Ab	Reproduction	NOEC ≥ 1000 µg FLCry1Ab/g diet $NOEC_{ba}$ ≥ 222.2 µg FLCry1Ab/g diet
Collembola	Cry1Ab maize leaves	Mortality and reproduction	NOEC ≥ 17.1 µg Cry1Ab/g diet
Daphnia[#]	Cry1Ab maize pollen	2-d neonate survival	NOEC ≥1.8540 µg Cry1Ab/L
Catfish	Microbial FLCry1Ab	30-d juvenile survival	NOAEC ≥ 8.13 µg Cry1Ab/g feed $NOAEC_{ba}$ ≥ 1.81 µg Cry1Ab/g feed

[†]*Coleomegilla maculata*

[#]NOEC based on density of 150 mg pollen/L water; concentration of Cry1Ab in pollen = 12.36 µg/g

21. **p 293** Provide justification for the use of lyophilized maize leaf (LLBt-0100) and maize pollen (PHO176-0194) as test substances.

    Syngenta response:
    LLBt11-0100 and PHO176-0194 contain truncated Cry1Ab molecules that have the same active region as the FLCry1Ab contained in COT67B cotton (Figure 4.1, page 74 of the Petition). Also, when FLCry1Ab is ingested by animals it is likely to be cleaved by proteases to produce truncated Cry1Ab molecules similar in size to those in LLBt11-0100 and PHO176-0194. The sequence identity of the active sites, and the probable proteolytic cleavage of FLCry1Ab when ingested, mean that the trCry1Ab and FLCry1Ab are likely to have a similar spectrum of activity, and hence test substances containing truncated Cry1Ab are useful for predicting the sensitivity of non-target organisms to FLCry1Ab. The studies using truncated Cry1Ab test substances are not intended to be definitive, but to provide additional weight of evidence to test the hypothesis that at concentrations in transgenic plants the spectrum of activity of Cry1Ab is limited to Lepidoptera.

22. **p294** Provide a non-CBI brief description of testing methods used to test the toxicity of *cry1A(b),* as submitted in the CBI Supplement 12 D.

    Syngenta Response:
    A description of testing methods can be found at the end of this document after question 25.

    Question 22 continued: In addition, provide a discussion of previously published information that contributes to the weight of evidence that Cry1Ab in cotton will not harm non-target invertebrates. This information is needed since some of the submitted studies are not adequate by themselves to verify no toxicity. Examples of questions from the studies submitted as CBI Supplements include:

    Syngenta Response: (responses to the specific studies mentioned follow this response)
    In general, non-target arthropod studies were done to US EPA Guideline OPPTS 885.4340, which states with respect to insects inoculated with viruses, "Control and treated insects should be observed for a duration of at least 30 days after dosing, or in cases where an insect species cannot be cultured for 30 days, until control mortality rises above 20 percent". In non-target arthropod studies for Syngenta, tests of hypotheses are made using data collected from the sampling immediately before the time at which control mortality exceeded 20%. In some studies, data from measurements after the control mortality has exceeded 20% are reported, either because the study guideline differs from the EPA guideline, or because the data are believed to be informative; nevertheless, in these cases, analyses using data immediately prior to control mortality exceeding 20% are always reported. Control mortality exceeding 20% therefore does not invalidate a

study automatically; judgment must be made on whether the exposure prior to 20% control mortality being exceeded is sufficient for the study to achieve its purpose.

No study is adequate *by itself* to test the hypothesis of no toxicity in the field. The hazard studies are combined with exposure assessments to assess the risk to classes of organism, and the adequacy of the overall risk assessment must be determined by whether the tested species are sufficiently representative of the assessment endpoints (*e.g.*, biological control, pollination etc.). For the reasons above, Syngenta believes that the individual hazard studies were adequate to assess the toxicity of FLCry1Ab to the specific species tested, and that the range of species was adequate to assess risks of COT67B cotton to assessment endpoints of concern to the USDA. Additional weight of evidence for the low risk of FLCry1Ab in COT67B cotton comes from many laboratory toxicity studies of similar proteins (*e.g.*, truncated Cry1Ab and full-length Cry1Ac with Geiser fix) which have shown no effect on species other than Lepidoptera (US EPA, 2001). The predictive power of these studies to indicate negligible effects of these proteins to non-target organisms in the field have been confirmed by many field studies of plants expressing these proteins (*e.g.*, Naranjo *et* al., 2005; Romeis *et al*., 2006)

Naranjo, S.E., Head, G. and Dively, G.P., 2005. Field studies assessing nontarget effects in *Bt* transgenic crops: introduction. *Environmental Entomology* 34, 1178-1180.

Romeis, J., Meissle, M. and Bigler, F., 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nature Biotechnology* 24, 63-71.

- [Lady beetle study - Mortality in the negative control exceeded 20% and a decrease in body weight was observed]

  Syngenta response:
  There was no "decrease in body weight" in the negative control – the negative control individuals on average grew slightly slower than the individuals in the FLCry1Ab treatment. This is not considered an adverse effect and therefore the study does not indicate toxicity of FLCry1Ab at the concentration in the study.

  Due to the extended exposure times in this experiment, validity criteria were set for two phases of the experiment, namely pre-imaginal mortality and pupal emergence (Section 2.4.6 of the study protocol); the 20% control mortality criterion was met in both phases. If pupal emergence were considered an invalid endpoint because the cumulative mortality in the negative control exceeded 20%, larval mortality is still a valid

endpoint and sufficient to demonstrate low toxicity of FLCry1Ab to *Coleomegilla maculata*.

- [Rove beetle study – Mortality in the negative control exceeded 20% and mortality in the positive control was low.]

  Syngenta response:
  Adult mortality is not a test endpoint in this study – the endpoint is reproduction. Adult mortality data are collected to help interpret any differences in reproduction, but validity criteria are set for reproduction only.

- [Honey bee study – It is unclear how much Bt the honey bees were exposed to. Since activity was not verified at the end of the test, it is unclear if bees were exposed to active protein throughout the test and what quantity they were exposed to.]

  Syngenta response:
  1) The exposure of individual larvae was not determined; however, the NOEC is expressed in terms of brood development and therefore the important consideration is that the hives were exposed to the stated concentration of FLCry1Ab. The method of exposure simulates exposure of hives to FLCry1Ab *via* pollen and nectar and therefore it is legitimate to compare the concentration of FLCry1Ab in the sugar solution with EECs based on pollen concentration to characterize risk.

  2) The positive control had a significant effect on brood development indicating exposure of brood to test substances in the sugar solution.

23. **p298** Is the EPA document that is cited EPA 2005c and not 2005b?

    Syngenta response:
    Yes, the EPA document cited is 2005c.

24. **p302** What field trial data is section G.2.d referring to? Summarize over how many years and how many sites.

    Syngenta response:
    All trialists are required to note and report any differences observed between the events and the control – and each other. These observations are noted in the final field trial reports. No differences in pest or disease incidence were reported from 22 locations over 3 years.

25. [**Supplement 12, p 27** Provide a non-CBI description for the derivation of the EECs.]

Syngenta response:
Exposure of NTOs to FLCry1Ab *via* cultivation of COT67B cotton is assessed from protein expression studies and predictions of the dilution of FLCry1Ab in the diets of various groups of NTOs. Exposures are estimated in two ways: worst-case expected environmental concentrations (EECs), which represent exposure *via* a diet containing only tissue of COT67B cotton, and can be used to assess risk to individuals; and more realistic EECs, which represent exposure *via* a diet containing a mixture of potential food items, and can be used to assess risk to populations. The realistic EECs were calculated using the methods described in Raybould *et al*. (2007).

Raybould, A., Stacey, D., Vlachos, D., Graser, G., Li, X. & Joseph, R., 2007. Non-target organism risk assessment of MIR604 maize expressing mCry3A for control of corn rootworm. *Journal of Applied Entomology* (in press).
(A final version can be sent upon completion, if need be)

Description of Testing Methods for Non-Target Studies

Safety Assessment of FLCry1Ab in COT67B Cotton

The safety of FLCry1Ab is demonstrated if the cultivation of COT67B cotton represents low risk to NTOs. Risk is estimated as the HQ, the ratio of the EEC to the NOEC for representative indicator species (see Section B). EECs for groups of species potentially exposed to FLCry1Ab through the cultivation of COT67B cotton are detailed in a separate volume of this submission (Raybould, 2006a). The NOECs for representative indicator species were derived in a series of studies described in the Section D1. These hazard data are combined with the EEC estimates in Section D2 to evaluate the risk of COT67B cotton to NTOs.

1. The hazard of FLCry1Ab to non-target organisms

i. Overview

The insecticidal region of FLCry1Ab is similar to that of various truncated Cry1Ab molecules expressed in transgenic maize. The insecticidal regions of Cry1Ab and Cry1Ac share sequence identity over more than 400 amino acids (*ca*. 70%) of their active regions. In addition, Cry1Ac is a full-length protein that contains the Geiser motif found in the C terminal region of FLCry1Ab (see Reed, 2006, for a comparison of these proteins). Therefore the effects of truncated Cry1Ab and of Cry1Ac are considered predictive of the effects of FLCry1Ab to NTOs.

