Notices

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Animal and Plant Health Inspection Service

[Docket No. 95-023-1]

Receipt of Petition for Determination of Nonregulated Status for Genetically Engineered Cotton

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Notice.

SUMMARY: We are advising the public that the Animal and Plant Health Inspection Service has received a petition from the Monsanto Company seeking a determination of nonregulated status for cotton lines designated as 1445 and 1698 that have been genetically engineered for tolerance to

the herbicide glyphosate. The petition has been submitted in accordance with our regulations concerning the introduction of certain genetically engineered organisms and products. In accordance with those regulations, we are soliciting public comments on whether these cotton lines present a plant pest risk.

DATES: Written comments must be received on or before May 30, 1995.

ADDRESSES: Please send an original and three copies of your comments to Docket No. 95–023–1, Regulatory Analysis and Development, PPD, APHIS, Suite 3C03, 4700 River Road Unit 118, Riverdale, MD 20737–1228. Please state that your comments refer to Docket No. 95–023–1. A copy of the petition and any comments received may be inspected at USDA, room 1141, South Building, 14th Street and Independence Avenue SW., Washington, DC, between 8 a.m. and 4:30 p.m., Monday through Friday, except holidays. Persons wishing access to that room to inspect the petition or comments are asked to call in advance of visiting at (202) 690–2817.

FOR FURTHER INFORMATION CONTACT: Dr. Sivramiah Shantharam, Branch Chief, Biotechnology Permits, BBEP, APHIS, Suite 5B05, 4700 River Road Unit 147, Riverdale, MD 20737–1228; (301) 734–7612. To obtain a copy of the petition, contact Ms. Kay Peterson at (301) 734–7601.

SUPPLEMENTARY INFORMATION: The regulations in 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason to Believe Are Plant Pests," regulate, among other things, the introduction (importation, interstate movement, or release into the environment) of organisms and products altered or produced through genetic engineering that are plant pests or that there is reason to believe are plant pests. Such genetically engineered organisms and products are considered "regulated articles."

The regulations in § 340.6(a) provide that any person may submit a petition to the Animal and Plant Health Inspection Service (APHIS) seeking a determination that an article should not be regulated under 7 CFR part 340. Paragraphs (b) and (c) of § 340.6 describe the form that a petition for determination of nonregulated status must take and the information that must be included in the petition.

On February 14, 1995, APHIS received a petition (APHIS Petition No. 95–045–01p) from the Monsanto Company of St. Louis, MO, requesting a determination of nonregulated status under 7 CFR part 340 for cotton lines designated as 1445 and 1698 that have been genetically engineered for tolerance to the herbicide glyphosate. As described in the petition, cotton (*Gossypium hirsutum* L.) lines 1445 and 1698 contain the gene for CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) isolated from *Agrobacterium* sp. strain CP4, which encodes an enzyme conferring tolerance to glyphosate, the active ingredient in Roundup® herbicide. Cotton lines 1445 and 1698 also contain the *nptII* gene, which encodes the selectable marker neomycin phosphotransferase II, and the *aad* gene, which encodes the bacterial selectable marker 3"(9)-O-aminoglycoside adenylyltransferase. Expression of the *nptII* gene is driven by the 35S promoter derived from the plant pathogen cauliflower mosaic virus. The subject cotton lines were produced through the use of *Agrobacterium tumefaciens* transformation, a full description of which is provided in the petition.

The subject cotton lines are currently considered regulated articles under the regulations in 7 CFR part 340 because they contain gene sequences (vectors, vector agents, promoters, and terminators) derived from plant pathogens. Cotton lines 1445 and 1698 were evaluated in field trials conducted under APHIS permits or notifications in 1992, 1993, and 1994. In the process of reviewing the applications for those field trials, APHIS determined that the vectors were disarmed, and that the trials did not present a risk of plant pest introduction or dissemination.

In the Federal Plant Pest Act, as amended (7 U.S.C. 150aa *et seq.*), "plant pest" is defined as "any living stage of: Any insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof, viruses, or any organisms similar to or allied with any of the foregoing, or any infectious substances, which can directly or indirectly injure or cause disease, or damage in any plants or parts thereof, or any processed, manufactured or other products of plants." APHIS views this definition very broadly. The definition covers direct or indirect injury, disease or damage not just to agricultural crops, but also to plants in general, for example, native species, as well as to organisms that may be beneficial to plants, for example, honeybees, rhizobia, etc.

Cotton lines 1445 and 1698 are also currently subject to regulation by other agencies. The U.S. Environmental Protection Agency (EPA) is responsible for the regulation of pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended (7 U.S.C. 135 *et seq.*). FIFRA requires that all pesticides, including herbicides, be registered prior to distribution or sale, unless exempt by regulation. Plants that have been genetically modified for tolerance or resistance to herbicides are not regulated under FIFRA because the plants themselves are not considered pesticides.

In cases in which the genetically modified plants allow for a new use of an herbicide or involve a different use pattern for the herbicide, EPA must approve the new or different use. In conducting such an approval, EPA considers the possibility of adverse effects to human health and the environment from the use of this herbicide.

When the use of the herbicide on the genetically modified plant would result in an increase in the residues of the herbicide in a food or feed crop for which the herbicide is currently registered, or in new residues in a crop for which the herbicide is not currently registered, establishment of a new tolerance or a revision of the existing tolerance would be required. Residue tolerances for pesticides are established by the EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 201 *et seq.*), and the Food and Drug Administration (FDA) enforces tolerances set by the EPA under the FFDCA.

The FDA published a statement of policy on foods derived from new plant varieties in the Federal Register on May 29, 1992 (57 FR 22984–23005). The FDA statement of policy includes a discussion of the FDA's authority for ensuring food safety under the FFDCA, and provides guidance to industry on the scientific considerations associated with the development of foods derived from new plant varieties, including those plants developed through the techniques of genetic engineering.

In accordance with § 340.6(d) of the regulations, we are publishing this notice to inform the public that APHIS will accept written comments regarding the Petition for Determination of Nonregulated Status from any interested person for a period of 60 days from the date of this notice. The petition and any comments received are available for public review, and copies of the petition may be ordered (see the ADDRESSES section of this notice).

After the comment period closes, APHIS will review the data submitted by the petitioner, all written comments received during the comment period,

and any other relevant information. Based on the available information, APHIS will furnish a response to the petitioner, either approving the petition in whole or in part, or denying the petition. APHIS will then publish a notice in the Federal Register announcing the regulatory status of the Monsanto Company's cotton lines 1445 and 1698 and the availability of APHIS' written decision.

Authority: 7 U.S.C. 150aa–150jj, 151–167, and 1622n; 31 U.S.C. 9701; 7 CFR 2.17, 2.51, and 371.2(c).

Done in Washington, DC, this 24th day of March 1995.

Terry L. Medley,

Acting Administrator, Animal and Plant Health Inspection Service.

[FR Doc. 95–7835 Filed 3–29–95; 8:45 am]

BILLING CODE 3410–34–P

95-045-01p

Monsanto

Monsanto Company
700 Chesterfield Parkway North
St. Louis, Missouri 63198
Phone: (314) 694-1000

February 9, 1995

Michael A. Lidsky, Esq.
Deputy Director, BBEP, APHIS, USDA
6505 Belcrest Road
Federal Building
Hyattsville, MD 20782

Subject: Petition for Determination of Non-Regulated Status: Cotton with the Roundup™ Ready gene Lines 1445 and 1698
Monsanto #95-001

Dear Mr. Lidsky:

The Agricultural Group of Monsanto Company is submitting a Petition for Determination of Non-Regulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding Cotton with the Roundup Ready gene lines 1445 and 1698. This petition requests a determination from APHIS that Cotton with the Roundup Ready gene lines 1445 and 1698 and any progenies derived from crosses between Cotton with the Roundup™ Ready gene lines 1445 and 1698 and traditional cotton varieties no longer be considered a regulated article under regulations in 7 CFR part 340. These cotton lines have been tested for 3 years at over 65 locations.

This Petition for Determination contains no confidential business information.

We appreciate your attention to this matter. Should you have any questions, please feel free to contact either Dr. Dickerson at 202-783-2460 or myself (314-537-7054).

Sincerely,

Frank Serdy Ph.D.
Regulatory Affairs Director

cc: C.T. Dickerson, Jr. Ph.D. - Monsanto

Petition for Determination of Non-regulated Status:

Cotton with the Roundup Ready™ gene, Lines 1445 and 1698

The undersigned submits this petition of 7 CFR 340.6 to request that the Director, BBEP, make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by:

Frank S. Serdy, Regulatory Affairs
The Agricultural Group of Monsanto Company, BB3A
700 Chesterfield Parkway North
Chesterfield, MO 63198
Tel: 314-537-7054
FAX: 314-537-7085

February 10, 1995
#95-001

Prepared by:
Frank S. Serdy, and Debbie L. Nida

Contributors:
Robert E. Buehler, Bruce G. Hammond, Eric M. Johnson, Roy Fuchs and Kathryn Kolacz

Contains No Confidential Business Information

Summary

The Agricultural Group of Monsanto is submitting this Petition for Determination of Non-regulated Status to the United States Department of Agriculture (USDA) Animal Plant Health Inspection Service (APHIS) regarding Cotton with the Roundup Ready™ gene, lines 1445 and 1698 which express a form of the 5-enolpyruvylshikimate-3-phosphate synthase protein which imparts tolerance to Roundup® herbicide to these cotton plants (*Gossypium hirsutum* L.). This petition requests a determination from APHIS that Cotton with the Roundup Ready™ gene, lines 1445 and 1698 and any progeny derived from crosses between Cotton with the Roundup Ready™ gene, lines 1445 and 1698 and traditional cotton varieties no longer be considered regulated articles under regulations in 7 CFR part 340.

Cotton is the leading plant fiber crop produced in the world and the most important in the United States. Cotton production in the United States is located primarily in the tier of 15 southern states stretching from North Carolina to California, with approximately 13 M acres grown. Control of weeds in the cotton crop is essential, as they compete with the crop for sunlight, water and nutrients. Failure to control weeds within the crop will result in decreased yields and reduced crop quality. In addition, many weeds, if present at harvest, reduce the efficiency of the mechanical harvest of the crop and can reduce the quality of the lint since the green vegetation stains the lint which reduces its potential uses and value. Present weed management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. On the average weeds must be removed by 6 to 8 weeks after crop emergence to avoid yield loss. Total weed control costs may range from a low of nearly $20/acre to $67/acre for full season weed control depending on location and weed infestation severity. Total losses due to weeds, including cost of control and yield loss, is estimated to be $406 M annually in the United States. The introduction of Cotton with the Roundup Ready™ gene is expected to alleviate at least part of the numbers and costs of herbicide application in current use, both soil applied at or before planting, and applied post-emergence, with considerable advantage accruing to conservation-tillage cotton. Such a system should enhance utilization of integrated weed management (IWM) practices as well.

Roundup Ready™ is a registered trademark of Monsanto Company
Roundup® is a registered trademark of Monsanto Company

Roundup® herbicide is a non-selective herbicide which is applied to the green tissue of growing plants to be controlled. Cotton, like most plants, is susceptible to injury from this herbicide, therefore, it cannot be safely used within the growing crop. Monsanto has developed genetically modified cotton plants that are tolerant to sprays of Roundup herbicide. These cotton plants, named Cotton with the Roundup Ready™ gene, lines 1445 and 1698 (Cotton with Roundup Ready), offers cotton growers the opportunity to reduce the numbers of herbicides and herbicide applications now used to control weeds which reduce cotton yield and quality. This change in weed control practice will result in equal or better control of the weeds at reduced cost to the grower. In addition, Roundup herbicide is considered to be essentially non-toxic and is environmentally friendly, posing little or no threat to wildlife, groundwater, or to the development of resistant weeds.

Results from field experiments were conducted in the years 1992, 1993 and 1994 at over 65 locations throughout the United States cotton growing region have demonstrated that Cotton with Roundup Ready, are tolerant to topical applications of Roundup herbicide (up to the 4-leaf stage) and most weeds contacted by the spray are controlled. Growers planting Cotton with Roundup Ready are likely to be able to reduce the numbers of herbicides used to control the economically destructive weeds which grow in their fields and realize a savings in the cost of weed control. This reduction in herbicide use will not only benefit the environment but also make it possible to implement Integrated Weed Management practices in their fields, a practice generally not possible when pre-plant or pre-emergent herbicides are used.

The commercialization of Cotton with Roundup Ready, following receipt of all required approvals, (including this Determination of Non-regulated Status), will represent an efficacious and environmentally compatible addition to the existing options for cotton weed control. In addition, it will provide significant benefits to growers, the general public and the environment, including:

1. Replace currently used herbicides - growers will substitute Roundup herbicide for more expensive and possibly less reliable pre-plant incorporated and pre-emergent herbicides.

2. Replace costly directed sprays - growers will replace high cost early post directed sprays with Roundup herbicide. Post directed sprays require ground equipment which must be driven very slowly to prevent damage which will result if the products are not precisely applied. With Cotton with Roundup Ready, growers will be able to use a variety of application methods (high speed broadcast booms, etc.). This will provide a strong economic advantage in labor, timeliness and management costs.

3. Soil Conservation Compliance - federal soil conservation laws mandate growers raising crops on fields which are prone to soil erosion, to implement cultural and agronomic practice to reduce soil erosion. One of the most effective ways to reduce soil erosion is to reduce the amount of tillage. Substituting herbicides for mechanical weed control accomplishes the reduced tillage benefit. Roundup herbicide can be used effectively in all conservation or no-tillage systems.

4. Insurance against pre-emergent herbicide inconsistency - Zorial, Prowl, Cotoran are all very dependent on rainfall for activation and weed control and thus become inconsistent in performance. Roundup herbicide is applied to the foliage of the growing plants, and thus is not dependent upon rainfall for activation.

5. Control herbicide resistant weeds - growers will select Roundup herbicide to control weeds which have become resistant to current herbicides. Some examples include DNA resistant goosegrass and pigweed, MSMA resistant cocklebur and aryl-oxy-phenoxy resistant johnsongrass. After 20 years of use worldwide in many situations, there are no known reports of weed resistance to Roundup herbicide, and because of its unique mode of action and lack of soil persistence, resistance is not likely.

6. Stale seed bed applications - eliminate the application of pre-plant herbicide applications to stale seed beds until after planting when the maximum number of weeds may have emerged. This will allow growers more flexibility in preparing the field for planting earlier (reduce management and equipment costs), as well as allowing planting in a more timely fashion (planting when the conditions are most ideal, instead of being delayed because of the need for additional tillage operations.

7. Herbicide combinations - combine a pre-plant herbicide with Roundup herbicide to eliminate the need for a second treatment.

8. Roundup herbicide use in directed sprays - substitute Roundup herbicide in second post-directed sprays replacing MSMA/Cotoran mixtures. The advantage will be broad spectrum effectiveness on grasses and broadleaf weeds; control of unmet needs such as sicklepod, spurred anoda, and morningglory; and management/suppression of perennial species. In addition, use of Roundup herbicide will provide a more economical treatment based on the current herbicide costs.

9. Lay by applications - use of Roundup herbicide at layby applications. This may be applied in combination with residual products like Karmex or Bladex because of the need to eliminate subsequent weed flushes which might interfere with lint quality due to trash and staining.

10. Pre-harvest sprays - one additional use of Roundup herbicide in Cotton with Roundup Ready will be pre-harvest sprays for management of perennial weed escapes. The current application window for use of Roundup herbicide, pre-harvest, is limited to after 60% boll opening has occurred. With Cotton with Roundup Ready, the grower may be able to move to earlier applications such as first boll crack. This provides the opportunity to control weeds like silverleaf nightshade, redvine, or rhizome johnsongrass when they are most susceptible to Roundup herbicide. Additionally, it will allow applications during a period of time which is free of the threat of frost. By applying at this earlier time, the risk of green staining on the lint from these weeds will be reduced.

11. Reliable and economic - Roundup herbicide will provide a more reliable, economical and less labor intensive means to control economically important weeds.

12. Human and environmental safety - use of Roundup herbicide reduces the potential for adverse effects to humans and the environment.

13. Reduction in herbicide use - use of Roundup herbicide will provide growers the means to significantly reduce the amount of chemical herbicides now applied to the crop while maintaining comparable yields and quality.

14. A reduction in the manufacturing, shipment and storage of chemical herbicides used on cotton - use of Roundup herbicide will reduce over all herbicide production, shipment and storage.

15. Reduced spray solution exposure - use of Roundup herbicide will result in an overall reduction in the exposure to workers to the herbicide and herbicide spray solution.

16. Reduced numbers of containers for disposal - use of Roundup herbicide will lead to a reduction in the number of empty herbicide containers and amount of herbicide spray solution that must be disposed of according to applicable environmental regulations.

17. Integrated weed management systems - use of Roundup herbicide provides an ideal fit with IWM and sustainable agricultural systems.

In conclusion, weeds are a severe constraint in the production of cotton worldwide. Cotton cannot compete effectively in its early growth stages and must be protected from the invasion of aggressive weeds. Present management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. On average, weeds must be removed by 6 to 8 weeks after crop emergence to avoid yield loss. Total losses due to weeds, including cost of control and yield loss, is estimated to

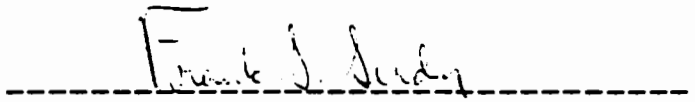
be $406 M annually in the United States. Control costs include early disking and pre-plant herbicide incorporation, a pre-emergence herbicide application at planting (excluding much of the Southwest), and one to three cultivations, either alone or in combination with one to three post-directed herbicide applications. One to two herbicide applications may be applied over-the-top broadcast or in spot treatment to control grass weeds. Total weed control costs may range from a low of approximately $20/acre to a high of $67/acre for full season weed control depending on location and weed infestation severity. The introduction of Cotton with Roundup Ready, is expected to potentially reduce the numbers and costs of herbicide application in current use, both those soil applied at or before planting, and those applied post-emergence, with considerable advantage accruing to conservation-tillage cotton. Such a system should enhance utilization of IWM practices as well. Roundup herbicide is environmentally friendly, posing little or no threat to wildlife, groundwater, or to the development of resistant weeds. The adoption of such a system should also lead to soil improvement since fewer trips across a field will be necessary, and further economical stabilization of cotton production through reduced purchased inputs is expected.

Safety studies summarized in this submission demonstrate that Cotton with Roundup Ready are equivalent, with the exception of the expression of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (CP4-EPSPS) and neomycin phosphotransferase II (NPTII) proteins, to cotton varieties presently grown in the United States. There is no evidence that growing these plants will result in any adverse effects to the environment, nor will any animals such as fish, birds and mammals be affected by these proteins and cotton plants. In addition, agronomic evaluations consisting of plant vigor, growth habit characteristics and general disease susceptibility, have shown Cotton with Roundup Ready to be equivalent to the parental Coker 312 cotton variety. Finally, data generated to support the registration of Roundup herbicide, other available data and 20 years of use experience, demonstrate that this herbicide is essentially non-toxic to humans, mammals and other organisms, and its use in cotton is not expected to cause any adverse environmental effects.

Therefore, the Agricultural Group of Monsanto requests a determination from APHIS that Cotton with the Roundup Ready™ gene, lines 1445 and 1698 and any progenies derived from crosses between Cotton with the Roundup Ready™ gene, lines 1445 and 1698 and traditional cotton varieties no longer be considered regulated articles under regulations in 7 CFR part 340.

Certification

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



Frank S. Serdy, Regulatory Affairs
The Agricultural Group of Monsanto Company, BB3A
700 Chesterfield Parkway North
Chesterfield, MO 63198
Tel: 314-537-7054
FAX: 314-537-7085

Abbreviations Used in this Petition for the Determination of Non-Regulated Status of Cotton with the Roundup® Ready gene, Lines 1445 and 1698

aad	Gene for 3"(9)-O-aminoglycoside adenylyltransferase
AAD	3"(9)-O-aminoglycoside adenylyltransferase
APHIS	Animal Plant Health Inspection Service
C	Centigrade
C312	Coker cotton variety 312
CFR	Code of Federal Regulations
DNA	Deoxyribonucleic Acid
CMoVb	Promoter for EPSPS and GOX genes
CP4 EPSPS	Gene for CP4 EPSPS
CTP1	Transit peptide for directing GOX to the chloroplasts
CTP2	Transit peptide for directing CP4 EPSPS to the chloroplasts
E9 3'	Poly A termination signal for the CP4 EPSPS gene
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
F	Fahrenheit
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
g	gram
GLP	Good Laboratory Practice
GOX	The glyphosate metabolizing enzyme oxidoreductase
IPM	Integrated Pest Management
IWM	Integrated Weed Management
Kb	Kilobase pairs
M	Million
m	meter
mg/kg	milligram per kilogram
ng	nanogram
NOS 3'	Poly A termination signal for *nptII*
NPTII	Neomycin phosphotransferase II
nptII	Gene for neomycin phosphotransferase II
oriV	*Agrobacterium* origin of replication
P-35S	Promoter for *nptII* gene
ppb	part per billion
ppm	part per million
sp	species
T-DNA	Transfer-DNA
µg	microgram
USDA	United States Department of Agriculture
w/w	weight/weight

Petition for Determination of Non-Regulated Status: Cotton with the Roundup Ready gene, lines 1445 and 1698

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Part I. Introduction

A. Rationale For Development of Roundup Ready Cotton

Cotton is the leading plant fiber crop produced in the world and the most important in the United States. Cotton production in the United States is located primarily in the tier of 15 southern states stretching from North Carolina to California, with approximately 13 M acres grown. Control of weeds in the cotton crop is essential, as they compete with the crop for sunlight, water and nutrients. Failure to control weeds within the crop will result in decreased yields and reduced crop quality. In addition, many weeds, if present at harvest, reduce the efficiency of the mechanical harvest of the crop and can reduce the quality of the lint since the green vegetation stains the lint reducing its potential uses and value. Present weed management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. On the average weeds must be removed by 6 to 8 weeks after crop emergence to avoid yield loss. Total weed control costs may range from a low of nearly $20/acre to $67/acre for full season weed control depending on location and weed infestation severity. Total losses due to weeds, including cost of control and yield loss, are estimated to be $406 M annually in the United States. The introduction of Cotton with the Roundup Ready™ gene is expected to alleviate at least part of the numbers and costs of herbicide application in current use, both soil applied at or before planting, and applied post-emergence, with considerable advantage accruing to conservation-tillage cotton. Such a system should enhance utilization of integrated weed management (IWM) practices as well.

Roundup® herbicide is a non-selective herbicide which is applied to the green tissue of growing plants to be controlled. Cotton, like most plants, is susceptible to injury from contact with this herbicide, and therefore, it cannot be safely used within the growing crop. Monsanto has developed genetically modified cotton plants that are tolerant to sprays of Roundup herbicide. These cotton plants, named Cotton with the Roundup Ready™ gene (Cotton with Roundup Ready), offer cotton growers the opportunity to reduce the numbers of herbicides and herbicide applications now used to control weeds which reduce cotton yield and quality. This change in weed control practice will result in equal or better control of the weeds at reduced cost to the grower. In addition, Roundup herbicide is considered to be essentially non-toxic and is environmentally friendly, posing little or no threat to wildlife, groundwater, or to the development of resistant weeds.

Roundup Ready™ is a registered trademark of Monsanto Company
Roundup® is a registered trademark of Monsanto Company

Field experiments were conducted in the years 1992, 1993 and 1994 at over 65 locations throughout the United States cotton growing region under permits from the United States Department of Agriculture (USDA) (#91-347-01, 93-012-03, 93-012-02, 94-027-01 and 94-027-02), in addition to winter seed increases in Puerto Rico during the winter of 1993-94 (USDA permit #93-210-02 and 93-223-02). Results from these experiments have demonstrated that Cotton with Roundup Ready (Lines 1445 and 1698), is tolerant to topical applications of Roundup herbicide (up to the 4-leaf stage), and most weeds contacted by the spray are controlled. Growers planting Cotton with Roundup Ready are likely to be able to reduce the numbers of herbicides used to control the economically destructive weeds which grow in their fields and realize a savings in the cost of weed control. This reduction in herbicide use will not only benefit the environment but also make it possible to implement Integrated Weed Management practices in their fields, a practice generally not possible when pre-plant or pre-emergent herbicides are used.

Safety studies summarized in this submission demonstrate that Cotton with Roundup Ready is equivalent, with the exception of the expression of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (CP4-EPSPS) and neomycin phosphotransferase II (NPTII) proteins, to cotton varieties presently grown in the United States. There is no evidence that growing these plants will result in any adverse effects to the environment, nor will any animals such as fish, birds and mammals be affected by these proteins and cotton plants. In addition, agronomic evaluations consisting of plant vigor, growth habit characteristics and general disease susceptibility, have shown Cotton with Roundup Ready to be equivalent to the parental Coker 312 cotton variety. Finally, data generated to support the registration of Roundup herbicide, other available data and 20 years of use experience, demonstrate that this herbicide is essentially non-toxic to humans, mammals and other organisms, and its use in cotton is not expected to cause any adverse environmental effects.

The commercialization of Cotton with Roundup Ready, following receipt of all required approvals, (including this Determination of Non-regulated Status), will represent an efficacious and environmentally compatible addition to the existing options for cotton weed control. In addition, it will provide significant benefits to growers, the general public and the environment, including:

1. Replace currently used herbicides - growers will substitute Roundup herbicide for more expensive and possibly less reliable pre-plant incorporated and pre-emergent herbicides.

2. Replace costly directed sprays - growers will replace high cost early post directed sprays with Roundup herbicide. Post directed sprays require ground equipment which must be driven very slowly to prevent damage which will result if the products are not precisely applied. With Cotton with Roundup Ready, growers will be able to

use a variety of application methods (high speed broadcast booms, etc.). This will provide a strong economic advantage in labor, timeliness and management costs.

3. Soil Conservation Compliance - federal soil conservation laws mandate growers raising crops on fields which are prone to soil erosion, to implement cultural and agronomic practice to reduce soil erosion. One of the most effective ways to reduce soil erosion is to reduce the amount of tillage. Substituting herbicides for mechanical weed control accomplishes the reduced tillage benefit. Roundup herbicide can be used effectively in all conservation or no-tillage systems.

4. Insurance against pre-emergent herbicide inconsistency - Zorial, Prowl, Cotoran are all very dependent on rainfall for activation and weed control and thus become inconsistent in performance. Roundup herbicide is applied to the foliage of the growing plants, and thus is not dependent upon rainfall for activation.

5. Control herbicide resistant weeds - growers will select Roundup herbicide to control weeds which have become resistant to current herbicides. Some examples include DNA resistant goosegrass and pigweed, MSMA resistant cocklebur and aryl-oxy-phenoxy resistant johnsongrass. After 20 years of use worldwide in many situations, there are no known reports of weed resistance to Roundup herbicide, and because of its unique mode of action and lack of soil persistence, resistance is not likely.

6. Stale seed bed applications - eliminate the application of pre-plant herbicide applications to stale seed beds until after planting when the maximum number of weeds may have emerged. This will allow growers more flexibility in preparing the field for planting earlier (reduce management and equipment costs), as well as allowing planting in a more timely fashion (planting when the conditions are most ideal, instead of being delayed because of the need for additional tillage operations.

7. Herbicide combinations - combine a pre-plant herbicide with Roundup herbicide to eliminate the need for a second treatment.

8. Roundup herbicide use in directed sprays - substitute Roundup herbicide in second post-directed sprays replacing MSMA/Cotoran mixtures. The advantage will be broad spectrum effectiveness on grasses and broadleaf weeds; control of unmet needs such as sicklepod, spurred anoda, and morningglory; and management/suppression of perennial species. In addition, use of Roundup herbicide will provide a more economical treatment based on the current herbicide costs.

9. Lay by applications - use of Roundup herbicide at layby applications. This may be applied in combination with residual products like Karmex or Bladex because of the need to eliminate subsequent weed flushes which might interfere with lint quality due to trash and staining.

10. Pre-harvest sprays - one additional use of Roundup herbicide in Cotton with Roundup Ready will be pre-harvest sprays for management of perennial weed escapes. The current application window for use of Roundup herbicide, pre-harvest, is limited to after 60% boll opening has occurred. With Cotton with Roundup Ready, the grower may be able to move to earlier applications such as first boll crack. This provides the opportunity to control weeds like silverleaf nightshade, redvine, or rhizome johnsongrass when they are most susceptible to Roundup herbicide. Additionally, it will allow applications during a period of time which is free of the threat of frost. By applying at this earlier time, the risk of green staining on the lint from these weeds will be reduced.

