EXPERIMENTAL USE PERMIT REQUEST

For

"*Bacillus thuringiensis* Cry2Ae Insecticidal Protein as Expressed in Cotton Plants", and "*Bacillus thuringiensis* Cry1Ab and Cry2Ae Insecticidal Proteins as Expressed in Combined Trait Cotton (TwinLink cotton) Plants"

Replacement Pages for 264-EUP-RUG

Administrative materials

May 22, 2008

Author:

Joe Kepiro
Registration Manager, USA

SUBMITTED BY:

Bayer CropScience LP- BioScience
P.O. Box 12014
2 T.W. Alexander Dr.
Research Triangle Park, NC 27709

Total pages 59

STATEMENT OF CONFIDENTIALITY CLAIMS

Information claimed confidential that does not fall within the scope of FIFRA §10(d)(1)(A), (B),or (C) has been identified as confidential business information (CBI), under the 40 CFR 174.9 "Confidential Business Information Claims for Plant Incorporated Protectants" and 40 CFR Part 2, subpart B, has been removed and placed in a Confidential Appendix as follows:

Information claimed as CBI in this application:

Experiment Managers -Pages 2 to 56 Confidential Appendix to section G

The information claimed confidential is cited by cross reference number in the body of the study.

Justification. Certain information about Bayer CropScience's cooperators, contained in Volume I has been marked Confidential Business Information. Bayer CropScience must keep this information confidential in this EUP request to maintain its competitive position in a highly competitive market, as well as to ensure the safety of those involved. Disclosure of this information would cause substantial competitive harm to Bayer CropScience by allowing other companies to unfairly compete.

Company: Bayer CropScience LP- BioScience
P.O. Box 12014
2 T.W. Alexander Dr.
Research Triangle Park, NC 27709

Company Agent: Joe Kepiro
_____
Joe Kepiro Ph.D.
Registration Manager, USA

Date: May 22, 2008

STATEMENT CONCERNING GOOD LABORATORY PRACTICES

The information contained in this request for an Experimental Use Permit for "Experimental Use Permit Request for *Bacillus thuringiensis* Cry2Ae Insecticidal Protein as Expressed in Cotton Plants, and *Bacillus thuringiensis* Cry1Ab and Cry2Ae Insecticidal Proteins as Expressed in Combined Trait Cotton (TwinLink cotton) Plants" is presented as preliminary results and in summary form in the volumes supporting this submission. The experiments to produce the data were NOT conducted in compliance with Good Laboratory Practices (GLP) as described in 40 CFR 160, unless otherwise stated in the GLP Statement within each individual report.

Company: Bayer CropScience LP- BioScience
P.O. Box 12014
2 T.W. Alexander Dr.
Research Triangle Park, NC 27709

Submitter: _____
Joe Kepiro Ph.D.
Registration Manager, USA

Sponsor: _____
Ali Scott Ph.D.
Manager, Regulatory Affairs Region Americas

Study Director: _____
Ali Scott Ph.D.
Manager, Regulatory Affairs Region Americas

Date: May 22, 2008

Ms. Sheryl Reilly
Biopesticides and Pollution Prevention Division (7511C)
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard,
2777 S. Crystal Dr.,
Alexandria, VA 22202

Attn: Shanaz Bacchus

May 22, 2008

Subject: Request for changes/replacement in the Experimental Use Permit to allow the field testing of BCS Cry2Ae Cotton and BCS Cry1Ab x Cry2Ae combined Trait Cotton (TwinLink cotton). **Code- 264-EUP-RUG**

Dear Ms. Bacchus:

Bayer CropScience (BCS) respectfully requests the replacement of certain pages in the Experimental Use Permit 264-EUP-RUG for *Bacillus thuringiensis* Cry2Ae protein and the genetic material necessary for its production in event GHB119 and GHB714 cotton plants, and for the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) plant lines, under section 5 of the Federal Insecticide, Fungicide and Rodenticide Act.

With this letter and enclosed materials, BCS is requesting three changes:
1. the label (Section B of the EUP) is replaced per formatting changes suggested by the Agency. This will also allow harmonization with the label of another BCS EUP (264-EUP-140) for a separate product.
2. the Section G of the EUP be expanded to include further acres as initially proposed in the 264-EUP-RUG document of 2006, and to include the territory of Puerto Rico (PR), as the only addition of states.
3. the EUP is requested between the dates of Sept 01, 2008 and December 31, 2010.

The purpose of these changes are to update the acreage requirements since the original submission, and most importantly, to harmonize the requests of 264-EUP-RUG and 264-EUP-140. The trials to be conducted will have the two products listed in this EUP (Cry2Ae cotton and Cry1Ab x Cry2Ae cotton (TwinLink cotton)) planted in the same location as different treatments within the trial. In some cases, the combination product- TwinLink cotton- may be planted in the absence of Cry2Ae cotton, but no Cry2Ae cotton will be planted in the absence of TwinLink.

We request that all previous versions of Section G of this EUP are replaced by the attached document, including the Original 264-EUP-RUG request on 2006, the letter submitted on April 25, 2008 and May 19, 2008. The endangered species evaluation for PR submitted in May 19, 2008 is still applicable.

The Experimental Use Permit (264-EUP-RUG) will allow further evaluation of cotton plant lines in a wider range of environmental conditions during the next growing seasons. All cotton plants to be evaluated under this EUP contain the Cry2Ae protein and have been derived from either transformation event GHB119 or GHB714 or are combinations derived from either transformation event T303-3 or T304-40 and event GHB119 or GHB714. Cotton derived from transformation events T303-3 or T304-40 express the Cry1Ab protein and are the subject of a previously granted experimental use permit, EPA EUP No. 264-EUP-140. Several different experiments are

planned: insect efficacy trials, agronomic performance evaluation, and herbicide efficacy evaluations, as well as the production of sample material for regulatory studies. In addition to these experimental plans, introgression trials are also included for the combined trait product, and seed may be produced for future plantings of experimental field trials.

The primary goal of this experimental research program is to evaluate the Cry2Ae protein containing cotton plants, and the Cry1Ab x Cry2Ae protein containing cotton plants for their efficacy against insect pests of cotton, as well as to produce plant material for regulatory studies to support global event registration. These plants also contain herbicide tolerant inert ingredient as a selectable marker, the phosphinothricin acetyltransferase (PAT) protein that confers tolerance to glufosinate-ammonium herbicides.

Some plant material will be retained for further scientific research and planting purposes. All other plant material will be destroyed. There will be no unintentional exposure to humans or domestic animals since the program will be conducted using confinement precautions and under strict internal compliance oversight. Isolation will be maintained in order to prevent any inadvertent outcrossing (pollination) from transgenic plants to non-transgenic cotton plants that are not part of the covered trials. No environmental impact issues related to the testing of these transgenic cotton plants have been identified. The Cry1Ab and Cry2Ae proteins have specific ranges of toxicity to the target lepidopteran pests and are not expected to have an adverse effect on non-target, beneficial insects.

In total, the program will be carried out at a maximum of 47 counties across 13 states/territories. A maximum of 1,919 acres will be planted as part of the experimental use permit program of which 152 acres will be planted to Cry2Ae and 307 acres will be planted to Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton). Our proposed experimental research program will thus total 3.32 to 26.71 g of Cry1Ab protein (or 0.008 to 0.059 pounds of Cry1Ab protein) from the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) and 4.59 to 9.18 g (or 0.011 to 0.021 pounds of Cry2Ae protein) from the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) and the Cry2Ae cotton. The level of Cry1Ab and Cry2Ae protein in the different plant material is only an estimation based on our current level of information.

Competent Bayer CropScience employees will supervise the program which will be conducted by them, in addition to public and private cooperators. All responsible researchers listed are professionally qualified to accomplish their stated duties.

Certain information about BCS' cooperators, contained in this document has been marked Confidential Business Information. BCS must keep this information confidential in this EUP amendment request to maintain its competitive position in a highly competitive market. Disclosure of this information would cause substantial competitive harm to BCS by allowing other companies to unfairly compete. A confidential appendix to Section G is provided. The non-confidential amendment application is available for public review.

BCS respectfully requests that BPPD evaluate this application in time to grant this EUP by September 1, 2008. This timing will allow experiments to begin at the most opportune time for 2008 PR plantings. Please do not hesitate to contact me at (919) 549 2710, or FAX: (919) 549 3929 or Email: joe.kepiro@bayercropscience.com.

Sincerely,

Joe Kepiro

Joe Kepiro Ph.D.

TABLE OF CONTENTS

EXPERIMENTAL USE PERMIT APPLICATION FORM

Replace this sheet by appropriate form

Section A Confidential Statement of Formula and Product Chemistry

BCS Cry2Ae Cotton

Cotton derived from one of two events, GHB119 or GHB714, carrying the *cry2ae* gene and transformed using the same transformation plasmid, will be evaluated under this Experimental Use Permit.

The *cry2ae* gene was modified for expression in plants and is based upon the wild type gene isolated from *Bacillus thuringiensis.* It encodes the Cry2Ae protein, an insecticidal protein, whose effects are specific to lepidopteran insects.

Characteristics of cotton plants derived from the transformation events GHB119 and GHB714 have been described in the preliminary report cited below. Standard RFLP data for each event has been updated and copies are included with this submission and cited as supplemental information. Both events appear to carry one copy of the *bar* gene and one copy of the *cry2ae* gene.

In addition to the Confidential Statement of Formula, a summary of the Product Chemistry information for Cry2Ae protein, is described in the following studies.

Volume	*Study Title*	*MRID*
NA	Characteristics of Cry2Ae cotton plants derived from transformation events GHB119 and GHB714. Preliminary report.	46708901
NA	Characteristics of Cry2Ae cotton plants derived from transformation events GHB119 and GHB714. Preliminary Report and supplemental information.	47125101
NA	Description of the amino acid sequence of the Cry2Ae protein.	46708902

Author: Veerle Habex
Title: Scientist, Molecular and Biochemical Analytical Services

Cry1Ab x Cry2Ae Combined Trait Cotton (TwinLink cotton)

A combined trait cotton known as TwinLink cotton has been developed through conventional breeding by crossing BCS Cry1Ab Cotton, Event T303-3 or Event T304-40, with BCS Cry2Ae Cotton, either Event GHB119 or Event GHB714. Diagram 1 outlines the breeding development of Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton).

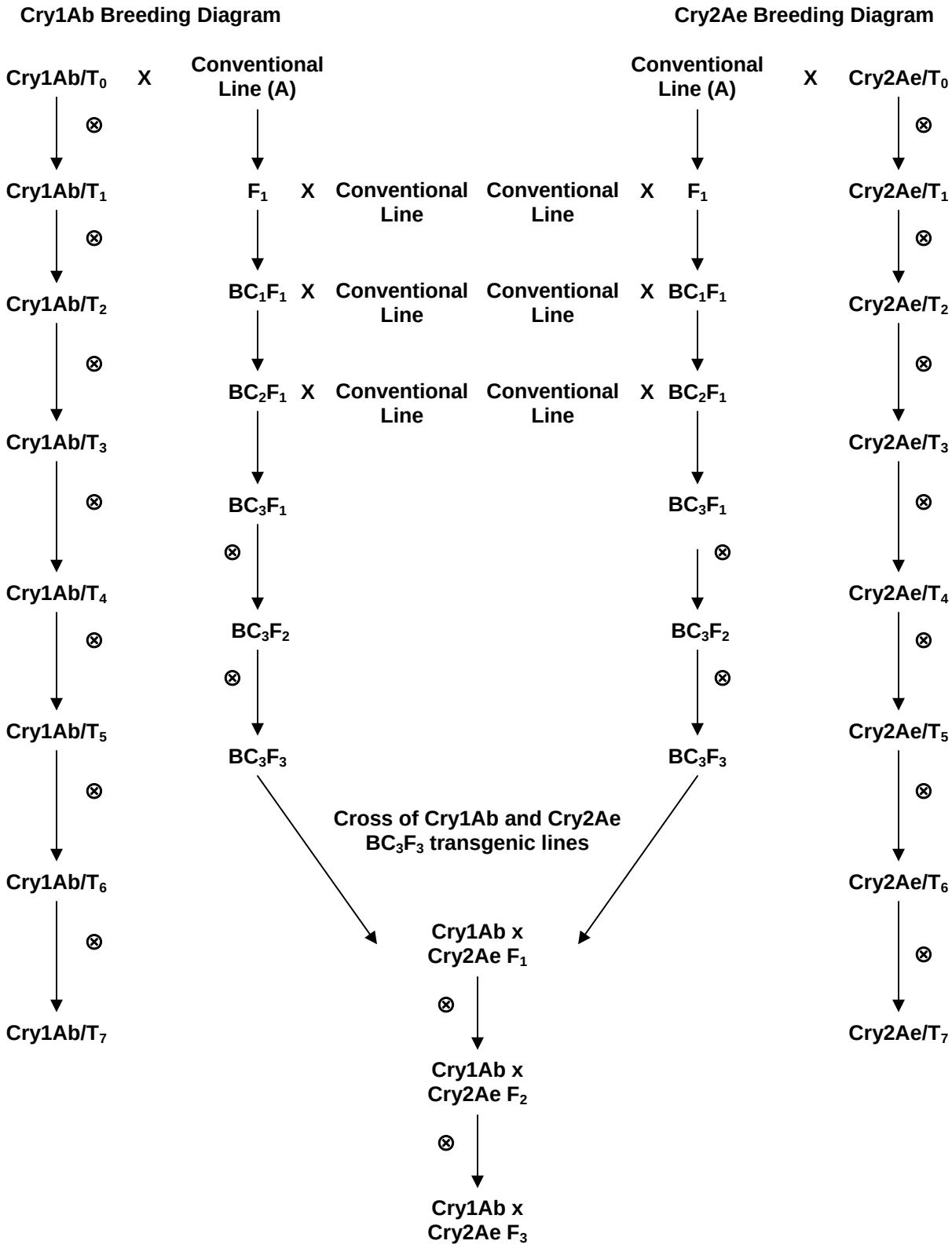
Characterization information on Cry1Ab events T303-3 and T304-40 was provided with the application for EUP No. 264-EUP-140 and is cited below. Both of these events carry the *cry1ab* gene and the *bar* gene. The *cry1ab* gene was isolated from *Bacillus thuringiensis* and modified for expression in plants. It encodes an insecticidal protein whose effects are specific to lepidopteran insects.

The combined trait cotton (TwinLink cotton) evaluated under this experimental use permit will include the genetic elements from a combination of one Cry1Ab cotton event (T303-3 or T304-40) and one Cry2Ae cotton event (GHB119 or GHB714). It is expected that the inserted genetic elements derived from any one event comprising a particular BCS Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) will be found to be identical when part of the stack.