The mode of action of Cry1Ab, and laboratory studies and field studies of transgenic Cry1Ab-expressing maize and Cry1Ac-expressing cotton (*e.g.*, Schnepf *et* al., 1998; US EPA, 2001; Naranjo *et al*., 2005; Romeis *et al*., 2006) provide a large weight of evidence that at concentrations found in transgenic plants, Cry1Ab is toxic to

Lepidoptera only, and therefore it is expected that toxicity of FLCry1Ab in COT67B cotton will be limited to Lepidoptera.

To test this hypothesis, the toxicity (hazard) of FLCry1Ab to several representative indicator species of NTOs in cotton was assessed in the laboratory. These studies used a microbial test substance, FLCRY1AB-0103, which has been shown to be a suitable surrogate for FLCry1Ab produced in COT67B cotton (Graser, 2005; Kramer, 2006). In addition, several studies that exposed representative indicator species to truncated Cry1Ab contained in maize tissues are also considered. These studies satisfy certain data requirements for PIPs and allow a broader risk assessment for certain categories of NTOs. The test substances used in the hazard assessments are summarised in Table 4 and the results are summarized in Table 5.

Table 4. Test substances used to assess the hazard of FLCry1Ab to NTOs

Test substance	Type	Concentration of active ingredient
FLCRY1AB-0103	Microbial	*ca.* 800 mg FLCry1Ab1/g
LP176-0194	Maize leaf protein	*ca.* 0.70 mg trCry1Ab/g
LLBt-0100	Lyophilized maize leaves	34.20 µg trCry1Ab/g
PHO176-0194	Maize pollen	12.36 µg trCry1Ab/g

ii. The effects of FLCry1Ab on mice

Five male and five female mice were exposed to a single dose of the microbial test substance FLCRY1AB-0103, containing 1830 mg FLCry1Ab/kg body weight, by oral gavage. The effects on mortality and growth were compared with a control group for 14 days after exposure, and many histological and biochemical endpoints were assessed at end of the test.

No adverse effects were observed in the treatment or control groups, and the NOEL was shown to be at least 1830 mg FLCry1Ab/kg body weight (Barnes, 2005, MRID 457665-05).

iii. The effects of truncated Cry1Ab on bobwhite quail

Five male and five female juvenile bobwhite quail (*Colinus virginianus*) were exposed to a single dose of the maize leaf protein test substance LP176-0194, containing 140 mg Cry1Ab/kg body weight, by oral gavage. The effects on mortality and growth were compared with a control group exposed to maize leaf protein that did not contain Cry1Ab, and with a group that received the dosing capsule without leaf material, for 14 days after exposure.

No adverse effects were observed in the treatment or control groups, and the NOEL was shown to be at least 140 mg Cry1Ab/kg body weight (Campbell, 1994, MRID 433236-09).

iv. The effects of truncated Cry1Ab on poultry

Broiler chicks were exposed to Cry1Ab *via* diets prepared from Bt11 maize grain, or non-transgenic, near-isogenic maize grain. The broilers were fed the diets for 42 days. A total of 1600 birds were used in the study, which were housed in pens of 25 individuals of the same sex that received the same diet. Various endpoints were measured, including mortality and feed conversion ratio.

There were no deleterious effects of the Cry1Ab-containing feed on broiler survival or carcass yield in males or females (Guyer, 2002, MRID 456521-01; Brake *et al*., 2003). The concentration of Cry1Ab in Bt11 grain used to formulate the diet was 0.8 µg/g, and the maize grain comprised about 48% of the starter diet, 57% of the grower diet and 63% of the finisher diet. The concentration of Cry1Ab in the feed was not determined; however, in a similar study to evaluate the effect of Bt176 maize, Cry1Ab was detected in the diet even though the concentration of Cry1Ab in Bt176 grain is less than 0.005 µg/g (Brake and Vlachos, 1999). Therefore the 42-d NOEC for broilers cannot be determined, but exposure to Cry1Ab during the experiment is likely if Cry1Ab in Bt11 grain behaves similarly to Cry1Ab in Bt176 grain during preparation of broiler diets.

v. The effects of FLCry1Ab on honeybees

Honeybees (*Apis mellifera*) were exposed to FLCry1Ab *via* the microbial test substance FLCRY1AB-0103. The test substance was dissolved in 50 mM Tris-HCl buffer (pH 9.5) and added to the diet of 50% sucrose solution at a concentration of 76.98 µg FLCry1Ab/g diet; this concentration was intended to represent approximately 10 times the concentration of FLCry1Ab in COT67B pollen. The negative control was 50% sucrose solution with Tris-HCl buffer, and the positive control was 2.0 µg diflubenzuron/g diet.

The treatment and control groups each comprised three queen-right hives, each supplied with 1 L of the appropriate treatment diet through a commercial bee feeder. All diet was consumed within 5 days. In each hive, brood development was assessed in 100 cells containing eggs, 100 cells containing 1-3-day old larvae, and 100 cells containing 4-6-day old larvae. The cells were monitored for development of adults or death of the larvae for up to 24 days. Other test endpoints were mortality of adult bees within the hive for 24 days, and the condition of the hives at the beginning and end of the test.

Survival of larvae was over 90% in all ages classes in hives supplied with FLCRY1AB-0103-treated diet and negative control diet. There were no statistically significant differences between the FLCRY1AB-0103 treatment and the negative control. Larval survival was statistically significantly lower in hives receiving the positive control diet. There were no statistically significant differences between the FLCRY1AB-0103 treatment and the negative control in any other endpoint. The NOEC to bee brood exposed *via* a sugar solution diet was shown to be at least 76.98 µg FLCry1Ab/g diet (Jeker, 2006).

vi. The effects of FLCry1Ab on pink spotted ladybird beetles

Five-day old, second instar ladybird beetles (*Coleomegilla maculata*) were exposed to FLCry1Ab *via* the microbial test substance FLCRY1AB-0103. The test substance was ncorporated into a diet of bee pollen and moth eggs at 1000 µg FLCry1Ab/g diet; this concentration was intended to represent approximately 10 times the concentration of FLCry1Ab in COT67B leaves. The negative control was the pollen and moth egg diet only, and the positive control was 250 µg potassium arsenate/g diet.

The treatment and control groups each comprised 40 beetles, and were fed fresh diet every other day. Several endpoints were measured and analyzed statistically: survival to pupation, days to pupation, survival to adult emergence, days to adulthood, adult weight and adult survival. There were no statistically significant differences in survival between the treatment and negative control groups. The mean weight of adults in the FLCry1Ab-treatment group was significantly lower than that of the negative control group; the difference was 14%, and was not considered an adverse effect for the purposes of risk assessment. All larvae died before pupation in the positive control group and the negative control group met the validity criterion of at least 80% survival between developmental stages (Patnaude, 2006).

Analysis of the diet by ELISA, Western blot and bioassay of first instar European corn borer (ECB; *Ostrinia nubilalis*) confirmed the presence of intact bioactive FLCry1Ab in the treatment diet. ELISA analysis showed that the protein was distributed heterogeneously in the diet, so some larvae may have been exposed to higher or lower concentrations than the nominal concentration. Nevertheless, for the purposes of risk assessment the larvae were all considered to have been exposed 1000 µg FLCry1Ab/g diet. Therefore the no observable adverse concentration (NOAEC) was taken to be at least 1000 µg FLCry1Ab/g diet (Patnaude 2006).

Re-characterization of FLCRY1AB-0103 shortly after the completion of the experimental phase of the *C. maculata* study showed a statistically significant loss of bioactivity against first-instar ECB compared with the initial characterization of the test substance: the initial LC_{50} was 3.7 ng FLCry1Ab/cm^2 diet surface, and the LC_{50} at re-characterization was 16.5 ng FLCry1Ab/cm^2 diet surface (Kramer, 2006). A conservative interpretation of these data is that the measured NOAEC should be reduced by a factor of 4.5 for the purposes of the risk assessment to reflect the loss of bioactivity in the test substance. The NOAEC based on bioactivity, the $NOAEC_{ba}$, is therefore at least 222.2 µg FLCry1Ab/g diet.

vii. The effects of FLCry1Ab on *Orius*

Second instar *Orius laevigatus* were exposed to FLCry1Ab *via* the microbial test substance FLCRY1AB-0103. The test substance was dissolved in 50 mM Tris-HCl buffer (pH 9.5) and incorporated into an artificial liver-based diet at 1003.9 µg FLCry1Ab /g diet; this concentration was intended to represent approximately 10 times the concentration of FLCry1Ab in COT67B leaves. The negative control diet was treated with Tris-HCl only, and the positive control was 10 µg teflubenzuron/g diet. The treatment and control groups each comprised 40 nymphs, which were fed

fresh diet daily until they developed into adults; all individuals had become adults or died 12 days after the start of the test.

Survival was 17.95% in the FLCRY1AB-treated group and 12.82% in the control group; this difference was not statistically significant. ELISA, Western blotting and a bioassay with first instar ECB demonstrated that FLCry1Ab was present in the diet at the nominal concentration[1]. The NOEC was shown to be at least 1003.9 µg FLCry1Ab/g diet (Stacey, 2006a).