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13. Reduction in herbicide use - use of Roundup herbicide will provide growers the means to significantly reduce the amount of chemical herbicides now applied to the crop while maintaining comparable yields and quality.

14. A reduction in the manufacturing, shipment and storage of chemical herbicides used on cotton - use of Roundup herbicide will reduce over-all herbicide production, shipment and storage.

15. Reduced spray solution exposure - use of Roundup herbicide will result in an overall reduction in the exposure to workers to the herbicide and herbicide spray solution.

16. Reduced numbers of containers for disposal - use of Roundup herbicide will lead to a reduction in the number of empty herbicide containers and amount of herbicide spray solution that must be disposed of according to applicable environmental regulations.

17. Integrated weed management systems - use of Roundup herbicide provides an ideal fit with IWM and sustainable agricultural systems.

B. Benefits of Roundup Ready Cotton

The following are summaries of the Agronomic and Economic Benefits of Cotton with Roundup Ready as prepared by Frans *et al.* (1994) and Spurlock (1994) respectively. Copies of these full papers are found in Appendices I and II, respectively.

1. Summary

Weeds are a severe constraint in the production of cotton worldwide. Cotton cannot compete effectively in its early growth stages and must be protected from the invasion of aggressive weeds. Present management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. On the average, weeds must have been removed by 6 to 8 weeks after crop emergence to avoid yield loss. Total losses due to weeds, including cost of control and yield loss, is estimated to be $406 M annually in the United States. Control costs include early disking and pre-plant herbicide incorporation, a pre-emergence herbicide application at planting (excluding much of the Southwest), and one to three cultivations, either alone or in combination with one to three post-directed herbicide applications. One to two herbicide applications may be applied over-the-top broadcast or in spot treatment to control grass weeds. Total weed control costs may range from a low of approximately $20/acre to a high of $67/acre for full season weed control depending on location and weed infestation severity. The introduction of Cotton with Roundup Ready, is expected to have potential for the alleviation of at least part of the numbers and costs of herbicide application in current use, both those soil applied at or before planting, and those applied post-emergence, with considerable advantage accruing to conservation-tillage cotton. Such a system should enhance utilization of IWM practices as well. Roundup herbicide is environmentally friendly, posing little or no threat to wildlife, groundwater, or to the development of resistant weeds. The adoption of such a system should also lead to soil improvement since fewer trips across a field will be necessary, and to further economical stabilization of cotton production through reduced purchased inputs.

2. Agronomic Benefits

The growth of cotton in rows constitutes the creation of ecological niches in which weeds can, and do, flourish (Buchanan and Frans, 1979). These niches, or open areas, are rapidly invaded by aggressive weed species early in the season. Cotton, in its early stages of growth, is not competitive with these invading weeds and must be protected throughout a fairly long establishment period of several weeks. Shading of the soil by the crop canopy, in itself a good weed control measure, does not occur as soon as in other major field crops such as soybeans or corn. Therefore, systems of weed management in cotton have been directed towards controlling the weed pests primarily in the germination, emergence and early establishment stages of growth.

Tillage has long been an important part of these weed management systems. Indeed, tillage interwoven with herbicide practices has been the mainstay of cotton weed control for many years. It is important to note, however, that a good balance must exist between too much or too little of either set of practices. Too much herbicide may stress cotton adversely during this early growth phase in addition to giving rise to intolerable herbicide residue levels in soil and water. Also, too much tillage may cause stress if root pruning occurs from excessive or too deep cultivations. Too little of each, obviously, will result in insufficient weed control (Frans and Chandler, 1989).

Just six weeds or genera of weeds are responsible for the major part of crop loss from weed competition. They are: morninglories (*Ipomoea* spp.), common cocklebur (*Xanthium strumarium* L.), pigweeds (*Amaranthus* spp.), johnsongrass (*Sorghum halepense* [L.] Pers.), nutsedges (*Cyperus* spp.), and prickly sida (*Sida spinosa* L.). Annual grass weeds are also frequently noted as problems, including barnyardgrass (*Echinochloa crus-galli* L. Beauv.), crabgrass (*Digitaria* spp.), goosegrass (*Eleusine indica* [L.] Gaertn.), junglerice (*Echinochloa colonum* [L.] Link), panicum (*Panicum* spp.), and broadleaf signalgrass (*Brachiaria platyphylla* [Griseb.] Nash). Weeds allowed to grow for 5 to 6 weeks after crop emergence and before removal, dramatically reduce growth and ultimate yield of the crop. This early competition results in stand loss, weak and unhealthy plants, and greatly reduced set of fruit (Frans and Chandler, 1989). Systems of control must not only be effective against these species, but also must have the ability to protect the developing crop during the critical period of establishment noted above. The magnitude of crop loss varies according to the weeds present and their respective densities in the field and has been measured as high as 40%.

As previously noted, inter-row cultivation is still a necessary part of weed management schemes in cotton (Buchanan, 1992). Combined with various herbicide practices, including preplant, preemergence, and postemergence, these full ranges of practices are necessary to bring the cotton crop to the point of full canopy closure (Frans and Chandler, 1989; Chandler and Cooke, 1992). Although many studies have been done attempting to substitute herbicide control for cultural control, little evidence exists today that would indicate that inter-row cultivation can be significantly reduced. Hand-hoeing, once extensively used in cotton, has declined dramatically in recent decades, presumably because of the introduction of specific herbicides which gave better and more economical control (Frans and Chandler, 1989), and the lack of availability of personnel and rising labor costs (ranging from $10 to $20 per acre - Chandler and Cooke, 1992)

Conventional Tillage Practices - Under conventional culture, several herbicide practices are combined with inter-row cultivation to achieve maximum protection from weed infestations. The following are examples of these programs used in the southeast and midsouth cotton growing regions:

Pre-plant applications - Treflan or Prowl, will be mixed shallowly in the top 2 to 3 inches of soil (called a preplant incorporated application - PPI) either alone (Southeast), or in combination with other residual herbicides such as Zorial (Midsouth, with Command possibly substituting for Zorial in such future combinations in both areas).

Pre-emergence applications - herbicides (Cotoran/Meturon or Zorial - Southeast), or various combinations of herbicides, including Dual-Cotoran/Meturon, Command-Cotoran/Meturon and Bladex-Zorial (Midsouth and Southwest) are those that are applied in bands over the row, usually in the same operation as planting

Post Emergence - multiple post-directed herbicide applications (those applied to the base of the crop plant to cover small emerging weeds) typically are used to supplement early control obtained with the preplant and pre-emergence herbicides. These usually begin when cotton is approximately in the V1 stage of growth (3 to 4 inches) and continue with second applications made approximately in the V3 to V4 stages of cotton growth (6 to 8 inches and later - see Elsner *et al.*, 1978, for a description of these growth stages). The earliest directed applications may include either Cotoran\Meturon or Caparol\Cotoran pre mixed with either MSMA or DSMA. Second or follow-up applications may include Karmex\Direx plus MSMA or DSMA, Bladex or Bladex plus MSMA, Goal, Cobra or Cobra plus MSMA, or Linex.

Layby applications (those applied to both emerged weeds and to the soil at the time of last cultivation) - are also commonly applied after cotton attains a height of at least 15 inches - these may include Bladex, Karmex\Direx, or Linex.

A variation in irrigated cotton of the Southwest would be the use of the pre-plant incorporated herbicides Treflan or Prowl, but not always pre-emergence applications. However, where specific problem weeds exist additional preemergence herbicides such as Caparol and Karmex are utilized. Nevertheless, fewer than 10% of the Southwestern cotton acres receive pre-emergence herbicide applications. Up to three mechanical cultivations are used annually for weed control in this region.

The above herbicide programs have been in use for the past two to three decades and usually give satisfactory control of most weed infestations under "average" climatic conditions. As stable as this program has been, there have been newer herbicide practices utilized to supplement or replace some of the above practices. Grass-weed specific herbicides are now available for control of johnsongrass, bermudagrass (*Cynodon dactylon* [L.] Pers), and annual grasses. These include Poast, Fusilade, Assure, and Select. Because of their grass selectivity, they are sometimes used to substitute for the preplant-incorporated materials (Treflan or Prowl). Nevertheless, the reasonable cost and dependability of the latter herbicides favors their continued use (Nastasi *et al.*, 1986).

Conservation tillage. In contrast to the above traditional systems of cotton culture, conservation-tillage cotton is rapidly gaining in popularity, stimulated in part by the Conservation Compliance Provision of the 1985 Food Security Act. Under this program, farmers with highly erodible cropland are expected to develop a Soil Conservation Service-approved plan for all highly erodible fields. Under the various conservation measures possible, little or no soil stirring is done prior to planting. Control of existing vegetation (burndown applications) is usually accomplished with applications of either Roundup herbicide or Gramoxone alone, or mixed with residual herbicides, such as Goal or Bladex. Cotton is then planted through existing mulch on the soil surface. Under this scenario, the preplant-incorporated herbicides are not used, and preemergence herbicides are depended upon for continued early control.

Grass-specific herbicides such as Dual or Command are mixed with Cotoran\Meturon (Midsouth) to partially substitute for the incorporated materials. Conservation-tillage production of cotton is expected to continue to increase in use and will likely become the dominant pattern of production in the very near future. In irrigated Southwestern cotton production, control of weeds has been the limiting factor in conservation tillage. These systems in the Southwest involve the planting of winter wheat into cotton stalks in the fall, chemical termination of the wheat in the spring with Roundup herbicide, and cotton planted into the wheat residue in April or May. In these wheat residue systems, Treflan and Prowl herbicides are applied through sprinkler irrigation systems prior to the planting of the cotton. Following cotton planting, herbicides such as Caparol, Dual, Command or Cotoran are used on sandy loam or heavier soil types. On loamy fine sands or lighter soils which are predominant in much of the Texas High Plains, pre-emergence herbicide use is very limited due to potential cotton injury.

For the conservation tillage systems of the Southwest, the availability of Cotton with Roundup Ready would be very beneficial to the producers. This availability would also allow more producers to practice conservation tillage and comply with wind and water erosion provisions of the 1985 and 1990 Farm Bills. In addition to conservation tillage, crop rotations, strip crops, and deep moldboard tillage are also accepted practices for meeting these provisions.

Integrated Weed Management (IWM) - The key to effective cotton weed control is management. Most often, the best approach to controlling weeds in cotton is to use two or more methods employed in a systematic fashion. For example, the early tillage practice of seed bed preparation and destruction of existing weed infestations with a disk, is often combined with application of a preplant-incorporated herbicide. Such a systems approach has been referred to as IWM, and is compatible with the overall concept of integrated pest management (IPM) originally developed by entomologists in dealing with insect pests (Buchanan, 1992). Producers must be alert not only to kinds and species of weeds present in their fields, but also to the

timing of their appearance in relationship to control practices available on the farm. This means that periodic surveys or examinations of fields must be performed to stay ahead of the weed problem. Alert management can help producers move away from soil-applied herbicides (put out before weeds or crop emerges) to post-emergence applications which might be applied only as needed. Such an IWM scheme might well result in reduced herbicide applications, greater protection of the environment and increased profitability to the producer. The availability of Roundup herbicide coupled with a tolerant cultivar fits the IWM concept very well. Since Roundup herbicide should control the major cotton weeds referred to above (and many more), its broad-spectrum aspects can be utilized to advantage in cotton. In conservation-tillage cotton, where before-planting applications of Roundup herbicide are used on existing weeds. A second application after planting and when cotton is in the V1 to V2 stage of growth might well suffice for this early-season period, thus potentially eliminating the use of preplant and pre-emergence herbicides entirely. Depending on weeds present, over-the-top applications of Roundup herbicide early in the life of cotton, might well be at least the equal of the newer practices just coming to the market place (Buctril and Staple).

Presently available herbicide systems, particularly in conventionally grown cotton, fit the IWM concept only partially. Soil-applied herbicides (pre-plant incorporated and pre-emergence) are used before one knows what the years' weed infestation will be. Post-directed applications are somewhat more amenable to IWM, since producers must be aware of the weeds present before making these applications. The early Roundup herbicide application in tolerant cotton is truly an IWM practice - used as needed, or on weeds identified as potential problems in the field if not controlled.

Environmental Impacts. Negative environmental impacts due to weed management in cotton come principally from two sources: soil erosion promoted by tillage operations and chemicals applied for weed control which may end up in ground or surface water. Soil erosion may be managed effectively by reducing or eliminating tillage operations and with the management of plant residues. However, preventing herbicide movement into water is more complicated. In general, those herbicides which are applied to recently tilled bare soil as pre-plant or pre-emergent treatments are more likely to become surface water contaminants than herbicides that are applied to emerged weeds. Non-point source groundwater contamination is more likely to occur in areas with very coarse soil texture, shallow groundwater, moderate to high rainfall or high volume sprinkler or furrow irrigation, and from herbicides applied at relatively high rates, with high leaching potential and moderate to long persistence characteristics.

Roundup herbicide offers several advantages over presently available herbicides in terms of environmental protection. First, it is a very broad spectrum herbicide, controlling basically all of the weeds likely to infest a cotton field. This broad spectrum of activity means that fewer different

types of herbicides will be necessary for use in the crop, resulting in fewer herbicide applications in any given year. Second, Roundup herbicide is applied to emerged weeds, which allows the grower to treat only those fields or parts of fields in which weeds are known to exist at economically damaging populations. This post-emergence approach utilizing economic thresholds for determining the need for herbicide use embodies the essence of the integrated pest management (IPM) philosophy in weed management and lessens the need for growers to use insurance type treatments. Third, Roundup herbicide degrades very rapidly in soil and does not leach. Therefore, it does not pose a ground or surface water threat and leaves no soil residue to interfere with rotational crop selection. In addition, Roundup herbicide has very low mammalian toxicity and poses no chronic health effects. Therefore, it would not cause problems in areas where endangered animal species are a consideration. It is not volatile, and does not move off target after application. Finally, the likelihood of development of Roundup herbicide-resistant weeds is very low. After 20 years of use worldwide in many situations, there are no known reports of weed resistance to Roundup herbicide, and because of its unique mode of action and lack of soil persistence, resistance is not likely.

Other advantages. Because the number of herbicide applications in cotton would be reduced with the adoption of Cotton with Roundup Ready, there would be a concomitant reduction in containers for disposal and a reduction in herbicide exposure to handlers and applicators. Roundup herbicide is currently approved for many uses, so the problem of disposal of excess tank mixes and reinstates would be lessened since any excess could likely be used in another approved manner.

A final benefit to the environment with Roundup herbicide availability for use in cotton is the potential for a reduction in trips over the field. This is an advantage not only economically, but may indirectly lead to less soil tillage. Each trip across a field with equipment increases soil compaction. Since crops grow better in areas with less compacted soil, most growers use tillage to ensure that soils do not become excessively compacted. This increased tillage places the soil at higher risk of erosion. Fewer trips across a field with equipment would mean fewer tillage operations.

Replacement advantage of the Roundup herbicide option. In the cotton production areas of the United States where currently available weed control options do not provide satisfactory control of specific annual and perennial weed species, the availability of Cotton with Roundup Ready will provide a clear economic advantage over spot spraying, cultivation, and hand labor. Where spot spraying, cultivation, or hand labor is utilized, yields are often lost not only to the weed competition but also to the control process. It is very difficult to achieve spot spraying or hand labor weed control of problem weeds without injury to adjacent cotton plants.

Sustainability with Roundup herbicide. This technology will provide economic advantages as mentioned earlier in conservation tillage systems and allow producers to achieve a higher level of sustainable systems. In some areas, spot spray technology has almost eliminated the use of hand labor. With Cotton with Roundup Ready, the labor requirements associated with spot spraying will be reduced for an economic advantage to the producer.

Environmental safety. The availability and utilization of Cotton with Roundup Ready will provide environmentally safe technology for all producers, regardless of size of operation. The availability of this technology should not provide economic advantage to any one group of producers per se, and the anticipated cost of seed and herbicide should allow large or small producers equal access to the technology.

In addition, the availability of Cotton with Roundup Ready should ensure the availability of an environmentally safe weed control mechanism for use in cotton production which adjoins urban areas. This technology will also help the cotton producer meet the expectations of the 1985, 1990, and anticipated 1995 Farm Bills which require control of wind and water erosion and use of environmentally safe materials. This technology should also be more compatible with areas of Endangered Species concern. Furthermore, this technology is not expected to have a significant net change in any areas of employment.

3. Economic Benefits

The introduction of Cotton with Roundup Ready will have significant impact on the profitability of some cotton farms and related agribusinesses. This Cotton will allow cotton growers to apply Roundup herbicide (a broad-spectrum herbicide) after cotton plants have emerged and thus be able to eliminate the application of some currently used herbicides. It is expected that growers who adopt Cotton with Roundup Ready will do so in an attempt to reduce their overall weed control costs. However, it is expected that the cost of Cotton with Roundup Ready seed (used for planting) will be greater than the cost of conventional cotton seed. Thus, the grower's decision to adopt Cotton with Roundup Ready will be impacted by the expected profitability of conventional cotton relative to that of Cotton with Roundup Ready.

Weed control costs. In a recent survey of weed control costs across the cotton belt (Chandler and Cooke, 1992), it was found that total costs for full-season weed control ranged from $19.31 per acre in the Southern High Plains of Texas to $67.18 per acre in Arkansas. In the Southeastern states of Alabama, Georgia, Florida, and North Carolina, the average cost for full-season weed control was $41.87 per acre, and South Carolina and Tennessee averaged $53.20. In the Mid-South, the average cost for Missouri and Louisiana was $42.23 and for Arkansas and Mississippi - $64.62. In Oklahoma and Texas, costs were greatly influenced by availability of

moisture, and ranged from $25.58 dry land (disking twice with an application of a preplant incorporated herbicide and two cultivations) to $46.13 per acre on irrigated areas (Oklahoma). Texas costs were lower, ranging from $19.31 dry land (High Plains) to $37.17 per acre in the Coastal Bend area where rainfall is higher. In the western states of Arizona, California, and New Mexico, cotton is irrigated, and weed control costs averaged $58.19 per acre. These costs include the cost of mechanical cultivation, cost of the herbicide, application costs and hand hoeing costs.

Weed control systems incorporating Cotton with Roundup Ready have yet to be developed, thus the exact savings over the above costs cannot be estimated at this time. Since Roundup herbicide is priced competitively with other herbicides currently used, one would expect a savings to the grower as both the number of applications and herbicides used are replaced. However, it is estimated that the economic impacts on cotton growers who adopt Cotton with Roundup Ready could be significant in some regions of the country. One application of Roundup herbicide at the broadcast rate of 24 ounces per acre would cost about $7.50 per acre (excluding application cost). Banded application rates would be less expensive than broadcast rates. Use of Roundup herbicide in place of conventional weed control programs, which could include chemical herbicides, mechanical cultivation, and/or hand hoers, will likely reduce a grower's weed-control cost.

In recent years weed control practices in cotton have changed due to the loss of some commonly used herbicides due to regulation and the introduction of newer and more effective products. New cotton varieties that are designed to be tolerant to Roundup herbicide will allow cotton growers to control weeds with less chemical herbicides than are now used. Cotton with Roundup Ready allows the use of Roundup herbicide (a broad-spectrum herbicide) during the growing season in place of other conventional weed control chemical herbicides. Farmers who adopt Cotton with Roundup Ready, would expect to see slight revenue increases and possible cost decreases; if expectations are correct, then profits would increase.

Cotton lint yields are expected to be similar under both conventional and Cotton with Roundup Ready production systems. However, it is possible that Cotton with Roundup Ready will allow for more-effective weed control, leading to marketable lint that has lower levels of trash contamination from grasses and broadleaf weeds than conventional cotton. Lint having lower trash levels will generally command a slightly higher price.

Per-acre production costs of Cotton with Roundup Ready are expected to be impacted due to the changes in herbicides used and the substitution of Cotton with Roundup Ready seed for conventional cotton seed. Growers who adopt Cotton with Roundup Ready will simply substitute this Cotton seed for conventional cotton seed and then alter their weed control practices. Thus, the added cost of the Cotton with Roundup Ready seed must be compared with the savings obtained from the adoption of new weed control practices.

Due to the diverse and complex interactions throughout the agricultural sector and other sectors of the economy, it is difficult (if not impossible) to predict future magnitudes of key variables with a high degree of accuracy. However, speculation about the direction of change in these variables may be beneficial. For instance, pesticide regulations in the United States will likely become more restrictive over time. Imposed reductions in herbicide use without Cotton with Roundup Ready will cause cotton yields to decline, farm profits to decline, and acres devoted to cotton production to decline, especially in those regions where herbicide use is an integral production practice. A scenario which allows for the introduction of Cotton with Roundup Ready results in a very different forecast. Reductions in herbicide use can be had without yield reductions, farm profits will increase, and acres devoted to cotton will remain constant or even increase in some regions.

It is often argued that some new technologies have characteristics which promote a more rapid rate of adoption by large farms than by small farms (Kuchler, 1990). For instance, large initial investment costs or high levels of management may preclude small farms from adopting the technology. However, the adoption of Cotton with Roundup Ready will not have a negative impact on small farms. No specialized equipment will need to be purchased and both small and large farms will have the same per acre costs and benefits from the adoption of Cotton with Roundup Ready. Thus, adoption rates should be equal for all size farms.

Conclusions - The adoption rate of Cotton with Roundup Ready will be influenced by economic factors. Cotton growers will evaluate the profit potential of Cotton with Roundup Ready relative to that of conventional cotton. Due to varying weed infestation levels and weed control practices in different regions of the country, some growers will be able to increase profits by adopting Cotton with Roundup Ready, whereas other growers will not. Where profitable, one can expect a variety of benefits to accrue to the grower, the surrounding community and the environment. These include, use of Roundup herbicide proven properties of low toxicity and environmental safety, reduced numbers of herbicides used and reduced numbers of applications. Use of Cotton with Roundup Ready will allow the grower to reduce soil erosion lose from fields and finally, growers may be able to control some weeds which are difficult to control with present herbicides, thus reducing the need for other control measures.

C. Regulatory Approvals

Before commercializing Cotton with Roundup Ready, Monsanto will obtain the following regulatory approvals:

1. This determination from USDA/APHIS that Cotton with Roundup Ready (Lines 1445 and 1698), and all progenies derived from crosses between Lines 1445 and 1698 and other cotton cultivars, are no longer regulated articles according to 7CFR §340.6.

2. Regulatory approval from the EPA for the use of Roundup herbicide over the top of Cotton with Roundup Ready.

In addition, we will complete our consultations with the FDA under their May 29, 1992 policy statement concerning foods derived from new plant varieties.

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Part II. Description of the Biology of the Cotton Family

A. Cotton as a Crop in the United States.

According to Niles and Feaster (1984), cotton production in the United States is located primarily in the tier of 15 states stretching from North Carolina to California. The primary producing states are: Alabama, Arkansas, Arizona, California, Georgia, Florida, Louisiana, Mississippi, Missouri, New Mexico, North Carolina, South Carolina, Oklahoma, Tennessee and Texas. Of these states, the largest producers in 1993 were (in order of production); Texas, Mississippi, California, Arkansas and Louisiana, which, in 1993, accounted for approximately three quarters of the total United States production.

Two species of cotton are grown commercially in the United States: *Gossypium barbadense*, commonly called Pima or Egyptian cotton, and *Gossypium hirsutum*, commonly called upland cotton. *G. hirsutum* is noted for its general adaptability and high productivity and is the predominant species in the United States and the world (Lee, 1984). Upland fiber is used for cordage and other non-woven products, as well as for textiles. In addition, upland cotton linters, which are the short fibers removed from seeds prior to crushing, are a major source of industrial cellulose. *G. barbadense* is noted for the length and quality of its fiber and its production in the United States is primarily restricted to Arizona, New Mexico and West Texas (Niles and Feaster, 1984). Pima fiber, because of its high quality, is used primarily for sewing threads and luxury fabrics.

Niles and Feaster (1984) have classified the upland cultivars grown in the United States into four major types: Acala, Delta, Plains and Eastern.

The **Eastern** type is of special interest since it includes the Coker cultivar which provides the genetic background for the transformant containing the protein that is the subject of this application.

The **Acala** type cultivars are produced primarily in the irrigated areas in West Texas, New Mexico, Arizona and California. In the first of these states, the Acala cultivars grown are predominantly of the Acala 1517 family, whereas production in California is confined to cultivars derived from the Acala SJ series. The Acalas account for approximately 11% of the total United States production.

The **Delta** types account for approximately one-third of the total United States production, primarily of the Deltapine and Stoneville series. Adaptation of Delta-type cultivars, generally, is quite broad and representative cultivars are grown in every cotton-producing state.

The **Plains** type comprises a rather heterogeneous group of cultivars essentially confined to Texas and Oklahoma, with limited production in eastern New Mexico. They account for more than 40% of the total United States production.

B. Taxonomy of cotton

Cotton is of the genus *Gossypium* of the tribe Gossypieae of the family Malvaceae of the order Malvales (Fryxell, 1979; Munro, 1987). The genus *Gossypium* is comprised of 39 very diverse species which occur in widely separated parts of the world, typically in relatively arid parts of the tropics and subtropics (Fryxell, 1984). Worldwide, four species of cotton are of agronomic importance: the two diploid Old World (or Asiatic) species, *G. arboreum* and *G. herbaceum*; and the two allotetraploid New World species, *G. barbadense* and *G. hirsutum*. Although the old world species remain important in restricted areas of India, Africa and Asia, the two new world species account for about 98% of the world's cotton fiber production. Of this amount *G. hirsutum* accounts for 90% while *G. barbadense* accounts for 8% (Lee, 1984).

Wild species of *Gossypium* typically occur in arid parts of the tropics and subtropics. Fryxell (1984) subdivides the wild diploid species into the following three geographical groups: the Australian group (11 species), the Afro-Arabian group (8 species) and the American group (12 species). Two species of the American group occur in Peru and in the Galapagos, and the remaining 10 occur in western Mexico with one (*G. thurberi* Todaro) extending into Arizona.

In addition to the wild diploids, the following wild tetraploid species of *Gossypium* occur in the New World (Fryxell, 1984): *G. tomentosum* (Hawaii); *G. mustelinium* (northeastern Brazil); *G. darwinii* (the Galapagos); *G. lanceolatum* (Mexico, in house yard cultivation); *G. barbadense* originally from the Antilles, South and Central America (Fryxell, 1984) and now growing wild on the coasts of Peru, Ecuador and possibly the Galapagos Islands (Lee, 1984); and *G. hirsutum* (indigenous to Middle America), the Antilles and certain Pacific islands (Fryxell, 1984) and now growing in its wild or commensal forms in the drier areas of Middle America, Northern South America, the West Indies, the southern tip of Florida, Polynesia, North Africa and southern Asia (Lee, 1984). The wild populations of *G. hirsutum* are relatively rare and tend to be widely dispersed. All grow on beach strands or on small islands (Lee, 1984).

There are four species of cotton in the United States. Two of them, *Gossypium hirsutum* (upland cotton), and *Gossypium barbadense* (sea island cotton, pulpulu haole), are used commercially and escaped plants can be found growing in the wild climates where they can survive in the winter, *i.e.* southern Florida and Hawaii. In addition, only two native species of *Gossypium* occur in the United States: *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seeman (Brown and Ware, 1958; Fryxell, 1979; Munro, 1987). The former has been described by Kearney and Peebles (1952).

Gossypium thurberi Todaro (*Thurberia thespesiodes* Gray) is found in southern Arizona in mountainous regions. It is found in the following counties: Graham, Gila, Pinal, Maricopa, Cochise, Santa Cruz and Pima. It has also been found in the Bradshaw Mountains (Yavapai County). It is generally found at elevations of 2,500 to 5,000 feet and is common on rather rocky slopes and sides of canyons in the late summer and fall. It has been described as a handsome shrub, known in Sonora as algodoncillo (little cotton), reaching a height of 4.2 m. Petals are normally spotless, but plants with faint crimson basal spots are not rare. Any gene exchange between this species and *G. hirsutum*, if it did occur, would result in triploid (3x=39), sterile plants because *G. hirsutum* is an allotetraploid (4x=52) and *G. thurberi* is a diploid (2x=26). Such sterile hybrids have been produced under controlled laboratory conditions, but they cannot persist in the wild; in addition, fertile allohexaploids (6x=78) have not been reported in the wild (Stewart 1991).

G. tomentosum is a tetraploid and is found on Hawaii (Degener, 1946). The local range is on the larger islands as well as on Nihau and Kahoolawe. It grows on arid, rocky or clay plains not far from the sea. Thus, on the larger islands, it is found chiefly on the dry, leeward side. On Oahu it is common near Koko Crater, and grows scattered between Honolulu and Markus Balley. On Molokai it is extremely common on the southwestern end; elsewhere it is rare except near Kamalo. Specimens growing near Kaunakakai differ from the typical. On Maui the species may be found from the sea in one of the valleys south of Wailuku.