Volume	*Study Title*	*MRID*
NA	Characteristics of Cry1Ab cotton plants derived from transformation events number T303-3 and T304-40. Preliminary report.	46788801

Author: Stefan Jansens, Ph.D.
Title: Research Program Leader Insect Control Cotton

Diagram 1: Schematic of the Breeding Development of Cry1Ab x Cry2Ae Combined Trait Cotton (also known as TwinLink cotton)



Section B Proposed labels

MASTER LABEL

BCS Cry2Ae Cotton
And
BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton)
INSECT RESISTANT COTTON SEED

FOR EXPERIMENTAL USE ONLY

Sub-label 1. BCS Cry2Ae Cotton INSECT RESISTANT COTTON SEED (Event GHB119)

Active Ingredient:
Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB119 cotton……0.0002-0.0004%*

Inert Ingredients:
A substance produced by a marker gene and its controlling sequences in cotton.....……..……....0.010- 0.014%*

* Percent protein on a dry weight basis as expressed in cotton seeds.

Sub-label 2. BCS Cry2Ae Cotton INSECT RESISTANT COTTON SEED (Event GHB714)

Active Ingredient:
Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB714 cotton……0.0002-0.0004%*

Inert Ingredients:
A substance produced by a marker gene and its controlling sequences in cotton.....……..……....0.010- 0.014%*

* Percent protein on a dry weight basis as expressed in cotton seeds.

Sub-label 3. BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton) INSECT RESISTANT COTTON SEED (Event T303-3 x Event GHB119)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004) in Event T303-3 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB119 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……..……..0.010- 0.02%*

* Percent protein on a dry weight basis as expressed in cotton seeds.

Sub-label 4. BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton) INSECT RESISTANT COTTON SEED (Event T303-3 x Event GHB714)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004) in Event T303-3 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB714 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……..……..0.010- 0.02%*

* Percent protein on a dry weight basis as expressed in cotton seeds.

Sub-label 5. BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton) INSECT RESISTANT COTTON SEED (Event T304-40 x Event GHB119)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL008) in Event T304-40 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB119 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……0.010- 0.02%*

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* Percent protein on a dry weight basis as expressed in cotton seeds.

Sub-label 6. BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton) INSECT RESISTANT COTTON SEED (Event T304-40 x Event GHB714)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL008) in Event T304-40 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB714 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……0.010- 0.02%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _____ pounds of cotton seed.

Bayer CropScience
2 T.W. Alexander Dr.
RTP, NC 27709

Sub-label 1.

BCS Cry2Ae Cotton

INSECT RESISTANT COTTON SEED (Event GHB119)

This package contains cotton seeds for insect-resistant cotton that produces an insecticidal protein, Cry2Ae, from *Bacillus thuringiensis* for protection against lepidopteran cotton pests. The insect-resistant cotton seed is derived from event GHB119 that contains the gene encoding the Cry2Ae insecticidal protein transformed with vector pTEM12.

FOR EXPERIMENTAL USE ONLY

For use only at an application site of a cooperator or participant and in accordance with the terms and conditions of the Experimental Use Permit. Not for sale to any person other than a participant or cooperator of the EPA-approved Experimental Use Program. This label must be in the possession of the user at the time of planting the cotton seed.

For use in the following states or US territories only:

Alabama, Arkansas, Arizona, California, Florida, Georgia, Louisiana, Mississippi, North Carolina, Puerto Rico, South Carolina, Tennessee and Texas. (AL, AR, AZ, CA, FL, GA, LA, MS, NC, PR, SC, TN and TX)

Active Ingredient:
Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB119 cotton……0.0002-0.0004%*

Inert Ingredients:
A substance produced by a marker gene and its controlling sequences in cotton.....……...……....0.010- 0.014%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _____ pounds of cotton seed.

Bayer CropScience
2 T.W. Alexander Dr.
RTP, NC 27709

DIRECTIONS FOR USE

Use of this seed in any manner inconsistent with the terms of the Experimental Use Permit is a violation of Federal Law.

The contents may only be used according to the approved EUP program. Cooperators and participants must have at least one copy of each applicable protocol prior to initiating any research with these contents.

Test plots must not be located within ¼ of a mile from the habitats of endangered/threatened Lepidoptera species as listed by the U.S. Fish and Wildlife Service. For example, the 264-EUP-RUG test plots must not be within ¼ mile of Francis' Satyr Butterfly habitat.

USE PATTERN

For evaluation of the control of the following insects in cotton:
Cotton bollworm (CBW, *Helicoverpa zea*)
Tobacco budworm (TBW, *Heliothis virenscens*)
Pink bollworm (PBW, *Pectinophora gossypiella)*
Fall armyworm (FAW, *Spodoptera frugiperda)*
Beet armyworm (BAW, *Spodoptera exigua)*

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

Storage: Store in a cool dry place inaccessible to children.

Seed and Plant Disposal: Any seeds, plants or plant materials not used in these experiments must be returned to Bayer CropScience or disposed of as specified in the field protocols. All plant material that is not saved for further research analyses or future plantings must be destroyed as specified in the field protocols. None of the plants or plant material may be sold or allowed to enter commerce.

Container Disposal: Do not reuse bag. Discard bag in trash. Ensure that the bag is completely empty of seed before disposal.

Sub-label 2.

BCS Cry2Ae Cotton

INSECT RESISTANT COTTON SEED (Event GHB714)

This package contains cotton seeds for insect-resistant cotton that produces an insecticidal protein, Cry2Ae, from *Bacillus thuringiensis* for protection against lepidopteran cotton pests. The insect-resistant cotton seed is derived from event GHB714 that contains the gene encoding the Cry2Ae insecticidal protein transformed with vector pTEM12.

FOR EXPERIMENTAL USE ONLY

For use only at an application site of a cooperator or participant and in accordance with the terms and conditions of the Experimental Use Permit. Not for sale to any person other than a participant or cooperator of the EPA-approved Experimental Use Program. This label must be in the possession of the user at the time of planting the cotton seed.

For use in the following states or US territories only:

Alabama, Arkansas, Arizona, California, Florida, Georgia, Louisiana, Mississippi, North Carolina, Puerto Rico, South Carolina, Tennessee and Texas. (AL, AR, AZ, CA, FL, GA, LA, MS, NC, PR, SC, TN and TX)

Active Ingredient:
Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB714 cotton……0.0002-0.0004%*

Inert Ingredients:
A substance produced by a marker gene and its controlling sequences in cotton.....……...……....0.010- 0.014%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _____ pounds of cotton seed.

Bayer CropScience
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RTP, NC 27709

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USE PATTERN

For evaluation of the control of the following insects in cotton:
Cotton bollworm (CBW, *Helicoverpa zea*)
Tobacco budworm (TBW, *Heliothis virenscens*)
Pink bollworm (PBW, *Pectinophora gossypiella)*
Fall armyworm (FAW, *Spodoptera frugiperda)*
Beet armyworm (BAW, *Spodoptera exigua)*

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

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Container Disposal: Do not reuse bag. Discard bag in trash. Ensure that the bag is completely empty of seed before disposal.

Sub-label 3.

BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton)

INSECT RESISTANT COTTON SEED (Event T303-3 x Event GHB119)

This package contains cotton seeds for insect resistant cotton that produces two insecticidal proteins, Cry1Ab and Cry2Ae, from *Bacillus thuringiensis* for protection against lepidopteran cotton pests. The insect resistant cotton seed is derived from events T303-3 and GHB119 that contain the genes encoding the Cry1Ab and Cry2Ae insecticidal proteins respectively, transformed with vector pTDL004 (Cry1Ab) and pTEM12 (Cry2Ae).

FOR EXPERIMENTAL USE ONLY

For use only at an application site of a cooperator or participant and in accordance with the terms and conditions of the Experimental Use Permit. Not for sale to any person other than a participant or cooperator of the EPA-approved Experimental Use Program. This label must be in the possession of the user at the time of planting the cotton seed.

For use in the following states or US territories only:

Alabama, Arkansas, Arizona, California, Florida, Georgia, Louisiana, Mississippi, North Carolina, Puerto Rico, South Carolina, Tennessee and Texas. (AL, AR, AZ, CA, FL, GA, LA, MS, NC, PR, SC, TN and TX)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004) in Event T303-3 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB119 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……0.010- 0.02%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _____ pounds of cotton seed.

Bayer CropScience
2 T.W. Alexander Dr.
RTP, NC 27709

DIRECTIONS FOR USE

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The contents may only be used according to the approved EUP program. Cooperators and participants must have at least one copy of each applicable protocol prior to initiating any research with these contents.

Test plots must not be located within ¼ of a mile from the habitats of endangered/threatened Lepidoptera species as listed by the U.S. Fish and Wildlife Service. For example, the 264-EUP-RUG test plots must not be within ¼ mile of Francis' Satyr Butterfly habitat.

USE PATTERN

For evaluation of the control of the following insects in cotton:
Cotton bollworm (CBW, *Helicoverpa zea*)
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Pink bollworm (PBW, *Pectinophora gossypiella)*
Fall armyworm (FAW, *Spodoptera frugiperda)*
Beet armyworm (BAW, *Spodoptera exigua)*

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

Storage: Store in a cool dry place inaccessible to children.

Seed and Plant Disposal: Any seeds, plants or plant materials not used in these experiments must be returned to Bayer CropScience or disposed of as specified in the field protocols. All plant material that is not saved for further research analyses or future plantings must be destroyed as specified in the field protocols. None of the plants or plant material may be sold or allowed to enter commerce.

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Sub-label 4.

BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton)

INSECT RESISTANT COTTON SEED (Event T303-3 x Event GHB714)

This package contains cotton seeds for insect resistant cotton that produces two insecticidal proteins, Cry1Ab and Cry2Ae, from *Bacillus thuringiensis* for protection against lepidopteran cotton pests. The insect resistant cotton seed is derived from events T303-3 and GHB714 that contain the genes encoding the Cry1Ab and Cry2Ae insecticidal proteins respectively, transformed with vector pTDL004 (Cry1Ab) and pTEM12 (Cry2Ae).

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Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004) in Event T303-3 cotton………………………………………..……………………0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB714 cotton…………………………………………………………………0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.......……………………………...………………………..……………......0.010- 0.02%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _________ pounds of cotton seed.

Bayer CropScience
2 T.W. Alexander Dr.
RTP, NC 27709

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For evaluation of the control of the following insects in cotton:
Cotton bollworm (CBW, *Helicoverpa zea*)
Tobacco budworm (TBW, *Heliothis virenscens*)
Pink bollworm (PBW, *Pectinophora gossypiella)*
Fall armyworm (FAW, *Spodoptera frugiperda)*
Beet armyworm (BAW, *Spodoptera exigua)*

STORAGE AND DISPOSAL

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Storage: Store in a cool dry place inaccessible to children.

Seed and Plant Disposal: Any seeds, plants or plant materials not used in these experiments must be returned to Bayer CropScience or disposed of as specified in the field protocols. All plant material that is not saved for further research analyses or future plantings must be destroyed as specified in the field protocols. None of the plants or plant material may be sold or allowed to enter commerce.

Container Disposal: Do not reuse bag. Discard bag in trash. Ensure that the bag is completely empty of seed before disposal.

Sub-label 5.

BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton)

INSECT RESISTANT COTTON SEED (Event T304-40 x Event GHB119)

This package contains cotton seeds for insect resistant cotton that produces two insecticidal proteins, Cry1Ab and Cry2Ae, from *Bacillus thuringiensis* for protection against lepidopteran cotton pests. The insect resistant cotton seed is derived from events T304-40 and GHB119 that contain the genes encoding the Cry1Ab and Cry2Ae insecticidal proteins respectively, transformed with vector pTDL008 (Cry1Ab) and pTEM12 (Cry2Ae).

FOR EXPERIMENTAL USE ONLY

For use only at an application site of a cooperator or participant and in accordance with the terms and conditions of the Experimental Use Permit. Not for sale to any person other than a participant or cooperator of the EPA-approved Experimental Use Program. This label must be in the possession of the user at the time of planting the cotton seed.

For use in the following states or US territories only:

Alabama, Arkansas, Arizona, California, Florida, Georgia, Louisiana, Mississippi, North Carolina, Puerto Rico, South Carolina, Tennessee and Texas. (AL, AR, AZ, CA, FL, GA, LA, MS, NC, PR, SC, TN and TX)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL008) in Event T304-40 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB119 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……0.010- 0.02%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _____ pounds of cotton seed.

Bayer CropScience
2 T.W. Alexander Dr.
RTP, NC 27709

DIRECTIONS FOR USE

Use of this seed in any manner inconsistent with the terms of the Experimental Use Permit is a violation of Federal Law.

The contents may only be used according to the approved EUP program. Cooperators and participants must have at least one copy of each applicable protocol prior to initiating any research with these contents.

Test plots must not be located within ¼ of a mile from the habitats of endangered/threatened Lepidoptera species as listed by the U.S. Fish and Wildlife Service. For example, the 264-EUP-RUG test plots must not be within ¼ mile of Francis' Satyr Butterfly habitat.

USE PATTERN

For evaluation of the control of the following insects in cotton:
Cotton bollworm (CBW, *Helicoverpa zea*)
Tobacco budworm (TBW, *Heliothis virenscens*)
Pink bollworm (PBW, *Pectinophora gossypiella)*
Fall armyworm (FAW, *Spodoptera frugiperda)*
Beet armyworm (BAW, *Spodoptera exigua)*

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

Storage: Store in a cool dry place inaccessible to children.

Seed and Plant Disposal: Any seeds, plants or plant materials not used in these experiments must be returned to Bayer CropScience or disposed of as specified in the field protocols. All plant material that is not saved for further research analyses or future plantings must be destroyed as specified in the field protocols. None of the plants or plant material may be sold or allowed to enter commerce.

Container Disposal: Do not reuse bag. Discard bag in trash. Ensure that the bag is completely empty of seed before disposal.

Sub-label 6.

BCS Cry1Ab x Cry2Ae Cotton (TwinLink cotton)

INSECT RESISTANT COTTON SEED (Event T304-40 x Event GHB714)

This package contains cotton seeds for insect resistant cotton that produces two insecticidal proteins, Cry1Ab and Cry2Ae, from *Bacillus thuringiensis* for protection against lepidopteran cotton pests. The insect resistant cotton seed is derived from events T304-40 and GHB714 that contain the genes encoding the Cry1Ab and Cry2Ae insecticidal proteins respectively, transformed with vector pTDL008 (Cry1Ab) and pTEM12 (Cry2Ae).