Re-characterization of FLCRY1AB-0103 shortly after the completion of the experimental phase of the *O. laevigatus* study showed a statistically significant loss of bioactivity against first-instar ECB compared with the initial characterization of the test substance: the initial LC_{50} was 3.7 ng FLCry1Ab/cm^2 diet surface, and the LC_{50} at re-characterization was 16.5 ng FLCry1Ab/cm^2 diet surface (Kramer, 2006). A conservative interpretation of these data is that the measured NOEC should be reduced by a factor of 4.5 for the purposes of the risk assessment to reflect the loss of bioactivity in the test substance. The NOEC based on bioactivity, the $NOEC_{ba}$, is therefore at least 223.1 µg FLCry1Ab/g diet.

viii. The effects of FLCry1Ab on rove beetles

Adult *Aleochara bilineata* were exposed to FLCry1Ab *via* the microbial test substance FLCRY1AB-0103. The test substance was dissolved in 50 mM Tris-HCl buffer (pH 9.5) and incorporated into an artificial meat-based diet at 1000 µg FLCry1Ab/g diet; this concentration was intended to represent approximately 10 times the concentration of FLCry1Ab in COT67B leaves. The negative control diet was treated with Tris-HCl only, and the positive control was 10 µg diflubenzuron/g diet.

The treatment and control groups each comprised four replicates of 10 male and 10 female adult beetles. The beetles were fed fresh diet daily for 35 days. After the exposure phase, the beetles were allowed to parasitize pupae of the onion fly (*Delia antiqua*). The number of second-generation beetles that emerged from the pupae was recorded until the average emergence dropped below 2 per day.

A mean of 317.0 beetles per replicate emerged from the FLCry1Ab treatment, 428.3 emerged from the negative control, and 160.0 emerged from the positive control. The difference in emergence between the FLCry1Ab treatment and the negative control was not statistically significant; the difference in emergence between the negative and positive controls was statistically significant. The positive and negative control validity criteria of the study guideline (Grimm *et al*., 2000) were met, and analysis of the diet by ELISA, Western blot and bioassay confirmed that intact, bioactive FLCry1Ab was present in the diet at the nominal concentration. The NOEC was shown to be at least 1000 µg FLCry1Ab/g diet (Stacey, 2006b).

[1] ELISA showed 56.1% recovery of FLCry1Ab from the *Orius* diet. This was considered to be due to low extractability because the Western blot showed no degradation of FLCry1Ab. See Raybould (2006b) for further discussion of this study.

Re-characterization of FLCRY1AB-0103 shortly after the completion of the experimental phase of the *A. bilineata* study showed a statistically significant loss of bioactivity against first-instar ECB compared with the initial characterization of the test substance: the initial LC_{50} was 3.7 ng FLCry1Ab/cm^2 diet surface, and the LC_{50} at re-characterization was 16.5 ng FLCry1Ab/cm^2 diet surface (Kramer, 2006). A conservative interpretation of these data is that the measured NOEC should be reduced by a factor of 4.5 for the purposes of the risk assessment to reflect the loss of bioactivity in the test substance. The NOEC based on bioactivity, the $NOEC_{ba}$, is therefore at least 222.2 µg FLCry1Ab/g diet.

ix. The effects of truncated Cry1Ab on collembola

Juvenile collembola were exposed to FLCry1Ab *via* the lyophilized Bt11 maize leaf test substance LLBt11-0100. The test substance was mixed with an equal weight of yeast to form the treatment diet containing 17.1 µg Cry1Ab/g diet. A control diet containing equal parts yeast and lyophilized leaves of non-transgenic, near-isogenic maize was also prepared. A diet to control for the effects of maize leaves consisted of yeast only, and the positive control diet was yeast with 500 µg thiodicarb/g diet. The treatment and control groups each comprised four replicates of 10 collembola provided with fresh diet daily.

After 28 days, mean survival was 83% in the LLBt11-0100-treated group and 78% in the non-transgenic maize leaf-treated group; mean survival was 80% in the group fed yeast only, and 3% in the positive control group. The differences in survival among the maize leaf-treated groups and the yeast only group were not statistically significant; survival was statistically significantly lower in the positive control group.

The mean number of juveniles was 446.5 in the LLBt11-0100-treated group and 343.5 in the non-transgenic maize leaf-treated group. The mean number of juveniles was 218.5 in the group fed yeast only, and 3.8 in the positive control group. The difference between the LLPACHA-0100-treated group and the non-transgenic maize leaf-treated group was not statistically significant; and the LLBt11-0100-treated group was statistically significantly different from the yeast only control, possibly because of nutritional benefits from the maize leaves. The positive control was significantly different from the other groups. The NOEC was shown to be at least 17.1 µg Cry1Ab/g diet (Privalle, 2002b).

x. The effects of truncated Cry1Ab on *Daphnia*

Neonate *Daphnia magna* were exposed to Cry1Ab *via* the Bt176 maize pollen test substance PHO176-0194, which contained 12.36 µg Cry1Ab/g pollen (Privalle, 1997, MRID 442742-01). The test substance was suspended in water at five concentrations up to 150 mg pollen/L, representing 1.8540 µg Cry1Ab/L. The negative control was non-transgenic, near-isogenic maize pollen suspended in water at 150 mg/L. A water only control for the effects on maize pollen was also included. The treatment and control groups each comprised three replicates of 10 daphnids exposed for 48 hours, with complete renewal of test medium after 24 hours.

Survival was 100% in each group, and no there was no sign of immobilization or other sub-lethal effects. The NOEC was shown to be at least 150 mg PHO176-0194/L, representing 1.8540 µg Cry1Ab/L (Collins, 1994, MRID 433236-10).

xi. The effects of FLCry1Ab on catfish

Juvenile channel catfish (*Ictalurus punctatus*) were exposed to FLCry1Ab *via* the microbial test substance FLCRY1AB-0103. The test substance was dissolved in 50mM Tris-HCl buffer (pH 9.5) and incorporated into a standard catfish diet at a nominal concentration of 15.4 µg FLCry1Ab/g feed, which was intended to represent at least 1X the worst-case exposure of farmed fish to FLCry1Ab *via* feed containing COT67B cottonseed meal. A control diet formulated in the same way, but incorporating Tris-HCl only, was also prepared. The treatment and control groups both comprised 4 groups of 20 fish, which were exposed to the respective diets for 28 days.

There were no deaths, and no fish showed abnormalities, in either treatment. There were no statistically significant differences in total feed consumption or the feed: weight-gain ratio between the treatment and control groups. The 28-d mean biomass and mean weight gain were both statistically significantly lower in the FLCRY1AB-0103 diet treatment group, but the respective 12.5% and 18.1% reductions associated with the FLCRY1AB-0103 treated diet were not considered adverse effects for the purposes of risk assessment.

ELISA showed that an average of 52.8% of the nominal concentration of FLCry1Ab could be recovered from FLCRY1AB-0103-treated diet. Loss of FLCry1Ab was assumed to have occurred during feed manufacture, although it is possible that there is low extractability of the protein from the diet. Western blotting and an ECB bioassay showed that intact bioactive protein was present in the diet. Therefore the NOAEC was shown to be 8.13 µg FLCry1Ab/g feed (Hoberg, 2006). Correcting for loss of bioactivity of the test substance, the $NOAEC_{ba}$ is 1.81 µg FLCry1Ab/g feed.

2. Risk assessment for COT67B cotton and non-target organisms

The risk of FLCry1Ab in COT67B cotton to NTOs is calculated as the HQ for a series of representative indicator organism (Table 6). In most cases this is the ratio the EEC and the NOEC for FLCry1Ab; for organisms exposed to an acute oral dose of protein, the NOEL is compared with the predicted daily dietary dose (DDD) of FLCry1Ab the organism would receive from consumption of COT67B cotton.

The derivation of EECs and DDDs is described in a separate summary volume (Raybould, 2006a). For each group of organisms, two EECs or DDDs are calculated: a worst-case estimate, which represents exposure *via* a diet containing only the potential food item with the highest concentration of the relevant protein; and a more realistic estimate, which represents exposure *via* a diet containing a mixture of potential food items.

i. Wild mammals

The risk to wild mammals can be estimated from the NOEL of at least 1830 mg FLCry1Ab/kg body weight from the mouse study. The worst-case DDD of FLCry1Ab to wild mammals is 9.06 mg/kg body weight, and therefore the HQ under worst-case exposure is no greater than 9.06/1830 = 0.0050. Under more realistic exposure, the DDD is 0.13 mg/kg body weight, and the HQ is no greater than 0.66/1830 = 0.0004. The worst-case HQ is well below 1, indicating negligible risk to wild mammals from FLCry1Ab in COT67B cotton.

ii. Wild birds

The acute risk to birds can be estimated from the NOEL of at least 140 mg Cry1Ab/kg body weight from the bobwhite quail study. The worst-case DDD of FLCry1Ab to wild birds is 8.82 mg/kg body weight, and therefore the HQ under worst-case exposure is no greater than 8.82/140 = 0.0630. Under more realistic exposure, the DDD is 0.12 mg/kg body weight, and the HQ is no greater than 0.81/140 = 0.0058. The worst-case HQ is well below 1, indicating negligible acute risk to wild birds from FLCry1Ab in COT67B cotton.