Hence, only 2 wild species of cotton are known to inhabit the United States, the *G. thurberi* Todaro as previously listed and the *G. tomentosum* which is endemic to Hawaii. Only the *G. tomentosum* is considered to be capable of crossing with the domesticated *G. hirsutum* and *G. barbendense* and produce fertile offspring.

C. Genetics of Cotton

Based on cytological evidence, seven genomic types, A through G inclusive, many with subtypes, have been identified for the genus *Gossypium* (Endrizzi *et al.*, 1984). Diploid species, AA, BB, etc (2n=2x=26), are distributed among tropical and subtropical regions worldwide. As noted above, two of the diploid species, *G. herbaceum* and *G. arboreum,* are of regional agronomic importance.

Worldwide, there are six allotetraploid species (2n=4x=52). All of these are of the genomic group AD and euploids are frequently represented as AADD. The allotetraploid species appear to represent the fusion of the A genomic group from the old world with the D genomic group from the new world. Both *G. barbadense* and *G. hirsutum* are of the AD genomic group. Other members of this group are *G. tomentosum* (Hawaii); *G mustilinum* (Brazil), *G. darwinii* (Galapagos Islands) and *G. lanceolatum* (Mexico).

D. Pollination of Cotton

Although natural crossing can occur, cotton is normally considered to be a self-pollinating crop (Niles and Feaster, 1984). The pollen is heavy and sticky and transfer by wind is unlikely. Pollen is transferred instead by insects, in particular by various wild bees, bumble bees (*Bombus* sp.), and honeybees (*Apis mellifera*).

The range over which natural crossing occurs appears to be limited. McGregor (1976) traced movement of pollen by means of fluorescent particles and found that, even among flowers located only 150 to 200 feet from a cotton field which was surrounded by a large number of bee colonies to ensure ample opportunity for transfer of pollen, fluorescent particles were detected on only 1.6% of the flowers. For the sake of comparison, the isolation distances for foundation, registered and certified cotton seed are 1320 feet, 1320 feet and 660 feet respectively (7CFR§201).

E. Weediness of Cotton

G. hirsutum is ineffective as a weed. Wild populations are rare, widely dispersed and confined to beach strands or to small islands (Lee, 1984). It appears to be somewhat opportunistic towards disturbed land and appears not to be especially effective in invading established ecosystems. In the continental United States, wild populations of *G. hirsutum* exist only in the southern tip of Florida, due at least in part to the fact that cotton cannot over-winter in those areas where freezing conditions occur.

F. Potential Routes of Gene Escape in Cotton

Three potential routes of gene escape in cotton are considered: (1) by vegetative material; (2) by seed; and (3) by pollen. Cotton does not commonly propagate from vegetative material, and, even if it did, it would be unlikely to survive the freezing winters which occur throughout most of the cotton growing regions of the United States. Gene escape via seed is unlikely since voluntarism is very rare for cotton. It should also be noted that cotton bolls, due to their size and general properties, are unlikely to be dispersed by any of the common mechanisms of seed dispersal such as wind, birds or terrestrial animals.

Escape of genes by pollen is possible only if the pollen finds a *Gossypium* species of the correct chromosomal type. In the case of pollen from *G. hirsutum*, the recipient must be an allotetraploid of AADD genome. *G. thurberi*, the native cotton indigenous to Arizona and nearby Mexico, is not a suitable recipient since it is a diploid of DD genotype.

In the United States there are, in fact, only three *Gossypium* species which can serve as recipients for *G. hirsutum*. These are *G. hirsutum* itself, *G. barbadense*, and *G. tomentosum*, which grows only in Hawaii. *G. barbadense* has not been found growing wild in the United States and, thus,

only cultivated plants would be available to be pollinated by *G. hirsutum*. Seed which is intended for planting usually comes from plants which have been segregated from other cotton plants to prevent out-crossing. Thus, if there were such an out-cross, it would almost certainly involve plants whose seed was intended for processing rather than planting, since seed production fields are isolated from commercial cotton fields, and any such escape of genes into *G. barbadense* would be very short-lived and of no significance. This would also be true if the genes escaped from *G. hirsutum* into another strain of cultivated *G. hirsutum*. As noted above, *G. hirsutum* grows wild in southern Florida and, while it is possible that genes could escape to a wild *G. hirsutum*, it is unlikely since there is no commercial cotton production within several hundred miles of this area.

Escape of genes to *G. tomentosum* in Hawaii is possible; however, this is also not likely to occur since there is no commercial cotton production on these islands. In addition, although *G. tomentosum* and *G. hirsutum* are chromosomally compatible, cross pollination is unlikely. First, the flowers of *G. tomentosum* are pollinated by moths rather than by bees as is the case for *G. hirsutum*. Second, the flowers of *G. tomentosum* are receptive at night rather than during the day. In view of these two factors, cross pollination would appear to be unlikely. Nevertheless, the potential for cross pollination of these species will be controlled by maintaining the appropriate isolation distances between any cotton plantings and the wild *G. tomentosum* species.

Additional support for the low out-crossing potential of cotton is found in a paper prepared by Dr. James McD. Stewart of the University of Arkansas on the possible introgression between cultivated cotton and wild relatives contained in Appendix III. The same conclusion was reached by the Environmental Fate and Ground Water Branch of the Environmental Fate Effects Division of the EPA as part of the review to support the Experimental Use Permit under the Federal Fungicide, Insecticide and Rodenticide Act (FIFRA) of these insect resistant cotton plants, EPA Reg. No. 524-EUP-73 (Appendix IV).

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Part III. Description of the Method of Transformation and the Molecular Biology of the Plant

Introduction

Cotton with the Roundup Ready™ gene, Lines 1445 and 1698 (Lines 1445 and 1698), contain the following three genes which were inserted into the genomic DNA using genetic engineering techniques.

- The gene for CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), isolated from *Agrobacterium* sp. strain CP4, encodes an enzyme which is naturally tolerant to glyphosate, the active ingredient in Roundup® herbicide.

- The *nptII* gene, isolated from the Tn5 transposon, encodes the selectable marker neomycin phosphotransferase II (NPTII). This enzyme is important to identify cells transformed with CP4 EPSPS which grow on media containing kanamycin.

- The *aad* gene, isolated from the Tn7 transposon, encodes the bacterial selectable marker, 3"(9)-O-aminoglycoside adenylyltransferase (AAD). This enzyme allows bacteria which contain a plasmid having the *aad* gene to grow in media containing spectinomycin or streptomycin.

Lines 1445 and 1698 are the result of *Agrobacterium tumefaciens* transformation which is described below.

A. Characteristics of the Non-transformed Cultivar

The genetically modified cotton lines presented here have the cotton (*Gossypium hirsutum* L.) cultivar, Coker 312, as the parental line. Coker 312 was released in 1974 by the Coker Pedigree Seed Company and the variety is currently owned by the SeedCo. Corporation of Lubbock, Texas. This older variety of cotton is grown today on a very limited basis. Therefore, Monsanto Company does not intend to introduce Cotton with the Roundup Ready ™ gene (Cotton with Roundup Ready), lines 1445 and 1698, but will allow our seed company partners to transfer the trait into their commercial cotton varieties by traditional breeding techniques.

The Coker 312 cultivar was used because of its positive response to the tissue culture system used in the process to produce transgenic plants. Several researchers (Trolinder and Goodin, 1987; Umbeck, *et al.,* 1987) have demonstrated that Coker 312 and a family of cultivars related to that line have a genetic precondition to respond favorably to tissue culture. Coker 312, although no longer widely grown, is still a commercially acceptable cultivar. Cotton with Roundup Ready,.lines 1445 and 1698, generated with a Coker 312 background is acceptable from an agronomic perspective for testing purposes.

B. *Agrobacterium* Vectors and Transformation

The plant expression vectors described below were assembled, transformed in *E. coli,* and mated into the ABI *Agrobacterium* strain by the triparental conjugation system as described by Ditta *et al.,* using the helper plasmid pRK2013 (Ditta, *et al.,* 1980). The binary ABI strain contains the disarmed (*i.e.,* lacking the T-DNA phytohormone genes) pTiC58 plasmid pMP90RK (Koncz and Schell, 1986), in a chloramphenicol-resistant derivative of the *Agrobacterium tumefaciens* strain A208. The disarmed pMP90RK Ti plasmid does not carry the T-DNA phytohormone genes and is unable to cause crown gall disease. The pMP90RK Ti plasmid was engineered to provide the *trfA* gene functions required for autonomous replication of the plasmid vector after conjugation into the ABI strain. When the plant tissue is incubated with the ABI::plasmid vector conjugate, the vector is transferred to the plant cells via the *vir* functions encoded by the disarmed pMP90RK Ti plasmid (Klee, *et al.,* 1983; Stachel and Nester, 1986). The Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium*. Additional information on this system can be found in the Klee and Rogers, 1989, review.

Usually, only the T-DNA is transferred and integrated into the plant genome (Zambryski, 1982). It is accepted that T-DNA transfer into plant cells by *Agrobacterium* is irreversible (Huttner, *et al.,* 1992). The border sequence itself is not entirely transferred during the process of insertion of the T-DNA into the plant genome (Bakkeren, *et al.,* 1989). This means that the inserted DNA is no longer a functional T-DNA, *i.e.*, once integrated, it cannot be remobilized into the genome of another plant even if acted on again by *vir* genes.

Agrobacterium transformation of cotton hypocotyl sections was performed with modifications described by Umbeck, *et al.* (1987). Plants were regenerated with modifications of those described by Trolinder and Goodin (1987).

C. Plant Expression vectors - PV-GHGT06 and PV-GHGT07

The CP4 EPSPS, *nptII* and *aad* genes were introduced into Coker 312 cotton derived tissue using *Agrobacterium tumefaciens* binary single border transformation vectors (Bevan, 1984; Wang, *et al.,* 1984). The plasmid vectors, PV-GHGT06 (Figure III-1) and PV-GHGT07 (Figure III-2), contain well-characterized DNA segments required for selection and replication of the plasmid in bacteria as well as a right border for initiating the region of DNA (T-DNA) transferred into plant genomic DNA. The plasmids are composed of several genetic elements listed in Table III-1. The 0.70Kb fragment from the RK2 plasmid (Stalker, *et al.,* 1981) provides the origin of replication (0.39Kb *ori-V*) for maintenance in *Agrobacterium tumefaciens* and is fused to the 3.0Kb SalI to PvuI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (0.43Kb *ori*-322) and the *bom*

site for the conjugational transfer into *Agrobacterium tumefaciens* (Bolivar, *et al.,* 1977 and Sutcliffe, 1978). This was fused to 0.09Kb DNA fragment from the pTiT37 plasmid which contains the 0.025Kb nopaline-type T-DNA right border (Depicker, *et al.,* 1982, Zambryski, *et al.,* 1982 and Bevan, *et al.,* 1983). The remaining portion of plasmid DNA consists of two genes engineered for plant expression, CP4 EPSPS and *nptII* in both plasmids and the *gox* gene in PV-GHGT07. [The *gox* gene, which encodes the glyphosate metabolizing enzyme oxidoreductase (GOX) was cloned from *Achromobacter* sp. strain LBAA (Barry *et al.*, 1992; Barry *et al.,* 1994), was not transferred into line 1445.] The *aad (3")* gene is also present and is under the control of a bacterial promoter.

D. Inserted Genes

1. The CP4 EPSPS gene

The gene encoding for CP4 EPSPS (Figure III-3) is driven by the CMoVb promoter (Gowda, *et al.,* 1989; Richins, *et al.,* 1987; Sanger, *et al.,* 1990). The chloroplast transit peptide coding region from *Arabidopsis thaliana* EPSPS (Klee, *et al.,* 1987) is fused to the coding region of CP4 EPSPS (Barry, *et al.,* 1992; Padgette, *et al.,* 1994), to target the CP4 EPSPS to the chloroplast, the site of aromatic amino acid biosynthesis. The 3' region of the gene is from the 3' non-translated region of the nopaline synthase gene of the Ti plasmid, pTiT37 from *Agrobacterium tumefaciens* strain T37 (Bevan, *et al.,*1983; Fraley, *et al.,* 1983). The native CP4 EPSPS gene contains sequences that could be inimical to high expression of the gene in some plants. These sequences include potential polyadenylation sites that are often A+T rich, a higher G+C% than that frequently found in dicotyledonous plant genes (63% versus ~50%), concentrated stretches of G and C residues, and codons that may not be used frequently in dicotyledonous plant genes. A plant preferred version of the gene was synthesized and used in the vectors used for cotton transformation. This coding sequence was expressed in *E. coli* from a *PRecA-gene 10L* vector (Olins, *et al.,* 1988) and the EPSPS activity was compared with that from the native CP4 EPSPS gene. The results established that enzyme expressed from the synthetic gene was unaltered. CP4 EPSPS is expressed in Cotton with Roundup Ready lines 1445 and 1698 and confers tolerance to Roundup® herbicide to both lines.

2. The *nptII* gene

The *nptII* gene, isolated from the Tn5 transposon (Beck, *et al.,* 1982), is driven by 35S promoter (Kay, *et al.,* 1985; Odell, *et al.,* 1985). The *nptII* gene encodes neomycin phosphotransferase type II (NPTII) which confers resistance to the aminoglycoside antibiotics kanamycin and neomycin. The 3' region of the gene is from the 3' non-translated region of the nopaline synthase gene of the Ti plasmid, pTiT37 from *Agrobacterium tumefaciens* strain T37. The *nptII* gene functions as a selectable marker in the initial

laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock, *et al.*, 1984). Cells that contain the *nptII* and CP4 EPSPS genes can be selected for plant regeneration. The *nptII* gene is expressed in Cotton with Roundup Ready lines 1445 and 1698, (See Part V, paragraph A, in this Petition for Determination).

3. The *aad* gene

The *aad* gene, isolated from the Tn7 transposon (Fling, *et al.*, 1985), is under control of its own bacterial promoter. This marker gene encodes 3"(9)-O-aminoglycoside adenylyltransferase (AAD) which allows for of bacteria containing the PV-GHGT06 or PV-GHGT07 to grow on medium containing spectinomycin or streptomycin. This *aad* gene is not expressed in Cotton with Roundup Ready lines 1445 and 1698 (See Part V, paragraph A, in this Petition for Determination).

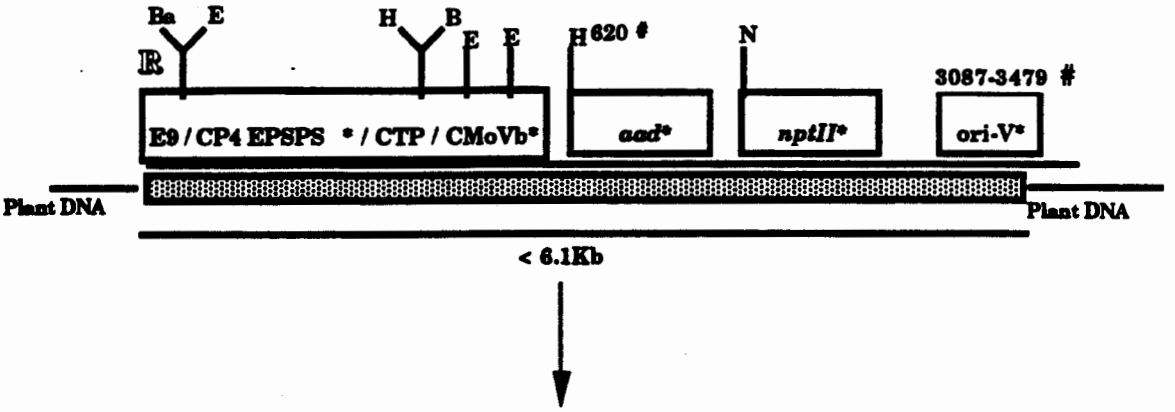
E. Genetic Analysis

1. Cotton with Roundup Ready line 1445

a. Genetic elements

Southern blots with Cotton with Roundup Ready line 1445 (line 1445) have been probed with the ^{32}P labelled DNA sequence corresponding to the CMoVb promoter as well as with ^{32}P labelled coding sequences of GOX, CP4 EPSPS, *aad*, and *nptII* genes, to determine which genetic elements are present within the genome. The CMoVb promoter is present in this line (Figure III-4, lane 3) however, GOX was shown not to be present (Figure III-5, lane 3). The CP4 EPSPS, *aad*, and *nptII* genes are all present (Figures III-6, III-7 and III-8). Southern blots were also probed with ^{32}P labelled *ori-322* and *ori-V* sequences. *Ori-322* (Figure III-9, Panel C) was shown not to be present, but a portion of the *ori-V* is present in line 1445 (Figure III-9, Panels A and B). In order to determine the amount of the *ori-V* which was transferred into line 1445, the following strategy was employed. Two identical blots were performed using PV-GHGT07 plasmid DNA, genomic Coker 312, and genomic line 1445 DNAs all cut with HindIII: one blot was probed with *ori-V* DNA (Figure III-9, Panel B), and the other blot was probed with the coding region of *nptII* (Figure III-8, Panel B). The results show two bands of similar size (Figures III-9, Panel B and Figure III-8, Panel B). The plasmid map of PV-GHGT07, Figure III-2, shows that there is a HindIII fragment (map numbers 620 to 3611) of 3Kb which contains the *nptII* gene and the *ori-V* element. When probed with *ori-V*, a predicted 3Kb band is present in the PV-GHGT07 positive control. Line 1445 (Figure III-9, Panel B, lane 3) shows a 2.7Kb band; the same size bands are present in a similar blot when probed with *nptII* (Figure III-8, Panel B). This establishes that all of the *nptII* gene is present. However, not all of the *ori-V* is present because the map numbers

(Figure III-2) for *ori-V* are 3087 to 3479: the 2.7Kb size fragment observed on the gel indicates that the end of the DNA transferred from PV-GHGT07 is in the area of map number 3300. The actual number may be somewhat less because the second HindIII site is located in the genomic DNA. The schematic diagram below shows this pictorially for line 1445.



Area of plasmid that integrated into genomic DNA (Map not to scale)

*These DNAs were used for probes.

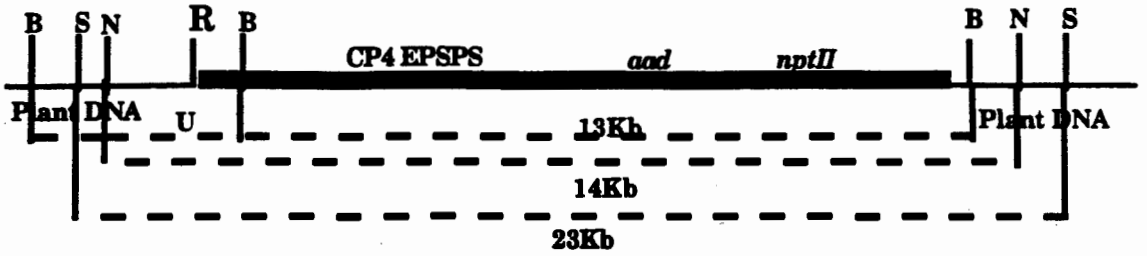
Abbreviations: R=right border, Ba=BamHI, B=BglII, E=EcoRI, H=HindIII

= map number from Figure 2

b. Number of loci

The Southern blot in Figure III-10, which contains Coker 312 genomic DNA (lanes 2, 5, and 8), line 1445 genomic DNA (lanes 3, 6, and 9), cut with BamHI, or NdeI, or SpeI respectively, and PV-GHGT06 DNA cut with NotI (lane 1) all which were probed with ^{32}P labelled PV-GHGT06 DNA. BamHI and NdeI cut once within PV-GHGT07 at map numbers 7828 and 2080 respectively, while SpeI does not cut PV-GHGT07. The results of the BamHI digest (lane 3) show a single band of 13Kb. However on a very long exposure of this same gel, (exposure in data file), a band of 10.8Kb is detectable. These are identified as border fragments. The NdeI digestion (lane 6) shows a single band of 14Kb indicating that the NdeI site (map number 7851, Figure III-2) has not been transferred into line 1445. The SpeI digest (lane 9) shows a single band of 23Kb. All of the above information support the conclusion that line 1445 has a single locus containing DNA from PV-GHGT07. The schematic diagram below illustrates the basis for this conclusion.

Schematic diagram of T-DNA in Line 1445 (not to scale)



Abbreviations: R=right border, S=SpeI, N=NdeI, B=BamHI, U=undetected

c. Copy number of CP4 EPSPS

The analyses of the genetic elements show that the DNA transferred from PV-GHGT07 includes CP4 EPSPS, CMoVb, *aad*, *nptII*, and a portion of the *ori-V*. The analysis of the number of loci show that there is only one locus into which PV-GHGT07 DNA integrated into the genome of line 1445. It can be concluded that there is a single copy of CP4 EPSPS present in this line. See the schematic diagram pictured immediately above this paragraph.

d. Stability of the glyphosate tolerance gene and the T-DNA

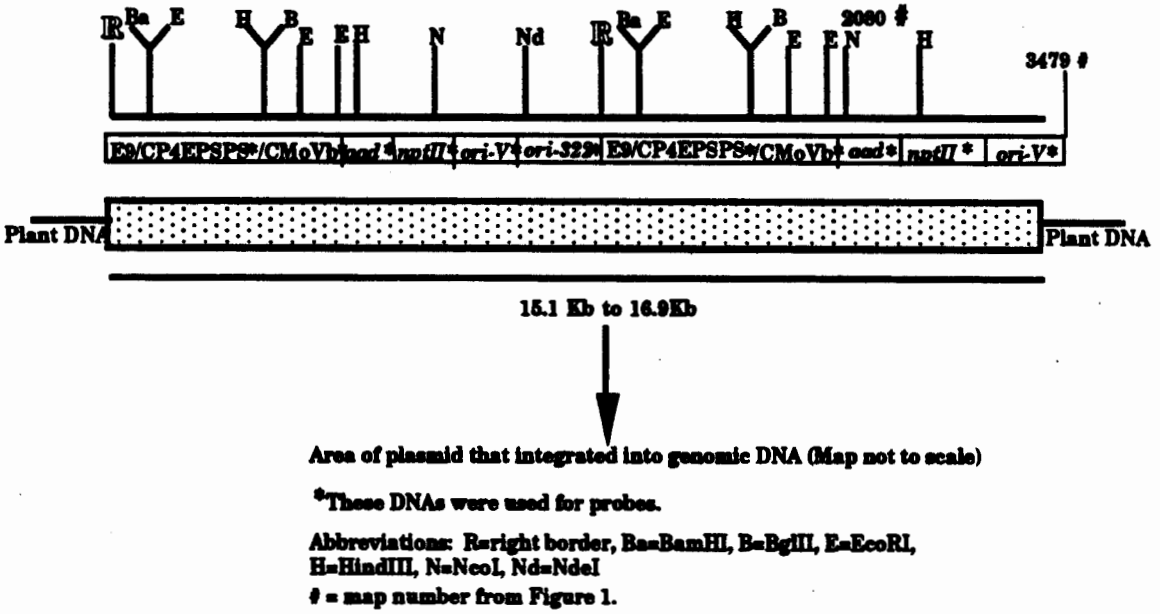
The Southern blot shown in Figure III-11, Panel A, lanes 3 and 4, shows that CP4 EPSPS is stably maintained from the R_3 generation through the R_5 generation. In Panel B, lanes 3 and 4, the blot shows a similar banding pattern when probed with the *nptII* gene. Given this evidence, it is concluded that the T-DNA has been stably maintained for these three generations.

2. Cotton with Roundup Ready line 1698

a. Genetic elements

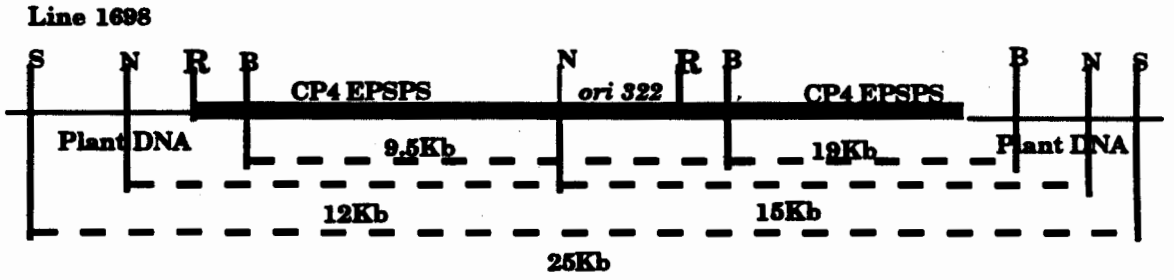
Southern blots with Cotton with Roundup Ready line 1698 (line 1698) were performed with the following genetic elements as probes: CMoVb (Figure III-4), CP4 EPSPS (Figure III-6), *aad* (Figure III-7), *nptII* (Figure III-8), *ori-V*, and *ori-322* (Figure III-9). All of the elements gave bands of the predicted sizes. When *aad* DNA (Figure III-8, Panel B) was used to probe line 1698 cut with BglII (cuts within PV-GHGT06 once) two bands were generated: one of the predicted 9.5Kb and a larger band of 10.5Kb which is identified as a border fragment. Using *aad* DNA as a probe (Figure III-7, Panel B), line 1698 was cut with HindIII (cuts three

times in PV-GHGT06) and generated two bands, one of which was the predicted size of 8.9Kb and a smaller band of 8.0Kb, which is identified as a border fragment. When line 1698 was cut with EcoRI and NcoI, then probed with *nptII* DNA (Figure III-8, Panel A), three bands were observed: the 5.8Kb band and the 1.6Kb band are of the predicted size, but the third band of 3Kb is identified as a border fragment. On a Southern blot with line 1698 cut with EcoRI and NcoI which was probed with *ori-V* (Figure III-9, Panel A), a band of 5.8Kb, the predicted size, was observed as well as a band of 2.9Kb, which is considered a border fragment. A pattern emerges here: the 3Kb bands identified in the Southern blots probed with *nptII* and *ori-V* are of similar size. This establishes that all of the *nptII* is on this fragment as well as all or part of the *ori-V*. Starting from map number 2080 (Figure III-1), the NcoI site, plus the 3Kb EcoRI, NcoI fragment, an integration site at about map number 5080 would be generated. This is well past the end of the ori-V element which is located at map site 3479. However, it is not known if the integration of the PV-GHGT06 occurred in the *ori-V* region or beyond it. What is known is that the site of integration of PV-GHGT06 into line 1698 occurred well before the NdeI site at map number 5359. From this analysis, it can be stated that all of the plasmid PV-GHGT06 integrated into the genome of line 1698 plus an additional piece of plasmid DNA of not more than 7.4Kb or less than 5.6Kb. The schematic diagram below shows the plasmid DNA that was integrated into genomic Coker 312 to generate line 1698.



b. Number of loci

In Figure III-10, the Southern blot, which contained Coker 312 genomic DNA (lanes 2, 5, and 8) and line 1698 genomic DNA (lanes 4, 7, and 10b) cut with BamHI, or NdeI, or SpeI respectively, and PV-GHGT06 DNA cut with NotI (lane 1), which cuts once within the plasmid, was probed with ^{32}P labelled PV-GHGT06. In the BamHI digested lane of line 1698 (lane 4), a band of 9.5Kb, the predicted size, is observed. An additional band of 19Kb is also seen and is identified as a border fragment. The fact that a 9.5Kb band is seen in this digestion indicates that the entire plasmid was integrated into the genomic DNA; the presence of the 19Kb fragment indicates the presence of the additional portion of the plasmid. In the NdeI digestion, which cuts once in PV-GHGT06, two bands (lane 7) are detected: one of 15Kb and the other of 12Kb. Both of these are considered border fragments. There is no band of 9.5Kb detected as there would be if the integrated plasmid DNA had continued around to the NdeI site for a second time. This confirms what is determined in the analyses of the genetic elements. In the SpeI digestion, which does not cut PV-GHGT06, a single band is observed in line 1698 (lane 10b). With the information presented in the genetic elements discussion and the information presented here, it can be concluded that the plasmid PV-GHGT06 integrated into the genome of line 1698 at a single locus. The schematic diagram below illustrates this conclusion.