FOR EXPERIMENTAL USE ONLY

For use only at an application site of a cooperator or participant and in accordance with the terms and conditions of the Experimental Use Permit. Not for sale to any person other than a participant or cooperator of the EPA-approved Experimental Use Program. This label must be in the possession of the user at the time of planting the cotton seed.

For use in the following states or US territories only:

Alabama, Arkansas, Arizona, California, Florida, Georgia, Louisiana, Mississippi, North Carolina, Puerto Rico, South Carolina, Tennessee and Texas. (AL, AR, AZ, CA, FL, GA, LA, MS, NC, PR, SC, TN and TX)

Active Ingredients:

Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL008) in Event T304-40 cotton……0.0002-0.0017%*

Bacillus thuringiensis Cry2Ae protein and the genetic material necessary for its production (pTEM12) in Event GHB714 cotton……0.0002-0.0004%*

Inert Ingredients:

A substance produced by a marker gene and its controlling sequences in cotton.....……0.010- 0.02%*

---

* Percent protein on a dry weight basis as expressed in cotton seeds.

KEEP OUT OF REACH OF CHILDREN
CAUTION

EPA EXPERIMENTAL USE PERMIT NUMBER: 264-EUP-RUG
EPA ESTABLISHMENT NUMBER: 000264-TX-004
NET WEIGHT: _____ pounds of cotton seed.

Bayer CropScience
2 T.W. Alexander Dr.
RTP, NC 27709

DIRECTIONS FOR USE

Use of this seed in any manner inconsistent with the terms of the Experimental Use Permit is a violation of Federal Law.

The contents may only be used according to the approved EUP program. Cooperators and participants must have at least one copy of each applicable protocol prior to initiating any research with these contents.

Test plots must not be located within ¼ of a mile from the habitats of endangered/threatened Lepidoptera species as listed by the U.S. Fish and Wildlife Service. For example, the 264-EUP-RUG test plots must not be within ¼ mile of Francis' Satyr Butterfly habitat.

USE PATTERN

For evaluation of the control of the following insects in cotton:
Cotton bollworm (CBW, *Helicoverpa zea*)
Tobacco budworm (TBW, *Heliothis virenscens*)
Pink bollworm (PBW, *Pectinophora gossypiella)*
Fall armyworm (FAW, *Spodoptera frugiperda)*
Beet armyworm (BAW, *Spodoptera exigua)*

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

Storage: Store in a cool dry place inaccessible to children.

Seed and Plant Disposal: Any seeds, plants or plant materials not used in these experiments must be returned to Bayer CropScience or disposed of as specified in the field protocols. All plant material that is not saved for further research analyses or future plantings must be destroyed as specified in the field protocols. None of the plants or plant material may be sold or allowed to enter commerce.

Container Disposal: Do not reuse bag. Discard bag in trash. Ensure that the bag is completely empty of seed before disposal.

Section C Toxicology data

1. Toxicity of Expressed Proteins to Mammals

The Cry2Ae protein expressed in cotton events GHB119 and GHB714 is described within Habex, V. 2005. *Description of the amino acid sequence of the Cry2Ae protein*. MRID No. 46708902. This protein is encoded by the *cry2ae* gene which is derived from *Bacilius thuringiensis*.

Bacillus thuringiensis (*B.t.*) is a bacterium that occurs naturally in the soil and on plants. Various subspecies of this bacterium produce crystal proteins that are insecticidal to specific groups of insects. *B.t.* has been available in North America as a commercial microbial insecticide since the 1960s and these products contain various subspecies of *B.t.* depending on the targeted insect pests. *B.t.* products have an excellent safety record and can be used on crops until close to the day of harvest (EPA 2005). *B.t.* genes have also been transferred to plants for the production of insect-protected crops. Mammalian safety of pesticidal proteins incorporated into plants is described in the EPA Biopesticides Registration Document for the *Bacillus thuringiensis* (*B.t.*) Plant-Incorporated Protectants (EPA, 2001).

Specific toxicology and mammalian safety information for the *B.t*. insecticidal protein Cry2Ae is available in the following reports.

- Cry2Ae (GEM2) Protein: Overall Amino Acid Sequence Homology Search with Known Toxins and Allergens. Rouquie, D. (2005). BCS internal report. (see table below)
- Cry2Ae (GEM2) Protein: Epitope Homology and N-glycosylation Searches. Rouquie, D. (2005). BCS internal report. (see table below)
- Cry2Ae (GEM2) Protein: In Vitro Digestibility Study in Simulated Gastric Fluid. Rouquie, D. (2005). BCS internal report. (see table below)
- Cry2Ae (GEM2) Protein: Acute Toxicity by Intravenous Injection in the Mouse. Rouquie, D. (2005). BCS internal report. (see table below)
- Cry2Ae (GEM2) protein: Acute Toxicity by Oral Gavage in Mice. Rouquie, D. 2006. BCS Internal Report. (see table below)
- Analysis to Determine if the Cry2Ae (GEM2) Protein from Cotton Leaves is Glycosylated. Currier, T. (2005) BCS internal report. (see table below)

Volume	*Study Title*	*MRID*
NA	Cry2Ae (GEM2) protein: Overall amino acid sequence homology search with known toxins and allergens.	46708903
NA	Cry2Ae (GEM2) protein: Epitope homology and N-glycosylation searches	46708904
NA	Cry2Ae (GEM2) protein: In vitro digestibility study in simulated gastric fluid	46708905
NA	Cry2Ae (GEM2) protein: Acute toxicity by intravenous injection in the mouse	46708906
NA	Analysis to Determine if the Cry2Ae (GEM2) Protein from Cotton Leaves is Glycosylated	46708907
NA	Cry2Ae (GEM2) protein: Acute Toxicity by Oral Gavage in Mice	47125102

The results of these studies support the lack of mammalian toxicity or allergenic potential for Cry2Ae. The *in silico* studies show that there is no homology between Cry2Ae protein and any known toxins or allergens. Cry2Ae is not stable in an acidic environment. Digestibility testing of

the protein, in its activated form, shows that the protein is quickly broken down (within 2 minutes) in simulated gastric fluid.

In an acute toxicology study in mice, in which the Cry2Ae protein was administered intravenously, no adverse effects were seen after a high dose administration of Cry2Ae. The study included a 14 day observation period and a macroscopic post-mortem examination at study termination.

The acute oral toxicity of Cry2Ae protein was assessed in mice. Groups of five female OF1 mice were administered Cry2Ae protein by oral gavage at a dose level of 2000 mg/kg body weight. All animals were observed for clinical signs daily for fourteen days and their body weights were measured weekly. At termination of the study, the mice were necropsied and subjected to gross macroscopic examination. There were no mortalities, clinical signs or treatment-related effects on body weight evolution. Treatment with Cry2Ae protein at 2000 mg/kg body weight *via* the oral route did not produce signs of systemic toxicity. Thus, the acute oral LD50 of Cry2Ae was found to be greater than 2000 mg/kg body weight when administered by oral gavage to OF1 female mice. These results combined with the high digestibility of the protein support the lack of potential toxicity for Cry2Ae by dietary exposure.

Full sequence and epitope homology searching did not reveal homology between Cry2Ae and any allergens across several internationally recognized databases. In addition, experimental testing showed that Cry2Ae is not post-translationally glycosylated in plants. It is concluded that the potential for Cry2Ae to cause an allergenic reaction is very unlikely. Because the insecticidally active component of *B.t.* proteins is known to be stable to trypsin, a digestibility study on Cry2Ae using simulated intestinal fluid was considered to be unnecessary and was not conducted. Overall, the results of the studies conducted using Cry2Ae show that Cry2Ae has no structural similarity to known toxins or allergens, it does not possess intrinsic toxic properties and is highly unlikely to exhibit an allergenic response.

These studies are in agreement with the numerous mammalian safety studies conducted over the past 40 years on microbial preparations which have demonstrated the safety of *B.t.* microbial insecticide mixtures containing *B.t* proteins. Betz (2000) and McClintock (1995) report that collectively, these studies demonstrate the absence of any acute, sub-chronic and chronic toxicity associated with *B.t*. microbial pesticides. These findings are relevant to the safety assessment of *B.t.* protected plants because the microbial preparations contain the same classes of *B.t*. proteins that have been introduced into insect resistant plants. The mammalian safety of *B.t.* protected crops is supported by the long history of safe use of microbial pesticides around the world. EPA has concluded that there is a reasonable certainty of no harm resulting from the use of numerous *B.t*. insecticidal proteins and the genetic material necessary for their production in plants (EPA BRAD, 2001).

The genetic material necessary for the production of the Cry2Ae protein is DNA which is common to all forms of plant and animal life. There are no known instances where nucleic acids have caused toxic effects as a result of dietary exposure.

Based upon the information cited above, the Cry2Ae protein expressed in material derived from transformation events GHB119 and GHB714 is expected to be as safe to mammals as other *B.t.* proteins that may be found in food and feed as the result of applications of microbial *B.t.* products or from a plant expressing assessed and approved *B.t* proteins.

Cotton derived from transformation events GHB119 and GHB714 also contains the marker protein, PAT (phosphinothricin-acetyl-transferase) enzyme, encoded by the *bar* gene. This is the same protein that is in Bayer CropScience LLCotton25. PAT and the genetic material necessary for its production in plants are exempt from the requirement of a tolerance, as indicated within Section F of this application (40 CFR part 180, Sec. 180.1173). Detailed information regarding

the toxicology and safety of the PAT enzyme encoded by the *bar* gene is contained in the reports listed below. In addition, an extensive overview of the evaluation of the safety of the PAT protein is available in a 2005 article published in Regulatory Toxicology and Pharmacology (Hérouet, *et al.*, 2005).

Volume	*Study Title*	*MRID*
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: Overall amino acid sequence homology search with known toxins and allergens.	46455105
NA	Phosphinothricin Acetyltransferase (PAT) - *bar* gene product: Epitope homology and glycosylation searches.	47076903
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: Epitope homology and glycosylation searches	46455106
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: *In vitro* digestibility test in simulated gastric fluid	46455107
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: *In vitro* digestibility test in simulated intestinal fluid.	46455108
N/A	PAT (Phosphinothricin-Acetyl-Transferase) protein derived from *bar* gene: Acute toxicity by intravenous injection in the mouse.	46455109

The results of these studies show that the PAT protein has no homology with any known allergens or toxins. It has no glycosylation sites, which can be present on food allergens. It is not stable in an acidic environment. It is quickly degraded and denatured in gastric and intestinal fluids of domestic animals and humans. The PAT enzyme is highly substrate specific. There were no effects found in the acute mouse test, even at a high dose level of the PAT protein. Taken together, this information indicates there is a reasonable certainty of no harm resulting from the inclusion of the PAT protein in food and feed.

Based on all the information above, the Cry2Ae protein expressed in material derived from transformation events GHB119 and GHB714 is expected to be as safe as other *B.t.* proteins in food and feed. In addition, the PAT protein safety database does not show any evidence for toxicological concern. Based on the above information, there is reasonable certainty of no harm resulting from the inclusion of the Cry2Ae protein and the PAT protein in cotton plants and resulting food and feed.

Author: Helen Cunny, Ph.D. DABT
Title: Senior Regulatory Toxicologist

References:

- Betz, F.S., Hammond, B.G., Fuchs, R.L. 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regulatory Toxicol. Pharamacol. 32: 156-173. (MRID# 464551-10)
- EPA, 2001. Biopesticides Registration Action Document (BRAD) – *Bacillus thuringiensis* Plant-Incorporated Protectants, US EPA October 15 2001. http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm
- EPA, 2005. biopesticide active ingredient FACT sheets - http://www.epa.gov/pesticides/biopesticides/ingredients/
- Hérouet C., Esdaile, D., Mallyon, B., Debruyne, E., Schulz, A., Currier, T., Hendrickx, K., van der Klis, R., Rouan, D. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory Toxicol. Pharmacol. 41 (2005) 134-149. (MRID# 46600908)

- McClintock, J.T., C.R. Schaffer, Sjoblad, R.D. 1995. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. 45: 95-105. (MRID# 464551-10)

Cry1Ab x Cry2Ae Combined Trait Cotton (TwinLink cotton) – Toxicity of Expressed Proteins to Mammals

In addition to expressing the Cry2Ae and PAT proteins, Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) also expresses the Cry1Ab protein. The safety of the Cry1Ab protein as expressed in the cotton Events T303-3 and T304-40, either of which may make up a part of the combined trait cotton (TwinLink cotton) stack that will be evaluated under this EUP, was evaluated as part of the application for EUP NO. 264-EUP-140. As discussed in that application, studies submitted and cited show that there is no homology between the Cry1Ab protein and known toxins or allergens. The Cry1Ab protein does not possess N-glycosylation sites often found on allergens and it is quickly broken down in simulated gastric and intestinal fluids. Acute oral toxicity studies in mice have not shown any evidence of toxicity.

An extract from the EPA BRAD, *Bacillus thuringiensis* Cry2Ab2 protein and its genetic material necessary for its production in cotton (Chemical PC Code 006487) AMENDED, noted that:
"A *B. thuringiensis* strain expressing more than one type of Crystal protein could be expected to have synergistic or additive effects on the intended target pest insect. However, there is no indication from the testing of microbial *B. thuringiensis* strains registered and known to express an array of crystal proteins that human dietary safety has been adversely changed."

Based upon the data provided with this application or cited, there will not be a significant risk of toxic or allergenic effects to humans or other animals if exposed to the Cry1Ab or the Cry2Ae protein as expressed in cotton derived from either a Cry1Ab event or a Cry2Ae event respectively. Similar to the situation as noted above for microbial *B. thuringiensis* products which express an array of crystal proteins, toxic or allergenic effects are also not anticipated if exposure would be to the proteins together, such as when expressed within Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton).

Author: Helen Cunny, Ph.D. DABT
Title: Senior Regulatory Toxicologist

2. Toxicity to non-target organisms

In the study, Cry2Ae (GEM2) Protein Overall Amino Acid Sequence Homology Search with Known Toxins and Allergens, MRID No. 46708903, a high similarity of the Cry2Ae protein to other Cry2 proteins was shown (Rouquie, D., 2005). In particular, there is a high similarity of the Cry2Ae protein with the Cry2Aa and Cry2Ab proteins, 86 to 87% identical.

The Cry2Aa protein is present in commercial *Bacillus thuringiensis* var. *kurstaki* (*Btk*), microbial pesticide products, such as DiPel® (Abbott Laboratories), which expresses Cry2Aa as well as several Cry1 proteins (Betz et al, 2000).