The risk to birds of long-term exposure to FLCry1Ab cannot be estimated as an HQ because the feed used to assess the effects of Bt11 maize in the 42-d broiler study (Brake *et al*., 2003) was not analyzed for Cry1Ab content. However, the starting grain was shown to contain 0.8 µg Cry1Ab/g and the grain comprised about 50% of the diet. Cry1Ab was detected in poultry feed formulated from Bt176 maize grain, which contains much lower amounts of Cry1Ab than does Bt11 grain (Brake and Vlachos, 1999); unfortunately loss of Cry1Ab during diet preparation cannot be quantified because Cry1Ab was below the limit of quantification in the Bt176 grain and diets. Therefore, it is likely that Cry1Ab was present in the diet prepared from Bt11 grain, but it is not possible to estimate the concentration.

If no Cry1Ab were lost during preparation, the concentration of Cry1Ab in the broiler diets would be about 0.4 µg Cry1Ab/g diet, which is below the EECs for birds exposed to COT67B seeds in their diet (Raybould, 2006a). However, the EECs are very conservative because wild birds are not common in cotton fields (Cederbaum *et al*., 2004) and hence cotton seeds are unlikely to form a significant proportion of a bird's long-term diet. Therefore, the low acute HQ from the quail study, and the likely exposure to Cry1Ab in the Bt11 maize poultry study, are sufficient to indicate low chronic risk to birds from consumption of COT67B seeds.

iii. Pollinators

The risk to pollinators can be estimated from the NOEC of at least 76.98 µg FLCry1Ab/g diet from the honeybee brood study. The EEC for pollinators is 5.45 µg FLCry1Ab/g pollen, and hence the HQ is no greater than 5.45/76.98 = 0.0708. The HQ is well below 1, indicating negligible risk to pollinators from FLCry1Ab in COT102 cotton.

iv. Above-ground non-target arthropods

The risk to above-ground non-target arthropods can be estimated from the *Orius* and *Coleomegilla* studies: the NOEC in the *Orius* study was at least 1003.9 µg FLCry1Ab/g diet, and the NOAEC[2] in the *Coleomegilla* study was 1000 µg FLCry1Ab/g diet. If adjustment is made for loss of bioactivity of FLCRY1AB-0103, the *Orius* $NOEC_{ba}$ is 223.1 µg FLCry1Ab/g diet, and the *Coleomegilla* $NOAEC_{ba}$ is 222.2 µg FLCry1Ab/g diet. The HQs calculated from the adjusted hazard estimates are denoted HQ_{ba}.

The worst-case EEC for above ground non-target arthropods is 142.01 µg/g diet, the concentration of FLCry1Ab in leaves of COT67B cotton, and the realistic EEC is 18.36 µg FLCry1Ab/g diet. The resulting HQs are all below 1:

Orius worst-case	$HQ \leq 142.01/1003.9 = 0.1414$
Orius realistic	$HQ \leq 18.36/1003.9 = 0.0183$
Orius worst-case	$HQ_{ba} \leq 142.01/223.1 = 0.6365$
Orius realistic	$HQ_{ba} \leq 18.36/223.1 = 0.0823$
Ladybeetle worst-case	$HQ \leq 142.01/1000 = 0.1420$
Ladybeetle realistic	$HQ \leq 18.36/1000 = 0.0184$
Ladybeetle worst-case	$HQ_{ba} \leq 142.01/222.2 = 0.6391$
Ladybeetle realistic	$HQ_{ba} \leq 18.36/222.2 = 0.0826$

The low HQ values indicate minimal risk to above-ground non-target arthropods from FLCry1Ab in COT67B cotton.

v. Soil-dwelling non-target invertebrates

The risk to soil-dwelling non-target invertebrates can be estimated from the rove beetle and collembola studies: the NOEC in the rove beetle study was at least 1000 µg FLCry1Ab/g diet, and the NOEC in the collembola study was 17.1 µg Cry1Ab/g diet. If adjustment is made for loss of bioactivity of FLCRY1AB-0103, the rove beetle $NOEC_{ba}$ is 222.2 µg FLCry1Ab/g diet.

The worst-case EEC for soil-dwelling non-target invertebrates is 14.08 µg/g diet, the concentration of FLCry1Ab in roots of COT67B cotton, and the realistic EEC is 0.17 µg FLCry1Ab/g soil. The resulting HQs are all below 1:

Rove beetle worst-case	$HQ \leq 14.08/1000 = 0.0141$
Rove beetle realistic	$HQ \leq 0.17/1000 = 0.0002$
Rove beetle worst-case	$HQ_{ba} \leq 14.08/222.2 = 0.0634$
Rove beetle realistic	$HQ_{ba} \leq 0.17/222.2 = 0.0008$
Collembola worst-case	$HQ \leq 14.08/17.1 = 0.8234$
Collembola realistic	$HQ \leq 0.17/17.1 = 0.0099$

[2] NOAEC used because there was a small, but statistically significant difference in the weight of adults

The low HQ values indicate minimal risk to soil-dwelling non-target invertebrates from FLCry1Ab in COT67B cotton.

vi. Aquatic invertebrates

Exposure of aquatic organisms to FLCry1Ab will be minimal because cotton tissue, including pollen, is unlikely to be deposited in ponds, lakes or watercourses in significant quantities. Therefore the risk to aquatic organisms can be judged negligible from exposure arguments alone. A study of *Daphnia magna* exposed to high densities of maize pollen containing Cry1Ab showed that in the unlikely event of small quantities of COT67B material entering a water body, the likelihood of harmful effects to aquatic organisms is minimal.

vii. Farmed fish

The risk to farmed fish can be estimated from the NOAEC[3] of at least 8.13 µg FLCry1Ab/g feed, and the $NOAEC_{ba}$ of at least 1.81 µg FLCry1Ab/g feed from the catfish study. The worst-case and realistic EECs for farmed fish from COT67B cottonseed meal are 14.99 and 7.50 µg FLCry1Ab/g feed, respectively. Several HQs can be calculated:

Worst-case	$HQ \leq 14.99/8.13 = 1.84$
Realistic	$HQ \leq 7.50/8.13 = 0.92$
Worst-case	$HQ_{ba} \leq 14.99/1.81 = 8.28$
Realistic	$HQ_{ba} \leq 7.50/1.81 = 4.14$

Apart from the realistic HQ, the values are above 1; this does not indicate risk to fish exposed to feed made from COT67B cottonseed meal, simply that the concentration used in the fish study is below the conservative exposure estimates.

Three highly conservative assumptions were made in the calculation of the EECs for farmed fish. First, the concentration of FLCry1Ab was estimated from untoasted cottonseed meal; fish feed is likely to be manufactured from toasted meal, which will contain much lower concentrations of FLCry1Ab than does the raw meal. Secondly, it is assumed that no loss of bioactivity of FLCry1Ab will occur during the manufacture of fish feed; this is unlikely as high temperatures are used during pelleting of commercial feeds. Finally, the EEC calculations assume that feed will be made from batches of meal derived from COT67B seeds only; it is likely that COT67B seeds will be mixed with other seed of other cotton varieties before use in feed manufacture. These considerations show that the HQs are very conservative, and the risk to farmed fish from feed made from COT67B cottonseed meal is low.

3. Summary Risk Assessment

[3] NOAEC used because there was a small, but statistically significant difference in the weight of the fish

i. NTOs in cotton fields

No adverse effects were seen in studies of the effects of full-length and truncated Cry1Ab on indicator species representative of NTOs found in cotton fields. Comparison of the NOECs (or NOAECs) or NOELs with EECs or estimated DDDs indicated that in most studies, organisms were exposed to amounts of Cry1Ab far greater than they are likely to be exposed to *via* cultivation of COT67B cotton (Table 6). Therefore the lack of adverse effects in the studies indicates low risk to NTOs with high certainty.

ii. NTOs outside cotton fields

The accompanying environmental fate assessment (Raybould, 2006a) indicates that significant exposure to FLCry1Ab is highly likely to be limited to cotton fields. Therefore, FLCry1Ab in COT67B cotton presents low risk to NTOs outside cotton fields. The exception is farmed fish that may be exposed to FLCry1Ab *via* fish feed manufactured from cottonseed meal. The HQs for FLCry1Ab and fish are approximately 1 or greater. However, the HQs represent very conservative EECs, rather than high risk of FLCry1Ab. When more realistic exposures are considered, the risk to farmed fish is low.