Abbreviations: R=right border, S=SpeI, N=NdeI, B=BamHI

c. Copy number of CP4 EPSPS

It has been established from the foregoing analyses that plasmid PV-GHGT06 DNA integrated into the genomic DNA of line 1698 as one complete plasmid plus between 5.6Kb and 7.4Kb more plasmid DNA. The total amount of plasmid DNA is between 15.1Kb and 16.9Kb. The number of copies of CP4 EPSPS in this line is 2 copies. There is one copy that is incorporated as a complete plasmid DNA, and an additional copy on the extension of the plasmid DNA that was integrated into the genome at this same location. The schematic diagram above this paragraph shows this concept.

d. Stability of the glyphosate-tolerant gene and the T-DNA

In the Southern blot shown in Figure III-11, Panel A, the line 1698 DNA (lanes 5 and 6) show that CP4 EPSPS is stably maintained during the life cycle of the plant from the R_3 generation through the R_5 generation. In Panel B, lanes 5 and 6, the blot shows a similar banding pattern between the R_3 and R_5 generations when probed with the *nptII* gene. Given this evidence, it is concluded that the T-DNA has been stably maintained for these three generations.

F. Conclusions

No more than 6.1Kb of PV-GHGT07 plasmid DNA integrated into the genomic DNA of line 1445. This T-DNA contains the CMoVb promoter, CP4 EPSPS, the *aad* gene, the *nptII* gene, and a portion of the *ori-V* genetic element. There is a single locus into which the T-DNA integrated and that T-DNA has a single copy of CP4 EPSPS. The stability of the CP4 EPSPS and the T-DNA has been stably maintained from the R_3 through the R_5 generations of line 1445.

Between 15.1Kb and 16.9Kb of PV-GHGT06 plasmid DNA integrated into the genomic DNA of line 1698. The T-DNA contains the CMoVb promoter, CP4 EPSPS, the *aad* gene, the *nptII* gene, the *ori-V* and the *ori-322*. There is a single locus into which the T-DNA has integrated and that DNA contains two copies of CP4 EPSPS. The stability of the CP4 EPSPS and the T-DNA has been maintained from the R_3 through the R_5 generations of line 1698.

Table III-1. Summary of the genetic elements contained in PV-GHGT06 and PV-GHGT07.

Genetic Element	Size, Kb	Function
Right border	0.025	Initiates the T-DNA transfer event
E9 3'	0.63	Poly A termination signal for the CP4 EPSPS gene
CP4 EPSPS	1.36	Gene for CP4 EPSPS
CTP2	0.23	Transit peptide for directing CP4 EPSPS to the chloroplasts
CMoVb	0.57	Promoter for the CP4 EPSPS gene (In PV-GHGT07, promoter for the GOX gene)
aad (3")	0.79	Confers bacterial resistance to spectinomycin/streptomycin
NOS 3'	0.26	Poly A termination for *nptII* (In PV-GHGT07, poly A termination signal for the GOX gene)
nptII	0.79	Plant selectable marker
P-35S	0.32	Promoter for *nptII*
ori-V	0.39	Origin of replication
ori-322	0.43	Origin of replication
GOX	1.3	Only in PV-GHGT07, gene for GOX
CTP1	0.16	Only in PV-GHGT07, transit peptide for directing GOX to the chloroplasts

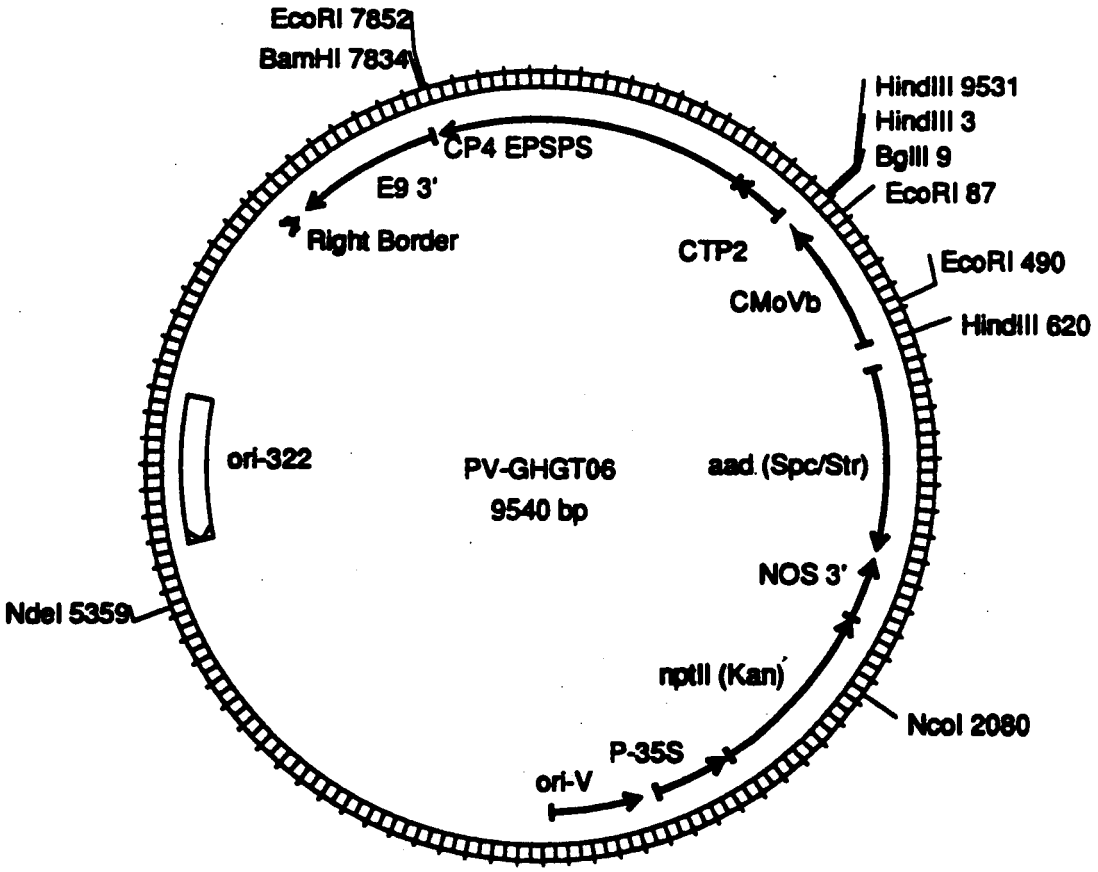


Figure III-1. Plasmid map of PV-GHGT06.

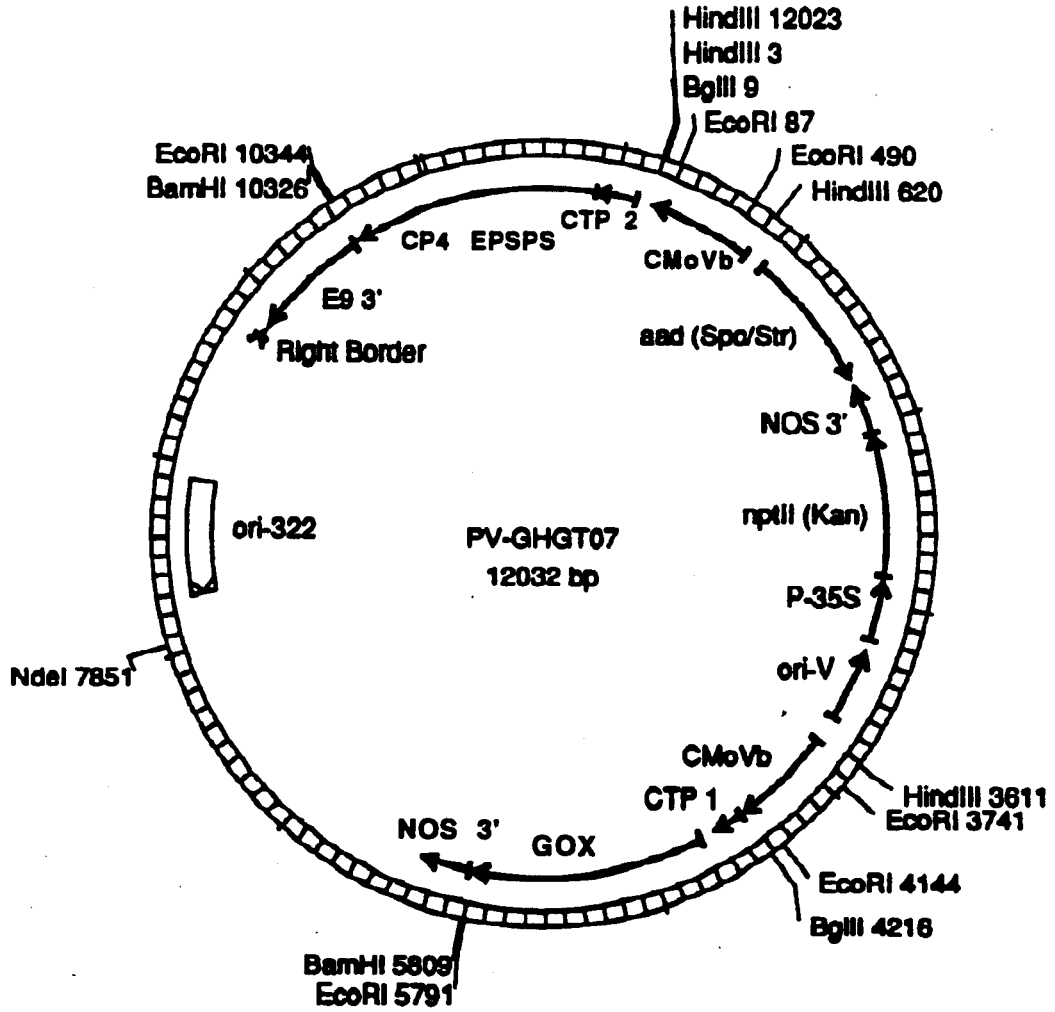


Figure III-2. Plasmid map of PV-GHGT07

95-045-01p

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Figure III-3. The nucleotide sequence and the ammino acid sequence of CP4 EPSPS in Cotton with Roundup Ready Lines 1445 and 1698.

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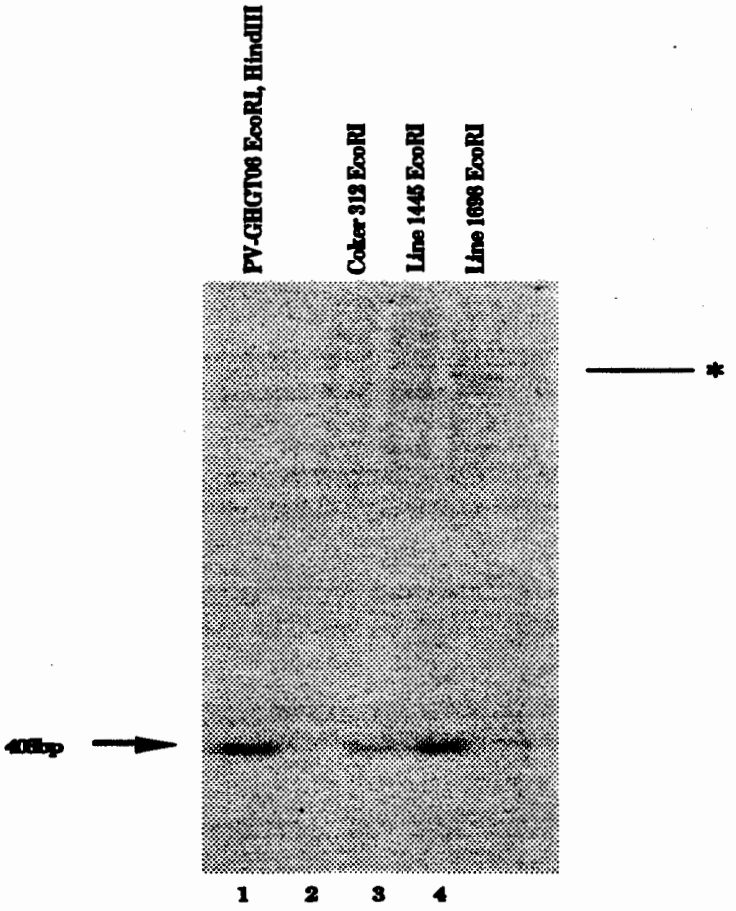
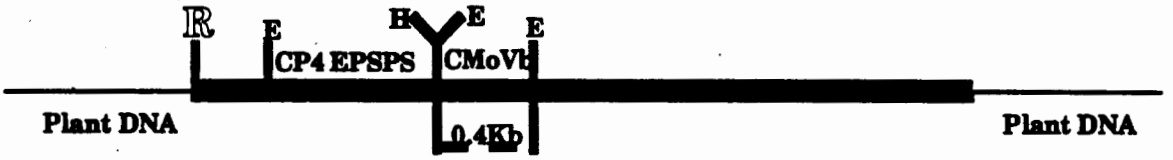
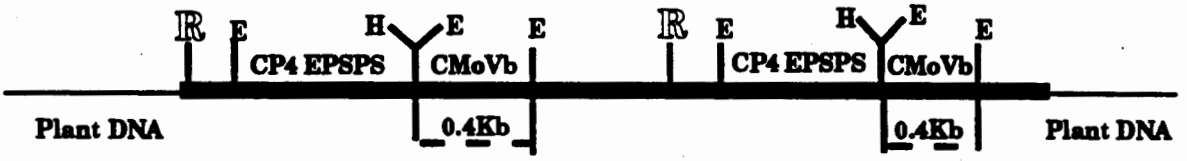


Schematic diagram of T-DNA in line 1445 (not to scale)



Schematic diagram of T-DNA in line 1698 (not to scale)



Probe: CMoVb

Figure III-4. Southern Blot Probed for the CMoVb Promoter in Cotton with Roundup Ready Lines 1445 and 1698. Each lane represents approximately 100 pg of plasmid DNA or approximately 5 ug genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P labelled CMoVb DNA and subjected to autoradiography. Abbreviations: R=right border, E=EcoRI, H=HindIII, *=bands common to all three DNAs.

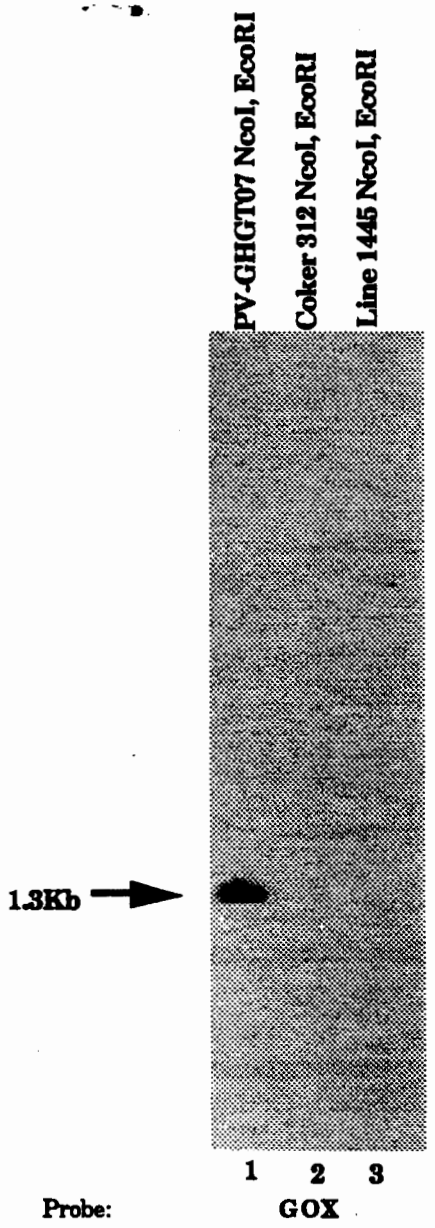
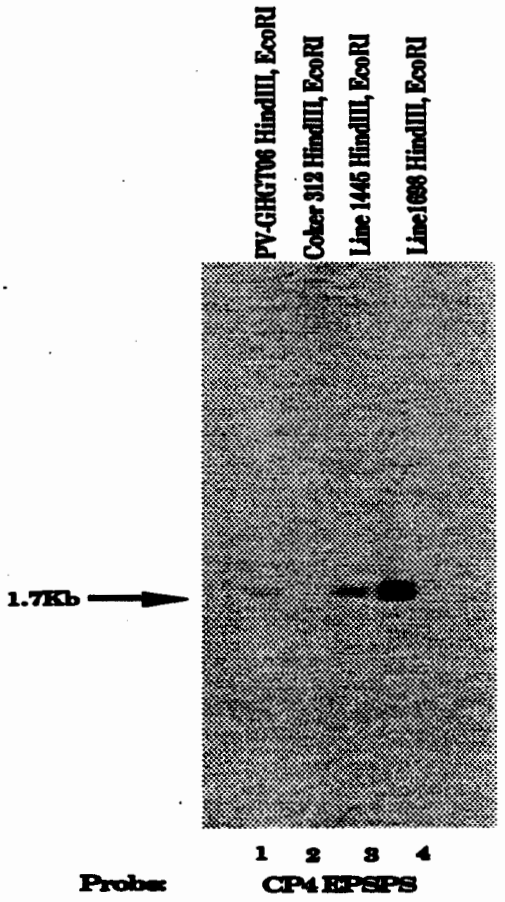


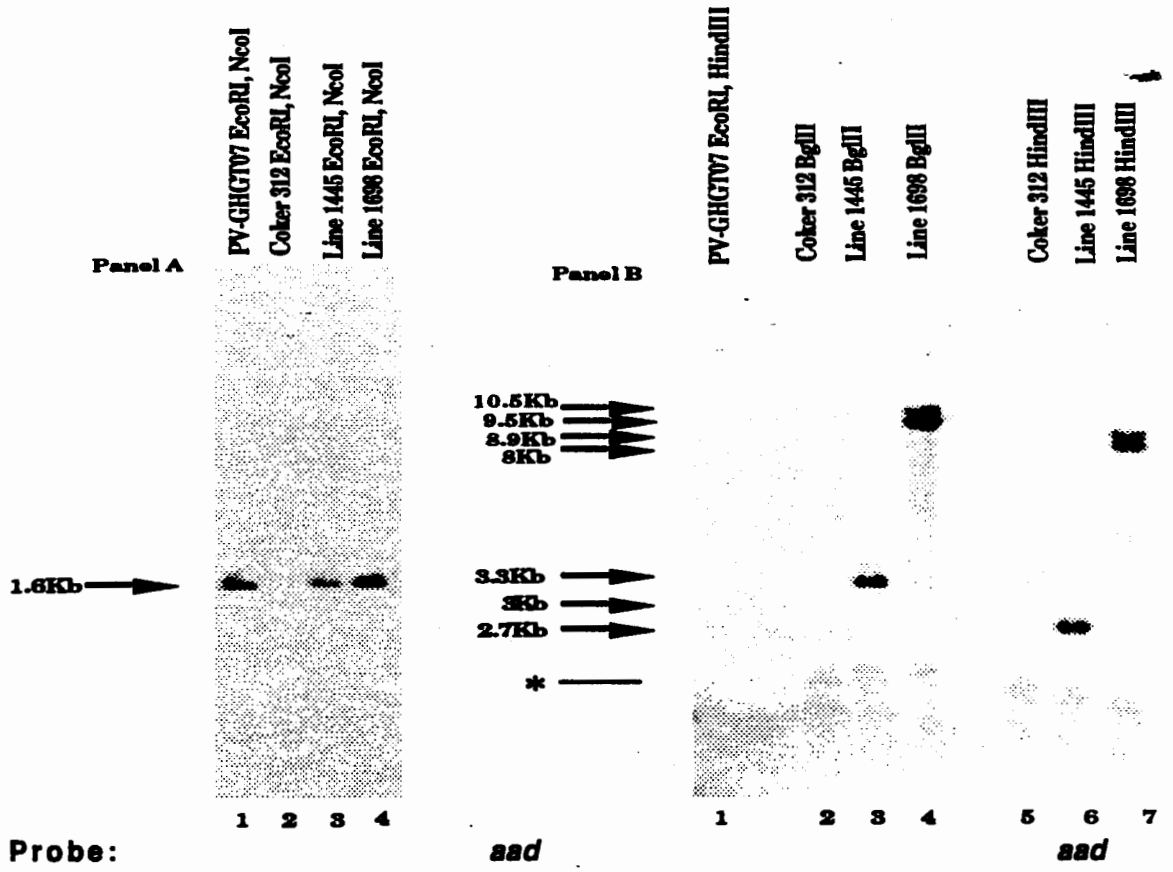
Figure III-5. Southern Blot Probed with GOX in Cotton with Roundup Ready Line 1445. Lane 1 represents approximately 100 pg plasmid DNA. Lanes 2 and 3 represent approximately 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P labelled coding region of GOX and then subjected to autoradiography.



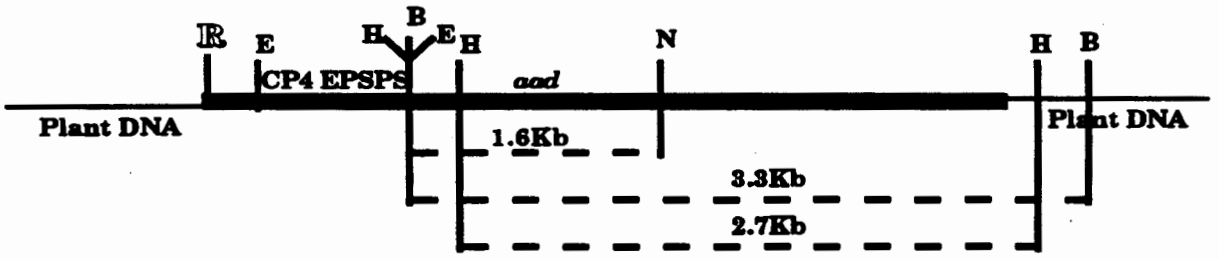
Schematic diagram of T-DNA in Line 1445 (not to scale)

Schematic diagram of T-DNA in line 1698 (not to scale)

Figure III-6. Southern Blot Probed for CP4 EPSPS in Cotton with Roundup Ready Lines 1445 and 1698. Each lane represents approximately 100 pg of plasmid DNA or approximately 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P labelled coding region of CP4 EPSPS and then subjected to autoradiography. Abbreviations: R=right border, E=EcoRI, H=HindIII.



Schematic diagram of T-DNA in Line 1445 (not to scale)



Schematic diagram of T-DNA in Line 1698 (not to scale)

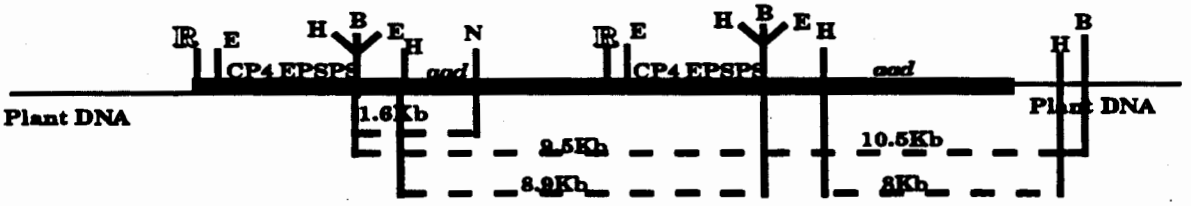


Figure III-7. Southern Blots Probed for the *aad* Gene in Cotton with Roundup Ready Lines 1445 and 1698. Each lane represents approximately 100 pg plasmid DNA or approximately 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P labelled *aad* DNA and then subjected to autoradiography. Abbreviations: R=right border, E=EcoRI, H=HindIII, N=NcoI, B=BglII, *=bands common to all three DNAs.

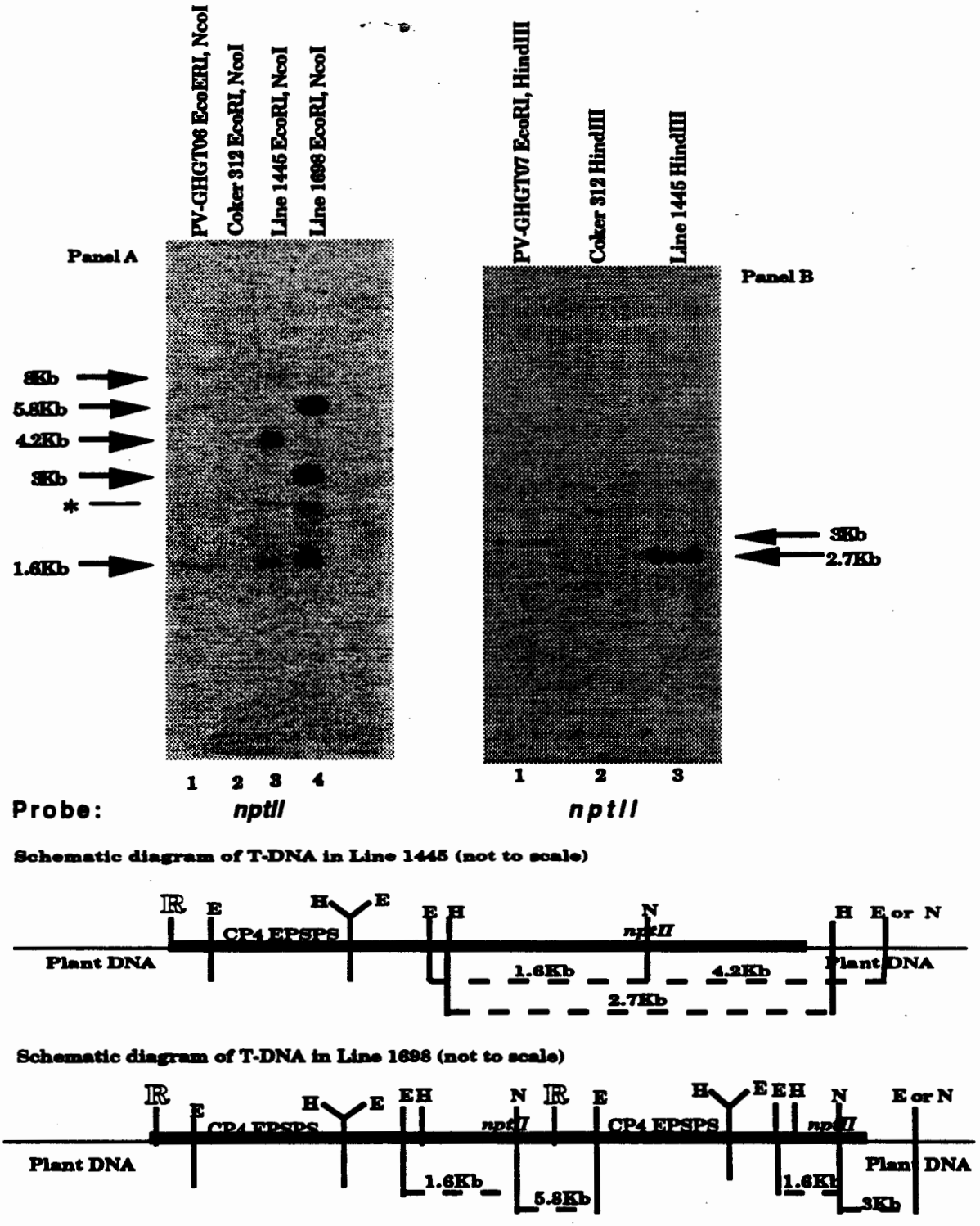


Figure III-8. Southern Blots Probed for *nptII* in Cotton with Roundup Ready Lines 1445 and 1698. Each lane represents approximately 100 pg plasmid DNA. Each lane of genomic DNA in Panel A represents approximately 5 µg. Each lane of genomic DNA in Panel B represents approximately 10 µg. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P labelled *nptII* and then subjected to autoradiography. Abbreviations: R=right border, E=EcoRI, H=HindIII, N=NcoI, *= bands common to all three DNAs.