The Cry2Ab protein is expressed as a plant incorporated pesticide (PIP) in cotton, EPA Reg. No. 524-522. (http://www.epa.gov/pesticides/biopesticides/pips/pip_list.htm).

Microbial *Bacillus thuringiensis* pesticides have been registered with the U.S. Environmental Protection Agency since 1961. In its Reregistration Eligibility Decision for *Bacillus thuringiensis*, issued in March, 1998, the Agency concluded that "toxicity and infectivity risks due to delta-endotoxin effects to non-target avian, freshwater fish, freshwater aquatic invertebrates, estuarine and marine animals, arthropod predators/parasites, honey bees, annelids and mammalian wildlife

will be minimal to nonexistent at the label use rates of registered *B. thuringiensis* active ingredients" (EPA, 1998, page 16). Among the active ingredients reviewed was *B. thuringiensis* subsp. *kurstaki*, which expresses the Cry2Aa protein.

In addition to evaluating the ecological toxicity of microbial *Bacillus thuringiensis* pesticides which contain the Cry2Aa protein, the ecological toxicity of the plant incorporated Cry2Ab protein has also been evaluated. In the BRAD for *Bacillus thuringiensis* Cry2Ab protein and its genetic material necessary for its production in cotton, Chemical PC Code 006487 Amended, the U.S. Environmental Protection Agency concluded that "the weight of evidence indicates no unreasonable adverse effects of … Cry2Ab singularly or jointly expressed in cotton to non-target wildlife, plants, beneficial invertebrates, or listed endangered/threatened species from the proposed … registration." (EPA, 2003, page 20). Specifically considered for *B. thuringiensis* subsp. *kurstaki* were effects on the following non-target organisms, evaluated in various studies, including those noted in Table 1.

Table 1: Non Target Organism Data – *B. thuringiensis* subsp. *kurstaki* microbial pesticide

Organism	*Test Guideline No.*	*Study Result*
Mallard duck	154-16	Practically nontoxic after 2.9 g/kg/day for 5 days
Bobwhite quail	154-16	Practically nontoxic after 2.9 g/kg/day for 5 days
Predaceous neuropteran	154-23	NOEL = 3000 ppm
Parasitic hymenoptera	154-23	NOEL = 3000 ppm
Predaceous coleopteran (Ladybird beetles)	154-23	NOEL = 1500 ppm, slightly toxic
		NOEL = 2.4 x 10^8 spores/ml diet, practically non-toxic
Predatory mite (*M. occidentalis*) and Twospotted spider mite (*T. urticae*)	154-23	Slightly toxic
Honey bee	154-24	NOEL = 7.7 μg/bee

Specifically considered for Cry2Ab protein as expressed in cotton, were potential effects on the following non-target organisms as evaluated in various studies, noted in Table 2.

Table 2: Non Target Organism Data – Cry2Ab as expressed in cotton

Organism	Test Guideline No.	Study Result
Bobwhite quail	885.4050	NOEC (Cry2Ab protein in diet w/10% ground cottonseed) is greater than 100,000 ppm.
Freshwater fish	885.4200	LC50 and NOEC of Cry2Ab protein is greater than 20% cottonseed meal in diet
Earthworm	850.6200	LC50 and NOEC for Cry2Ab protein is greater than 330 mg/kg dry soil
Honey bee adult and larvae	885.4380	Larvae NOEC for Cry2Ab protein is greater than 100 μg/mL (ppm) Adult NOEC is greater than 68 μg/mL Cry2Ab protein
Green lacewing larvae	885.4340	NOEC for Cry2Ab protein is greater than 1,100 ppm LD50 is greater than 4,500 ppm.
Lady beetle adults	885.4340	LC50 for Cry2Ab protein is greater than 4,500 ppm.
Collembola	885.4340	NOEC greater than 69.5 g Cry2Ab protein (in cotton leaf tissue)/g diet

Given the degree of similarity of the Cry2Ae protein to Cry2Aa and to Cry2Ab and the lack of adverse effects to non-target organisms of Cry2Aa and Cry2Ab, it is expected that Cry2Ae will similarly lack adverse effects to non-target organisms and will not present an environmental hazard under the conditions of the proposed experimental use permit.

Cry1Ab x Cry2Ae Combined Trait Cotton (TwinLink cotton) - Toxicity of Expressed Proteins to Non-Target Organisms

In addition to expressing the Cry2Ae and PAT proteins, Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) also expresses the Cry1Ab protein. The safety of the Cry1Ab protein to non-target organisms was evaluated as part of the application for EUP NO. 264-EUP-140. As indicated in that submission, Cry1Ab protein has been extensively tested for potential ecological effects, including effects on a wide range of non-target species. A detailed reassessment of all registered crops containing *Bt* was carried out in 2001 and the results were published in the *Biopesticides Registration Action Document (BRAD) – Bacillus thuringiensis Plant Incorporated Protectants*. While much of the data was generated to support the registration of corn containing the Cry1Ab protein, a majority of the experiments on non-target organisms were carried out using bacterially derived purified protein and are, therefore, also relevant to cotton containing a similar protein.

Based upon the lack of adverse effects to non-targets of Cry2A proteins and of Cry1Ab protein and the specificity of the proteins, it is not expected that cotton expressing both the Cry2Ae and the Cry1Ab protein will have an adverse impact on non-target organisms.

References:

- Betz, F.S., Hammond, B.G., Fuchs, R.L. 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regulatory Toxicol. Pharamacol. 32: 156-173. (MRID# 464551-10)

- EPA, 1998, Reregistration Eligibility Decision (RED) *Bacillus thuringiensis*, EPA 738-R098-004, March 1998

- EPA, 2001. Biopesticides Registration Action Document (BRAD) – *Bacillus thuringiensis* Plant-Incorporated Protectants, US EPA October 15 2001. http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm

- EPA, 2003*, BRAD (Bacillus thuringiensis* Cry2Ab2 protein and its genetic material necessary for its production in cotton) (Chemical PC Code 006-487) Amended

- EPA, 2005. Biopesticide Active Ingredient FACT Sheets - http://www.epa.gov/pesticides/biopesticides/ingredients/

- Hérouet C., Esdaile, D., Mallyon, B., Debruyne, E., Schulz, A., Currier, T., Hendrickx, K., van der Klis, R., Rouan, D. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory Toxicol. Pharmacol. 41 (2005) 134-149. (MRID# 46600908)

- McClintock, J.T., C.R. Schaffer, Sjoblad, R.D. 1995. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. 45: 95-105. (MRID# 464551-10)

Author: Helen Cunny, Ph.D. DABT
Title: Senior Regulatory Toxicologist

Section D Residue and Environmental data

Cry2Ae Cotton - Environmental Fate and Expression Levels (Residue) of Expressed Proteins

Environmental Fate

In addition to evaluating potential toxicity to non-target organisms, the environmental fate of *Bacillus thuringiensis* microbial pesticides as well as the Cry2Ab PIP protein have also been considered. Indicated in the *Bacillus thuringiensis* Re-registration Eligibility Document (EPA, 1998), "formal environmental fate data is not generally required for microbial pesticides because it is not usually needed and it is difficult to evaluate due to the potential for microbial growth under suitable environmental conditions. However, the behavior of *Bacillus thuringiensis* and related bacilli has been thoroughly studied and is well known. With regard to risk characterization it is known that *B. thuringiensis* toxins degrade rapidly in the phyllosphere as a result of exposure to UV light. *B. thuringiensis* toxins may persist in soil for several months, yet a half-life for typical B. thuringiensis products on foliage is approximately 1-4 days. As a result, exposure to most above-ground non-target organisms is expected to be minimal. *B. thuringiensis* spores, which are non-toxic, may persist in the environment, yet infection of insects from environmental dose levels is minimal" (EPA, 1998).

With regard to PIPs, the Agency has noted that "several studies indicate that Cry proteins bind to clays and humic acids, thus, slowing the rate of microbial degradation of these toxins compared to when these soil components are not present". A "Cry protein DT50 (time to 50% degradation) study was submitted for registration of Bollgard II cotton containing Cry2Ab and Cry1Ac (MRID 453371-01). According to this study, Cry2Ab + Cry1Ac proteins degrade rapidly in this sandy loam soil (typical soil type for cotton production). The DT50 was 2.3 days, DT90 was 15 days, and 75% of the protein degrades in the first week of incubation." However, EPA concluded that an "accurate degradation time (DT50) could not be determined from that study since the dose of Cry2Ab or Cry1Ac expressed was not high enough to control the cotton bollworm." Additional studies were requested by the Agency, in conjunction with an unlimited full Section 3 registration. Additional environmental fate data was not required in conjunction with the registration evaluation under the limited exposure considered in the BRAD for the Bt Cry2Ab protein (EPA, 2003).

It is not expected that the degradation profile of Cry2Ae protein will be significantly different from that of other Cry proteins. Exposure of soil to the Cry2Ae protein expressed in cotton under this EUP will be very limited, given the relatively small number of acres proposed for planting to cotton derived from either Event GHB119 or GHB714.

Protein Expression Levels

As indicated in the study Saey, B. and Jansens, S., 2005, PAT and Cry2Ae protein in cotton tissues of events GHB119 and GHB714. Preliminary report (MRID No. 46708908), the percentage of Cry2Ae protein in seed, on a fresh weight basis, is approximately 0.00019 to 0.00035%. The percentage in leaves, bolls, squares and flowers, as a percent total soluble protein basis is 0.026 to 0.068%, 0.007 to 0.036%, 0.008 to 0.021% and 0.018 to 0.021% respectively.

Cry2Ab expression levels in seed range from 0.00247 to 0.00507 % on a fresh weight basis. The highest expression levels were in seed, compared to other reported plant parts (EPA, 2003).

To calculate the protein content on a percentage dry weight basis, 5% moisture was used in the conversion. A dry weight equivalent range was calculated based upon the lowest and highest average fresh weight of Cry2Ae and PAT.

The percentage dry weight equivalent range for the Cry2Ae and PAT content in seeds are thus calculated to be: Cry2Ae: 0.0002 – 0.00037% and PAT: 0.01 – 0.014%.

In comparing the expression levels of the Cry2Ae protein in cotton seed with that of Cry2Ab protein in cotton seed, the expression level of the Cry2Ab protein is higher. If the cotton seed levels are used as the maximum levels expressed in cotton, an evaluation of Cry2Ab ecological toxicity or environmental fate would encompass the Cry2Ae protein levels expressed.

Cry1Ab x Cry2Ae Combined Trait Cotton (TwinLink cotton) - Environmental Fate and Expression Levels (Residue) of Expressed Proteins

Environmental Fate

The environmental fate of Cry1Ab protein expressed in crops and cotton in particular was evaluated as part of the application for EUP No. 264-EUP-140. As summarized in that application, an extensive assessment of the environmental fate of Cry1Ab protein as expressed in *B.t*. corn plants is contained in the EPA Biopesticides Registration Action Document for the *Bacillus thuringiensis* (*B.t*.) Plant-Incorporated Protectants, dated October 15, 2001. Also included in this assessment are evaluations of the potential for horizontal gene transfer to soil microbes, and the fate of *B.t.* Cry proteins in soil.

The biodegradability of Cry1Ab was evaluated in preliminary experiments, where *Bacillus thuringiensis* subsp. *kurstaki* cotton plants were placed in natural soils and decomposed. It was recorded that *B.t. kurstaki* endotoxin persisted and retained its immunological and biological activity at levels similar to those observed with microbial produced *B.t. kurstaki endotoxins* (Pratt *et al*., 1993).

A risk assessment was performed in 1998, to study the effect of transgenic cotton expressing the *B.t. kurstaki* endotoxin on soil microorganisms. The aim of the study was to determine the impact of the *Bacillus thuringiensis* subsp. *kurstaki* endotoxin in decomposing transgenic plants on soil microorganisms. The results showed that changes occurred in the levels of culturable, aerobic soil bacteria, fungi and protozoa. The populations were significantly higher in the transgenic cotton treatments relative to the parental cotton treatment. It was suggested that transgenic plants decomposed faster then the parent plants, and thus more rapidly provided nutrients for microbial growth (Donegan *et al*., 1998).

The environmental fate of highly similar purified *B.t*. proteins has been extensively studied. The published literature has demonstrated that *B.t*. protein adsorption to soil is rapid and complete within 30 minutes (Venkateswerlu and Stotzky, 1992). Numerous other studies of the biodegradation and binding of *B.t*. proteins in soil have been conducted, which demonstrate that isolated *B.t.* proteins could bind to clay particles and humic acids in artificial soil mixes. Exposure of organisms in soil to *B.t.* residues may also occur as a result of root exudations, as has been observed in *B.t*. corn expressing Cry1Ab (Saxena & Stotzky, 2000). However, the mechanism for this is not clear, and it is not known whether a similar process occurs for cotton.

With regard to the PAT protein, we do not expect that the biodegradability of the plants will change because they contain the PAT protein. The protein is a natural component of soil bacteria and is continually turned over along with the microbial populations in the soil.

It is not expected that the environmental fate profile of Cry1Ab protein or Cry2Ae protein will be different whether expressed singularly in cotton or if present in cotton as a result of trait stacking *via* conventional breeding, such as in the Combined Trait cotton (TwinLink cotton). There is no indication that the proposed experimental plots of transgenic cotton will pose any significant environmental hazard related to the environmental fate of the Cry1Ab, Cry2Ae or PAT proteins.

Protein Expression Levels

Preliminary information on Cry2Ae protein expression levels in Cry2Ae cotton events GHB119 and GHB714 was summarized above. As indicated in the study Saey, B. and Jansens, S., 2004, PAT and Cry1Ab protein in cotton tissues of T303-3 and T304-40 Events. Preliminary Report (MRID No.46708804), the percentage of Cry1Ab protein in seed, on a dry weight basis, is approximately 0.0002 to 0.0017%. The percentage of PAT protein in seed, on a dry weight basis, is approximately 0.013 to 0.014%. The percentage of Cry1Ab protein in leaves, bolls, squares and flowers, as a percent total soluble protein is 0.003 to 0.08%, 0.02 to 0.03%, 0.03 to 0.09% and 0.04% respectively.

Samples will be taken from field trials established under this EUP to further evaluate expression levels in tissue of the individual events and the combined trait cotton (TwinLink cotton).