Table 5. Summary of ecotoxicology studies on FLCry1Ab and Cry1Ab

Species	Test substance	Endpoints	Result
Mouse	Microbial FLCry1Ab	14-d mortality and growth	NOEL ≥ 1830 mg FLCry1Ab/ kg bw
Bobwhite quail	Cry1Ab leaf protein	14-d mortality and growth	NOEL ≥ 140 mg Cry1Ab/ kg bw
Poultry		49-d mortality and growth	Not determined
Honeybee	Microbial FLCry1Ab	34-d larval survival	NOEC ≥ 76.98 µg FLCry1Ab/g diet
Ladybeetle[†]	Microbial FLCry1Ab	Larval and adult mortality	NOAEC ≥ 1000 µg FLCry1Ab/g diet $NOAEC_{ba}$ ≥ 223.1 µg FLCry1Ab/g diet
Orius	Microbial FLCry1Ab	Pre-imaginal survival	NOEC ≥ 1003.9 µg FLCry1Ab/g diet $NOEC_{ba}$ ≥ 223.1 µg FLCry1Ab/g diet
Rove beetle	Microbial FLCry1Ab	Reproduction	NOEC ≥ 1000 µg FLCry1Ab/g diet $NOEC_{ba}$ ≥ 222.2 µg FLCry1Ab/g diet
Collembola	Cry1Ab maize leaves	Mortality and reproduction	NOEC ≥ 17.1 µg Cry1Ab/g diet
Daphnia[#]	Cry1Ab maize pollen	2-d neonate survival	NOEC ≥1.8540 µg Cry1Ab/L
Catfish	Microbial FLCry1Ab	30-d juvenile survival	NOAEC ≥ 8.13 µg Cry1Ab/g feed $NOAEC_{ba}$ ≥ 1.81 µg Cry1Ab/g feed

[†]*Coleomegilla maculata*

[#]NOEC based on density of 150 mg pollen/L water; concentration of Cry1Ab in pollen = 12.36 µg/g

Table 6. Hazard quotients for NTOs potentially exposed to FLCry1Ab via COT67B cotton

Species	NTO Group Represented	Worst-case EEC	Realistic EEC
Mouse	Wild mammals	HQ ≤ 0.0050	HQ ≤ 0.0004
Bobwhite quail	Wild birds	≤ 0.0630	HQ ≤ 0.0058
Poultry	Wild birds	N.D.	N.D.
Honeybee	Pollinators	HQ ≤ 0.0708	HQ ≤ 0.0708
Ladybeetle†	Above-ground arthropods	HQ ≤ 0.1420	HQ ≤ 0.0184
		HQ_{ba} ≤ 0.6391	HQ_{ba} ≤ 0.0826
Orius nymphs	Above-ground arthropods	HQ ≤ 0.1414	HQ ≤ 0.0183
		HQ_{ba} ≤ 0.6365	HQ_{ba} ≤ 0.0823
Rove beetle	Soil invertebrates	HQ ≤ 0.0141	HQ ≤ 0.0002
		HQ_{ba} ≤ 0.0634	HQ_{ba} ≤ 0.0008
Collembola	Soil invertebrates	HQ ≤ 0.8244	HQ ≤ 0.0099
Daphnia	Aquatic invertebrates	No exposure	No exposure
Catfish	Farmed fish	HQ ≤ 1.84	HQ ≤ 0.92
		HQ_{ba} ≤ 8.28	HQ_{ba} ≤ 4.14

†*Coleomegilla maculata*
ND = not determined

iii. Endangered and threatened species

The hazard data summarized above, and studies of the pest spectrum and mode of action of FLCry1Ab (*e.g.*, Gill *et* al., 1992; Schnepf *et al*., 1998), indicate that FLCry1Ab is highly unlikely to be toxic to non-lepidopteran species at concentrations produced by COT67B cotton. A recent assessment by the US EPA (2005) indicated that the only endangered or threatened lepidopteran species known to occur in a cotton-growing county in the United States is the Kern primrose sphinx moth (*Euproserpinus euterpe*). This species does not feed on cotton, nor do its food plants, the contorted suncup (*Camissonia contorta*) and red-stem filaree (*Erodium cicutarium*), occur near cotton fields. Therefore, FLCry1Ab in COT67B cotton is predicted to have no adverse effects on endangered or threatened species in the United States.

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Stacey, D.A. (2006a) FLCry1Ab: A Laboratory Study to Determine the Effects of Full-Length Cry1Ab Protein on the Predatory Bug *Orius laevigatus* (Heteroptera: Anthocoridae). Syngenta Ltd. Report # T004346-05. Unpublished.

Stacey, D. (2006b) FL Cry1Ab: A Laboratory Study to Determine Effects of Full-Length Cry1Ab Protein on the Rove Beetle *Aleochara bilineata* (Coleoptera: Staphylinidae). Syngenta, Jealott's Hill International Research Centre, UK Study # T004347-05. Unpublished. (Included as Supplement 17 in COT67B USDA petition application no. 07-108-01p)

US EPA (2001) Biopesticides Registration Action Document – *Bacillus thuringiensis* Plant Incorporated Protectants.

US EPA (2005) Biopesticides Registration Action Document: *Bacillus thuringiensis* var. *aizawai* Cry1F and the Genetic Material (from the Insert of Plasmid PGMA281) Necessary for its Production in Cotton and *Bacillus thuringiensis* var. *kurstaki* Cry1Ac and the Genetic Material (from the Insert of Plasmid PMYC3006) Necessary for its Production in Cotton.

Petition 07-108-01p
References (missing/problems)

Chapters 1-9

- Pg. 72 – Shimada, 2006? (2005 in references)
  - 2006 is the correct year
- Pg. 82 and 127 – US EPA, 2001 (a, b, or both?)
  - Should be 2001a, b
- Pg. 82 – US EPA, 2005 (a, b, c, or all?)
  - Should be 2005a
- Pg. 110 – Rily, 1997 (is this Reily?)
  - Reily is correct
- Pg. 134 – Williams, 2005 (a or b?)
  - The correct year is 2005a
- Pg. 144 – GRIN website (not referenced)
  - GRIN, 2006 http://www.ars-grin.gov/npgs/searchgrin.html
- Pg. 149 – Bravo, 2002 and 2004 (unable to locate in text) *Remove from References*
- Pg. 151 – Federal Register, 1989 (unable to locate in text) *Remove from References*
- Pg. 157 – Noteborn, 1994 (unable to locate in text) *Remove from References*
- Pg. 158- SAS, 2000 (unable to locate in text) *Remove from References*
- Pg. 159 – Shimada, 2005 (unable to locate in text) *Remove from References*
- Pg. 160 – US EPA 1996a, b, c (unable to locate in text) *Remove from References*
- Pg. 161 – US EPA 2005a (unable to locate in text) *Remove from References*
- Pg. 162 – Van Mellaert, 1988 (unable to locate in text) *Remove from References*

Appendices 1-7

References for App. 1-5 appear to be located at the end of the petition (chapters 1-9)
References for App. 6-7 appear to be at the end of each report within each appendix. Why are these separate?
Appendix 6 is the Public Interest Document (PID) submitted to EPA December 15, 2006. The PID contains appendices comprised of stand-alone expert reports, each one containing its own reference section.
Appendix 7 is a Data Summary for the CBI Supplemental reports and contains no references

App 1-5:

- Pg. 286 – US EPA, 2001a, b, c (there is no c in references)
  - Should be EPA, 2001 a, b.
- Pg. 287 – US EPA, 2001 (a, b, or both?)
  - Should be EPA, 2001 a
- Pg. 299 – US ERS, 2006? (2007 in references)
  - Should be 2006 in References

- Pg. 301 – Wells, 2002 (not referenced)
  - Wells, R. (2002) Stem and root carbohydrate dynamics of two cotton cultivars bred fifty years apart. *Agronomy Journal* **94**: 876-882.

App 6-7:

All of the below refer to App. 6 (PID). There were no references for App. 7.

- Pg. 304 – USDA AMS, 2006 (referenced in petition, but not at the end of public interest document)
  - US Department of Agriculture, AMS (Agricultural Marketing Service) (2006), U. S. Dept. of Agriculture http://www.ams.usda.gov
- Pg. 305 – FIFRA and Federal Register Notice, 1986 (not referenced). All other Fed Reg referenced throughout the petition and supporting documentation.
  - Federal Register, 51 FR, 5 March 1986.
- Pg. 308 – NCAP, 2005 (not referenced)
  - NCAP (National Center for Food & Agricultural Policy) (2005) Biotechnology-Derived Crops Planted in 2004 – Impacts on U.S. Agriculture http://www.ncfap.org/
- Pg. 326 – Sankula, 2005 (unable to locate in text) *Remove from References*
- Pg. 332 – US EPA, 2000? (no year in references)
  - 2000 is correct.
- Pg. 343 – Boquet, 2006 (unable to locate in text) *Remove from References*
- Pg. 345 – May and Lege, 1999? (no year in references)
  - Should be 1999 in References
- Pg. 349 – Rummel, 1978 (not referenced) *Remove from References*
- Pg. 360 – Kurtz et al., 2006 (not referenced)
  - Kurtz, R., A. McCaffery, D. O'Reilly and T. Stone. (2006). Insect resistance management considerations for VipCot™ cotton. Syngenta Seeds Biotechnology Report No. SSB-170-06.
- Pg. 372 – Goldman et al., 1998? (1996 in references)
  - Should be 1996
- Pg. 373 – Anderson, 2006 (unable to locate in text) *Remove from References*
- Pg. 374 – Proceedings Beltwide Cotton Conferences (unable to locate in text) *Remove from References*
- Pg. 375 – McCaffery, 2006 (unable to locate in text) *Remove from References*