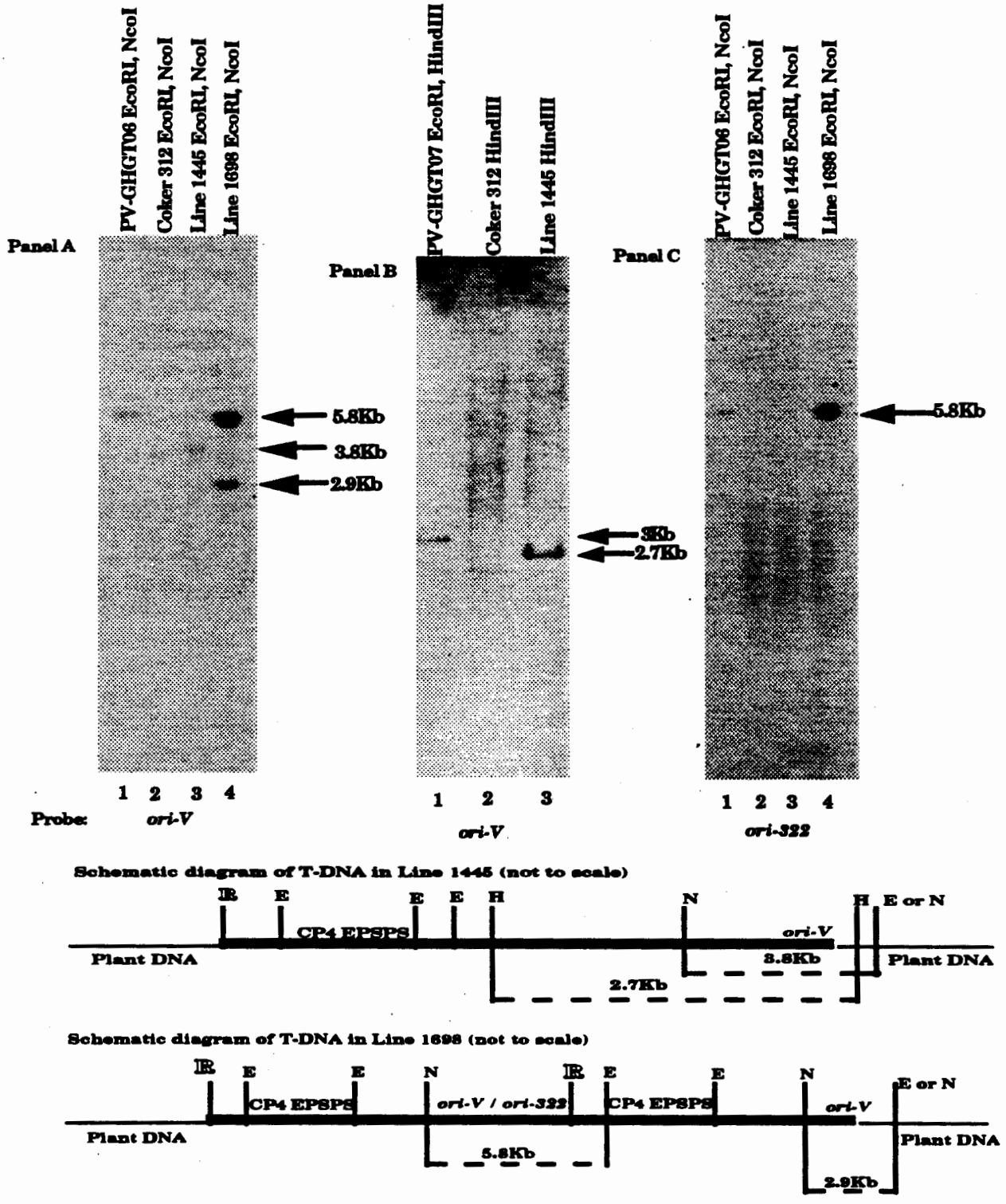
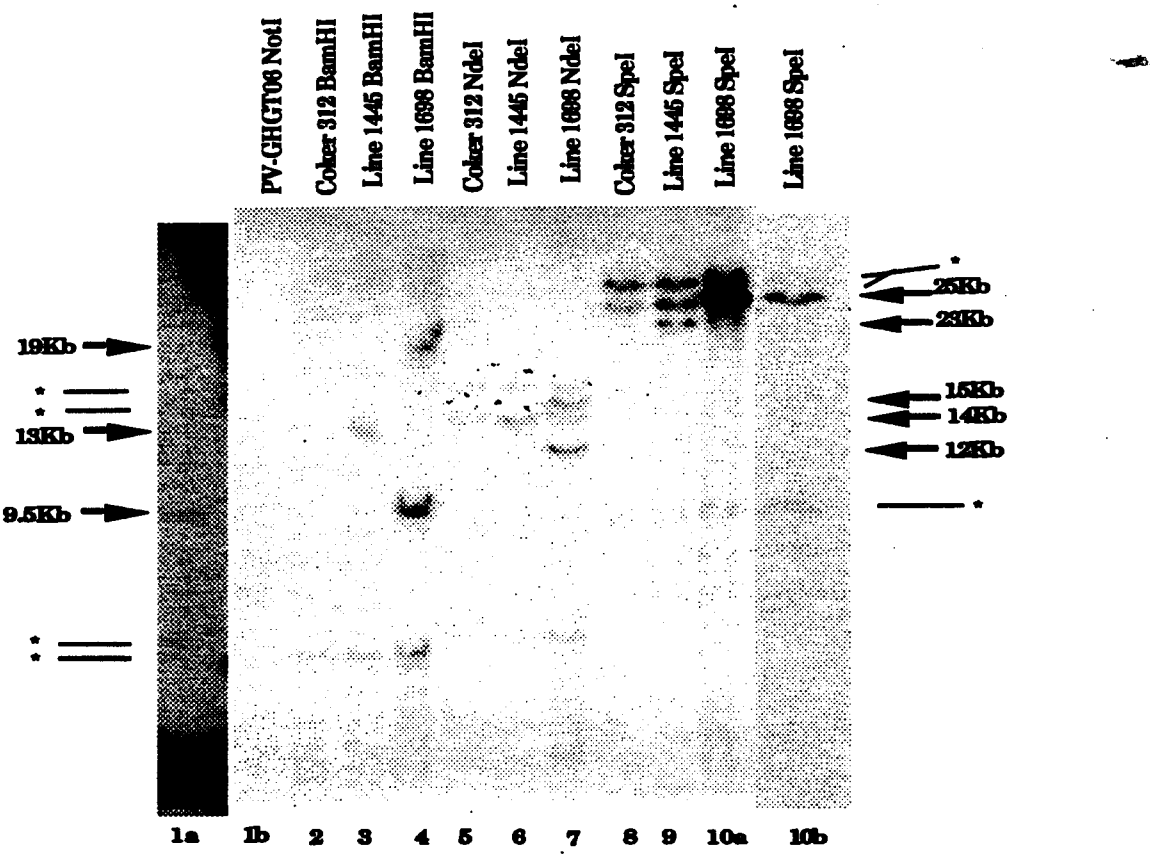


Figure III-9. Southern Blots Probed with *ori-V* and *ori-322* in Cotton with Roundup Ready lines 1445 and 1698. Each lane of plasmid DNA represents approximately 100 pg while the genomic DNA lanes of Panels A and C represent approximately 5 µg and the genomic lanes in Panel B represent 10µg. The digest were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane were probed with ^{32}P labelled *ori-V* (Panels A and B) or *ori-322* (Panel C) and then subjected to autoradiography. Abbreviations: R=right border, E=EcoRI, H=HindIII, and N=NcoI.



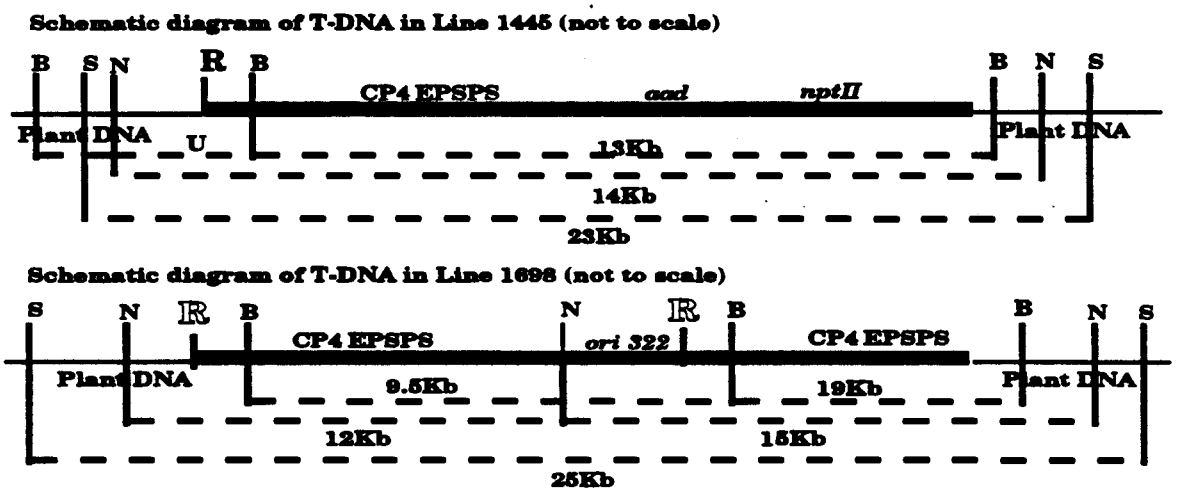


Figure III-10. Southern Blots Probed with PV-GHGT06 to Determine the Number of Loci of Plasmid Integration in Cotton with Roundup Ready Lines 1445 and 1698. Lane 1a is a longer exposure of lane 1b. Lane 10b is a shorter exposure of lane 10a. Each lane represents approximately 100 pg plasmid DNA or approximately 10 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.6% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P labelled PV-GHGT06 plasmid DNA and then subjected to autoradiography. Abbreviations: R=right border, S=SpeI, N=NdeI, B=BamHI, U=Undetected, *=bands common to all three DNAs.

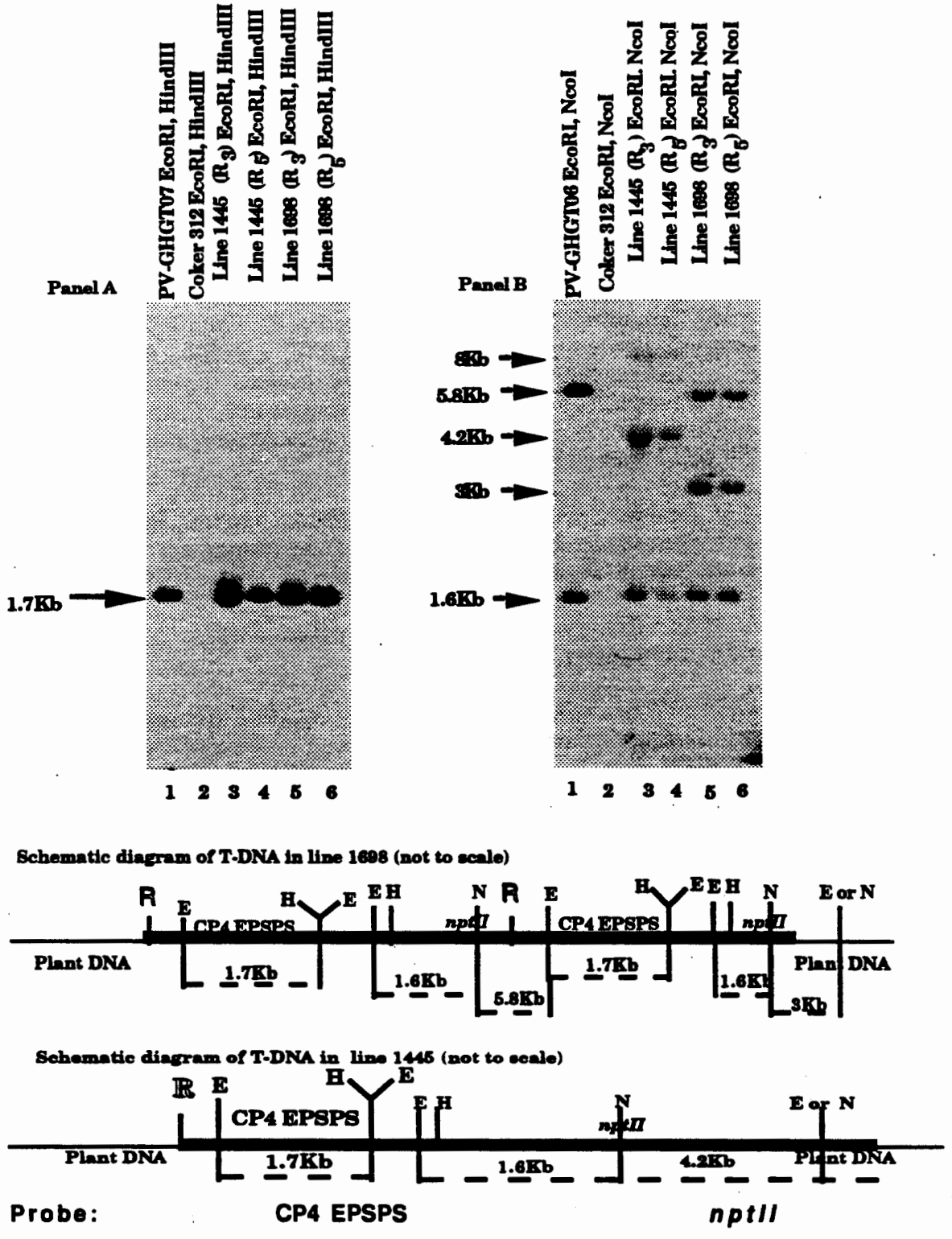


Figure III-11. Southern Blots Probed With CP4 EPSPS and *nptII* to Determine the Stability of the T-DNA. Each lane represents approximately 100 pg plasmid DNA or approximately 5 µg genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P CP4 EPSPS coding sequence (Panel A) or *nptII* DNA (Panel B) and then subjected to autoradiography. Abbreviations: R=right border, E=EcoRI, H=HindIII, N=NcoI.

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Part IV. Results of Field Trials

A. Field Test Permits and Locations

Cotton with the Roundup Ready™ gene lines 1445 and 1698, (lines 1445 and 1698), have been field tested in 1992, 1993 and 1994 at approximately 65 locations throughout the mainland United States and Puerto Rico.

The following are the USDA/APHIS permit or notification numbers under which these trials were conducted: 91-347-01, 93-012-03, 93-012-02, 93-210-02, 93-223-02, 94-027-01, 94-027-02, and 94-273-03. The final reports for USDA permitted studies numbers 91-347-01, 93-012-03, 93-012-02, 93-210-02 and 93-223-02, are included in Appendix V of this Determination. The final reports for the 1994 field trials (94-027-01 and 94-027-02) will be completed early in 1995. Experiments under notification 94-273-03 are still in progress, hence, the final report will not be available until later in 1995.

At all of these sites the following information was collected:

- Weediness Characteristics.
- Differences in morphology, plant growth characteristics and crop development.
- Susceptibility of lines 1445 and 1698 to attack by non-target insects.
- Susceptibility of lines 1445 and 1698 to disease infection.
- Monitoring for volunteers.

B. Plant growth and general observations

Lines 1445 and 1698 were grown and observed at multiple locations during the 1992 and 1993 field seasons, as well as at a winter nursery site in Puerto Rico in 1993-94. The following summary of these measurements and observations for weediness, plant growth characteristics, susceptibility to non-target insects, and susceptibility to disease infection show no meaningful differences between these lines and non-transgenic cotton.

No significant differences in weediness or survival characteristics were noted between lines 1445 and 1698 and non-transgenic cotton (Appendix V). Results for other Cotton lines with the Roundup Ready™ gene, than lines 1445 and 1698 are not addressed in this report. Most locations reported no differences in germination, days to flowering, or number of flowers between lines 1445, 1698, and non-transgenic cotton.

Some locations reported minor differences between the lines and non-transgenic cotton. For example, the researcher in West Sinton, Texas reported that lines 1445 and 1698 flowered approximately 7 days later than Coker 312. Similarly, at Scott, Mississippi, the researcher indicated that all transgenic lines except for Bollgard™ Cotton line 531 were later in maturity and appeared less productive. Visual observations at Starkville, Mississippi indicated that less fruit and less open bolls may have been present on the transgenic versus the non-transgenic lines. Cotton germplasm contains considerable genetic variability for maturity and productivity. The maturity and productivity of these lines fall well within expected ranges for cotton germplasm.

The source of these differences is unknown. It could be due to the initial plant selection of Coker 312 (C312), for transformation with these lines as considerable genetic diversity exists among plants within the C312 variety. The variation could also be due to genetic changes during the tissue culture process unrelated to the transformation event. Also, the differences could be due to genetic changes caused by the insertion of the transgene.

The importance of this delayed flowering/delayed maturity depends upon the degree of the differences and whether these differences are present in all breeding material produced with these lines. This cannot be determined in the field tests which reported these differences since this material was generally selfed progeny of the original transformant. The commercial acceptability of backcrossed derivatives will require lines without significant delay in maturity. As mentioned previously, the maturity of these lines falls well within expected ranges for cotton germplasm.

Three locations also reported male sterility and/or reduced boll set of the transgenic lines following treatment with Roundup® herbicide. This is not a function of the lines *per se*, but rather a function of the rate and timing of the Roundup herbicide application. Applications of Roundup herbicide at excessive rates or late timings (i.e. after square initiation) can induce male sterility which can reduce boll set. Applications at commercial rates within the primary timing window do not cause male sterility.

No differences in susceptibility to non-target insects were noted between lines 1445 and 1698 and C312 (Appendix V). Specific notations were made for similar responses of the Cotton with Roundup Ready and the non-transgenic cotton for aphids, fleahoppers, boll weevils, tobacco budworm, cotton bollworm, and thrips.

Similarly, no differences in susceptibility to diseases were noted between lines 1445 or 1698 and C312 (Appendix V). Specific notations were made for similar responses of the lines and non-transgenic cotton for *Verticillium* wilt, "boll rot," bacterial blight, and *Ascochyta* blight.

All plots were monitored for volunteer plants for one year following harvest. The results of the post-harvest monitoring programs demonstrated that the survival of the cottonseed remaining in the field was not different than what was expected for current varieties. Some regrowth was observed in the fall at one location, but was easily destroyed prior to seed production. One location (San Patricio County Texas) reported survival of seed through the winter to the following spring. San Patricio County is in the southern portion of Texas which makes survival more likely due to the relatively warm winters at this location. None of the other locations reported survival to the following spring. This trial contained both transgenic and non-transgenic lines. The volunteers were not tested to determine whether they were transgenic or non-transgenic, thus the effect of the transgene on survivability could not be determined. A protocol was initiated at several locations at the conclusion of the 1994 growing season to ascertain whether differences exist between the over-wintering ability of Cotton with Roundup Ready and C312.

Cotton is not considered to have seed which can persist in the environment for long periods of time. If planted before the soil temperature reaches 60° F, it is likely to rot in the soil. Following germination, the seedling is relatively "tender" and may not be able to push its way through the soil and emerge (Hughes and Nelson, 1957). Thus, in most cotton growing areas of the United States, some of the seed remaining in the field following harvest and cultivation may germinate in the autumn if conditions are favorable. The seeds not germinating are likely to rot and die. Except in the extreme southern cotton growing regions, such as Arizona and southern Texas, and only during mild and dry winters can cotton seed be expected to over-winter and germinate the following spring. Results of the monitoring program support this since no cotton plants outside of the extreme southern cotton region were reported to have survived. Additionally, integrated pest management practices in cotton recommend that all volunteers be destroyed as part of recommended cropping practices.

Based on results of the field monitoring program, there were no significant differences between lines 1445 or 1698 and C312. The major difference noted was in maturity. Maturity differences are common between cotton varieties and these differences do not cause concern in the commercialization of the crop. Furthermore, this does not impart any special adaptive, competitive, or survival characteristics to lines 1445 or 1698. Finally, no new cotton variety with the Roundup Ready™ gene will be commercialized unless it meets all morphological, yield, and quality characteristics of cotton varieties produced in the United States.

Reference

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Part V. Detailed Description of the Phenotype of Cotton With The Roundup Ready™ Gene, Lines 1698 and 1445

Introduction

Data and information supplied in this Petition for Determination of Non-Regulated Status demonstrate that Cotton with Roundup Ready, lines 1445 and 1698 (lines 1445 and 1698), are equivalent to the non-modified cotton line, Coker 312 (*Gossypium hirsutum*), except for the inserted genetic sequences, the expressed proteins [5-enolpyruvyl-shikimate 3-phosphate synthase from *Agrobacterium sp.* strain, CP4 (CP4 EPSPS) and neomycin phosphotransferase (NPTII)], and the tolerance to Roundup® herbicide. The information supplied in this section and referenced from other sections demonstrates that lines 1445 and 1698, are not likely to pose a greater plant pest risk than the parental control cotton line, Coker 312 (C312) from which it was derived. This conclusion is based on evaluation of phenotypic characteristics and safety of the inserted proteins and cottonseed products.

A variety of studies were conducted to characterize the unique traits of lines 1445 and 1698 and to establish that these lines are equivalent to C312. The inserted genetic material and herbicide tolerance were described in previous sections. Additional characterization of lines 1698 and 1445 are described in this section, as follows:

- expression levels of CP4 EPSPS and NPTII proteins in lines 1445 and 1698.
- comparison of the composition of lines 1445 and 1698 to C312 to determine levels of nutrients and anti-nutrients in cottonseed and cottonseed products.
- demonstration of the wholesomeness of cottonseed food/feed products.
- comparison of disease susceptibility of lines 1445 and 1698 to C312.
- the potential for outcrossing and weediness.

Roundup Ready™ is a registered trademark of Monsanto Company
Roundup® is a registered trademark of Monsanto Company

A summary of the methods utilized to conduct the protein extraction, analysis and quantitation, compositional analysis, cottonseed processing, preparation of seeds for gossypol and fatty acid analyses, moisture determination, gossypol levels, quantitation of fatty acid levels are found in Appendix VI. The following sections summarize these investigations.

A. Expression of the Introduced Genes in Tissues from lines 1445 and 1698

Cotton, *Gossypium hirsutum*, has been modified to confer tolerance to Roundup® herbicide. Glyphosate is the active ingredient of Roundup herbicide, a non-selective, post-emergent weed control agent. The biochemical target site of action of glyphosate is the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) which is present in plants, bacteria, and fungi as a component of the shikimate pathway of aromatic amino acid biosynthesis (Levin and Sprinson, 1964). Lines 1445 and 1698 were produced to express *Agrobacterium* sp. strain CP4 5-enolpyruvyl shikimate-3-phosphate synthase (CP4 EPSPS), which is naturally highly tolerant to inhibition by glyphosate (Padgette *et. al.*, 1993). Expression of CP4 EPSPS confers tolerance to Roundup herbicide to lines 1445 and 1698. In addition to the CP4 EPSPS gene, genes encoding neomycin phosphatase II (NPTII) as a plant selectable marker and aminoglycoside adenylyltransferase (AAD) as a bacterial selectable marker are also present in both lines. The *aad* gene is controlled by a bacterial promoter; therefore, the protein was not expected to be expressed in the cotton leaf or seed from lines 1698 or 1445. Cotton line 1445 was transformed with plasmid vector PV-GHGT07, and line 1698 was transformed with plasmid vector PV-GHGT06. Both of these vectors contain the genes encoding CP4 EPSPS, NPTII and AAD. In addition, vector PV-GHGT07 contains the genes that encode the enzyme glyphosate oxidoreductase (GOX). Molecular analysis confirmed that the GOX coding region was not transferred to the plant genome, therefore, the GOX protein would not be expected to be present. C312 does not contain the genes encoding the CP4 EPSPS, NPTII, or AAD proteins. A complete discussion of the molecular characteristics of lines 1445 and 1698 is found in Part III of the Request for a Determination of Non-Regulated Status.

Lines 1445 and 1698 were grown in the field at six locations throughout the cotton-growing region of the United States (USDA permit # 93-012-03). Field sites were located at West Sinton, Texas; Starkville, Mississippi; Bossier City, Louisiana; Maricopa, Arizona; Loxley, Alabama; and Tifton, Georgia. Levels of the CP4 EPSPS, NPTII, GOX, and AAD were evaluated in young leaf samples collected from the field plots. CP4 EPSPS, NPTII, and AAD were evaluated in seed samples. Enzyme linked immunosorbent assays (ELISA) were developed and optimized to quantitate CP4 EPSPS and NPTII proteins in cotton leaf and seed matrices. Seed were harvested and processed to produce full fat flour and toasted meal, which were used for other analyses discussed later in this section.

ELISA results for the mean, range of individual assays, and standard deviations for CP4 EPSPS, GOX, NPTII, and AAD expression in leaf tissue are shown in Table V-1. CP4 EPSPS and NPTII proteins were detected in the lines 1445 and 1698 and were not detected in the C312 parental line. The mean leaf expression of CP4 EPSPS in lines 1445 and 1698 was 0.052 µg/mg and 0.311 µg/mg, tissue, respectively, on a fresh weight basis. These expression levels are considered extremely low and, therefore, are not considered a macroconstituent.

The mean leaf expression for NPTII in lines 1445 and 1698 was 0.045 µg/mg and 0.031 µg/mg tissue, respectively, on a fresh weight basis. Since the coding region for the AAD protein is driven by a bacterial promoter it was not expected to be expressed in plant cells, and was not detected in either leaf or seed samples. The thresholds of detection were determined during AAD ELISA validation and were 0.136 ng/ml leaf extract and 0.160 ng/ml seed extract.

GOX was not detected in line 1698, as expected based on the fact that the transformation vector did not contain the GOX gene, or in line 1445 as Southern analysis indicated that the GOX gene present in the transformation vector was not transferred to this line. The threshold of detection was determined during validation of the GOX ELISA and was determined to be 0.021 OD units.

Boll samples were collected prior to harvest. The seed-cotton was ginned and delinted at Monsanto. The mean seed expression of CP4 EPSPS in lines 1445 and 1698 was 0.082 µg/mg and 0.204 µg/mg, tissue, respectively. The mean seed expression of NPTII in lines 1445 and 1698 was 0.0067 µg/mg and 0.0044 µg/mg, tissue, respectively. The means, ranges of individual assays, and standard deviations of ELISA values from seed samples are provided in Table V-2.

B. Compositional Analyses of the Cottonseed from lines 1445 and 1698

Cottonseed is primarily used as cattle feed, with smaller proportions of meal fractions used in feed for poultry, sheep, catfish, and swine. Cottonseed serves as an excellent source of fiber and protein, particularly due to its high lysine content. Cottonseed oil is also used in the food industry as frying oil and salad dressings.

Compositional (proximate) analyses were performed on the cottonseed from lines 1445, 1698 and C312. Components measured were protein, fat, moisture, and ash. Carbohydrate and calories were calculated from these values.

Proximate analysis results for cottonseed from lines 1445, 1698, and C312 are shown in Table V-3, and are expressed on a dry weight basis. No significant differences between lines 1445, 1698 and C312 were observed for:

% Fat, % Ash, Calories/100g and % Moisture. Significant differences in the protein and carbohydrate levels between the Cotton with Roundup Ready and C312 were observed at the 5% level using a pairwise t-test. The per cent protein levels of lines 1445 and 1698 were found to be 29.59 and 29.53 respectively, as compared to 27.76 in C312. A wide range has been reported for cottonseed protein levels: 12-32% (Kohel, *et al.*, 1985) and 18.8-22.9% and 23.5-29.5% (Turner, *et al.*, 1976; Cherry, *et al.*, 1978). All values determined for lines 1445, 1698 and C312 fall well within these reported ranges, and, therefore, the differences observed are not considered to be meaningful. Additionally, there were no significant differences in the amino acid profiles between lines 1445 and 1698 compared to the Coker 312. On per unit protein basis, the amino acid composition was similar among the lines and within ranges previously reported in the literature for cottonseed (Lawhon, 1977). The carbohydrate levels were determined by calculation. Therefore, the significant increases in protein levels for lines 1445 and 1698 previously discussed, resulted in the significantly reduced levels of the percent carbohydrates in lines 1445 and 1698, and, therefore, are not considered meaningful.

Gossypol is a terpenoid substance that is produced in discrete glands present in various tissues, including the seed (Abou-Donia, 1976). It can cause discoloration and toxicity problems in food and feed products of cottonseed (Berardi and Goldblatt, 1980). Gossypol levels detected in seed of C312 and lines 1445 and 1698 are presented in Table V-4. Gossypol levels of line 1698 were significantly lower than the levels in C312, and gossypol levels in line 1445 were significantly higher than levels in C312. The levels of gossypol were highly variable among sites, as previously reported (Berardi and Goldblatt, 1980). The total gossypol results for the test and control lines are within the previously reported range of 0.39 and 1.7% total gossypol for cotton varieties grown under various field conditions (Berardi and Goldblatt, 1980; Abou-Donia, 1976), and, therefore, the differences observed are not considered meaningful.

Level of the toxicant cyclopropenoid fatty acids (dihydrosterculic, sterculic, and malvalic) were determined for cottonseed oil from the six field sites. No statistically significant differences were detected between the lines 1445 and 1698 and C312. The determined values were within the previously reported ranges and are reported in Table V-5.

Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* that may contaminate food and feed products (Jorgensen and Price, 1981). Cottonseed is one of the commodities most commonly contaminated by aflatoxin (Bagley, 1979). Contamination can be very difficult to prevent or control because it may occur either in the field before harvest or after harvest during storage (Goldblatt and Dollear, 1977). The detection and detoxification of aflatoxin in food and feed products is critical due to the human and animal health risks (Scott, 1991). The maximum action level allowed by the FDA is 20 µg/kg (20 ppb) (Jorgensen and Price, 1981). To determine the levels of aflatoxin in C312 and lines 1445

and 1698 grown under the same field conditions, seed samples were evaluated for B1, B2, G1, and G2 aflatoxins. The four primary aflatoxins in cottonseed were not detectable at the sensitivity of 1 ppb in all three lines evaluated.

Compositional analyses establish that lines 1445 and 1698 are equivalent to C312 or other cotton varieties currently available, except for the expression of CP4 EPSPS and NPTII proteins.

C. Cottonseed Processing

Fuzzy cottonseed from each of the six sites was composited by line and processed, under conditions that mimic commercial processing, at Texas A&M University Food Protein Research and Development Center. The cottonseed was processed into the following products: full fat flour, toasted meal, and refined oil. Whole seed and toasted meal are primary fractions used for animal feed, and refined oil serves as the precursor of refined, bleached, deodorized oil used for human consumption.

The full fat flour and toasted meal were analyzed for total gossypol levels. Alpha-tocopherols and fatty acid profile were estimated in the refined oil samples.

Reduction of the free gossypol in the toasted meal and oil is a measure of food/feed quality and processing efficiency. During the processing, the gossypol that partitions into the oil, is essentially completely eliminated during the subsequent refining of the oil (Cottonseed Oil, 1993). Under typical conditions of high heat and moisture used to process cottonseed meal, most of the gossypol is removed by solvent extraction or detoxified to non-extractable (bound) form of gossypol. As expected there was no detectable gossypol in refined oil and toasted meal in all three of the lines evaluated (Table V-4 and Table V-5).

Alpha tocopherol analysis: Tocopherols are naturally present in cottonseed oil and serve as antioxidants providing good storage properties. Alpha tocopherols in particular have vitamin E potency. The levels of tocopherols vary in nature and are affected by processing. They are lost primarily during the steps of refining and deodorizing (Cottonseed Oil, 1993). Rossel reported a wide range of 136-543 mg/kg of alpha tocopherol present in cottonseed oil (Rossel, 1991). Martha Dicks reported an alpha tocopherol level as high as 660 mg/kg of refined oil (Dicks, 1965). Alpha tocopherol was measured in refined oil prepared from lines 1445, 1698 and C312. The level in lines 1445, 1698, and C312 was 588 mg/kg, 624 mg/kg, and 670 mg per kg oil, respectively. Therefore the levels of alpha-tocopherols were similar in all lines as well as those previously reported in the literature.

The fatty acid profile of refined oil from lines 1445, 1698 and C312 were similar and within reported ranges (Cherry and Leffler, 1984; Cherry, 1983; Phelps, 1965; Cottonseed Oil, 1993). The major fatty acids detected in the samples were linoleic, oleic and palmitic as anticipated for cottonseed and refined oil (Table V-5).

The cyclopropenoid fatty acids, sterculic and malvalic acid, are unique fatty acids common in cotton. Sterculic and malvalic acids are 18 and 17 carbons long respectively and contain a double bond at the propene ring. The levels of cyclopropene acids must be minimized due to undesirable effects which result in unsafe food and feed products (Cherry and Leffler, 1984; Phelps, *et. al.*, 1965). The cyclopropenoid fatty acids inhibit the desaturation of stearic to oleic acid, which alters membrane permeability and increases the melting point of fats. The levels of cyclopropenoid fatty acids are greatly decreased during processing, with the greatest point of deactivation during the deodorization of the refined oil (Cottonseed Oil, 1993).

These data establish that the gossypol levels in the processed fractions of cottonseed from lines 1445 and 1698 are comparable and equivalent to processed cottonseed fractions from C312 (Table V-5). The fatty acid profile of refined oil (including the cyclopropenoid fatty acids), from all three lines were similar and within reported ranges (Table V-5). Therefore, insertion of the genes to provide glyphosate tolerance did not alter these components following processing.

D. Allelochemical Levels in Vegetative Tissues

Cotton contains allelochemicals, in addition to gossypol, that may be involved in pest control (Hedin, *et al.*, 1988; Hedin, *et al.*, 1991; Hedin, *et al.*, 1992). Three of the most important are flavonoids, tannins, and anthocyanin (Hedin *et al.*, 1992). The levels of these classes of compounds in cotton squares and the terminal leaves were determined from samples obtained from the 1993 field tests in Loxley, Alabama. As expected, the levels of gossypol, flavonoids, tannins, and anthocyanins detected in the Cotton with Roundup Ready lines were similar to levels detected in C312 (Table V-6).

E. Disease and Pest Susceptibilities

All test sites were monitored on a regular basis for differences in disease susceptibility between transformed and non-transformed plants. Survey methods (i.e. number of plants examined and specific timing of plant examination) were not standardized across the various test locations to allow for regional and temporal differences in development of symptom expression in these cotton disease complexes. Both above and below ground plant parts were examined for the presence of disease development. Plant examination was not restricted to obviously diseased specimens. Healthy

plants were examined for abnormal growth and development and the presence of sub-chronic disease symptomatology. Because the cotton plants were transformed using a disarmed *Agrobacterium tumefaciens* vector, plants were specifically examined for the development of crown gall throughout the growing season.

The major diseases affecting cotton are the Seedling Disease Complex (*Rhizoctonia solani*, *Pythium* spp., *Ascochyta gossypii*, *Fusarium* spp. and *Glomerella gossypii*), Verticillium Wilt (*Verticillium dahliae*), Fusarium Wilt (*Fusarium oxysporum*), Phymatotricum Root Rot (*Phymototrichum omnivorum*), Bacterial Blight (*Xanthomonas campestris*), Boll Rots (various saprophytic fungi), and Nematodes (Root Knot, Lance, Reniform, and Sting). In addition, there are about 25 other fungi, viruses, and bacteria which may develop as localized epidemics in the various cotton growing regions of the United States.

The data presented in part IV and Appendix V of this Petition for Determination of Non-Regulated Status support the conclusion that lines 1445 and 1698 possess no disease or pest susceptibilities different than C312.

F. Plant Pest Risk

In all field and greenhouse trials, lines 1445 and 1698 plants were repeatedly inspected for any signs of *Agrobacterium* infection. None was found (see part IV). None of the gene sequences inserted into the cotton plant are capable of causing lines 1445 and 1698 to express any plant disease (See part III). Lines 1445 and 1698 do not exhibit any different agronomic or morphological characteristics which may give it an advantage over other species within the ecosystem in which it is grown (see part IV). The compositional and toxicant analyses comparing lines 1445 and 1698 to the parental C312 showed no meaningful differences (see section B, above). Therefore, it is concluded that lines 1445 and 1698 do not pose any different plant pest risk to other plants and the environment than non-transformed cotton varieties.

G. Weediness

G. hirsutum is ineffective as a weed. Wild populations are rare, widely dispersed and confined to beach strands or to small islands (Lee, 1984). It appears to be somewhat opportunistic towards disturbed land and appears not to be especially effective in invading established ecosystems. In the continental United States, wild populations of *G. hirsutum* exist only in the southern tip of Florida, due at least in part to the fact that cotton cannot over winter in those areas where freezing conditions occur.

There is little probability that lines 1445 and 1698 or any *Gossypium* species crossing with these transformed lines could become a weed. All wild and feral relatives of cotton are tropical, woody, perennial shrubs other than a few herbaceous perennials in NW Australia. With the exception of *G. thurberi* and *G. sturtianum* in Australia, these cannot naturally exist even in the milder temperate regions. In most instances the distribution of these species is determined by soil and climatic conditions rather than insect pressure. As perennials the plants are not particularly programmed to produce seed each year. In fact, they tend to drop fruit in response to stress. It is unlikely that expression of the CP4 EPSPS protein would impact survival either way. The only species that approaches the designation of pest is the arborescent *G. aridum* in parts of central western Mexico where it grows in fence rows much like sassafras in parts of the United States.

In those areas of the United States where feral or wild cottons occur (south Florida, Hawaii) the problem is not potential proliferation of plants but loss of the germplasm resource. If the CP4 EPSPS gene were transferred to a wild population of a tetraploid, the only advantage that could be gained by the wild population would be tolerance to Roundup herbicide. This gene transfer would only make it difficult to control the cotton new variety with Roundup herbicide. Any other herbicide which now controls *Gossypium sp.* would still be effective.

Cotton is not considered to have weedy characteristics as an annual plant grown in the United States. It does not possess any of the attributes commonly associated with weeds such as seed dormancy, long soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersal and long distance dispersal of seeds. These characteristics of weeds are controlled by multiple not single genes.

The only difference one would expect between the modified and non-modified cultivated cotton would be that the modified cotton would be tolerant to Roundup herbicide. This tolerance is not expected to lead to an advantage for these plants for the following reasons:

- The seed is not dormant and is not able to persist in the soil for long periods of time. In fact, only in the southern most parts of the cotton growing regions can the seed successfully over-winter and germinate the next spring.

- As discussed in Part II, the plant has no weedy relatives in the continental United States to which it can cross, and therefore it is not expected to cross with other species.

- Monitoring of plots during and after harvest for the past 2 years has not revealed any differences in survivability and competitiveness of the modified versus the non-modified cotton.

Therefore, there is no indication that the weediness of the modified cotton plant has changed as a result of the insertion of the CP4 EPSPS and *nptII* genes. Expression of the gene products (CP4 EPSPS and NPTII proteins) in the modified cotton plant would not change any of the above listed attributes.

H. Germination and Vigor Results for Lines 1445 and 1698

Seed from lines 1445, 1698, and C312 grown in the Dominican Republic were analyzed to determine the germination of these lines. Seeds were placed in moist germination paper and results were recorded after seven days.

Parameters analyzed included % germination in warm (31° C day, 24° C night) and cool (19° C constant) conditions, % diseased seedlings, and the % of seedlings with a radicle greater than one inch. This final measurement was used as an indication of seedling vigor.

Lines 1445, 1698 and C312 were of excellent quality as indicated by results in both warm and cool conditions (Table V-7). Additionally, vigor was excellent for all three lines and seedling disease was essentially non-existent. Thus, seed quality of lines 1445 and 1698 was excellent and equivalent to C312.

In addition to the evaluation of lines 1445 and 1698 relative to C312, "iso-lines" of lines 1445 and 1698 were also analyzed. For these iso-line comparisons, seeds from plants homozygous for the Roundup Ready™ gene (referred to as "positives") were compared to seeds from plants lacking the gene (referred to as "negatives"). The populations of positives and negatives should be nearly identical genetically except for the Roundup Ready™ gene insert (and gene(s) tightly linked to the insert).

Seed quantities were limited for "iso-lines" of line 1445, so results were obtained only for cool conditions. These results indicate no differences in the cool germination or vigor of "iso-lines" of line 1445 positives versus negatives (Table V-8). For "iso-lines" of line 1698, germination in both warm and cool conditions was excellent. Also, none of the seedlings were diseased. Significant differences existed between the positives and negatives in terms of % of seedlings with a radicle > 1 inch (Table V-8). These results indicate that the negative seeds were slightly more vigorous than the positive seeds. This would not be expected to have commercial implications since 85% of the positive seedlings had a radicle greater than one inch; a result of 85% is an indication of excellent seed quality.

These data indicate that seed from "iso-lines" of lines 1445 and 1698 were of excellent quality. Both lines should produce excellent quality seed which performs as well as commercially available varieties.

I. Out-Crossing Potential

The potential for pollen transfer from cotton to other species and for lines 1445 and 1698 to become a weed or pest is addressed in Part II and Appendix IV of this Petition for Determination of Non-Regulated Status. The following is a summary of the conclusions reached in these sections.

1. Pollen Transfer to Wild Species

For gene flow to occur via normal sexual transmission certain conditions must exist: the two parents must be sexually compatible, their periods of fecundity must coincide, a suitable pollen vector must be present and capable of transferring pollen between the two parents and resulting progeny must be fertile and ecologically fit for the environment in which they find themselves.

Based upon these criteria, out-crossing to wild species is not considered possible on the mainland United States and not likely in all of the 50 states for the following reasons:

a. All *Gossypium* species are self-fertile but can be cross-pollinated by certain insects. Wind transport of pollen is not a factor.

b. Lines 1445 and 1698 (*Gossypium hirsutum)* are not expected to hybridize with any wild species within the contiguous 48 United States. This conclusion is supported by the following:

   i. No other genera in the Gossypieae tribe are endemic to the United States.

   ii. The wild diploid, *G. thurberi,* occurs in the mountains of southern Arizona (Fryxell, 1979) and *G. hirsutum* is not grown in the vicinity where the *G. thurberi* is found. Secondly, cultivated cotton is an allotetraploid, whereas *G. thurberi* is a diploid, so these are incompatible and would not produce fertile offspring (Fryxell, 1979).

   iii. A relative of cotton (*Gossypium tomentosum*) grows in Hawaii (Stephens, 1964) however pollen transfer to this species is not anticipated to occur since cotton is not grown commercially in this state. *G. tomentosum* is morphologically and temporally incompatible with commercial cotton varieties. Should lines 1445 and 1698 be grown in Hawaii for testing or winter nursery seed increases, possible gene transfer can be prevented via the use of isolation distances.

In conclusion, there is no reasonable mechanism for out-crossing the introduced genes present in lines 1445 and 1698 into wild cotton species on the mainland United States. Out-crossing to other cultivated species *G. hirsutum* and *G. barbadense*, is expected but can be prevented by isolation practices common to the production of certified seed.

2. Pollen Transfer to Cultivated Genotypes.

In as much as similar cotton genotypes are fully compatible, any pollen that is transferred has the potential to produce a hybrid seed. The degree of out-crossing in a production field is strongly dependent upon the geographic location of the field (Simpson, 1954), which depends upon the crop ecology. The most important factors are the kinds and numbers of insect pollen vectors. Bumble bees *(Bombus* spp.) and honey bees *(Apis mellifera)* are the most significant (Theis, 1953; McGregor, 1959; Moffett and Stith, 1972; Simpson and Duncan, 1956) with the former being the most efficient pollinator. Typical out-crossing percentages for a number of locations in the cottonbelt range from 0 to 28%. Almost without question, the transgenic material can be expected to be transferred to other cultivated genotypes over time.

While some out-crossing to cultivated cotton (*Gossypium hirsutum* and *G. barbadense*) can be expected, such out-crossing would not be expected to cause any adverse effects for the following reasons:

- No adverse effects have been identified that may result from releasing the modified plants into the environment.
- If cross pollination to other cultivated cotton were to occur, the gene would only be present in the seed, and the plant would not express the CP4 EPSPS and NPTII proteins.
- Crossing with cotton grown for seed can be controlled with appropriate isolation distances (1/4 mile) or the use of border rows or both.

3. Results of Out-Crossing Studies

Under permits granted by the United States Department of Agriculture, Monsanto conducted several field studies on cotton containing the *Bacillus thuringiensis* var. *kurstaki* HD-73 *cryIA(c)* gene, (*B.t.k.*) in 1990. In two of these field trials, a study on the out-crossing potential of these cotton plants was conducted. These trials were conducted in Casa Grande, AZ (Permit # 90-90-025-01), and at: Maricopa, Arizona; Bossier City, Louisiana; Starkville, Mississippi; Brawley, California; Plainview, Texas and College Station, Texas (Permit # 90-032-02). The use of cotton containing the *B.t.k.* gene versus the Roundup Ready gene is not considered to reduce the usefulness of these results. The out-crossing potential of cotton would not be impacted by the presence or absence of these genes.

The experiments of the insect resistant cotton were surrounded by border rows of non-transgenic cotton. Seed from these border areas were evaluated to ascertain the frequency of out-crossing. Seed was harvested from every other row surrounding each field. Since 24 border rows were used, there were a total of 12 samples from each of the 6 test sites committed

to this evaluation. The seed was analyzed for the presence of the *B.t.k.* protein by ELISA. The ELISA method, developed by Monsanto, is used routinely to identify seed/plants that are expressing the *B.t.k.* protein. The assay is specific to the *B.t.k.* protein and very sensitive to small quantities of the protein. The results are presented in Tables V-9 (Permit #90-25-01), and V-10 (Permit #90-032-02).

The data indicate that the levels of out-crossing are low and well within the previously observed, normal frequency of out-crossing for plants in fairly close proximity. In fact, at three sites (College Station, Casa Grande and Maricopa), no out-crossed seed were detected. At those sites where out-crossing occurred, most of it was found in rows adjacent to the test field. Beyond the twelfth border row (40'), out-crossing events were extremely rare. Out-crossed seed was detected at the extremities of the border area at only one site (Bossier City). No out-crossed seeds were identified in the samples collected in adjacent cotton fields at the College Station, Texas site.

J. Transfer of Genetic Information to Species to which it cannot Interbreed

We are not aware of any other species within the United States with which *Gossypium hirsutum* is able to successful exchange pollen and produce viable hybrid plants. There is no evidence that plants can exchange genes with any other living species in nature.

K. Lack of Effect to Non-target Organisms

1. EPSPS

Lines 1445 and 1698 encode the enzyme EPSP synthase (EPSPS). EPSPS is an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants (including cotton) and microorganisms (Levin and Sprinson, 1964), and is thus ordinarily present in food derived from plant sources. Genes for numerous EPSPS's have been cloned (Padgette *et al.*, 1989, 1991, and references therein), and active site domains are conserved among the known EPSPSs (Padgette *et al.*, 1988, 1991). Bacterial EPSPSs have been well-characterized with respect to the 3-dimensional X-ray crystal structure (Stallings *et al.*, 1991) and the detailed kinetic and chemical reaction mechanism (Anderson *et al.*, 1990). EPSPSs from a number of bacteria exhibit tolerance to glyphosate (Schulz *et al.*, 1985). CP4 EPSPS thus represents one of many different EPSPSs found in nature. EPSPS is considered to be ubiquitous in nature since it is present in all plants and microorganisms. Therefore, all organisms which presently feed on plants and/or microbes, historically have been exposed to EPSPS.

2. Non-Target Birds and Fish

A study was conducted to assess the wholesomeness of lines 1445 and 1698 cottonseed meal when fed to bobwhite quail since birds may feed on cottonseed left in the field after harvest. No mortality occurred in birds fed up to 100,000 ppm (10% w/w) raw cotton seed meal in the diet. This feeding level approximates consumption of 400 seeds/kg body weight per bird of cottonseed. The "no observed effect level" was considered to be greater than 100,000 ppm. Based on the parameters measured, the wholesomeness of meal from insect resistant cotton seed was comparable to that of the parental line when fed in the diet to quail.

It is unlikely that fish in their natural environment would be exposed to cottonseed. Based on the fact that EPSPS is ubiquitous in nature and fish have been exposed to these proteins as a part of their diet and the unlikely event of exposure, a study with cottonseed in fish was not considered necessary.

3. Lack of Exposure to Fish and Wildlife

Cotton is a unique field crop in that mammals and other species which consume vegetation avoid feeding on the plant due to both the gossypol content and the morphology of the plant. The seed is within the boll and covered with lint. The seed will not be normally found in a lint-free condition in the field. Therefore, avian species are not expected to feed on the large lint covered seed. In addition, since the seed is not expected to enter aquatic habitats, fish should not be exposed.

Since the naturally occurring EPSPS proteins are considered innocuous in nature and non-toxic to fish, avian species, insects, mammals and other species and exposure to these species is not likely due their feeding preferences, no adverse effects to wildlife are expected from the commercialization of these plants.

4. Conclusion

EPSPS is ubiquitous in nature and there is no know toxicity of this enzyme to any species. Cotton is a unique field crop in that mammals and other species which consume vegetation avoid feeding on the plant due to both gossypol in the plant and the morphology of the plant. The seed is within the boll and covered with lint. The seed will not be normally found in a lint-free condition in the field. Therefore, avian, fish, and other wildlife are not expected to be significantly exposed in nature.

Based upon these facts, we are able to conclude that these is no expected adverse affects to any species as a result of the release of these cotton lines into nature.

L. Possible Impact on the Environment

1. **Persistence in the Environment Following Harvest** - As noted in part K above, EPSPS is ubiquitous in nature and there is no know toxicity of this enzyme to any species. The enzyme would, therefore, be expected to degrade within the environment as do all plant EPSPS's and plant proteins in general.

2. **Roundup Herbicide** - Glyphosate (N-(phosphonomethyl) glycine) is the active ingredient of Roundup herbicide and is an extremely effective broad spectrum, post-emergent herbicide. The primary mode of action of the herbicide is competitive inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway of aromatic amino acid biosynthesis. Roundup herbicide provides effective control for the majority of the world's worst weeds. It is translocated in the plant via both phloem and xylem. The broad spectrum herbicidal activity is only evident when it is applied to foliage, as there is little penetration of bark or woody stems (Franz, 1983). Roundup herbicide is not active when applied to the soil (i.e., it has no pre-emergence or residual soil activity). Its degradation appears to be mainly microbial (Atkinson, 1985). Glyphosate is essentially non-toxic to mammals and birds (Atkinson, 1985). Environmental impact studies indicate that the herbicide has little direct effect on animal communities (Sullivan and Sullivan, 1979, 1981, 1982). Glyphosate is only slightly toxic to fish and invertebrates, although some of the commercial formulations are more toxic due to the presence of a surfactant (Atkinson, 1985). Effects of the herbicide on soil invertebrates in field situations are minor (Eijsackers, 1985). Although there are numerous reports on the effect of glyphosate on microbial respiration, nitrogen cycling, and cellulolytic activity in soils, no toxicity to any of these microbial processes should be observed at recommended field application rate of the herbicide (Carlisle and Trevors, 1988). There are no reports of problems which have been associated with the use of Roundup herbicide and groundwater contamination (Goldburg et *al.*, 1990). The EPA has classified glyphosate as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739).

   Current Uses of Roundup herbicide on Cotton - Roundup herbicide is foliar applied to growing vegetation. Effective for the control of both annual and perennial weeds, it is usually applied before planting to kill winter and early summer weeds. This use fits well in reduced-tillage systems. It is also used as a spot spray at any time during the growing season. Even though highly effective for weed control, the lack of crop selectivity limits widespread use except for spot sprays in the growing crop. Its characteristics of ready translocation in plants and lack of in-crop tolerance resulted in applications via selective equipment (rope wicks), but this technique is practical only on weeds growing taller than the crop. Roundup herbicide is also used for controlling weeds outside the crop field, and is currently labelled for pre-harvest application on cotton.

Cotton production in the United States is located primarily in the tier of 15 southern states stretching from North Carolina to California, with approximately 13 M acres grown. Weeds remain as a severe constraint in the production of cotton. Cotton cannot compete effectively in its early growth stages and must be protected from the invasion of aggressive weeds. Present management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. On the average weeds must have been removed by 6 to 8 weeks after crop emergence to avoid yield loss. Total losses due to weeds, including cost of control and yield loss, is estimated to be $406 M annually in the United States. Control costs include early disking and preplant herbicide incorporation, a preemergence herbicide application at planting (excluding much of the Southwest), and one to three cultivations, either alone or in combination with one to three post-directed herbicide applications. One to two herbicide applications may be applied over-the-top broadcast or in spot treatment to control grass weeds. Total weed control costs may range from a low of nearly $20/acre to $67/acre for full season weed control depending on location and weed infestation severity. The introduction of Cotton with the Roundup Ready™ gene is expected to have potential for the alleviation of at least part of the numbers and costs of herbicide application in current use, both those soil applied at or before planting, and those applied postemergence, with considerable advantage accruing to conservation-tillage cotton. Such a system should enhance utilization of integrated weed management (IWM) practices as well. The adoption of such a system should also lead to soil improvement since fewer trips across a field will be necessary, and to further economical stabilization of cotton production through reduced purchased inputs.

Environmental impacts due to present weed control practices - Negative environmental impacts due to weed management in cotton come principally from two sources: soil erosion promoted by tillage operations, and chemicals applied for weed control which may end up in ground or surface water. Soil erosion may be managed effectively by reducing or eliminating tillage operations and with the management of plant residues. However, preventing herbicide movement into water is more complicated. In general, those herbicides which are applied to recently tilled bare soil as pre-plant incorporated or pre-emergence treatments are more likely to become surface water contaminants than herbicides that are applied to emerged weeds. Non-point source groundwater contamination is more likely to occur in areas with very coarse soil texture, shallow groundwater, moderate to high rainfall or high volume sprinkler or furrow irrigation, and from herbicides applied at relatively high rates, with high leaching potential and moderate to long persistence characteristics.

Positive environmental impacts - Roundup herbicide offers several advantages over presently available herbicides in terms of environmental protection. First, it is a very broad spectrum herbicide,

controlling basically all of the weeds likely to infest a cotton field. This broad spectrum of activity means that fewer different types of herbicides will be necessary for use in the crop, resulting in fewer herbicide applications in any given year. Second, Roundup herbicide is applied to emerged weeds, which allows the grower to treat only those fields or parts of fields in which weeds are known to exist at economically damaging populations. This postemergence approach utilizing economic thresholds for determining the need for herbicide use embodies the essence of the IPM philosophy in weed management, and lessens the need for growers to use insurance type treatments. Third, Roundup herbicide degrades very rapidly in soil and does not leach. Therefore, it does not pose a ground or surface water threat and leaves no soil residue to interfere with rotational crop selection. In addition, Roundup herbicide has very low mammalian toxicity and poses no chronic health effects. Therefore, it would not cause problems in areas where endangered animal species are a consideration. It is not volatile, and does not move off target after application. Finally, the likelihood of development of Roundup herbicide-resistant weeds is very low. After 20 years of use worldwide in many situations, there are no known reports of weed resistance to Roundup herbicide, and because of its unique mode of action and lack of soil persistence, resistance is not likely.

M. Summary

1. Expression of the Inserted Genes

Lines 1445 and 1698 have been modified by the insertion of the PV-GHGT07 and PV-GHGT06 plasmids respectively, which contain the gene imparting the tolerance to Roundup herbicide. These new cotton varieties express two new proteins, the CP4 EPSPS conferring tolerance to Roundup herbicide and the selectable marker, NPTII protein. The mean leaf expression of CP4 EPSPS in lines 1698 and 1445 was 0.311 µg/mg and 0.052 µg/mg, tissue, respectively, on fresh weight basis. The mean leaf expression for NPTII in lines 1698 and 1445 was 0.031 µg/mg and 0.045 µg/mg tissue, respectively, on a fresh weight basis. The mean seed expression of CP4 EPSPS in lines 1698 and 1445 was 0.204µg/mg and 0.082 µg/mg µg/mg tissue, respectively. The mean seed expression of NPTII in lines 1698 and 1445 was 0.0044 µg/mg and 0.0067 µg/mg tissue.

A second selectable marker gene encoding aminoglycoside adenylyltransferase (AAD) is present in lines 1445 and 1698; expression of the AAD protein is under the control of a bacterial promoter and was not detected in the cotton leaf or seed tissue from either line.

2. Composition, Quality, and Processing of the Seed

The cottonseed and processed cottonseed products from lines 1445 and 1698 are equivalent to the cottonseed and processed products from the C312 parental control on the basis of composition and quality.

The cottonseed from both lines were compared on the basis of major seed components (protein, fat, ash, carbohydrate, moisture and calories), fatty acid profile of the total lipid fraction from the seed, and the natural toxicant levels (gossypol, cyclopropenoid fatty acids, and aflatoxin). No differences in the seed were observed between lines 1445 and 1698 and the C312 control for % Fat, % Ash, Calories/100g and % Moisture. Significant differences in the protein and carbohydrate levels between the lines and the C312 control were observed at the 5% level using a pairwise t-test. The percent protein levels of lines 1698 and 1445 were found to be 29.53 and 29.59 respectively, as compared to 27.76 in C312. All values determined for the lines and C312 fall well within reported ranges in the literature, and therefore, the differences observed are not considered to be meaningful. The carbohydrate levels were determined by calculation. Therefore, the significant increases in protein levels in lines 1445 and 1698 previously discussed, resulted in the significantly reduced levels of carbohydrates.

Gossypol levels of line 1698 were significantly lower than the levels in C312, and gossypol levels in line 1445 were significantly higher than levels in C312. The levels of gossypol were highly variable among sites. The total gossypol results for the test and control lines are within the previously reported range of 0.39 and 1.7% total gossypol for cotton varieties grown under various field conditions. No significant differences in the levels of the toxicant cyclopropenoid fatty acids (dihydrosterculic, sterculic, and malvalic) were determined between all three lines. The four primary aflatoxins in cottonseed were not detectable at the sensitivity of 1 ppb for all three lines grown at any of the sites.

Compositional analyses indicate that the lines 1698 and 1445 are equivalent to C312 control line or other cotton varieties currently available, except for the expression of CP4 EPSPS and NPTII proteins.