Volume	*Study Title*	*MRID*
N/A	PAT and Cry1Ab protein in cotton tissues of T303-3 and T304-40 Events. Preliminary Report	46708804
N/A	PAT and Cry2Ae protein in cotton tissues of events GHB119 and GHB714. Preliminary report	46708908

References:

- Donegan, K.K., Seidler, R.J. 1998. Effect of Transgenic Cotton Expressing the *Bacillus thuringiensis* var *kurstaki* Endotoxin on Soil Micro-organisms–Risk Assessment Studies. Biotechnology in Agriculture and Forestry, Vol 42 Cotton. Bajaj, Y.P.S. (Ed). Springer-Verlag Berlin Heidelberg. (MRID No. 464551-12)

- EPA, 1998, Reregistration Eligibility Decision (RED) *Bacillus thuringiensis*, EPA 738-R098-004, March 1998

- EPA, 2001. Biopesticides Registration Action Document (BRAD) – *Bacillus thuringiensis* Plant-Incorporated Protectants, US EPA October 15 2001. http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm

- EPA, 2003, BRAD (*Bacillus thuringiensis* Cry2Ab2 protein and its genetic material necessary for its production in cotton) (Chemical PC Code 006-487) Amended

- Pratt, G.E., Royce, L.A., Croft, B.A. 1993. Measurements of toxicity of soils following incorporation of plant residues engineered with *Bacillus thuringiensis* var *kurstaki* endotoxin using *Heliothis virescens* growth bioassay, Proc 5th Investigators Meeting for the EPA's Environmental Release of Biotechnology Research program, College Park, Maryland.

- Saxena D, Stotzky G. 2000. Insecticidal toxin from *Bacillus thuringiensis i*s released from roots of transgenic *B.t.* corn *in vitro* and *in vivo*. FEMS Microbiology Ecology 33: 35-39. (MRID No. 464551-12)

- Venkateswerlu, G., G. Stotzky. 1992. Binding of the Protoxin and Toxin Proteins of *Bacillus thuringiensis* subsp*. kurstaki* on Clay Minerals. Curr. Microbiol. 25: 225-2 (MRID No. 464551-12)

Section E Effectiveness data

Cry2Ae Cotton Effectiveness data

Laboratory bioassays and preliminary field trial evaluations of small scale plots infested artificially indicate that BCS Cry2Ae cotton provides good control of infestations of cotton bollworm (CBW, *Helicoverpa zea*), tobacco budworm (TBW, *Heliothis virenscens*) and fall armyworm (FAW, *Spodoptera frugiperda*). An objective of the proposed experimental use permit is to extend the efficacy studies to additional locations and at a larger scale for the listed, as well as other lepidopteran cotton pests, relevant to the cotton growing area.

Volume	*Study Title*	*MRID*
NA	Preliminary Efficacy Report of Cry2Ae cotton plants derived from transformation events number GHB119 and GHB714.	46708909

Host Range

Non-target testing on Cry2Ab, as expressed in cotton, confirmed the expectation "that Cry1Ac and **Cry2Ab** (emphasis added) protein toxicity is confined to Lepidoptera species larvae; therefore, non-lepidopteran endangered or threatened species will not be affected by these proteins." (EPA).

Given the similarity of Cry2Ae protein to Cry2Ab and the latter's specificity to lepidopterans and the predominant activity of *Bacillus thuringiensis* subsp. *kurstaki* microbial pesticides against lepidopterans, we expect that the Cry2Ae host range is limited to lepidopterans. Studies are ongoing to confirm this.

Cry1Ab x Cry2Ae Combined Trait Cotton (TwinLink cotton) Effectiveness data

Preliminary field trial evaluations of small scale plots infested both naturally and artificially, indicate minimal damage of Cry1Ab cotton with infestations of cotton bollworm (CBW, *Helicoverpa zea*) and tobacco budworm (TBW, *Heliothis virenscens*). A summary report containing preliminary efficacy results was presented as part of the application for Experimental use permit 264-EUP-140 and is cited below.

Volume	*Study Title*	*MRID*
NA	Preliminary Efficacy Report of Cry1Ab cotton plants derived from transformation events number T303-3 and T304-40.	46708805

One objective of studies to be conducted under the proposed experimental use permit is to evaluate the efficacy of Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton), expressing both the Cry1Ab and the Cry2Ae proteins, against insect pests of cotton.

Author: Stefan Jansens, Ph.D.
Title: Research Program Leader Insect Control Cotton

Section F Tolerances

PAT, Cry1Ab, Cry2Ae
A tolerance exemption is in place for PAT protein in all crops, as well as for the marker gene. A tolerance exemption is in place for Cry1Ab protein in all crops.

A petition for a temporary exemption from the requirement of a tolerance for the Cry2Ae protein is being submitted at the same time as this request for an experimental use permit and as a separate document.

PAT Exemption from the Requirement of a Tolerance
40 CFR Part 180, Sec. 180.1151
Phosphinothricin Acetyltransferase (PAT) and the genetic material necessary for its production in all plants; exemption from the requirement of a tolerance.

Phosphinothricin Acetyltransferase (PAT) and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant-pesticide inert ingredients in all plant raw agricultural commodities. ``Genetic material necessary for its production'' means the genetic material which comprise genetic material encoding the PAT protein and its regulatory regions. ``Regulatory regions'' are the genetic material that control the expression of the genetic material encoding the PAT protein, such as promoters, terminators, and enhancers.

The documents described in Section C of this EUP application demonstrate the safety of the PAT protein, and support the existing tolerance exemption.

- Esdaile, D.J., 2002. Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: In vitro digestibility test in simulated gastric fluid. BCS internal report. (MRID #46455107)
- Esdaile, D.J., 2002. Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: In vitro digestibility test in simulated intestinal fluid. BCS internal report. (MRID # 46455108)
- Herouet, C., 2002. Phosphinothricin-Acetyl-Transferase (PAT)- *bar* gene product: Overall amino acid sequence homology search with known toxins and allergens. BCS internal report (MRID #46455105).
- Herouet-Guicheney, C., 2006. Phosphinothricin Acetyltransferase (PAT) - *bar* gene product: Epitope homology and glycosylation searches. BCS internal report (MRID47076903)
- Kennel, P., 2002. PAT (Phosphinothricin-Acetyl-Transferase) protein derived from *bar* gene: Acute toxicity by intravenous injection in the mouse. BCS internal report. (MRID #46455109)

Volume	*Study Title*	*MRID*
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: Overall amino acid sequence homology search with known toxins and allergens.	46455105
NA	Phosphinothricin Acetyltransferase (PAT) - *bar* gene product: Epitope homology and glycosylation searches	47076903*
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: In vitro digestibility test in simulated gastric fluid	46455107
N/A	Phosphinothricin-Acetyl-Transferase (PAT) - *bar* gene product: In vitro digestibility test in simulated intestinal fluid.	46455108
N/A	PAT (Phosphinothricin-Acetyl-Transferase) protein derived from *bar* gene: Acute toxicity by intravenous injection in the mouse.	46455109

*Same study also cited above under Cry2Ae cotton, Section C.

Cry1Ab Exemption from the Requirement for a Tolerance

40 CFR Part 180, Sec. 180.1173:

Bacillus thuringiensis Cry1Ab delta-endotoxin and the genetic material necessary for its production in all plants. *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant pesticides in all plant raw agricultural commodities. ``Genetic material necessary for its production'' means the genetic material which comprise genetic material encoding the Cry1Ab delta-endotoxin and its regulatory regions. ``Regulatory regions'' are the genetic material that control the expression of the genetic material encoding the Cry1Ab delta-endotoxin, such as promoters, terminators, and enhancers.

Volume	*Study Title*	*MRID*
N/A	Comparative analysis of the Cry1Ab protein amino acid sequence.	46708806

Cry2Ae Temporary Exemption from the Requirement for a Tolerance

As indicated previously, a petition for a temporary exemption from the requirement of a tolerance for the Cry2Ae protein is being submitted at the same time as this request for an experimental use permit and as a separate document.

Author: Diana Williams
Title: Global Registration Manager, Cotton

Section G Proposed experimental program.

1. Summary of participants

The experimental use program will be under the overall management of:

Dr. Linda Trolinder
Cotton Trait Manager
Bayer CropScience
Breeding and Product Development and

Dr. Jonathan Holloway
Field Trait Development Manager
Bayer CropScience BioScience
Contact information provided in Page 2 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Number 1.

Cooperators at specific locations are listed under the specific programs.

2. Acreage, seed and AI quantities by state

State	County/Parish, State	Protocol	Max. Acres	Cry2Ae Cotton Acres	Cry1Abx Cry2Ae Cotton Acres	Non-PIP Acres	Max. lb of Cry2Ae Cotton Seed	Max. lb of Cry1Ab x Cry2Ae Cotton Seed	Max. amt. of Cry2Ae protein in seed planted (g)	Max. amt. of Cry1Ab protein in seed planted (g)
AL	Lee, AL	A/E	20	2.5	2.5	15	30	30	0.10	0.218
	Limestone, AL	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
AR	Crittenden, AR	R	12	4.25	4.25	3.5	51	51	0.17	0.370
	Drew, AR	A/E, R	14	1.5	1.5	11	18	18	0.06	0.131
	Jackson, AR	R	4	0.25	0.25	3.5	3	3	0.01	0.022
	Lonoke, AR	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Washington, AR	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
AZ	La Paz, AZ	B	12.5	0	2.5	10	0	30	0.05	0.218
	Maricopa, AZ	A/E, B, R	37	6	8	23	72	96	0.28	0.696
	Pinal, AZ	A/E, B, R	140	6	22	112	72	264	0.56	1.914
	Yuma, AZ	A/E, B, R	37	6	8	23	72	96	0.28	0.696
CA	Fresno, CA	A/E	20	2.5	2.5	15	30	30	0.10	0.218
	Kern, CA	A/E, B, R	62	5	13	44	60	156	0.36	1.131
	Yolo, CA	A/E	20	2.5	2.5	15	30	30	0.10	0.218
FL	Escambia, FL	A/E, R	14	1.5	1.5	11	18	18	0.06	0.131
GA	Brooks, GA	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Tift, GA	A/E, R	32	6.75	6.75	18.5	81	81	0.27	0.587
	Turner, GA	A/E	20	2.5	2.5	15	30	30	0.10	0.218
LA	Bossier, LA	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Franklin, LA	A/E, R	65	5.5	8.5	51	66	102	0.28	0.740
	Madison, LA	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	St. Joseph, LA	R	4	0.25	0.25	3.5	3	3	0.01	0.022
	St. Landry, LA	R	4	0.25	0.25	3.5	3	3	0.01	0.022
MS	Chohoma, MS	A/E, R	14	1.5	1.5	11	18	18	0.06	0.131
	Oktibbeha, MS	A/E, R	65	5.5	8.5	51	66	102	0.28	0.740
	Tate, MS	R	4	0.25	0.25	3.5	3	3	0.01	0.022
	Washington, MS	A/E, B, R	223	10	41	172	120	492	1.02	3.567
NC	Halifax, NC	A/E, R	65	5.5	8.5	51	66	102	0.28	0.740
	Martin, NC	A/E, R	65	5.5	8.5	51	66	102	0.28	0.740
	Wake, NC	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
PR	Sabana Grande, PR	A, R, B	76	5	15	56	60	180	0.40	1.305
SC	Barnwell, SC	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Dillon, SC	A/E, B	105	4.25	17.25	83.5	51	207	0.43	1.501
	Marion, SC	A/E, B	105	4.25	17.25	83.5	51	207	0.43	1.501
TN	Madison, TN	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Shelby, TN	A/E, R	65	5.5	8.5	51	66	102	0.28	0.740
TX	Cameron, TX	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Gaines, TX	A/E, B	22.5	1.25	3.75	17.5	15	45	0.10	0.326
	Hidalgo, TX	A/E	65	5.5	8.5	51	66	102	0.28	0.740
	Hockley, TX	R	4	0.25	0.25	3.5	3	3	0.01	0.022
	Hunt, TX	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Lubbock, TX	A/E, B, R	223	10	41	172	120	492	1.02	3.567
	Nueces, TX	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Tom Green, TX	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
	Uvalde, TX	A/E, R	80	8.5	11.5	60	102	138	0.40	1.001
	Wharton, TX	A/E, R	80	8.5	11.5	60	102	138	0.40	1.001
	Willacy, TX	A/E	10	1.25	1.25	7.5	15	15	0.05	0.109
Total			1919	152	307	1460	1824	3684	9.18	26.709

*Protocols are abbreviated as follows: (A) Agronomic, (E) Efficacy, (R) Regulatory, (B) Breeding & Introgression.

3. Program overview

As indicated in Section A, Bayer CropScience (BCS) has developed cotton [*Gossypium hirsutum*] plants that express an insecticidal protein, Cry2Ae, from a common soil bacterium, *Bacillus thuringiensis*. The *cry2ae* gene was isolated from *B.t.* and its DNA sequence was modified for expression in plants.

The Cry2Ae protein is effective in controlling lepidopteran larvae such as bollworm (CBW, *Helicoverpa zea*), tobacco budworm (TBW, *Heliothis virenscens*) larvae (PBW, *Pectinophora gossypiella*) and fall armyworm (FAW, *Spodoptera frugiperda*) which are common pests of cotton. These pests cause severe economic damage to the cotton crop if not controlled. If controlled by chemical pesticides, there is the need for large input annually to control these pests. Small scale field trial experiments of cotton expressing Cry2Ae protein, conducted under notifications granted by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), and laboratory analyses have shown the plant's ability to protect itself against these pests.

BCS is applying for an Experimental Use Permit (EUP) to allow further evaluation of these cotton plants under a wider range of environmental conditions. All Cry2Ae cotton plants to be evaluated under the EUP have been derived from either transformation event GHB119 or GHB714. Several different field activities are planned: insect efficacy trials, agronomic performance evaluation and the production of sample material for regulatory feeding and analytical studies. Some seed produced as part of this program may be used for later plantings of experimental field trials.

In addition to Cry2Ae cotton, a Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) is also covered in this application. Cry1Ab cotton event T303-3 or T304-40 will be combined with Cry2Ae cotton event GHB119 or GHB714 to produce the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton). A review by EPA of the Cry1Ab cotton events was done in conjunction with the evaluation of EUP No. 264-EUP-140.

Several activities will be conducted on the combined trait cotton (TwinLink cotton); introgression (nurseries); evaluation (line trials), seed increases; evaluation of the insecticidal efficacy against cotton insect pests under different degrees of insect pressure, in different growing environments and in different genetic backgrounds; evaluation of agronomic performance in different genetic backgrounds and different growing regions; and possible generation of plant material and data to support future regulatory submissions in the United States and other countries.