Supplemental sections 1-8

- Pg. 3, sup 1 – Federal Register, 1989 (not referenced)
  - Federal Register, 40 CFR, Part 160, 17 August 1989
- Pg. 18, sup 1 – Kaster, 1983 (unable to locate in text) *Remove from References*
- Pg. 18, sup 2 – Tijssen, 1985 (unable to locate in text) *Remove from References*
- Pg. 13, sup 6 – Barton, 1987 (unable to locate in text) *Remove from References*
  Tijssen, 1985 (unable to locate in text) *Remove from References*

- Pg. 3, sup 8 – Fed Reg for GLPs are referenced in most sup sections, any reason why UK GLPs are not?
  - The citation is the reference. An internet search yielded the following website: http://www.opsi.gov.uk/si/si2004/20040994.htm
- Pg. 10, sup 8 – Redbook, 2000 (not referenced)
  - *http://www.cfsan.fda.gov/~redbook/red-toca.html*

Supplemental sections 9-14

- Pg. 3, sup 11 – Federal Register, 1989 (not referenced)
  - Federal Register, 40 CFR, Part 160, 17 August 1989
- Pg. 5, sup 11 – Accelrys, 2001 (not referenced)
  - http://www.accelrys.com/products/datasheets/gcg_data.pdf
- Pg. 13, sup 12 – FIFRA SAP, 2002 (not referenced)
  - FIFRA Scientific Advisory Panel (2002) Corn Rootworm Plant-Incorporated Protectant Insect Resistance Management and Non-Target Insect Issues US EPA August 27-29, 2002

Supplemental sections 15-20

- Pg. 60, sup 16 – Geiser, 1986 (unable to locate in text) *Remove from References*
- Pg. 49, sup 18 – Vinall, 2006 (unable to locate in text) *Remove from References*
  FIFRA SAP, 2004 (unable to locate in text) *Remove from References*
- Pg. 72, sup 18 – Geiser, 1986 (unable to locate in text) *Remove from References*
- Pg. 120, sup 18 – Crickmore, 2005 (unable to locate in text) *Remove from References*
- Pg. 24, sup 19 – Howald et al., 2003? (2002 in references)
  - Should say 2003 in text instead of 2002; 2003 in references is correct
- Pg. 29, sup 19 – Crickmore, 2005 (unable to locate in text) *Remove from References*
- Pg. 3, sup 20 – Federal Register, 1989 (not referenced)
  - Federal Register, 40 CFR, Part 160, 17 August 1989
- Pg. 7, sup 20 – Kurtz et al., 2006 (not referenced)
  - Kurtz, R., A. McCaffery, D. O'Reilly and T. Stone. (2006). Insect resistance management considerations for VipCot™ cotton. Syngenta Seeds Biotechnology Report No. SSB-170-06.

Supplemental sections 21-23

Changes below apply to section 22 (no page numbers in this section)

- Insect Resistance Management Considerations for VipCot™ Cotton:
  - MRID 45766501 (is this Artim, 2002?)
    - Yes
  - Nagamatsu et al., 1999 (not referenced)
    - Nagamatsu, Y., T. Koike, K. Sasaki, A. Yoshimoto, and Y. Furukawa. 1999. The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal Cry1Aa toxin. FEBS Lett. **460:**385–390.

- Blanco et al., 2002 (not referenced) also, MRID 45808417 belongs with Blanco?
  - This is a citation in Dow AgroScience's Widestrike IRM plan review; the review was quoted in the COT102 x COT67B EPA Sec.3 IRM volume (Supplement 22); the citation was in the quote. Blanco et al., 2002 is a study conducted for DAS and is MRID #45808417.
- Livingston et al., 2002? (2004 in references)
  - Should be 2004
- Artim, 2002 (are these two volumes? Both look the same, add a and b to distinguish)
  - There should be only one citation; the second citation was added inadvertently; remove one citation
- Caprio and Parker, 2000? (no year in references; also, submitted since 2000, hasn't been accepted? or wrong reference?)
  - Remove year and change to unpublished manuscript in text. Remove from References as it was never published.
- Banks, 2003 (unable to locate in text) *Remove from References*
- Bravo, 2004 (unable to locate in text) *Remove from References*
- Ferre, 1991 (unable to locate in text) *Remove from References*
- Gould, 2003 (unable to locate in text) *Remove from References*
- O'Reilly, 2006 (unable to locate in text) *Remove from References*

- Appendix 2
  - US EPA, 1998 (same as SAP in references?)
    - US EPA (1998) FIFRA Scientific Advisory Panel Subpanel on *Bacillus thuringiensis (Bt)* Plant-Pesticides and Resistance Management, February 9 and 10, 1998
- Appendix 3
  - Abbott, 1925 (not referenced)
    - Abbott, W.S. (1925) A method for computing the effectiveness of an insecticide. *J.Econ. Entomol.* 18:265-267.
- Appendix 6
  - Gould, 2006 (no year in references)
    - Add 2006 to reference
  - Peck, 1999 (no year in references)
    - Add 1999 to references

[This section contains Confidential Business Information]

This section contains Field Trial Reports from the following Notifications:

- 03-098-08n, 03-268-04n, 04-041-01n, 04-064-05n, 04-079-01n, 05-034-02n, 05-102-01n, 05-266-01n, 05-339-04n, 06-039-16n, and 06-060-04n.
- A CBI copy of Notification No. 06-039-16n (SYN2006-108) is also provided as requested in Question 1.
- Notification Nos. 06-223-109n and 06-223-110n refer to counter season trials currently underway in Hawaii. Field trial reports for these trials will be submitted immediately upon termination of the trials.

Janet N. Reed
Regulatory Affairs Manager
Syngenta Seeds, Inc.
P. O. Box 12257 (mail)
3054 East Cornwallis Road
Research Triangle Park, North Carolina 27709-2257

September 14, 2007

Ms. Margaret Jones
USDA-APHIS-Biotechnology Regulatory Services
4700 River Road, Unit 147
Riverdale, MD 20737

Re: Review for Technical Completeness of Petition Numbered 07-108-01p for a Determination of Non-Regulated Status for COT67B

Dear Ms. Jones:

This letter is in response to APHIS/BRS request dated September 7, 2007 concerning additional clarification on deficiencies noted June 9, 2007 in Petition No. 07-108-01p, application for determination of non-regulated status for COT67B. As done previously, Syngenta's responses are incorporated directly into the Agency letter. A CD of this letter and Syngenta's responses accompany a hard copy sent by overnight courier September 19, 2007.

If there are additional questions regarding the responses or the Petition please do not hesitate to contact me.

Sincerely,

Janet N. Reed
Regulatory Affairs Manager
Syngenta Seeds, Inc.
Tel. (919) 765-5076; Fax: (919) 541-8535; janet.reed@syngenta.com

cc: Terry B. Stone, Manager, Regulatory Affairs, Syngenta Seeds, Inc.
Lawrence W. Zeph, Regulatory Manager, NAFTA, Syngenta Seeds, Inc.
Lisa W. Zannoni, Head, Global Biotechnology Regulatory Affairs, Syngenta Seeds, Inc.

Addendum to Syngenta Petition Number 07-108-01p
September 14, 2007

USDA question/issue:

1. According to the field test reports submitted on July 20, 2007, the 05-339-04n field test site was not planted. However, on page 28 of the petition, Table 1-1 (Trials Conducted Under These Notifications), indicates that 05-339-04n was planted.

2. There are no indications from the July 20 2007 submitted field test reports for 06-039-16n or 06-060-04n that field trials were in fact conducted in CA and FL or TX, respectively, as indicated in Table 1-1 of the petition.

Syngenta response:

Table 1-1, USDA Notifications Approved for COT67B and Status of Trials Conducted Under These Notifications (page 28 of Petition), is amended as shown below with regard to trial status for notifications 05-266-01n, 05-339-04n, 06-039-16n, and 06-060-04n. The changes are bolded.

Table 1-1. USDA Notifications Approved for COT67B and Status of Trials Conducted Under These Notifications

USDA No.	Effective Dates	Release Site (State)	Trial Status
2003 Field Trials			
03-098-08n	5/8/2003	MS	Report Submitted to USDA July 28, 2004
03-268-04n	10/25/2003	PR	Report Submitted to USDA September 21, 2004
2004 Field Trials			
04-041-01n	3/26/2004	AR, FL, GA, LA, MS, NC, TN	Report Submitted to USDA August 24, 2005
04-064-05n	4/8/2004	AZ	Report Submitted to USDA October 12, 2005
04-079-01n	4/18/2004	TX	Report Submitted to USDA August 9, 2005
2005 Field Trials			
05-034-02n	3/28/2005	AL, AR, AZ, GA, LA, MO, MS, NC, SC, TX	Report Submitted to USDA October 30, 2006
05-102-01n	5/17/2005	CA	Report Submitted to USDA October 30, 2006
05-266-01n	11/7/2005	HI	**Submitted May 31, 2007**
2006 Field Trials			
05-339-04n	1/4/2006	MS, PR	**Sites not planted; Field Trial Termination Report Summary submitted February 20 and July 20, 2007 (Summary indicates that sites were not planted)**
06-039-16n	3/14/2006	AL, AR, AZ, CA, FL, GA, LA, MO, MS, NC, SC, TN, TX	**Reports Submitted to USDA July 16, 2007; CA and FL sites not planted**
06-060-04n	5/2/2006	NC, TX	**NC Report Submitted July 16, 2007; TX not planted**
06-223-109n	10/26/2006	HI	In Progress
06-223-110n	10/26/2006	HI	In Progress

USDA question/issue:

3. For each notification that was field tested with COT67B when there was a discrepancy between the line number that was planted (e.g. 43-67B, CE43-67B, 67B, 43-67), the genotype of COT67B (whether or not it contained the aph4 gene) or the promoter; Notify BRS compliance of the errors and changes in the nomenclature.