There was no detectable gossypol in refined oil and toasted meal in lines 1445, 1698 and C312. The levels of alpha-tocopherols were similar in all three lines as well as those previously reported in the literature. The fatty acid profile of refined oil from lines 1698 and 1445 and C312 were similar and within reported ranges. The levels of cyclopropenoid fatty acids are greatly decreased during processing. There were no detected differences in the levels of the allelochemicals, flavonoids, tannins, and anthocyanin.

These data also establish that the gossypol levels in the processed fractions of cottonseed from lines 1698 and 1445 are comparable and equivalent to processed cottonseed fractions from the C312 control. The fatty acid profile of refined oil from lines 1698 and 1445 and C312 were similar and within reported ranges. Therefore, insertion of the genes to provide glyphosate tolerance did not alter these components following processing.

3. **Plant Pest Risk**

Lines 1445 and 1698 do not pose any different plant pest risk to other plants and the environment than non-transformed cotton varieties.

In all field and greenhouse trials, lines 1445 and 1698 plants were repeatedly inspected for any signs of *Agrobacterium* infection and other disease symptoms, and none were found. Lines 1445 and 1698 possess no disease or pest susceptibilities different than non-transformed cotton and is not expected to have any different weedy characteristics than other cotton grown in the United States. Out-crossing to wild species on the mainland United States is not expected. Crossing of the glyphosate tolerance genes to cultivated cotton is possible should the plants be in proximity; however, this is expected to occur at a very low frequency and not considered to be a concern as it is unlikely to cause any unreasonable adverse impact to the environment.

We are not aware of any other species within the United States with which *Gossypium hirsutum* is able to successfully exchange pollen and produce viable hybrid plants.

4. **Safety and Environmental Effect**

Lines 1445 and 1698 and the expressed proteins CP4 EPSPS and NPTII are not expected to have any adverse effects on non-target organisms or the environment.

A study was conducted on Bobwhite Quail. No mortality occurred in birds fed up to 100,000 ppm (10% w/w) raw cotton seed meal in the diet. The "no observed effect level" was considered to be greater than 100,000 ppm. Based on the parameters measured, the wholesomeness of meal from insect resistant cotton seed was comparable to that of the parental line when fed in the diet to quail.

It is unlikely that fish would be exposed to cottonseed. Based on the ubiquitous nature of the EPSPS and NPTII proteins and no known toxicity of these enzymes to any species and the unlikely exposure of fish to cottonseed, a study with cottonseed in fish was not considered necessary.

Roundup herbicide has been thoroughly tested and shown to have very favorable toxicity and environmental properties. The use of this herbicide as a replacement for some of the presently used cotton herbicides is expected to provide benefits to growers in the form of improved weed control at lower cost and environmental benefits in the form of reduced soil persistence, reduced potential for contamination of ground and surface water and low levels of toxicity to non-target organisms.

Conclusions

A review of all available information including extensive field test results, safety studies and independent scientific research support the conclusion that the commercial use of this cotton will not result in any adverse effects to the environment. The low levels of expression of CP4 EPSPS and NPTII proteins do not pose safety risk for humans or animals. The compositional analyses indicate that lines 1698 and 1445 are not materially different from the parental line, C312.

In fact, the use of varieties of Cotton with Roundup Ready will likely lead to positive impacts to the environment, as Roundup herbicide is used as a replacement for some of the presently used cotton herbicides.

Therefore it is concluded that lines 1698 and 1445 do not pose any different plant pest risks to other plants and the environment than is now caused by non-modified cotton varieties currently available.

Table V-1. Levels of CP4 EPSPS, NPTII, GOX, and AAD Expression in Cotton Leaf Tissue Determined by ELISA

Analyte		µg/mg tissue fresh weight C312 [a]	Line 1698 [b]	Line 1445 [b]
CP4 EPSPS	mean[c]	ND [c]	0.311	0.052
	range[c]	NA [c]	0.169-0.522	0.027-0.101
	std dev.	NA	0.092	0.016
NPTII	mean	ND	0.031	0.045 [d]
	range	NA	0.011-0.071	0.019-0.084
	std dev.	NA	0.016	0.014
GOX	mean	ND	ND	ND
	range	NA	NA	NA
	std dev	NA	NA	NA
AAD	mean	ND	ND	ND
	range	NA	NA	NA
	std dev.	NA	NA	NA

[a] Single extract of leaf samples, one protein loading per sample, three replicate samples per line for five sites.

[b] Single extract of leaf samples, one protein loading per sample, four replicate samples per line for one site, three replicate samples per line for five sites. Of the five sites, the Arizona site with 3 replicate samples had two replicate samples from pooled adjacent sub-plots and the third sample was from a single sub-plot.

[c] ND=non-detectable; NA=not applicable; Mean and standard deviation calculated from site averages; Range denotes the lowest and highest individual assay across all plots.

Table V-2. Levels of CP4 EPSPS, NPTII, and AAD Expression in Cotton Seed Tissue Determined by ELISA

Analyte		µg/mg tissue fresh weight C312 [b]	Line 1698 [a]	Line 1445 [a]
CP4 EPSPS	mean	ND [c]	0.204	0.082
	range	NA [c]	0.122-0.259	0.058-0.117
	std dev.	NA	0.041	0.017
NPTII	mean	ND [b]	0.0044	0.0067
	range	NA	0.0021-0.0114	0.0050-0.0104
	std dev.	NA	0.0011	0.0010
AAD	mean	ND	ND	ND
	range	NA	NA	NA
	std dev.	NA	NA	NA

[a] Single extract of seed samples, one protein loading per sample, three replicate samples per line for five sites, four replicate samples per line for one site.

[b] Single extract of seed samples, one protein loading per sample with one exception that had two protein loadings, three replicate samples per line for five sites, four replicate samples per line for one site.

[c] ND=non-detectable; NA=not applicable; Mean and standard deviation calculated from site averages; Range denotes the lowest and highest individual assay across all plots.

Table V-3. Summary of Proximate Analysis of Cottonseed

Characteristic	C312 Mean	C312 Range	Line 1698 Mean	Line 1698 Range	Line 1445 Mean	Line 1445 Range
Protein %	27.76	24.6-28.9	29.53*	25.7-30.7	29.59*	25.6-31.3
Fat %	23.35	20.5-24.8	23.81	20.8-25.6	23.79	19.5-26.1
Ash %	4.54	4.1-4.9	4.60	4.1-5.1	4.70	4.2-5.2
Carbohydrate %	44.35	41.9-46.2	42.06*	39.2-44.1	41.91*	39.2-44.0
Calories/100g	498.59	483.0-504.6	500.76	483.8-509.7	500.17	477.0-511.8
Moisture%	11.55	9.1-14.1	11.08	9.0-13.8	11.05	9.0-13.0

Protein, fat, ash, carbohydrate, and calories reported on dry weight basis.
Six samples per line (one from each of six sites)
* Statistically significant from C312 control.

Table V-4. Gossypol levels determined in seed, raw meal, and toasted meal from Cotton lines 1698 and 1445 and C312

	% Total Gossypol Mean	% Total Gossypol Range	% Free Gossypol
Seed			
C312	1.19	(0.99-1.46) [a]	NA
1698	1.01*	(0.81-1.22)	NA
1445	1.32*	(1.13-1.63)	NA
Full Fat Flour			
C312	NA	1.05 [b]	0.695
1698	NA	0.97	0.661
1445	NA	1.35	0.830
Toasted meal			
C312	NA	0.99	ND
1698	NA	0.86	ND
1445	NA	1.30	ND

* Values are statistically significant compared to the C312 at p=0.05 using a pooled variance t-test..

[a] Values reported for seed samples are the means and ranges of six samples per line; One sample from each of six sites.

[b] Values reported from full fat flour (kernel) and toasted meal samples are one value obtained from processing fractions generated from the composite of seed from six sites.

NA = Not Applicable;

ND = Not Detectable

Table V-5. Summary of Oil Quality from Cotton Lines 1698 and 1445 and C312

Fatty Acid	Lit Range	Refined Oil (% of total fatty acids) C312	Line 1698	Line 1445
Myristic (14:0)	(0.5-2.5) [1] (0.68-1.16)[2]	0.95	0.93	0.84
Pentadecanoic (15:0)		0.40	0.40	0.43
Palmitic (16:0)	(17-29)[1] (21.63-26.18)[2]	25.54	25.42	25.14
Palmitoleic (16:1)	(0.5-1.5) [1] (0.56-0.82)[2]	0.64	0.63	0.61
Margaric (17:0)		0.16	0.12	0.20
Stearic (18:0)	(1.0-4.0)[1] (2.27-2.88)[2]	2.46	2.53	2.41
Oleic (18:1)	(13-44)[1] (15.17-19.94)[2]	15.03	14.51	14.53
Linoleic (18:2)	(33-58)[1] (49.07-57.64)[1]	50.10	50.44	51.27
Linolenic (18:3)	(0.1-2.1)[1] (0.23)[3]	0.14	0.14	0.16
Arachidic (20:0)	(<0.5)[1] (0.41)[3]	0.26	0.24	0.27
Behenic (22:0)	(<0.5)[1]	0.12	0.11	0.08
Sterculic	(0.08-0.56)[4]	0.44	0.53	0.50
Malvalic	(0.22-1.44)[4]	0.35	0.46	0.56
Dihydrosterculic (C-19)		0.23	0.36	0.23
Unidentified fatty acid		1.97	2.17	1.79
Total gossypol	≤0.01% (1ppm)[2]	ND	ND	ND
Free gossypol	≤0.01%(1ppm)[2]	ND	ND	ND

[1] Ranges adopted by the FAO/WHO Codex Alimentarius committee on fats and oils (Cottonseed Oil, 1993).
[2] Cherry and Leffler, 1984.
[3] Cherry, J.P., 1983 .
[4] Phelps, et.al., 1965 .
Values reported for crude cottonseed oil.

Table V-6. Allelochemical Levels in Leaf Tissues from Cotton lines 1698 and 1445 and C312

Line	Gossypol	Anthocyanin	Flavonoid	Tannin
Line 1445	0.135	0.11	0.56	3.838
Line 1698	0.0136	0.10	0.59	4.268
C312	0.144	0.11	0.58	3.710

Table V-7. Germination results for seed grown in the Dominican Republic of Cotton lines 1445, 1698, and C312.

	Warm results		Cold results	
Line	**% germ.**	**% diseased**	**%germ**	**% radicle> 1"**
Line 1445	98 a	2 a	97 a	97 a
Line 1698	100 a	0 a	100 a	100 a
C312	100 a	0 a	100 a	100 a

Means followed by same letter do not significantly differ (P=0.05, Duncan's MRT)

Table V-8. Germination results for positive and negative isolines of Cotton lines 1445 and 1698.

	Warm results		Cold results	
Line	**% germ.**	**% diseased**	**% germ.**	**% radicle> 1"**
Line 1445 pos.*	N/A	N/A	100 a	95 ab
Line 1445 neg.*	N/A	N/A	100 a	100 a
Line 1698 pos.	100 a	0 a	95 a	85 b
Line 1698 neg.	100 a	0 a	100 a	100 a

Means followed by same letter do not significantly differ (P=0.05, Duncan's MRT)

* Positive (pos.) lines are homozygous for the transgene while negative (neg.) isolines do not contain the insert.

Table V-9. Percent Outcrossing at varying distances from the Bollgard™ Cotton observed at Casa Grande, AZ in 1990, (USDA Permit # 90-025-01).

Approximate distance from test (ft)	%* Out-Crossing
3.3	0.0
9.9	0.0
16.7	0.0
23.3	0.0
30.0	0.0
36.7	0.0
43.3	0.0
50.0	0.0
56.7	0.0
63.3	0.0
70.0	0.0
76.7	0.0

* Values represent the percent of seed harvested at a given distance expressing the *B.t.k.* protein in ELISA assay. There were 150 seeds analyzed for each point on the table. Each seed was analyzed separately, none were pooled.

Table V-10. Percent Outcrossing at varying distances from the Bollgard™ Cotton observed at six sites in 1990, (USDA Permit # 90-032-02).

Approximate distance from test (ft)	College Station, TX %*	Plainview, TX %	S.D.†	Brawley, LA %	S.D.	Maricopa, AZ %	Bossier City, LA %	S.D.	Starkville, MS %	S.D.
				Location						
3.3	0.0	0.0		3.3	1.5	0.0	4.7	1.7	2.0	1.1
9.9	0.0	0.0		2.0	1.1	0.0	0.0		3.3	1.5
16.7	0.0	0.0		0.7	0.7	0.0	0.0		0.0	
23.3	0.0	0.0		0.0		0.0	0.0		0.7	0.7
30.0	0.0	1.3	0.9	0.0		0.0	0.0		0.0	
36.7	0.0	0.0		0.0		0.0	2.0	1.1	2.0	1.1
43.3	0.0	0.0		0.7	0.7	0.0	0.0		1.3	0.9
50.0	0.0	0.0		0.0		0.0	0.0		0.0	
56.7	0.0	0.0		0.0		0.0	0.0		0.0	
63.3	0.0	0.7	0.7	0.0		0.0	0.0		0.7	0.7
70.0	0.0	0.0		0.0		0.0	0.7	0.7	0.0	
76.7	0.0	0.0		0.0		0.0	0.7	0.7	0.0	
Adjacent Field 1	0.0	0.0								
Adjacent Field 2		0.0								
Adjacent Field 3		0.0								

* Values represent the percent of seed harvested at a given distance expressing the *B.t.k.* protein in ELISA assay. There were 150 seeds analyzed for each point on the table. Each seed was analyzed separately, none were pooled.

† Standard deviations were calculated when a positive event was observed using the binomial distribution (Snedecor and Cochran, 1967, Iowa State University Press, pp 207-209)

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Part VI. Environmental Consequences of Introduction of the Transformed Cultivar

A. The Herbicide Glyphosate

N-(phosphonomethyl) glycine (glyphosate) is an extremely effective broad spectrum, post-emergent herbicide. The primary mode of action of the herbicide is competitive inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway of aromatic amino acid biosynthesis. Glyphosate provides effective control for the majority of the world's worst weeds. It is translocated in the plant via both phloem and xylem. The broad spectrum herbicidal activity is only evident when glyphosate is applied to foliage, as there is little penetration of bark or woody stems (Franz, 1983). Glyphosate is not active when applied to the soil (i.e., glyphosate has no pre-emergence or residual soil activity). Its degradation appears to be mainly microbial (Atkinson, 1985). Glyphosate is essentially non-toxic to mammals and birds (Atkinson, 1985). Environmental impact studies indicate that the herbicide has little direct effect on animal communities (Sullivan and Sullivan, 1979, 1981, 1982). Glyphosate is only slightly toxic to fish and invertebrates, although some of the commercial formulations are more toxic due to the presence of a surfactant (Atkinson, 1985). Effects of the herbicide on soil invertebrates in field situations are minor (Eijsackers, 1985). Although there are numerous reports on the effect of glyphosate on microbial respiration, nitrogen cycling, and cellulolytic activity in soils, no toxicity to any of these microbial processes should be observed at recommended field application rate of the herbicide (Carlisle and Trevors, 1988). There are no reports of problems which have been associated with the use of glyphosate and groundwater contamination (Goldburg et *al.*, 1990). The EPA has classified glyphosate as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739).

B. Current Uses of Glyphosate and other Herbicides on Cotton

Glyphosate is used as a foliar-applied herbicide. Effective for the control of both annual and perennial weeds, it is usually applied before planting to kill winter and early summer weeds. This use fits well in reduced-tillage systems. It is also used as a spot spray at any time during the growing season. Even though highly effective for weed control, the lack of crop selectivity limits widespread use except for spot sprays in the growing crop. Its characteristics of ready translocation in plants and lack of in-crop tolerance resulted in applications via selective equipment (rope wicks), but this technique is practical only on weeds growing taller than the crop. Glyphosate is also used for controlling weeds outside the crop field. Roundup® herbicide is currently labelled for pre-harvest application on cotton.

Herbicides are used on close to 100% of the cotton acreage in the United States (Gianessi and Puffer, 1991). They are applied to cotton pre-plant (foliage or soil incorporated applications), at planting (pre-emergence applications), or after seedlings emerge (post-emergence directed or over-the-top).

The number of applications varies in each cotton production region, and is dependent upon weed species, population densities, weather, and production economics. See Part I, IV and Appendix I of this Determination for a more indepth discussion of the use of herbicides on cotton in the United States.

C. Cotton with the Roundup Ready™ gene

The use of cotton with the Roundup Ready™ gene, lines 1445 and 1698 (lines 1445 and 1698), provides an attractive alternative to cotton growers who wish to have additional options for effective weed control. Roundup herbicide is a foliar-applied, broad spectrum, non-selective, post-emergent herbicide (Baird *et al.*, 1971; Malik *et al.*, 1989). It is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Use of lines 1445 and 1698 would enable the cotton grower to utilize Roundup herbicide for control of weed pests and take advantage of this herbicide's well-known, very favorable environmental and safety characteristics. Cotton lines 1445 and 1698 can positively impact current agronomic practices in cotton by:

1. offering the farmer a new wide-spectrum weed control option;
2. allowing the use of an environmentally sound herbicide;
3. providing a new herbicidal mode of action for in-season cotton weed control;
4. increasing flexibility to treat weeds on an "as needed" basis;
5. offering less dependence on herbicides used before planting;
6. providing an excellent fit with no-till, and reduced tillage systems, which results in increased soil moisture, while reducing soil erosion and fuel use;
7. providing cost-effective weed control, not only because Roundup herbicide may be less expensive than most current options, but because total herbicide use may be reduced compared to the farmer's current weed management program.

Cotton lines 1445 and 1698 provides an excellent broad-spectrum weed control alternative to farmers. Currently, farmers are using up to four or five different herbicide families to manage cotton weed problems. Applications of Roundup herbicide at 24 to 32 oz/A will control both annuals and perennials which would reduce the time, cost (herbicide and application), and number of herbicide treatments per acre.

D. The Likelihood of the Appearance of Glyphosate-resistant Weeds

Several decades ago, herbicide resistant weeds were virtually unknown. Today there are some 109 herbicide resistant weed biotypes with over half of them resistant to triazines (LeBaron, 1991). Major factors which can contribute to the development of resistant weeds include: a single target site and a specific mode of action, broad spectrum of activity, long residual activity and the capacity to control weeds year-long, and frequent applications without rotation to other herbicides or cultural control practices. Using these criteria and based on current use data, glyphosate is considered to be a herbicide with low risk for weed resistance (Benbrook, 1991).

Roundup herbicide has been used for over 20 years in various preplant, directed, spot or post harvest weed management systems with no known reports of weed resistance. This is most likely due to biological and chemical properties demonstrated by glyphosate and the use patterns of the herbicide. Roundup herbicide has essentially no residual activity in the soil and is quickly broken down by microorganisms in the soil. Also, there is no other herbicide on the market today that has the same mode of action as glyphosate. The experts tend to agree that eventually one will see a shift in weed populations due to the use of Roundup herbicide in cotton; however, this would occur with any new herbicide. In fact, any significant change in weed management systems will cause a shift in weed species, but usually these shifts cannot be related to a single variable (combination of tillage, rotation, herbicides, etc.). Finally, cotton has no innate dormancy, therefore, over-wintering is rare. Due to this lack of dormancy, cotton seeds germinate quickly with adequate temperature and moisture, so all seed that might fall to the ground due to harvest losses eventually either germinate, emerge and be killed by frost during the fall/early winter of the year that they were produced or rot in the soil over the winter. Hence, Roundup herbicide is not used to control volunteer cotton plants the following year, a use if practiced, would be jeopardized by the introduction of lines 1445 and 1698. All field release permits stipulate that the field sites be monitored for one year after harvest for volunteers. Very few, if any, volunteers have been noted for lines 1445 and 1698 (Appendix V) and were destroyed by alternate means if observed.

E. Cross Pollination of Cultivated and Native Species of Cotton

Out-crossing to wild species on the mainland United States is not expected. The potential exists for out-crossing to the wild species *Gossypium tomentosum* in Hawaii. However, pollen transfer to this species is not anticipated to occur since cotton is not grown commercially in this state, and could be easily prevented via the use of isolation distances. Crossing to cultivated cotton is possible should lines 1445 and 1698 be grown in proximity, however, this is expected to occur at a very low frequency and is not considered to be a concern due to the demonstrated safety of lines 1445 and 1698.

A detailed discussion of the potential for gene escape via pollen transfer is addressed in Part V paragraph I, of this Petition for Determination of Non-Regulated Status.

F. Potential for Lines 1445 and 1698 to Become Weeds

Cotton lines 1445 and 1698 are not expected to have any different weedy characteristics than other cotton grown in the United States. A detailed discussion of the potential for lines 1445 and 1698 to become weeds is addressed in Part V paragraph G, of this Petition for Determination of Non-Regulated Status.

Conclusion

None of the environmental consequences identified are of a nature as to justify that cotton lines 1445 and 1698 should not be commercialized. Lines 1445 and 1698 are not expected to become weeds or have any other adverse impact on the environment or production agriculture in the United States. Gene transfer is only expected to occur with other cultivated cotton and then only at low levels. Such transfer is not expected to cause any adverse environmental effects due to the proven safety of the CP4 EPSPS and NPTII proteins and Line 1445 and 1698 cotton plants. The positive consequences of the use of Roundup herbicide, the reduction in the use of other herbicides, the potential for an over-all reduction in the use of herbicides in cotton production, the substantial equivalence of lines 1445 and 1698 as compared to conventionally bred cotton and the overall positive impacts to cotton production fully justifies approval of this request for a Determination of Non-Pest Status fully justified.

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Part VII. Statement of Unfavorable Grounds

The results of all field studies and laboratory tests establish that there are no unfavorable grounds associated with Cotton with the Roundup Ready™ gene lines 1445 and 1698 developed using the plasmid vectors PV-GHGT06 and PV-GHGT07. Therefore, on the basis of the substantial potential benefits to the farmer and the environment, Monsanto requests that lines 1445 and 1698, and any progenies derived from crosses between this line and other commercial cotton cultivars no longer be regulated under 7 CFR part 340.6 in order to provide the necessary flexibility required for the continued commercial development of cotton with Roundup Ready.

Appendix I

Agronomic Benefits of Cotton with the Roundup® Ready Gene

Benefits of Roundup® Herbicide in Cotton with the Roundup Ready™ Gene

Abstract. Weeds remain as a severe constraint in the production of cotton worldwide. Cotton cannot compete effectively in its early growth stages and must be protected from the invasion of aggressive weeds. Present management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect. On the average weeds must have been removed by 6 to 8 weeks after crop emergence to avoid yield loss. Total losses due to weeds, including cost of control and yield loss, is estimated to be $406 M annually in the U.S. Control costs include early disking and preplant herbicide incorporation, a preemergence herbicide application at planting (excluding much of the Southwest), and one to three cultivations, either alone or in combination with one to three post-directed herbicide applications. One to two herbicide applications may be applied over-the-top broadcast or in spot treatment to control grass weeds. Total weed control costs may range from a low of nearly $20/acre to $67/acre for full season weed control depending on location and weed infestation severity. The introduction of the Roundup Ready Cotton system is expected to have potential for the alleviation of at least part of the numbers and costs of herbicide application in current use, both those soil applied at or before planting, and those applied postemergence, with considerable advantage accruing to conservation-tillage cotton. Such a system should enhance utilization of integrated weed management (IWM) practices as well. Roundup herbicide is environmentally friendly, posing little or no threat to wildlife, groundwater, or to the development of resistant weeds. The adoption of such a system should also lead to soil improvement since fewer trips across a field will be necessary, and to further economical stabilization of cotton production through reduced purchased inputs.

WEED CONTROL

Production practices and competition. Under the usual systems for growing cotton in the United States, the crop is grown in fairly wide spaced rows (36 to 40 inches). Although some cotton is grown in narrower rows (30 inches), advantages have not been uniformly realized. The growth of cotton in rows constitutes the creation of ecological niches in which weeds can, and do, flourish (Buchanan and Frans, 1979). These niches, or open areas, are rapidly invaded by aggressive weed species early in the season. Cotton, in its early stages of growth, is not competitive with these invading weeds and must be protected throughout a fairly long establishment period of several weeks. Shading of the soil by the crop canopy, in itself a good weed control measure, does not occur as soon as in other major field crops such as soybeans or corn. Therefore, systems of weed management in cotton have been directed towards controlling the weed pests primarily in the germination, emergence and early establishment stages of growth.

Tillage has long been an important part of these weed management systems. Indeed, tillage interwoven with herbicide practices has been the mainstay of cotton weed control for many years. It is important to note, however, that a good balance must be struck between too much or too little of either set of practices. Too much herbicide may stress cotton adversely during this early growth phase in addition to giving rise to intolerable herbicide residue levels in soil and water. Too much tillage, also, may cause stress if root pruning occurs from excessive or too deep cultivations. Too little of each, obviously, will result in insufficient weed control (Frans and Chandler, 1989).

Cotton weeds and competition. Just six weeds or genera of weeds are responsible for the major part of crop loss from weed competition. They are: morningglories (*Ipomoea* spp.), common cocklebur (*Xanthium strumarium* L.), pigweeds (*Amaranthus* spp.), johnsongrass (*Sorghum halepense* [L.] Pers.), nutsedges (*Cyperus* spp.), and prickly sida (*Sida spinosa* L.). In the Southeast these account for 66% of the loss, in the Midsouth, 72% and in the Southwest, 70%. Various other weeds are problems in particular areas - it is interesting to note that of the six, four are broadleaves (Whitwell and Everest, 1984). Annual grass weeds are also frequently noted as problems, including barnyardgrass (*Echinochloa crus-galli* L. Beauv.), crabgrass (*Digitaria* spp.), goosegrass (*Eleusine indica* [L.] Gaertn.), junglerice (*Echinochloa colonum* [L.] Link), panicum (*Panicum* spp.), and broadleaf signalgrass (*Brachiaria platyphylla* [Griseb.] Nash). Weeds allowed to grow for 5 to 6 weeks after crop emergence and before removal, dramatically reduce growth and ultimate yield of the crop. This early competition results in stand loss, weak and unhealthy plants, and greatly reduced set of fruit (Frans and Chandler, 1989). The six weeds or groups listed above are among the most common and most competitive of all weeds infesting cotton fields. Therefore, systems of control must not only be effective against these species, but also must have the ability to protect the developing crop during the critical period of establishment noted above. However, weeds can be tolerated in cotton fields for varying lengths of time after crop establishment, and must be removed at a critical period after establishment to avoid yield loss. A weed-free maintenance period of 6 to 8 weeks was required in Alabama (Buchanan and Burns, 1970), but weeds could be tolerated for 6 to 7 weeks after cotton emergence. Weeds could be tolerated for a slightly longer period of 6 to 9 weeks in irrigated cotton in Arizona (Arle and Hamilton, 1973). In Mississippi, the weed-free period for prickly sida, velvetleaf (*Abutilon theophrasti* Medik.), or spurred anoda (*Anoda cristata* [L.] Schlecht.) was only 4 weeks (Chandler, 1977); that is, they had to be removed by 4 weeks to avoid yield loss. Generally, removal of weeds by 6 to 8 weeks after crop emergence will eliminate yield loss (Coble and Byrd, 1992).

The magnitude of crop loss varies according to the weeds present and their respective densities in the field. An indication of competitiveness, however, can be obtained on a comparative basis. In studies in which full-season competition was allowed, and for densities ranging from approximately 2 to

32 plants per 50 ft. of cotton row, common cocklebur caused the greatest average yield reduction - 40%. Johnsongrass was 28%, redroot pigweed (*Amaranthus retroflexus* L.) 25%, prickly sida 21%, and pitted morningglory (*Ipomoea lacunosa* L.) 18% (Snipes et al., 1982, Bridges and Chandler, 1987, Buchanan et al., 1980, Buchanan et al., 1977, Crowley and Buchanan, 1978). Put yet another way, losses due to weeds must take into account not only yield reductions, but also costs directly attributable to their control, including equipment, labor, and herbicides. Just over a decade ago, these costs amounted to a total of $377 million annually, with 34% due to herbicides, 39% to equipment, and 27% to labor. Total loss due to weeds at that period of time was $518 million annually in the cotton crop alone (Whitwell and Everest, 1984), although a more recent estimate puts the loss figure for the entire Cotton Belt at just over $406 M annually (Chandler and Cooke, 1992).