The entire program will be done on a "Crop Destruct" basis; no cotton seed will enter commerce. Some of the plant material will be retained for scientific research and/or future planting purposes. All other plant materials will be destroyed. There will be no unintentional exposure to humans or domestic animals since the program will be conducted using containment procedures and in a crop destruct fashion. Isolation will be maintained in order to prevent any inadvertent outcrossing (pollination) from transgenic plants to non- transgenic cotton plants that are not part of the trials. No environmental impact issues related to the testing of these transgenic cotton plants have been identified. The Cry2Ae protein is expected to have a limited and specific range of toxicity to target lepidopteran pests and is not expected to have an adverse effect on non-target, beneficial insects.

In total, the program will be carried out at a maximum of 47 counties across 13 states/terrritories. A maximum of 1,919 acres will be planted as part of the experimental use permit program, of which 152 acres will be planted to Cry2Ae cotton, 307 acres will be planted to Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) and 1460 acres will be planted to non-PIP cotton in close proximity to the transgenic cotton plants as border rows or control plots.

The Cry2Ae-containing cotton will be planted at a maximum rate of 60,000 seeds per acre (12lb/acre). The level of Cry2Ae protein in each seed is approximately 180-360 ng (0.18-0.36 µg dry weight); therefore the planting of these Cry2Ae-containing seeds represents an application rate of approximately 10 to 20 mg of Cry2Ae protein per acre (using 12 lb or 5,442 g seed/acre).

The Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) will also be planted at a maximum rate of 60,000 seeds per acres (12 lb/acre). The level of Cry1Ab protein in each Cry1Ab cotton seed is approximately 180-1450 ng (0.18–1.45 µg). Taken together, the combined trait cotton (TwinLink cotton) seed will contain approximately 180–1450 ng (0.18–1.45 µg dry weight) Cry1Ab protein, or 10.8 to 87 mg per acre, and 180–360 ng (0.18–0.36 µg dry weight) Cry2Ae protein, or 10 to 20 mg per acre (using 12 lb or 5,442 g seed/acre).

Our proposed experimental research program will have a total of 3.32 to 26.71 g of Cry1Ab protein (or 0.008 to 0.059 pounds of Cry1Ab protein) planted in 307 acres of the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton). The total Cry2Ae protein planted in 307 acres of Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) and 152 acres of Cry2Ae will be 4.59 to 9.18 g (or 0.011 to 0.021 pounds of Cry2Ae protein). The level of Cry1Ab and Cry2Ae protein in the different plant material is only an estimation based on our current level of information.

Competent Bayer CropScience employees will supervise the program which will be conducted by them, in addition to public and private cooperators. All responsible researchers listed are professionally qualified to accomplish their stated duties.

4. Program details

Experimental Protocol:
EFFICACY TESTING OF INSECT RESISTANT TRANSGENIC COTTON

Objectives
The purpose of these trials is to compare plant growth, morphology, and agronomic performance among insect resistant transgenic cotton lines in different genetic backgrounds and their respective non-transgenic counterparts when infested with (either naturally or artificially) or devoid of the target insect. Another objective of these trials is to evaluate insect resistance transgene efficacy and subsequently determine if transgene expression and resulting insect resistance affect plant growth, morphology, or any facet of agronomic performance including fiber characteristics.

Specific Objectives

- Insect Efficacy, Natural Infestations/ Herbicide Efficacy - These trials will be conducted to evaluate effectiveness of the transformants derived from events GHB119 or GHB714 and the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) in controlling the primary target pests CBW and TBW. The trials will include sites where the infestations occur only as natural infestations. We expect large differences in the level of severity of the infestation and damage that can be seen across locations and environments; and for this reason, the evaluation of efficacy across a broad spectrum of environmental and geographical conditions is needed to accurately evaluate efficacy of the Cry2Ae events and the combined traits cotton against the targeted pests under field conditions. Additionally, evaluation by independent experts, such as in trials conducted by university researchers and other cooperators, provides independently generated performance data.

- Insect Efficacy CBW, Artificial Infestations - These trials will be conducted to evaluate effectiveness of the transformants derived from events GHB119 or GHB714 and the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) in controlling the primary target

pest CBW. The trials will include sites where the infestations occur as natural infestations, as well as artificially infested to enhance the opportunity to observe extreme CBW pressure. We expect large differences in the level of severity of the infestation and damage that can be seen across locations and environments; and for this reason, the evaluation of efficacy across a broad spectrum of environmental and geographical conditions is needed to accurately evaluate efficacy of the Cry2Ae events and the combined traits cotton against the targeted pests under field conditions. Additionally, evaluation by independent experts, such as in trials conducted by university researchers and other cooperators, provides independently generated performance data.

- Insect Efficacy, other Leps - Though the evaluation of target pest for the Cry2Ae events has concentrated in TBW and CBW, the Cry2Ae protein has been shown to have activity against other lepidopteran pests of cotton, including Fall Armyworm, Pink Bollworm and Beet Armyworm. These pests can be significant in certain seasons and in certain geographical regions, though the overall incidence and damage attributable to these pests is probably less significant than for the two main pests. Since these and other lepidopteran pests can cause significant economic damage in some environments, the opportunity for Cry2Ae to provide control of additional lepidopteran pests will be evaluated. The expanded acreage available under an EUP will allow the flexibility to test for these additional insect pests in planned experiments.

- Agronomic Evaluation - These trials will be used to document yield and agronomic performance of the events containing Cry2Ae and the combined trait cotton (TwinLink cotton). Trials will be small plot evaluations to look at effects on flowering, maturity, seed size, herbicide sensitivity, and yield, for example.

Cooperators and Participants

- *Experimental Program Manager*
  Dr. Linda Trolinder-Cotton Trait Manager, Breeding and Product Development
  Dr. Jonathan Holloway–Cotton Trait Development Manager, BioScience
  Bayer CropScience
  Contact information provided in Page 2 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Number 1.

- *Experiment Managers*
  Contact information provided in Pages 3-10, 12-39 and 41-56 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Numbers 2-9, 11-38 and 40-55.

Locations

Trial locations, on a county and state basis, and the total maximum acreage planted with seed containing an event in the trial are listed on the next page.

These locations are the same and concurrent with those trials listed under the “agronomic” protocol. The same plots (with the exception of Sabana Grande, PR) will be used to obtain both efficacy and agronomic data.

Efficacy Testing Locations and Acreage

County, State	Total Acres	Non-PIP Acres	*Cry2Ae Acres*	*Cry1Ab x Cry2Ae Acres*	*Pounds (lb) of Seed per Acre*
Lee, AL	20	15	2.5	2.5	12 lb/acre
Limestone, AL	10	7.5	1.25	1.25	12 lb/acre
Drew, AR	10	7.5	1.25	1.25	12 lb/acre
Lonoke, AR	10	7.5	1.25	1.25	12 lb/acre
Washington, AR	10	7.5	1.25	1.25	12 lb/acre
Maricopa, AZ	20	12	4	4	12 lb/acre
Pinal, AZ	45	36	3	6	12 lb/acre
Yuma, AZ	20	12	4	4	12 lb/acre
Fresno, CA	20	15	2.5	2.5	12 lb/acre
Kern, CA	10	6	2	2	12 lb/acre
Yolo, CA	20	15	2.5	2.5	12 lb/acre
Escambia, FL	10	7.5	1.25	1.25	12 lb/acre
Brooks, GA	10	7.5	1.25	1.25	12 lb/acre
Tift, GA	20	10.5	4.75	4.75	12 lb/acre
Turner, GA	20	15	2.5	2.5	12 lb/acre
Bossier, LA	10	7.5	1.25	1.25	12 lb/acre
Franklin, LA	53	43	3.5	6.5	12 lb/acre
Madison, LA	10	7.5	1.25	1.25	12 lb/acre
Chohoma, MS	10	7.5	1.25	1.25	12 lb/acre
Oktibbeha, MS	53	43	3.5	6.5	12 lb/acre
Washington, MS	40	32	4	4	12 lb/acre
Halifax, NC	53	43	3.5	6.5	12 lb/acre
Martin, NC	53	43	3.5	6.5	12 lb/acre
Wake, NC	10	7.5	1.25	1.25	12 lb/acre
Barnwell, SC	10	7.5	1.25	1.25	12 lb/acre
Dillon, SC	55	43.5	4.25	7.25	12 lb/acre
Marion, SC	55	43.5	4.25	7.25	12 lb/acre
Madison, TN	10	7.5	1.25	1.25	12 lb/acre
Shelby, TN	53	43	3.5	6.5	12 lb/acre
Cameron, TX	10	7.5	1.25	1.25	12 lb/acre
Gaines, TX	10	7.5	1.25	1.25	12 lb/acre
Hidalgo, TX	65	51	5.5	8.5	12 lb/acre
Hunt, TX	10	7.5	1.25	1.25	12 lb/acre
Lubbock, TX	40	32	4	4	12 lb/acre
Nueces, TX	10	7.5	1.25	1.25	12 lb/acre
Tom Green, TX	10	7.5	1.25	1.25	12 lb/acre
Uvalde, TX	40	30	3.5	6.5	12 lb/acre
Wharton, TX	40	30	3.5	6.5	12 lb/acre
Willacy, TX	10	7.5	1.25	1.25	12 lb/acre

Genotypes and vectors

Transgenic cotton plants expressing the *cry2ae* gene (events GHB119 or GHB714).

Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) expressing the *cry1ab* gene (event T303-3 or T304-40) and the *cry2ae* gene (event GHB119 or GHB714).

Experimental lines with different backgrounds.

Non transgenic plants Coker 315, Coker 312 and/or other commercial controls.

Trial Design
The preferred statistical design is a split-plot where level of insect infestation (sprayed vs. non-sprayed) is the main plot and line/transgene is the subplot. A split-strip design may also be used which facilitates insecticide treatments. A randomized complete block design (RCBD) would suffice, but would not provide maximal precision.

Agronomic Treatments
Typical agronomic inputs for conventionally grown cotton for the area, including, but not limited to:

- Conventional herbicide treatments, both pre- and post-planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide applications for non-target and/or target insects only (see test treatments below)
- Growth regulator application
- Additional hand weeding as necessary
- Chemical defoliation

Test Treatments
Test treatments involve natural infestations of target insects, chemical control of target insects, and chemical control of non-target insects. Treatments include but are not limited to:

- Complete insect control, both target and non-target in sprayed plots
- Insect control of non-target insects that allows or encourages natural infestation of target insect, in non sprayed plots.

Border rows
The EUP test plants or the trial will be surrounded by one or more border rows of cotton.

Schedule
Planting dates: February-June
Harvest dates: July-November

Activities and Agronomic Practices
Plots will be harvested by hand or mechanically. If by hand, the bolls will be placed in cloth or paper bags of such construction to avoid loss of seed outside of the bags. If by machine, seed cotton will be harvested, transported and processed under conditions appropriate for the handling of regulated material. This includes separate, redundant labeled packaging of all regulated material leaving the location.

Use of GPS coordinates, stakes, markers or other methods will be used to identify the area where the transgenic plants are grown, and such an area will be subsequently monitored for volunteers for an appropriate period of time. Volunteer plants will be terminated by hand weeding, disking, herbicide spraying or other method.

Containment
EUP test plants will be isolated in accordance with USDA-APHIS Performance Standards for regulated cotton trials. Isolation methods will include one or more of the following: EUP test plants will be located at least 660 feet from other parties' sexually receptive cotton; (2) a 40 foot-wide perimeter of non-transgenic cotton will surround the transgenic plants planted at a density within 20% (greater or less than) the trial density to act as pollen sink for insect pollinators (the perimeter cotton would be disposed of by harvesting, disking and monitoring); (3) temporal isolation, where the flowering period for the EUP plants will not coincide with the presence of other parties' sexually receptive cotton within 660 feet of the EUP test plants. Open flowering

EUP test plants may be located within 660 feet of sexually receptive cotton provided such other cotton is used only for experimental purposes and/or destroyed.

Following the trial completion, all remaining plant debris will be destroyed by incorporation in the soil. All equipment used during crop destruction practices will be inspected and cleaned before leaving the field. Seed cotton not destined for further experimentation will be destroyed by incineration or deep burial.

If harvested material is to be ginned, seed cotton will securely be transported to a gin. Processing of harvested seed cotton will consist of either hand ginning or research/commercial scale ginning. Hand ginning will be on small, table-top gins. Machine ginning will occur at the ginning facility on-site on a limited number of research/commercial scale gins. All packaging and waste will be destroyed by devitalization. No seed cotton or ginning by-products will be used for food or feed. Ginned seed will be stored under containment practices for regulated materials.

Agronomic Data Collection

If available, the following data may be collected from the plots, using a 1-9 scale, where applicable:

- Strain uniformity: 1=uniform, 9=highly variable)
- Leaf pubescence: 1=highly pubescent, 5=semi-smooth, 9=glabrous
- Disease reaction (verticillium wilt, bacterial blight, bronze wilt, etc. If applicable): 1=no symptoms, 5=some symptoms apparent, 9=severe
- Stalk lodging: 1=upright, 9=severely lodged
- % open bolls as a visual average when uninfested recurrent parent is 40-60% open
- Yield in lb lint per acre
- % lint
- # seed per boll
- Boll size
- Seed index
- Fiber properties: length, length uniformity, strength, micronaire, elongation
- Plant mapping: plant map 10 plants per plot, each of 4 reps at maturity shortly before defoliation. Data will include plant height, number of nodes, and boll position. Boll damage ratings may be a part of the mapping data. Information collected will reflect overall plant architecture and maturity.

Target Insect Evaluation Data Collection

Data will be taken on insect damage for 6-8 weeks to measure resistance to the infested insect. Insect infestation and damage data are collected by examining the terminal and one square or boll on10 plants per row on each of the two center rows in each plot. Data collected may include:

- Number of live larvae in squares
- Number of live larvae in bolls
- Number of squares damaged by larvae
- Number of bolls damaged by larvae
- Number of damaged white flowers
- Number of live larvae in white flowers

Quality Control Sample Collection

Leaf samples are to be taken from two individual plants per plot for QC purposes.

Experimental Protocol:
AGRONOMIC EVALUATION OF INSECT RESISTANT TRANSGENIC COTTON

Objectives
The purpose of these trials is to compare total agronomic performance and fiber characteristics among the converted sister lines (per recurrent parent) and with their respective recurrent parent variety counterpart. The goal is to select lines that are equal to or better than the recurrent parent, to be advanced to additional testing.

Cooperators and participants

- *Experimental Program Manager*
  Dr. Linda Trolinder-Cotton Trait Manager, Breeding and Product Development
  Dr. Jonathan Holloway–Cotton Trait Development Manager, BioScience
  Bayer CropScience
  Contact information provided in Page 2 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Number 1.

- *Experiment Managers*
  Contact information provided in Pages 3-10 and 12-56 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Numbers 2-9 and 11-55.