Syngenta response:

The table below lists the designations used throughout the development of cotton event COT67B and the documents with which the designations are associated. Several different designations were used during the Research and Development phases prior to settling on the designation of COT67B during the Regulatory phase. Several typographical errors were also discovered and are likewise noted.

As indicated in the Petition, COT67B does not contain the *aph4* gene. As per 7 CFR Part 340 Sec.3B, Syngenta customarily includes in the USDA notifications information on the constructs used to generate the initial transformation event. Subsequent breeding activities to segregate a selectable marker gene such as *aph4* would not be captured in the notifications. As demonstrated in Chapter 3 of the Petition (molecular characterization), the two T-DNA system of transformation was employed specifically to enable the identification of transformation events in which the selectable marker gene had segregated away from the insecticide gene. Such was the case for event COT67B.

To reiterate, the information contained in the notifications listed below pertains to the constructs used in producing the initial transformation event. COT67B (otherwise known as CE43-67 and CE43-67B) does not contain the *aph4* gene or any of the functional elements contained in the transformation construct pNOV1914. This Syngenta response and the table below will be provided to BRS Compliance.

Table listing the designations used throughout the development of cotton event COT67B and the documents with which the designations are associated.

USDA Notification Number	Event Designation on Notification	Event Designation on Petition 07-108-01p	Event Designation on Field Trial Report	Aph4 gene, ubiquitin 3 promoter or pNOV1914 backbone present?
03-098-08n	CE43-67	COT67B	CE43-67B	No
03-268-04n	CE43-67B	COT67B	CE43-67B	No
04-041-01n	CE43-67	COT67B	CE43-67B, CE43-67	No
04-064-05n	CE43-67	COT67B	43-67	No
04-079-01n	CE43-67	COT67B	CE43-67B	No
05-034-02n	CE43-67	COT67B	CE43-67B	No
05-102-01n	CE43-67	COT67B	43-67B, CE43-67B, CE43-67, CE43-97*, 67B, 43-67B, 44-67B*	No
05-266-01n	CE43-67	COT67B	67B	No
05-339-04n	CE43-67	COT67B	Site not planted	No
06-039-16n	CE43-67	COT67B	67B, COT67B, 6TB*, 67B null	No
06-060-04n	CE43-67	COT67B	67B	No

* Typographical error

USDA question/issue:
4. Provide a copy of Reed, 2006 referenced in page 18 of the response to the deficiency letter.

Syngenta response:
The publication is attached.

USDA question/issue (bold) and Syngenta response:

5. The submitted honeybee studies are inadequate for APHIS to make a determination for the following reasons: (A, B, C below)

Therefore, please provide summary studies from scientifically rigorous honeybee studies. This may be garnered though scientific literature data mining and studies from previously deregulated events such as Event 176 and Bt11.

A. Cry1Ab activity was not verified at the end of the study.

The hypothesis that Cry1Ab is stable in sucrose solutions has been tested previously:

1. S. Sims. (1994) Stability of the CryIA(b) Insecticidal protein of *Bacillus thuringiensis* var. kurstaki (B.t.k. HD-1) in sucrose and honey solutions under non-refrigerated temperature conditions: Study Number IRC-91-ANA-11. Unpublished study prepared by Monsanto Co. 32 p. US EPA MRID 434680-02; cited in the US EPA Biopesticides Registration Document (2001) http://www.epa.gov/pesticides/biopesticides/pips/bt_brad2/3-ecological.pdf

   The study showed that a microbial test substance containing Btk HD-1 (deemed to be a suitable surrogate for Cry1Ab expressed in Events Bt11, Bt176 and MON810 maize) was stable for up to 7 days at room temperature in a 1:1 mixture of honey and sucrose solution; the test substance retained bioactivity.

2. J. Romeis, A. Dutton and F. Bigler (2004) *Bacillus thuringiensis* (Cry1Ab) has no direct effect on larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae). Journal of insect Physiology 50, 175-183.

   Bioassays using European corn borer showed no decrease in the bioactivity of purified Cry1Ab in 2M sucrose solution over 2 days at 23^{o}C.

Artificial diets containing sucrose and purified Cry1Ab are routinely used for screening insects for susceptibility or resistance to Cry1Ab:

1. F. Alinia, M.B. Cohen and F. Gould (2000) Heritability of tolerance to the Cry1Ab toxin of *Bacillus thuringiensis* in *Chilo suppressalis* (Lepidoptera: Crambidae). Journal of Economic Entomology 93, 14-17.
2. D. Santoso, T. Chaidamsari, S. Wiryadiptura and R.A. de Maagd (2004) Activity of *Bacillus thuringiensis* toxins against cocoa pod borer larvae. Pest Management Science 60, 735-838.

Syngenta believes there is a weight of evidence to conclude that FL Cry1Ab was bioactive in the sucrose solution supplied to the honeybee hives.

B. The analysis was done on a population of individuals (brood) and not on individuals, making a conclusion of a NOEC untenable.

There were several endpoints for this study. The most important endpoint (because it is probably the most sensitive) was brood development, which was assessed in cohorts of 100 individuals that were eggs, young larvae or old larvae at the beginning of the study. In each of the treated hives, cells in the comb were marked, so the fate of individuals could be followed. The hypothesis of no effect of FLCry1Ab was tested by comparing the proportion of individuals that developed successfully in hives exposed to sucrose solution containing FLCry1Ab with the number that developed successfully in hives exposed to untreated sucrose solution. The concept and analysis

is the same as laboratory experiments that estimate NOECs from effects on individuals in separate containers: the proportion of individuals showing a response in the treatment is compared with the proportion showing a response in the negative control.

A second endpoint was adult bee mortality, measured as the number of individuals collected in dead-bee traps. In this case, the hypothesis of no effect of FLCry1Ab was tested by comparing the distribution of dead bees between the FLCry1Ab-treated hives and the control hives with a random distribution of dead bees between the treatment and control hives. The hives were of similar size at the start of the experiment, so the numbers of dead bees can be used to infer whether the proportion of dead adults differed between the treatments. A third endpoint was the condition of the hives. The hypothesis of no effect of FLCry1Ab was tested by comparing the proportion of the combs in hives exposed to FLCry1Ab that were covered by bees (eggs, larvae and pupae), by stores (pollen, nectar and honey), or were empty, with the proportions in control hives.

The dead-bee counts and the colony condition endpoints are equivalent to population size and species diversity endpoints in mesocosm experiments: the effects of a treatment are estimated by changes in the number of individuals or species, or in the proportion of individuals of a certain type or species, not by the fate of known individuals. NOECs of crop protection products are routinely estimated by effects on population endpoints in mesocosms, and such estimates are used to infer NOECs for ecosystems (*e.g.*, Van Wijngaarden *et al.*, 2005):

R.P.A. van Wijngaarden, T.C.M. Brock and P.J. van den Brink (2005) Threshold levels for effects of pesticides in freshwater ecosystems: a review. Ecotoxicology 14, 355-380.

In summary, the bee brood study derives a NOEC in a manner equivalent to other regulatory studies. Syngenta believes the bee study provides a rigorous test of the hypothesis that the NOEC of FL Cry1Ab to honeybees is at least 77 µg/g sucrose solution.

C. There is lack of evidence that the test diet was in fact consumed, as it may have been stored.

It is unlikely that the test diet was stored. First, there were significant adverse effects on brood development in hives exposed to sucrose solution containing the toxic reference substance Dimilin® Flo: there were no survivors among cohorts aged between 1 and 3 days when exposure began. Secondly, as recommended by the guideline, the timing of the study was chosen specifically to minimize the availability other sources of food for the bees. The study site was in agricultural land that is a mixture of pasture and arable. Oilseed rape (*Brassica napus*), which is the main arable crop that could provide abundant food for bees in the area, had finished flowering before the start of the study. Despite the lack of abundant food sources other than the sucrose solution, the colonies appeared healthy and the brood developed normally. Finally, the guideline protocol is considered to be a suitable method for assessing the effects of plant protection products by the European and Mediterranean Plant Protection Organization (EPPO, 2003):

EPPO (2003) Environmental risk assessment scheme for plant protection products: honeybees. EPPO Bulletin 33, 141-145.

Syngenta believes that the hypothesis that the bees were exposed to the test item was adequately tested.

Additional weight of evidence of minimal toxicity of FL Cry1Ab to honeybees at concentrations in COT67B cotton

The realistic worst-case exposure of honeybees to FL Cry1Ab is 5.45 µg/g diet – the mean concentration of FL Cry1Ab in pollen of COT67B cotton. The following studies exposed honeybees to concentrations in excess of 5.45 µg Cry1Ab/g sucrose solution diet.

1. V. Maggi and S. Sims (1994) Evaluation of the Dietary Effects of Purified B.t.k. Endotoxin Proteins on Honey Bee Larvae: Lab Project Number: IRC-91-ANA-13. Unpublished study prepared by Monsanto Co. 51 p. US EPA MRID 434392-02.

Results: No effect of 20 µg Cry1Ab/g sucrose diet on the survival of honeybee larvae. Exposure to Cry1Ab was confirmed by bioassay of diet – see Simms (1994) cited above.

2. D. Babendreier, N.M. Kalberer, J. Romeis, P. Fluri, E. Mulligan and F. Bigler (2005) Influence of Bt-transgenic pollen, Bt-toxin and protease inhibitor (SBTI) ingestion on development of the hypopharyngeal glands in honeybees. Apidologie 36, 585-594.

Results: No effect of 10 µg Cry1Ab/g sucrose diet on the survival or hypopharyngeal gland development of adult honeybees. Exposure to Cry1Ab was not confirmed as part of the study, but the stability of the source of Cry1Ab in sucrose solution was tested by Romeis et al. (2004) cited above.

All references are provided.

Addendum to Syngenta Petition Number 07-108-01p
September 19, 2007

Submitted by Syngenta September 19, 2007 in response to September 7, 2007 request from USDA for additional clarification on items noted in June 19, 2007 deficiency letter.

USDA question/issue:

3. For each notification that was field tested with COT67B when there was a discrepancy between the line number that was planted (e.g. 43-67B, CE43-67B, 67B, 43-67), the genotype of COT67B (whether or not it contained the aph4 gene) or the promoter; Notify BRS compliance of the errors and changes in the nomenclature.

Syngenta response:

The table below lists the designations used throughout the development of cotton event COT67B and the documents with which the designations are associated. Several different designations were used during the Research and Development phases prior to settling on the designation of COT67B during the Regulatory phase. Several typographical errors were also discovered and are likewise noted.

As indicated in the Petition, COT67B does not contain the *aph4* gene. As per 7 CFR Part 340 Sec.3B, Syngenta customarily includes in the USDA notifications information on the constructs used to generate the initial transformation event. Subsequent breeding activities to segregate a selectable marker gene such as *aph4* would not be captured in the notifications. As demonstrated in Chapter 3 of the Petition (molecular characterization), the two T-DNA system of transformation was employed specifically to enable the identification of transformation events in which the selectable marker gene had segregated away from the insecticide gene. Such was the case for event COT67B.

To reiterate, the information contained in the notifications listed below pertains to the constructs used in producing the initial transformation event. COT67B (otherwise known as CE43-67 and CE43-67B) does not contain the *aph4* gene or any of the functional elements contained in the transformation construct pNOV1914. This Syngenta response and the table below will be provided to BRS Compliance.

Table listing the designations used throughout the development of cotton event COT67B and the documents with which the designations are associated.

USDA Notification Number	Event Designation on Notification	Event Designation on Petition 07-108-01p	Event Designation on Field Trial Report	Aph4 gene, ubiquitin 3 promoter or pNOV1914 backbone present?
03-098-08n	CE43-67	COT67B	CE43-67B	No
03-268-04n	CE43-67B	COT67B	CE43-67B	No
04-041-01n	CE43-67	COT67B	CE43-67B, CE43-67	No
04-064-05n	CE43-67	COT67B	43-67	No
04-079-01n	CE43-67	COT67B	CE43-67B	No
05-034-02n	CE43-67	COT67B	CE43-67B	No
05-102-01n	CE43-67	COT67B	43-67B, CE43-67B, CE43-67, CE43-97*, 67B, 43-67B, 44-67B*	No
05-266-01n	CE43-67	COT67B	67B	No
05-339-04n	CE43-67	COT67B	Site not planted	No
06-039-16n	CE43-67	COT67B	67B, COT67B, 6TB*, 67B null	No
06-060-04n	CE43-67	COT67B	67B	No

* Typographical error

<janet.reed@syngenta.com>
12/18/2007 10:18 AM

To <MargaretJones@aphis.usda.gov>
cc <terry-1.stone@syngenta.com>
bcc
Subject Syngenta Petition No. 07-108-01p

Dear Margaret,

Here is a summary describing efficacy of FLCry1Ab. As requested via voicemail, please replace this summary with that provided previously on December 11, 2007. I will call today but please leave a voicemail on either work or cell (919.812.1877) should we end up playing phone tag and you need to discuss further.

COT67B Efficacy Against Target Pests *Heliothis virescens* (TBW), *Helicoverpa zea* (CBW) and *Pectinophora gossypiella* (PBW)

Resistance of COT67B cotton plants to damage caused by *Heliothis virescens* (tobacco budworm, TBW) and *Helicoverpa zea* (cotton bollworm, CBW) has been evaluated in field studies since 2003. The data from field studies conducted in 2005 and 2006 is considered representative of that from previous years and is summarized below. A combination of field and laboratory "high dose" studies was used to assess the resistance of COT67B cotton plants to *Pectinophora gossypiella* (pink bollworm, PBW). The results are likewise described below.

COT67B Efficacy Against TBW and CBW

The purpose of the studies was to evaluate the resistance of COT67B cotton plants to damage caused by *Heliothis virescens* (tobacco budworm) and *Helicoverpa zea* (cotton bollworm). The trials were performed by both Syngenta scientists and university co-operators under conditions of artificial and/or natural infestation of *H. virescens* and *H. zea.* The trials were located in principal cotton production regions across the mid-south and southeastern US cotton belt.

Preliminary data (fruiting structure damage) from three locations obtained in 2005 from both natural and artificial infestations clearly demonstrated the efficacy of COT67B against these pests as the damage sustained by the fruiting structures of COT67B was clearly and substantially less than the nontransgenic control plants, Coker 312. Square, flower and boll damage ratings were taken in 2006 from between four and seven locations. Again, damage to the Coker 312 cotton plants was significantly greater (5% level) than that sustained by the COT67B cotton plants. These data confirm the preliminary indications obtained in 2005 that COT67B cotton has excellent activity and resistance to these key heliothine cotton pests in the US.

COT67B Efficacy Against PBW

The 1998 Scientific Advisory Panel Subpanel agreed with EPA that an "appropriate resistance management strategy is necessary to mitigate the development of insect resistance to Bt proteins expressed in transgenic crop plants" (EPA 1998, 2001). Furthermore, as extensively reviewed (EPA 1998), the Subpanel recognized that IRM programs should ideally be based on the use of a combination of a high expressed dose of the Bt plant-incorporated protectant (PIP) and structured refuges of conventional, non-transgenic variety(s) of the crop in question (Alstad and Andow 1995; Andow and Alstad 1998; Andow and Hutchison 1998; Gould 1998; Roush 1997; Tabashnik 1994).

Central to this strategy is the definition of "high dose". High dose expression is essential in order that any heterozygous (RS) individuals (deriving from crosses between [rare] homozygous resistant individuals and [abundant] susceptible insects from the refuge) are killed following feeding on the transgenic variety. The 1998 SAP Subpanel defined and the 2000 SAP Subpanel confirmed, (EPA 1998, 2001) a high dose for a Bt PIP as "25 times the protein necessary to kill susceptible larvae" and the EPA has adopted this 25x definition of high dose. To be able to demonstrate high dose, registrants are required to provide data generated by at least two of the following five laboratory and field approaches as set out by EPA in the 2001 Biopesticides Registration Action Document (EPA 2001).

1. 1. Serial dilution bioassay with artificial diet containing lyophilized tissues of Bt plants using tissues from non-Bt plants as controls.
2. 2. Bioassays using plant lines with expression levels approximately 25-lower than the commercial cultivar determined by quantitative ELISA or some other reliable technique
3. 3. Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed
4. 4. Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} similar to field strains; and
5. 5. Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of neonate larvae. If so the later stage could be tested on the Bt crop plants to determine if 95% or more of the later stage larvae were killed"

Methods 1, 4 and 5 are the most amenable to practical study and are the most widely used (e.g. EPA 2002, 2004) by registrants to demonstrate high dose of Bt-PIPs. Syngenta scientists have used Methods 1 and 4 to show high dose for COT67B against PBW.

Using EPA Method 1, neonate PBW were fed lyophilized transgenic plant tissue directly incorporated into the diet at a minimum 25-fold dilution as described in Method 1. Based on the results of seven tests with independent sources of boll material and insects, COT67B cotton contains a protein dose sufficient to kill 100% of neonate PBW when fed at least a 25-fold dilution of lyophilized plant tissue. This activity meets the standard for demonstrating a high dose through EPA Method 1.

Using EPA Method 4, field plots of transgenic COT67B and nontransgenic Coker 312 control cotton plants were artificially infested with laboratory strains of PBW larvae to determine if the dose of proteins in COT67B cotton was at or greater than the $LD_{99.9}$ for the key target pests as described in EPA Method 4. The data showed that COT67B cotton killed 99.92% of infested PBW while surviving larvae were observed in the nontransgenic Coker 312 control. This potency meets the standard for demonstrating a high dose through EPA Method 4 thereby demonstrating that COT67B cotton expresses a high dose of FLCry1Ab for PBW using two EPA prescribed Methods (1 and 4).

References

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Please contact me should further clarification be needed.

Best Regards, Janet

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