Locations
Trial locations, on a county and state basis, and the total maximum acreage planted with seed containing an event in the trial are listed on the next page

These locations are the same and concurrent with those trials listed under the “efficacy” protocol. The same plots (with the exception of Sabana Grande, PR) will be used to obtain both efficacy and agronomic data.

Agronomic Evaluation Locations and Acreage

County, State	Total Acres	Non-PIP Acres	*Cry2Ae Acres*	*Cry1Ab x Cry2Ae Acres*	*Pounds (lb) of Seed per Acre*
Lee, AL	20	15	2.5	2.5	12 lb/acre
Limestone, AL	10	7.5	1.25	1.25	12 lb/acre
Drew, AR	10	7.5	1.25	1.25	12 lb/acre
Lonoke, AR	10	7.5	1.25	1.25	12 lb/acre
Washington, AR	10	7.5	1.25	1.25	12 lb/acre
Maricopa, AZ	20	12	4	4	12 lb/acre
Pinal, AZ	45	36	3	6	12 lb/acre
Yuma, AZ	20	12	4	4	12 lb/acre
Fresno, CA	20	15	2.5	2.5	12 lb/acre
Kern, CA	10	6	2	2	12 lb/acre
Yolo, CA	20	15	2.5	2.5	12 lb/acre
Escambia, FL	10	7.5	1.25	1.25	12 lb/acre
Brooks, GA	10	7.5	1.25	1.25	12 lb/acre
Tift, GA	20	10.5	4.75	4.75	12 lb/acre
Turner, GA	20	15	2.5	2.5	12 lb/acre
Bossier, LA	10	7.5	1.25	1.25	12 lb/acre
Franklin, LA	53	43	3.5	6.5	12 lb/acre
Madison, LA	10	7.5	1.25	1.25	12 lb/acre
Chohoma, MS	10	7.5	1.25	1.25	12 lb/acre
Oktibbeha, MS	53	43	3.5	6.5	12 lb/acre
Washington, MS	40	32	4	4	12 lb/acre
Halifax, NC	53	43	3.5	6.5	12 lb/acre
Martin, NC	53	43	3.5	6.5	12 lb/acre
Wake, NC	10	7.5	1.25	1.25	12 lb/acre
Sabana Grande, PR	44	33	3	8	12 lb/acre
Barnwell, SC	10	7.5	1.25	1.25	12 lb/acre
Dillon, SC	55	43.5	4.25	7.25	12 lb/acre
Marion, SC	55	43.5	4.25	7.25	12 lb/acre
Madison, TN	10	7.5	1.25	1.25	12 lb/acre
Shelby, TN	53	43	3.5	6.5	12 lb/acre
Cameron, TX	10	7.5	1.25	1.25	12 lb/acre
Gaines, TX	10	7.5	1.25	1.25	12 lb/acre
Hidalgo, TX	65	51	5.5	8.5	12 lb/acre
Hunt, TX	10	7.5	1.25	1.25	12 lb/acre
Lubbock, TX	40	32	4	4	12 lb/acre
Nueces, TX	10	7.5	1.25	1.25	12 lb/acre
Tom Green, TX	10	7.5	1.25	1.25	12 lb/acre
Uvalde, TX	40	30	3.5	6.5	12 lb/acre
Wharton, TX	40	30	3.5	6.5	12 lb/acre
Willacy, TX	10	7.5	1.25	1.25	12 lb/acre

Genotypes and vectors

Transgenic cotton plants expressing the *cry2ae* gene (events GHB119 or GHB714).

Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) expressing the *cry1ab* gene (event T303-3 or T304-40) and the *cry2ae* gene (event GHB119 or GHB714).

Experimental lines with different backgrounds.

Non transgenic plants Coker 315, Coker 312 and/or other commercial controls.

Trial Design

The statistical design may be a RCBD. Plots may be 2- or 4-row with 3-4 replications. Multiple locations are necessary. Seed availability will dictate replications and number of locations. A lattice design may also be used. The precision of a split-plot is typically not practical at early stages of sister line evaluation due to the large number of converted sister lines, but is encouraged if possible. In this case, variety or genetic background would be the main-plot, and transgene +/- would be the split-plot.

Agronomic Treatments

Typical agronomic inputs for conventionally grown cotton for the area, including, but not limited to:

- Conventional herbicide treatments, both pre- and post-planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide applications
- Growth regulator application (this should be done sparingly if at all)
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants as this will mask maturity

Test Treatments

No special test treatments are required. If testing herbicide resistance transgene, the target herbicide is not to be applied for these trials. If testing insect resistance transgene, it is imperative that the target insect is completely controlled so that transgenic lines do not have an advantage over the unconverted recurrent parent lines.

Border rows

The EUP test plants or the trial will be surrounded by one or more border rows of cotton.

Schedule

Planting dates: February-June
Harvest dates: July-November
PR – Planting dates: October –November
Harvest dates: April-May

Activities and Agronomic Practices

Plots will be harvested by hand or mechanically. If by hand, the bolls will be placed in cloth or paper bags of such construction to avoid loss of seed outside of the bags. If by machine, seed cotton will be harvested, transported and processed under conditions appropriate for the handling of regulated material. This includes separate, redundant labeled packaging of all regulated material leaving the location.

Use of GPS coordinates, stakes, markers or other methods will be used to identify the area where the transgenic plants are grown, and such an area will be subsequently monitored for volunteers for an appropriate period of time. Volunteer plants will be terminated by hand weeding, disking, herbicide spraying or other method.

Containment

EUP test plants will be isolated in accordance with USDA-APHIS Performance Standards for regulated cotton trials. Isolation methods will include one or more of the following: EUP test plants will be located at least 660 feet from other parties' sexually receptive cotton; (2) a 40 foot-wide perimeter of non-transgenic cotton will surround the transgenic plants planted at a density within 20% (greater or less than) the trial density to act as pollen sink for insect pollinators (the perimeter cotton would be disposed of by harvesting, disking and monitoring); (3) temporal isolation, where the flowering period for the EUP plants will not coincide with the presence of other parties' sexually receptive cotton within 660 feet of the EUP test plants. Open flowering EUP test plants may be located within 660 feet of sexually receptive cotton provided such other cotton is used only for experimental purposes and/or destroyed. Details on Puerto Rico activities are described in pages 55 to 56 of this document.

Following the trial completion, all remaining plant debris will be destroyed by incorporation in the soil. All equipment used during crop destruction practices will be inspected and cleaned before leaving the field. Seed cotton not destined for further experimentation will be destroyed by incineration or deep burial.

If harvested material is to be ginned, seed cotton will securely be transported to a gin. Processing of harvested seed cotton will consist of either hand ginning or research/commercial scale ginning. Hand ginning will be on small, table-top gins. Machine ginning will occur at the ginning facility on-site on a limited number of research/commercial scale gins. All packaging and waste will be destroyed by devitalization. No seed cotton or ginning by-products will be used for food or feed. Ginned seed will be stored under containment practices for regulated materials.

Agronomic Data Collection

If available, the following data may be collected from the plots, using a 1-9 scale, where applicable:

- Strain uniformity: 1=uniform, 9=highly variable
- Plant type: 1=cluster, 9=open
- Leaf pubescence: 1=highly pubescent, 5=semi-smooth, 9=glabrous
- Disease reaction (verticillium wilt, bacterial blight, bronze wilt, etc. IF applicable): 1=no symptoms 5=some symptoms apparent, 9=severe
- Stalk lodging: 1=upright, 9=severely lodged
- Number days to first flower: as an average of the plot
- Number of days to first open boll: as an average of the plot
- Boll type: 1=loose, 5=intermediate, 9=storm proof
- Plant height: in inches at maturity in inches
- Total nodes: 10 plants per plot at maturity
- % open bolls as a visual average when recurrent parent is 40-60% open
- Yield in lb lint per acre
- % lint
- # seed per boll
- Boll size
- Seed index
- Fiber properties: length, length uniformity, strength, micronaire, elongation
- Plant mapping: plant map 10 plants per plot, each of 4 reps at maturity shortly before defoliation. Data will include plant height, number of nodes, and boll position. Boll damage ratings may be a part of the mapping data. Information collected will reflect overall plant architecture and maturity.

Experimental Program:
PRODUCTION OF SAMPLE MATERIAL FOR USE IN REGULATORY STUDIES

Objectives
The purpose of these trials is to produce sample materials for two types of regulatory trials, as part of the requirement of various regulatory agencies for the submission of registration packages all over the world. Material will be needed for:

- Production of sample material for use in poultry feeding studies.
- Production of sample material for use in composition studies.

Specific Objectives

- Production of sample material for use in poultry feeding studies. In order to support commercial use or importation of cotton derived from event GHB119 or GHB714 or from Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton), feeding studies are required by various governmental agencies. To conduct a poultry feeding study requires the production of approximately 2,000 pounds of cotton seed – per treatment – that can be used to prepare a meal based feed.
- Production of sample material for use in composition studies. Analytical data (*e.g*., composition and transgenic-expressed protein) must be obtained to fulfill regulatory requirements for transgenic crops in various countries. This production is designed to generate samples of cotton seed that will be used in the required analytical studies.
- Production of seed for use in future regulatory studies.

Cooperators and participants

- *Experimental Program Manager*
  Dr. Linda Trolinder-Cotton Trait Manager, Breeding and Product Development
  Dr. Jonathan Holloway–Cotton Trait Development Manager, BioScience
  Bayer CropScience
  Contact information provided in Page 2 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Number 1.

- *Experiment Managers*
  Contact information provided in Pages 6-10, 12-41 and 44-56 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Numbers 5-9, 11-40 and 43-55.

Locations
Trial locations, on a county and state basis, and the total maximum acreage planted with seed containing an event in the trial are listed on the next page

Regulatory Study Sample Material Generation Locations and Acreage

For material for a FRAC composition study and a poultry feeding study, 4 acres in two of the following locations will be used to generate Cry2Ae cotton samples and Cry1Ab x Cry2Ae cotton samples.

County, State	Total Acres	Non-PIP Acres	*Cry2Ae Acres*	*Cry1Ab x Cry2Ae Acres*	*Pounds (lb) of Seed per Acre*
Crittenden, AR	12	3.5	4.25	4.25	12 lb/acre
Drew, AR	4	3.5	0.25	0.25	12 lb/acre
Jackson, AR	4	3.5	0.25	0.25	12 lb/acre
Maricopa, AZ	12	8	2	2	12 lb/acre
Pinal, AZ	45	36	3	6	12 lb/acre
Yuma, AZ	12	8	2	2	12 lb/acre
Kern, CA	27	18	3	6	12 lb/acre
Escambia, FL	4	3.5	0.25	0.25	12 lb/acre
Tift, GA	12	8	2	2	12 lb/acre
Franklin, LA	12	8	2	2	12 lb/acre
St. Joseph, LA	4	3.5	0.25	0.25	12 lb/acre
St. Landry, LA	4	3.5	0.25	0.25	12 lb/acre
Chohoma, MS	4	3.5	0.25	0.25	12 lb/acre
Oktibbeha, MS	12	8	2	2	12 lb/acre
Tate, MS	4	3.5	0.25	0.25	12 lb/acre
Washington, MS	58	40	6	12	12 lb/acre
Halifax, NC	12	8	2	2	12 lb/acre
Martin, NC	12	8	2	2	12 lb/acre
Sabana Grande, PR	12	8	2	2	12 lb/acre
Shelby, TN	12	8	2	2	12 lb/acre
Hockley, TX	4	3.5	0.25	0.25	12 lb/acre
Lubbock, TX	58	40	6	12	12 lb/acre
Uvalde, TX	40	30	5	5	12 lb/acre
Wharton, TX	40	30	5	5	12 lb/acre

Genotypes and vectors
Transgenic cotton plants expressing the *cry2ae* gene (events GHB119 or GHB714).
Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) expressing the *cry1ab* gene (event T303-3 or T304-40).
Non transgenic plants Coker 315, Coker 312 and/or other commercial controls.

Trial Design
For the trial to produce material for the feeding studies, each plot will be approximately 4 acres in order to produce the required amount of seed per treatment. Thus, approx. 20 acres will be planted of the Cry2Ae cotton.

For the trial to produce material for the composition studies a maximum of 2 acres will be planted at each site for the sample production. Agronomic performance may also be evaluated at these sites. Of the 2 acres planted, approximately 0.5 acres will be planted with transgenic cotton seed. The remainder of the acreage per site covers border rows of non-transgenic cotton that are being considered as Cry2Ae cotton. The amount of border row material will vary depending upon final plot configuration at the site.

For seed increased to plant future regulatory studies, a block consisting of transgenic seed will be planted with rows of non transgenic border rows of cotton planted on all four sides of the perimeter of the seed block. The spacing between each row is approximately 38 inches.

Agronomic Treatments

Typical agronomic inputs for conventionally grown cotton for the area, including, but not limited to:

- Conventional herbicide treatments, both pre- and post-planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide applications
- Growth regulator application (this should be done sparingly if at all)
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants as this will mask maturity

Test Treatments

For the trial to produce material for the feeding study, a total of 4 transgenic plots will be planted. The transgenic plots and the non-transgenic control plots may be planted in the same field. One set of two plots will be planted to material derived from one of the two transformation events, GHB119 or GHB714. The other set of two transgenic plots will be planted to material derived from Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton). One set of two plots will also be planted to non-transgenic control plots of Coker 312 and Coker 315. Plot preparation and planting (e.g., row and plant spacing) will follow local commercial practice for cotton.

For the trial to produce material for the composition studies, a minimum of three treatments will be replicated three times at each site, for a minimum total of nine plots. One treatment will be the non-transgenic cotton, one will be transgenic cotton of one of the Cry2Ae cotton events treated with glufosinate ammonium and the other will be the transgenic cotton of the same event not treated with glufosinate ammonium. It is possible that plots will be established to include material from both Cry2Ae cotton and Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) at one site. Otherwise, separate sites will be established for the Cry1Ab x Cry2Ae combined trait cotton (TwinLink cotton) plots.

Glufosinate ammonium treatments will be made to three of the transgenic plots of each event or combined trait cotton (TwinLink cotton) (the transgenic cotton treated with glufosinate treatment). Each plot will contain at least six rows and be large enough to allow at least the required amount of samples to be obtained without sampling plants located on the edges of the plot. The plot(s) will be large enough to allow treatment with commercial type or small plot application equipment.

The plots established to produce material for composition studies may also be evaluated for agronomic performance.

Border rows

The EUP test plants or the trial will be surrounded by one or more border rows of cotton.

Schedule

Planting dates: February-June
Harvest dates: July-November
PR – Planting dates: October –November
Harvest dates: April-May

Activities and Agronomic Practices

After planting, transgenic and control seeds that were not planted will be collected. The transgenic seeds will be returned to the attention of the Study Director. Any non-transgenic

seeds that are not planted will be disposed of at the discretion of the Cooperator. The disposition of the seeds will be documented.

Plots will be harvested by hand or mechanically. The harvested seed cotton will be ginned at or near the field location to obtain required fuzzy seed samples. A minimum of 4 pounds of seed will be collected per treatment regimen. Lint obtained from the ginning will be disposed of. Seed samples will be packed, placed in frozen storage and ultimately shipped, frozen, to the Study Director and/or to a designated analytical laboratory for compositional analyses.

After the field phase of the study is complete, the remaining transgenic and non-transgenic cotton plants will be destroyed by plowing the crop into the ground.

Containment

EUP test plants will be isolated in accordance with USDA-APHIS Performance Standards for regulated cotton trials. Isolation methods will include one or more of the following: EUP test plants will be located at least 660 feet from other parties' sexually receptive cotton; (2) a 40 foot-wide perimeter of non-transgenic cotton will surround the transgenic plants planted at a density within 20% (greater or less than) the trial density to act as pollen sink for insect pollinators (the perimeter cotton would be disposed of by harvesting, disking and monitoring); (3) temporal isolation, where the flowering period for the EUP plants will not coincide with the presence of other parties' sexually receptive cotton within 660 feet of the EUP test plants. Open flowering EUP test plants may be located within 660 feet of sexually receptive cotton provided such other cotton is used only for experimental purposes and/or destroyed. Details on Puerto Rico activities are described in pages 55 to 56 of this document.

Following the trial completion, all remaining plant debris will be destroyed by incorporation in the soil. All equipment used during crop destruction practices will be inspected and cleaned before leaving the field. Seed cotton not destined for further experimentation will be destroyed by incineration or deep burial.

After completion of sampling, all perimeter cotton, if perimeters are planted, will be disposed of by mowing, followed by disking into the soil. The transgenic plots and any perimeter cotton fields will be monitored the following season for volunteers. Volunteer plants will be terminated by hand weeding, disking, herbicide spraying or other method.

If harvested material is to be ginned, seed cotton will securely be transported to a gin. All packaging and waste will be destroyed by devitalization. Ginned seed will be stored under containment practices for regulated materials.

Data Requirement

For the feeding study, a total of 2,000 pounds of BCS Cry2Ae cotton seed must be produced for each of two events or combination. The production will be duplicated as a back up precaution. In addition to the Cry2Ae cotton material, a non-genetically modified line of the same genetic background and another commercially available line will also be produced to provide material for comparative treatments.

Cotton seed will be harvested from each plot, including edge rows, at normal maturity. Material from the control non-transgenic plots will be harvested before harvesting the transgenic plots, if they were established in the same field. The harvested seed cotton will be ginned to obtain the required seed samples to prepare meal for feeding studies. Ginning will be done at or near the field site. If the seed cotton is transported to the gin, the samples will be contained in an enclosed or covered vehicle or module. Ginning may be performed on a commercial gin, under the supervision of the Cooperator.

The ginned seed samples will be stored for transport at ambient temperature in suitable containers such as large bulk bags. Sub-samples will be sent to Bayer CropScience. The bulk samples will be shipped to a processing facility for processing into meal. Transgenic cotton lint may be retained for Bayer CropScience after ginning for immediate storage by Bayer and possible future commercial use after registration. Alternatively, it will be disposed of. Any "waste" transgenic lint (*e.g.*, spillage or clean-out waste) will be disposed of.

After samples are collected, remaining transgenic crop (left in the field after harvesting) will be destroyed.

Future Plans
Transgenic seed harvested from the trial may be used for regulatory trials at a later date.

The following are details of the activities in Puerto Rico.

Background
Cotton is mainly self-pollinated. Reproductive maturity is reached 4-5 weeks after planting, with the formation of buds, followed 25 days later by anthesis. Cotton flowers antheses at dawn and remain open for 24 hours. Soon after anthesis, the anthers dehisce, releasing pollen which remains viable for approximately 24 hours. Cotton pollen is relatively large and heavy, and not easily dispersed by wind. Cotton is a facultative self-pollinator, and an opportunistic out-crosser when insect pollinators are present. Fertilization of ovules occurs about 12-30 hours after pollination.

The following measures and activities will be followed:

a. Surround the plot by border:
   The plot will be surrounded by rows of non-Bt cotton using the same conventional row spacing used in the experimental acreage (27"-40") to provide a minimum of 40 feet of border. The planting density and row spacing in border areas must be within 20% (greater than or less than) of that found in the experiment. These row spacing parameters will ensure that the prescribed border area serves as an effective pollen deposition site for cotton pollinators.
b. Insecticidal sprays:
   A normal insecticide spray regime will be used during the trial as required by standard agronomic practices. Sprays will conducted as necessary, following standard agronomic practices which depend on pest pressure that varies from season to season. Commercial thresholds are pest specific, and approximate thresholds are: aphids 90% infested (9 out of 10 plants have one aphid or more), two spotted mite 60% infested (six in 10 plants), and Lepidoptera 20% infested (two in 10 plants). Sprays are required for thrips. Treatments for sucking pests (whitefly and aphids) and mites are no different from conventional cotton (2-6 sprays per season). Sprays for Lepidopteran are reduced in Bt cotton but 1-2 supplemental sprays may still be needed for *Heliothis* and *Spodoptera* control.
c. Collection of data relevant to detection of pollen flow from the trial and reporting of results:
   BCS will conduct a survey of the land (that can be accessed) surrounding the trial after seed set. The survey will describe number of feral cotton plants found setting seed concurrently with the trial. If feral cotton with seed is found on land that can be accessed the seed will be collected for PCR analysis. All feral cotton plants found on land that can be accessed will be destroyed. The survey area will encompass a circle with a radius of one mile from the center of the trial. In

addition, BCS will design an appropriate experiment to test pollen flow from the trial into the border rows. All data collected from gene flow analyses from sampling of border rows and feral or indigenous cotton plants within a 1 mile radius of the test plots will be reported to EPA in conjunction with the annual EUP report, 3 months after harvest.

Bayer CropScience believes that pollen flow is unlikely due to:

- Cotton pollen is heavy and sticky, and is not readily moved by wind, which results in the pollen falling within feet of the plant.
- Cotton is a facultative self-pollinator, and an opportunistic out-crosser when insect pollinators are present. The distance provide by the border rows will prevent any small crawling insects from reaching fertile feral population within the 24 hours of the pollen remains viable.
- Feral/native/wild cotton flowering is temporally separated from that of the cotton planted in BCS trials. BSC anticipates few, if any, feral/native/wild cotton plants will flower during the same period as the BCS cotton plants minimizing the opportunity for pollen transfer.

Agronomic Evaluation

If it is possible, agronomic evaluation data will be collected from the plots, using a 1-9 scale, where applicable:

- Strain uniformity: 1=uniform, 9=highly variable)
- Leaf pubescence: 1=highly pubescent, 5=semi-smooth, 9=glabrous
- Disease reaction (verticillium wilt, bacterial blight, bronze wilt, etc. IF applicable): 1=no symptoms, 5=some symptoms apparent, 9=severe
- Stalk lodging: 1=upright, 9=severely lodged
- % open bolls as a visual average when uninfested recurrent parent is 40-60% open
- Yield in lb lint per acre
- % lint
- # seed per boll
- Boll size
- Seed index
- Fiber properties: length, length uniformity, strength, micronaire, elongation
- Plant mapping: plant map 10 plants per plot, each of 4 reps at maturity shortly before defoliation. Data will include plant height, number of nodes, and boll position. Boll damage ratings may be a part of the mapping data. Information collected will reflect overall plant architecture and maturity.

Experimental Program

BREEDING, INTROGRESSION, EVALUATION AND SEED INCREASE OF Cry1Ab x Cry2Ae COMBINED TRAIT COTTON (TwinLink cotton)

Objectives
The purpose of these activities is to introgress the Cry2Ae trait and Cry1Ab x Cry2Ae combined traits in elite cotton lines for purposes of elite line development, to evaluate new lines and to increase seed selected lines for further development trials.

Cooperators and participants

- *Experimental Program Manager*
  Dr. Linda Trolinder-Cotton Trait Manager, Breeding and Product Development
  Dr. David Becker– Station Manager, Breeding and Development
  Bayer CropScience
  Contact information provided in Page 2 and 46 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Number 1 and 45.

- *Experiment Managers*
  Contact information provided in Pages 11-17, 30-37, 40-44 and 46-56 of the *Confidential Appendix* to this EUP extension request, Section G, and listed as Cross Reference Numbers 10-16, 29-36, 39-43 and 45-55.

Locations
Trial locations, on a county and state basis, and the total maximum acreage planted with seed containing an event in the trial are listed on the next page

Locations: Acreage and states

County, State	Total Acres	Non-PIP Acres	*Cry2Ae Acres*	*Cry1Ab x Cry2Ae Acres*	*Pounds (lb) of Seed per Acre*
La Paz, AZ	12.5	10	0	2.5	12 lb/acre
Maricopa, AZ	5	3	0	2	12 lb/acre
Pinal, AZ	50	40	0	10	12 lb/acre
Yuma, AZ	5	3	0	2	12 lb/acre
Kern, CA	25	20	0	5	12 lb/acre
Washington, MS	125	100	0	25	12 lb/acre
Sabana Grande, PR	20	15	0	5	12 lb/acre
Dillon, SC	50	40	0	10	12 lb/acre
Marion, SC	50	40	0	10	12 lb/acre
Gaines, TX	12.5	10	0	2.5	12 lb/acre
Lubbock, TX	125	100	0	25	12 lb/acre

Genotypes and vectors
Transgenic cotton plants expressing the *cry2ae* gene (event GHB119 or GHB714).
Transgenic cotton plants expressing the *cry1ab* gene (event T303-3 or T304-40) and *cry2ae* gene (event GHB119 or GHB714) - Development and commercial lines of different backgrounds.

Trial Designs

Introgression (Nurseries)
The nurseries will be comprised of F2-derived F3 progeny rows from which individual plant selections will be made or F3-derived F4 progeny rows from which line selections will be made.

Plant to row progenies are generally 1-row plots, 20 to 30 feet long with 5 foot alleys between ranges. The transgenic cotton plots will be isolated from other cotton not part of the transgenic cotton nursery and surrounded by either fallow fields or a different crop.

Line Trials
Line trials will be designed as a replicated variety trial, with 2-row plots laid out in a randomized complete block design (RCBD) or lattice design. They will be established for evaluation purposes only and no seed will be saved.

Seed Increases
Seed increase plots will be established as 4-row plots per line being increased. The center two rows will be harvested. Plots of different pedigrees will be separated by at least 40 feet.

Agronomic Treatments
Typical agronomic inputs for conventionally grown cotton for the area, including, but not limited to:

- Conventional herbicide treatments, both pre- and post-planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide applications
- Growth regulator application (this should be done sparingly if at all)
- Additional hand weeding as necessary
- Chemical defoliation without boll-opening desiccants as this will mask maturity

Test Treatments
No special test treatments are required.

Border rows
No cotton border rows will be established around the plots. The transgenic cotton will be planted at least 660 feet from any other cotton not part of the transgenic cotton introgression, line evaluation or seed increase.

Schedule
Planting dates: February-June
Harvest dates: July-November
PR – Planting dates: October –November
Harvest dates: April-May

Activities and Agronomic Practices
Plots to be harvested will be harvested by hand or mechanically. If by hand, the bolls will be placed in cloth or paper bags of such construction to avoid loss of seed outside of the bags. If by machine, seed cotton will be harvested, transported and processed under conditions appropriate for the handling of regulated material. This includes separate, redundant labeled packaging of all regulated material leaving the location.

Use of GPS coordinates, stakes, markers or other methods will be used to identify the area where the transgenic plants are grown, and such an area will be subsequently monitored for volunteers for an appropriate period of time. Volunteer plants will be terminated by hand weeding, disking, herbicide spraying or other method.

Containment

EUP test plants will be isolated in accordance with USDA-APHIS Performance Standards for regulated cotton trials. Isolation methods will include one or more of the following: EUP test plants will be located at least 660 feet from other parties' sexually receptive cotton or temporal isolation, where the flowering period for the EUP plants will not coincide with the presence of other parties' sexually receptive cotton within 660 feet of the EUP test plants. Open flowering EUP test plants may be located within 660 feet of sexually receptive cotton provided such other cotton is used only for experimental purposes and/or destroyed. Border rows will not be used nor will plants be netted during pollen shed as that would be counterproductive to the activities described in this section. Details on Puerto Rico activities are described in pages 55 to 56 of this document.

Following the trial completion, all remaining plant debris will be destroyed by incorporation in the soil. All equipment used during crop destruction practices will be inspected and cleaned before leaving the field. Seed cotton not destined for further experimentation will be destroyed by incineration or deep burial.

If harvested material is to be ginned, seed cotton will securely be transported to a gin. Processing of harvested seed cotton will consist of either hand ginning or research/commercial scale ginning. Hand ginning will be on small, table-top gins. Machine ginning will occur at the ginning facility on-site on a limited number of research/commercial scale gins. All packaging and waste will be destroyed by devitalization. No seed cotton or ginning by-products will be used for food or feed. Ginned seed will be stored under containment practices for regulated materials.

Agronomic Data Collection

If available, the following data may be collected from the plots, using a 1-9 scale, where applicable:

- Strain uniformity: 1=uniform, 9=highly variable
- Plant type: 1=cluster, 9=open
- Leaf pubescence: 1=highly pubescent, 5=semi-smooth, 9=glabrous
- Disease reaction (verticillium wilt, bacterial blight, bronze wilt, etc. IF applicable): 1=no symptoms 5=some symptoms apparent, 9=severe
- Stalk lodging: 1=upright, 9=severely lodged
- Number days to first flower: as an average of the plot
- Number of days to first open boll: as an average of the plot
- Boll type: 1=loose, 5=intermediate, 9=storm proof
- Plant height: in inches at maturity in inches
- Total nodes: 10 plants per plot at maturity
- % open bolls as a visual average when recurrent parent is 40-60% open
- Yield in lb lint per acre
- % lint
- # seed per boll
- Boll size
- Seed index
- Fiber properties: length, length uniformity, strength, micronaire, elongation
- Plant mapping: plant map 10 plants per plot, each of 4 reps at maturity shortly before defoliation. Data will include plant height, number of nodes, and boll position. Boll damage ratings may be a part of the mapping data. Information collected will reflect overall plant architecture and maturity.