Control measures. As noted above, inter-row cultivation is still a necessary part of weed management schemes in cotton (Buchanan, 1992). Combined with various herbicide practices, both preplanting, preemergence, and postemergence, these full ranges of practices are necessary to bring the cotton crop to the point of full canopy closure (Frans and Chandler, 1989; Chandler and Cooke, 1992). Although many studies have been done attempting to substitute herbicide control for cultural control, little evidence exists today that would indicate that inter-row cultivation can be significantly reduced. Hand-hoeing, once extensively used in cotton, has declined dramatically in recent decades, presumably because of the introduction of specific herbicides which gave better and more economic control (Frans and Chandler, 1989). Also, the registration and use of Roundup herbicide as a spot spray treatment for escaped weed species has contributed to the reduction in use of hand labor for hoeing. These spot sprays have been utilized extensively in Southwestern cotton production, as well as elsewhere. The lack of availability of personnel and rising labor costs (ranging from $10 to $20 per acre - Chandler and Cooke, 1992), stimulated in part by minimum wage laws, have greatly reduced the dependence on hand labor (Murray, et al., 1992). To legally utilize contract labor, a certified crew chief must be employed, otherwise each individual will be considered an employee subject to Social Security and Federal and State income withholding. Under the law, it is almost impossible to hire contract labor. Several known cases of litigation are pending on this point.

Current herbicide practices. Under conventional culture, several herbicide practices are combined with inter-row cultivation to achieve maximum protection from weed infestations. Typically, in the rain-fed Southeast and Midsouth, herbicides such as Treflan or Prowl, will be mixed shallowly in the top 2 to 3 inches of soil (called a preplant incorporated application - PPI) either alone (Southeast), or in combination with other residual herbicides such as Zorial (Midsouth, with Command possibly substituting for Zorial in such future combinations in both areas). In the Southeast, these applications are applied broadcast in fields, either for flat-planted cotton or before bedding, but in the Midsouth they may be applied in bands on

partially-formed beds just before planting. Preemergence (PRE) herbicides (Cotoran/Meturon or Zorial - Southeast), or various combinations of herbicides, including Dual-Cotoran/Meturon, Command-Cotoran/Meturon and Bladex-Zorial (Midsouth and Southwest) are those that are applied in bands over the row, usually in the same operation as planting (a single nozzle behind each planter on approximately one-third the total row width). A variation in irrigated cotton of the Southwest would be the use of the preplant incorporated herbicides Treflan or Prowl, but not always preemergence applications. However, where specific problem weeds exist additional preemergence herbicides such as Caparol and Karmex are utilized. Nevertheless, fewer than 10% of the Southwestern cotton acres receive preemergence herbicide applications. Up to three mechanical cultivations are used annually for weed control in this region.

After crop emergence, post-directed herbicide applications (those applied to the base of the crop plant to cover small emerging weeds) typically are used to supplement early control obtained with the above-noted soil-applied herbicides. These usually begin when cotton is approximately in the V1 stage of growth (3 to 4 inches) and continue with second applications made approximately in the V3 to V4 stages of cotton growth (6 to 8 inches and later - see Elsner et al., 1978, for a description of these growth stages). The earliest directed applications may include either Cotoran\Meturon or Caparol\Cotoran pre mixed with either MSMA or DSMA. Second or follow-up applications may include Karmex\Direx plus MSMA or DSMA, Bladex or Bladex plus MSMA, Goal, Cobra or Cobra plus MSMA, or Linex. The success of these applications is based on having sufficient height differential between crop and weed. Obviously, if weeds are as tall as the crop, then only minimal control will be obtained. Layby applications (those applied to both emerged weeds and to the soil at the time of last cultivation) are also commonly applied after cotton attains a height of at least 15 inches - these may include Bladex, Karmex\Direx, or Linex.

Recent developments. The above herbicide programs have been in use for the past two to three decades and usually give satisfactory control of most weed infestations under "average" climatic conditions. As stable as this program has been, there have been newer herbicide practices utilized to supplement or replace some of the above practices. Grass-weed specific herbicides are now available for control of johnsongrass, bermudagrass (*Cynodon dactylon* [L.] Pers), and annual grasses. These include Poast, Fusilade, Assure, and Select. Because of their grass selectivity, they are sometimes used to substitute for the preplant-incorporated materials (Treflan or Prowl). Nevertheless, the reasonable cost and dependability of the latter herbicides favors their continued use (Nastasi et al., 1986). After several years of research, an petition has been filed for the approval of the herbicide Buctril for use with a tolerant transgenic cotton cultivar. It is expected that this practice will enjoy a certain amount of adoption if the cultivar becomes accepted. Buctril is useful for broadleaf weed control and can be applied over-the-top of the transgenic cultivar (BXN™). Still another new development for over-the-top use is Staple, which is being aggressively

developed across the cotton belt. It is selective on all cotton varieties and has utility for broadleaf control also, particularly for morningglory species. All of these latter practices (grass-weed herbicides, Buctril, and Staple) may alter or substitute for the more traditional post-directed applications described above.

One other over-the-top herbicide application should be mentioned. Often, the early soil-applied herbicides either are not used or used only partially, or they may not be sufficiently activated to give good control. Under those situations, and particularly in the rain-fed part of the cotton area, morningglories may gain such a foothold that they are difficult to control sufficiently so that directed applications may be made. Cotoran\Meturon can be applied when cotton is in the cotyledon stage to gain some control. This is only a salvage operation and is not always dependable, either from the standpoint of weed efficacy or crop tolerance.

Weed control costs. In a recent survey of weed control costs across the cotton belt (Chandler and Cooke, 1992), it was found that total costs for full-season weed control ranged from $19.31 per acre in the Southern High Plains of Texas to $67.18 per acre in Arkansas. In the Southeastern states of Alabama, Georgia, Florida, and North Carolina, the average cost for full-season weed control was $41.87 per acre, and South Carolina and Tennessee averaged $53.20. Practices included at least one disking and incorporation of a preplant herbicide, application of a preemergence herbicide in the planting operation, a single cultivation, followed by one or two cultivations with a post-directed herbicide application. South Carolina and Tennessee costs were higher because of an additional post-directed application, usually at layby. In the Mid-South, the average cost for Missouri and Louisiana was $42.23 and for Arkansas and Mississippi - $64.62. In the latter two states, two post-directed herbicides were used as well as a lay-by application. In Oklahoma and Texas, costs were greatly influenced by availability of moisture, and ranged from $25.58 dryland (disking twice with an application of a preplant incorporated herbicide and two cultivations) to $46.13 per acre on irrigated areas (Oklahoma). Texas costs were lower, ranging from $19.31 dryland (High Plains) to $37.17 per acre in the Coastal Bend area where rainfall is higher. In the High Plains, a preplant incorporated herbicide is used and a preemergence herbicide at planting, followed by spot spraying as needed. In the Coastal Bend area, both herbicide applications are used followed by two cultivations and by up to 1.5 hr/acre of hand-hoeing. In the western states of Arizona, California, and New Mexico, cotton is irrigated, and weed control costs averaged $58.19 per acre. Typically, a preplant incorporated herbicide is used followed by at least two cultivations, one of which may be accompanied by a post-directed herbicide application. Hoe labor is used extensively, ranging from 4 to 6 hrs/acre.

Conservation tillage. In contrast to the above traditional systems of cotton culture, conservation-tillage cotton is gaining in popularity, rapidly, stimulated in part by the Conservation Compliance Provision of the 1985 Food Security Act. Under this program, farmers with highly erodible cropland are expected to develop a Soil Conservation Service-approved plan for all highly erodible fields. The deadline for these plans to be fully implemented is January 1, 1995. Farmers who fail to comply may lose eligibility for most USDA farm support programs (Hutchinson, 1993; Wilcut, et al., 1993). Under various of the conservation measures possible, little or no soil stirring is done prior to planting. Burndown applications of either Roundup herbicide or Gramoxone alone, or mixed with residual herbicides, such as Goal or Bladex, are used to control weeds. Cotton is then planted through whatever mulch exists on the surface of the soil. Under this scenario, the preplant-incorporated herbicides are not used, and preemergence herbicides are depended upon for continued early control. Grass-specific herbicides such as Dual or Command are mixed with Cotoran\Meturon (Midsouth) to partially substitute for the incorporated materials. However, Dual only partially substitutes and Command, while effective for grass weeds, is subject to volatility loss when applied to the surface, with possible subsequent damage to nearby susceptible vegetation. Nevertheless, conservation-tillage production of cotton is expected to continue to increase in use and will likely become the dominant pattern of production in the very near future. In irrigated Southwestern cotton production, control of weeds has been the limiting factor in conservation tillage. These systems in the Southwest involve the planting of winter wheat into cotton stalks in the fall, chemical termination of the wheat in the spring with Roundup herbicide, and cotton planted into the wheat residue in April or May. In these wheat residue systems, Treflan and Prowl herbicides are applied through sprinkler irrigation systems prior to the planting of the cotton. Following cotton planting, herbicides such as Caparol, Dual, Command or Cotoran are used on sandy loam or heavier soil types. On loamy fine sands or lighter soils which are predominant in much of the Texas High Plains, preemergence herbicide use is very limited due to potential cotton injury. For the conservation tillage systems of the Southwest, the availability of Roundup Ready Cotton would be very beneficial to the producers. This availability would also allow more producers to practice conservation tillage and comply with wind and water erosion provisions of the 1985 and 1990 Farm Bills. In addition to conservation tillage, crop rotations, strip crops, and deep moldboard tillage are also accepted practices for meeting these provisions.

Roundup herbicide and IWM. The key to effective cotton weed control is management. Most often, the best approach to controlling weeds in cotton is to use two or more methods employed in a systematic fashion. For example, the early tillage practice of seedbed preparation and destruction of existing weed infestations with a disk, is often combined with application of a preplant-incorporated herbicide. Such a systems approach has been referred to as integrated weed management (IWM), and is compatible with the overall concept of integrated pest management (IPM) originally

developed by entomologists in dealing with insect pests (Buchanan, 1992). Producers must be alert not only to kinds and species of weeds present in their fields, but also to the timing of their appearance in relationship to control practices available on the farm. This means that periodic surveys or examinations of fields must be performed to stay ahead of the weed problem. Alert management can help producers move away from soil-applied herbicides (put out before weeds or crop emerges) to postemergence applications which might be applied only as needed. Such an IWM scheme might well result in reduced herbicide applications, greater protection of the environment and increased profitability to the producer. The availability of Roundup herbicide coupled with a tolerant cultivar fits the IWM concept very well. Since Roundup herbicide should control the major cotton weeds referred to above (and many more), its broad-spectrum aspects can be utilized to advantage in cotton. The many years of collective experience working with Roundup herbicide indicate that early applications are most efficacious. In soybeans, applications as early as V2 have been quite effective in controlling general weed populations and at rates as low as 3/8 lb/A. The greater the delay, the more of the material might be required for certain weeds. Sequential applications of low rates offer considerable potential - again, for weeds more difficult to control with single applications. Such a concept fits well with conservation-tillage cotton also, where before-planting applications of Roundup herbicide are used on existing weeds. A second application after planting and when cotton is in the V1 to V2 stage of growth might well suffice for this early-season period. Depending on weeds present, over-the-top applications of Roundup herbicide early in the life of cotton, might well be at least the equal of the newer practices just coming to the market place (Buctril and Staple).

Drawbacks to conventional systems. Presently-available herbicide systems, particularly in conventionally-grown cotton, fit the IWM concept only partially. Soil-applied herbicides (preplant incorporated and preemergence) are used before one knows what the years' weed infestation will be. Post-directed applications are somewhat more amenable to IWM, since producers must be aware of the weeds present before making these applications. Nevertheless, these applications are often scheduled as described above in order to insure maximum control. The early Roundup herbicide application in tolerant cotton is truly an IWM practice - used as needed, or on weeds identified as potential problems in the field if not controlled. A major weed in the Southeast, for example, is sicklepod (*Cassia obtusifolia* L.). Because of its severity, cotton is usually not grown in fields infested with this weed because of the lack of adequate means of control. Roundup herbicide offers the means of managing this weed pest, and would be of real benefit in this region. Silverleaf nightshade (*Solanum elaeagnifolium* Cav.) is the major perennial weed problem for cotton producers of the Southwest. Currently spot spray applications of Roundup herbicide are utilized extensively for control. Fall applications of Roundup herbicide over-the-top of cotton are also utilized in cotton fields with severe silverleaf nightshade populations. The availability of Roundup Ready cotton would provide producers with an excellent management tool for this particular weed pest.

ENVIRONMENTAL

Impacts. Negative environmental impacts due to weed management in cotton come principally from two sources: soil erosion promoted by tillage operations, and chemicals applied for weed control which may end up in ground or surface water. Soil erosion may be managed effectively by reducing or eliminating tillage operations and with the management of plant residues. However, preventing herbicide movement into water is more complicated. In general, those herbicides which are applied to recently tilled bare soil as PPI or PRE treatments are more likely to become surface water contaminants than herbicides that are applied to emerged weeds. Non-point source groundwater contamination is more likely to occur in areas with very coarse soil texture, shallow groundwater, moderate to high rainfall or high volume sprinkler or furrow irrigation, and from herbicides applied at relatively high rates, with high leaching potential and moderate to long persistence characteristics.

Environmental advantages. Roundup herbicide offers several advantages over presently available herbicides in terms of environmental protection. First, it is a very broad spectrum herbicide, controlling basically all of the weeds likely to infest a cotton field. This broad spectrum of activity means that fewer different types of herbicides will be necessary for use in the crop, resulting in fewer herbicide applications in any given year. Second, Roundup herbicide is applied to emerged weeds, which allows the grower to treat only those fields or parts of fields in which weeds are known to exist at economically damaging populations. This postemergence approach utilizing economic thresholds for determining the need for herbicide use embodies the essence of the IPM philosophy in weed management, and lessens the need for growers to use insurance type treatments. Third, Roundup herbicide degrades very rapidly in soil and does not leach. Therefore, it does not pose a ground or surface water threat and leaves no soil residue to interfere with rotational crop selection. In addition, Roundup herbicide has very low mammalian toxicity and poses no chronic health effects. Therefore, it would not cause problems in areas where endangered animal species are a consideration. It is not volatile, and does not move off target after application. Finally, the likelihood of development of Roundup herbicide-resistant weeds is very low. After 20 years of use worldwide in many situations, there are no known reports of weed resistance to Roundup herbicide, and because of its unique mode of action and lack of soil persistence, resistance is not likely.

Metabolic behavior. Glyphosate, the active ingredient of Roundup herbicide, acts as a competitive inhibitor of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSP synthase) in plants. Competitive inhibitors such as glyphosate act by replacing the substrate in enzyme catalyzed reactions such that the final product of the reaction cannot be produced. The enzyme involved is not inactivated, and increasing substrate concentration or increasing enzyme levels can overcome this competitive inhibition. Any genetic change in the enzyme

EPSP synthase which would lead to resistance to glyphosate would also be lethal to the plant, because such a change would also render the enzyme incapable of using the intended substrate for the reaction it catalyzes.

Genetically engineered tolerance to Roundup herbicide in cotton has been attained by two methods. One involves introduction of a gene from other plants which causes an increased production of the ESPS synthase enzyme, conferring partial tolerance. The other involves introduction of a gene from bacteria which causes the plant to produce an enzyme capable of degrading glyphosate into a non-active form. The gene responsible for the degradation enzyme is not found in plants, so there is no possibility of plants developing such a gene spontaneously.

Other advantages. Because the number of herbicide applications in cotton would be reduced with Roundup herbicide use, there would be a concomitant reduction in containers to be disposed of and a reduction in herbicide exposure to handlers and applicators. Roundup herbicide is approved for several uses already, so the problem of disposal of excess tank mixes and reinstates would be lessened since any excess could likely be used in another approved manner.

A final benefit to the environment with Roundup herbicide availability for use in cotton is the potential for a reduction in trips over the field. This is an advantage not only economically, but may indirectly lead to less soil tillage. Each trip across a field with equipment increases soil compaction. Since crops grow better in areas with less compacted soil, most growers use tillage to ensure that soils do not become excessively compacted. This increased tillage places the soil at higher risk of erosion. Fewer trips across a field with equipment would mean fewer tillage operations.

ECONOMIC

Replacement advantage of the Roundup herbicide option. In the cotton production areas of the United States where currently available weed control options do not provide satisfactory control of specific annual and perennial weed species, the availability of Roundup Ready Cotton will provide a clear economic advantage over spot spraying, cultivation, and hand labor. Where spot spraying, cultivation, or hand labor is utilized, many times cotton yields are lost not only to the weed competition but also to the control process. It is very difficult to achieve spot spraying or hand labor weed control of problem weeds without injury to adjacent cotton plants.

Economic impact of the cotton crop. Cotton is an important agricultural commodity in the United States. For example, the total value of the 1984 crop, including both lint and seed, was approximately $4 billion. Using an economic multiplier index developed by Texas economists (Jones and Williams, 1980), the total industry impact was estimated to be $15.1 billion.

The projected 1994 U.S. cotton crop is 19.45 million bales. At $0.65 per pound, the cash value will be $6.07 billion. Therefore, using the same multiplier index, the total impact for 1994 will be $22.9 billion. This index was developed for Texas and is used to approximate the U.S. economy since a precise index for all cotton states is not available. Total industry impact includes value not only to producers of the crop, but also to various suppliers of inputs necessary to produce cotton and the enterprises that process the crop.

SOCIAL

Sustainability with Roundup herbicide. This technology will provide economic advantages as mentioned earlier in conservation tillage systems and allow producers to achieve a higher level of sustainable systems. In some areas, spot spray technology has almost eliminated the use of hand labor. With Roundup Ready technology, the labor requirements associated with spot spraying will be reduced for an economic advantage to the producer.

Environmental safety. The availability and utilization of Roundup Ready technology will provide needed environmentally safe technology for all producers, regardless of size of operation. The availability of this technology should not provide economic advantage to any one group of producers per se, and the anticipated cost of seed and herbicide should allow large or small producers equal access to the technology.

In addition, the availability of Roundup Ready technology in cotton should insure the availability of an environmentally safe weed control mechanism for use in cotton production which adjoins urban areas. This technology will also help the cotton producer meet the expectations of the 1985, 1990, and anticipated 1995 Farm Bills which require control of wind and water erosion and use of environmentally safe materials. This technology should also be more compatible with areas of Endangered Species concern. Furthermore, this technology is not expected to have a significant net change in any areas of employment.

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Submitted by:

Robert Frans
Harold Coble
John Abernathy

Appendix II

Economic Impacts of Cotton with the Roundup® Ready Gene

Economic Impacts of Cotton with the Roundup Ready™ gene

Abstract

The introduction of genetically engineered cotton plants that are designed to be glyphosate tolerant will have significant impacts on the profitability of some cotton farms and related agribusinesses. Roundup Ready Cotton will allow some cotton growers to apply Roundup herbicide (a broad-spectrum herbicide) after cotton plants have emerged and thus be able to eliminate the application of some currently used herbicides. It is expected that growers who adopt Roundup Ready Cotton will do so in an attempt to reduce their overall weed control costs. However, it is expected that the cost of Roundup Ready Cotton seed (used for planting) will be greater than the cost of conventional cotton seed. Thus, the grower's decision to adopt Roundup Ready Cotton will be impacted by the expected profitability of conventional cotton relative to that of Roundup Ready Cotton. As the Roundup Ready Cotton seed market develops and grows during its adoption period, the demand for conventional cotton seed and some herbicides will decrease, resulting in a loss of profits for these agribusinesses.

Introduction

In recent years, public concern about the use of some agricultural chemicals has increased in the United States. Frequently, legal action has been taken to force the EPA to ban or severely restrict the use of some pesticides, particularly insecticides. Economic studies have been conducted to examine the likely impacts from such restrictive pesticide regulations. Taylor et al. developed a regional model and concluded that agricultural income in the South would be negatively impacted by more restrictive pesticide regulations. Richardson et al. analyzed the situation with a farm level model and concluded that the removal of pesticides would have a negative impact on Mississippi and Texas Southern High Plains cotton farms. However, neither of these studies allowed for the development of new technologies in response to increased pesticide regulations.

Even if increased regulations on herbicides are not forthcoming, technological developments such as new herbicides and transgenic cotton varieties have the potential to significantly impact the cotton industry. Genetically engineered cotton plants that are designed to be glyphosate tolerant will allow cotton growers to control weeds with less chemical herbicides than are now used. Roundup Ready Cotton is a transgenic variety that is glyphosate tolerant, allowing the use of Roundup herbicide (a broad-spectrum herbicide) during the growing season in place of other conventional weed control chemical herbicides. Farmers who adopt Roundup Ready Cotton would expect to see slight revenue increases and possible cost decreases; if expectations are correct, then profits would increase.

Cotton lint yields are expected to be similar under both conventional and Roundup Ready Cotton production systems. However, it is possible that Roundup Ready Cotton will allow for more-effective weed control, leading to marketable lint that has lower levels of trash contamination from grasses and broadleaf weeds than conventional cotton. Lint having lower trash levels will generally command a slightly higher price.

Per-acre production costs of Roundup Ready Cotton are expected to be impacted due to the changes in herbicides used and the substitution of Roundup Ready Cotton seed for conventional cotton seed. Growers who adopt Roundup Ready Cotton will simply substitute Roundup Ready Cotton seed for conventional cotton seed and then alter their weed control practices. Thus, the added cost of the Roundup Ready Cotton seed must be compared with the savings obtained from the adoption of new weed control practices.

Due to the diverse and complex interactions throughout the agricultural sector and other sectors of the economy, it is difficult (if not impossible) to predict future magnitudes of key variables with a high degree of accuracy. However, speculation about the direction of change in these variables may be beneficial. For instance, pesticide regulations in the U.S. will likely become more restrictive over time. Imposed reductions in herbicide use without Roundup Ready Cotton will cause cotton yields to decline, farm profits to decline, and acres devoted to cotton production to decline, especially in those regions where herbicide use is an integral production practice. A scenario which allows for the introduction of Roundup Ready Cotton results in a very different forecast. Reductions in herbicide use can be had without yield reductions, farm profits will increase, and acres devoted to cotton will remain constant or even increase in some regions.

It is often argued that some new technologies have characteristics which promote a more rapid rate of adoption by large farms than by small farms (Kuchler). For instance, large initial investment costs or high levels of management may preclude small farms from adopting the technology. However, the adoption of Roundup Ready Cotton will not have a negative impact on small farms. No specialized equipment will need to be purchased and both small and large farms will have the same per-acre costs and benefits from the adoption of Roundup Ready Cotton. Thus, adoption rates should be equal for all size farms.

Economic Impacts

The introduction of Roundup Ready Cotton will provide cotton growers with the choice of either maintaining or altering their current production practices. Each cotton grower will need to evaluate the profit potential of Roundup Ready Cotton relative to that of conventional cotton. Due to different weed pressures and weed control practices across the country, it is expected that some growers will be able to increase their profits by adopting Roundup Ready Cotton, whereas other growers will not. As adoption of this

new technology grows, some of the current supply-demand relationships in the cotton industry will change. As input prices and quantities adjust over time, the profitability of cotton growers and some associated agribusinesses will change.

Supply and demand relationships for Roundup Ready Cotton seed, conventional cotton seed, and some herbicides will shift over time as the Roundup Ready Cotton industry develops and grows. Shifts in supply of an input and demand for an input have a tendency to put upward or downward pressure on prices and quantities sold. Movements in an input's price are necessary to equate quantities supplied and demanded in order to allow the market to achieve a new equilibrium position. Directional impacts on an input's price and quantity sold from shifts in supply and demand may be summarized as follows:

Type of Shift in an Input's Market	Impact on an Input's Price	Impact on Quantity Sold
Increase in supply, holding demand constant	Decrease	Increase
Decrease in supply, holding demand constant	Increase	Decrease
Increase in demand, holding supply constant	Increase	Increase
Decrease in demand, holding supply constant	Decrease	Decrease

It is expected that the market for Roundup Ready Cotton seed that is used for planting will exhibit growth during the first few years after it is introduced. Market participants will gather information during this early stage of the adoption period. There will be much uncertainty in supply and demand conditions, generating an environment in which price discovery will evolve over time. As the Roundup Ready Cotton seed market matures over time, it is expected that a more stable supply-demand relationship will develop.

Cotton growers who decide to adopt Roundup Ready Cotton will replace conventional cotton seed with Roundup Ready Cotton seed. Seed companies will retain some of the Roundup Ready Cotton seed produced with the current year's Roundup Ready Cotton crop and make it available to growers for production of the next year's Roundup Ready Cotton crop. Thus, the supply of Roundup Ready Cotton seed is expected to increase during the first few years. As the Roundup Ready Cotton seed market grows, there will be a simultaneous decrease in the demand for and the supply of conventional cotton seed. These shifts will cause a decrease in the quantity of conventional cotton seed and either an increase or a decrease in its price. Over time, a new equilibrium position will be determined in the markets for both types of seed. It is expected that profits of seed producers will increase due to the introduction of Roundup Ready Cotton.

Growers who use Roundup Ready Cotton seed will be able to alter their applications of chemical herbicides that are used to control weed infestations after the cotton crop has emerged. It will be possible to apply Roundup herbicide in place of other herbicides. Application methods of Roundup herbicide may be either post-directed, over-the-top, or spot sprays. Thus, an increase in the demand for Roundup herbicide and a decrease in the demand for some herbicides will occur, causing changes in both the quantities and prices of these herbicides.

In some regions of the country, it is common for cotton growers to hire laborers to hand hoe the crop to remove weeds from cotton fields. It is possible that the use of Roundup Ready Cotton will allow growers to reduce the use of hand hoers, thus reducing production costs even further. The use of hand hoers in a given year is highly variable; estimates range from about 1 to 5 hours of labor per acre. At a wage rate of $5.75 per hour, cost reductions from elimination of hand hoers could range from $5.75 to $28.75 per acre.

Cotton consultants are often hired by cotton growers to help make management decisions throughout the growing season. It is expected that growers who adopt Roundup Ready Cotton will still utilize consultants. Therefore, the impact of the introduction of Roundup Ready Cotton on consultants is expected to be minor.

The economic impacts on cotton growers who adopt Roundup Ready Cotton could be significant in some regions of the country. Currently, it is not uncommon for growers in the southeast and mid-south regions to spend $20 to $50 per acre on herbicides. One application of Roundup herbicide at the broadcast rate of 24 ounces per acre would cost about $7.50 per acre (excluding application cost). Similarly, Roundup D-Pak herbicide, which is available in the Delta states, applied at the broadcast rate of 15 ounces per acre would cost about $4.70 per acre. Banded application rates would be less expensive broadcast rates. Use of a few applications of Roundup herbicide in place of conventional weed control programs, which could include chemical herbicides, mechanical cultivation, and/or hand hoers, might reduce a grower's weed-control cost. However, Roundup Ready Cotton seed will presumably command a higher price than conventional cotton seed, resulting in an increase in a grower's seed cost.

To entice a cotton grower to purchase Roundup Ready Cotton seed, the expected profits from Roundup Ready Cotton production must be greater than the expected profits from conventional cotton production. Thus, to assure adoption of Roundup Ready Cotton, the increased expense of Roundup Ready Cotton seed must be more than offset by the savings from reduced herbicide use. Supply and demand relationships in related markets will adjust over time until an equilibrium position exists between Roundup Ready Cotton and conventional cotton. It is expected that growers who adopt Roundup Ready Cotton will exhibit an increase in profitability.

In some regions of the country, cotton production on some types of cropland is less profitable than some other crops due to high production costs. Thus, this marginal acreage is currently best suited for uses other than cotton production in these regions. The introduction of Roundup Ready Cotton, with expected lower herbicide costs, could allow cotton production to become more profitable than current uses on these marginal acres, thereby providing an incentive for growers to convert this marginal acreage to cotton production. If a substantial increase in cotton acreage (and thus an increase in the supply of cotton) occurs in the U.S. and/or the world and the demand for cotton remains constant, the price of cotton should decrease, leading to lower farm-level cotton lint prices and lower wholesale and retail prices of cotton-related products. Lower prices and greater output increase the welfare of consumers.

Conclusions

The adoption rate of Roundup Ready Cotton will be influenced by economic factors. Cotton growers will evaluate the profit potential of Roundup Ready Cotton relative to that of conventional cotton. Due to varying weed infestation levels and weed control practices in different regions of the country, some growers will be able to increase profits by adopting Roundup Ready Cotton, whereas other growers will not. As cotton growers increase their use of this new technology, some of the current supply-demand relationships in the cotton industry will be altered. As the Roundup Ready Cotton seed market grows, it is expected that the markets for conventional cotton seed and some herbicides will exhibit a decline in demand.

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Submitted by Stan R. Spurlock

Appendix III

Gene Transfer Between Contiguous Cultivated Cotton and Between Cultivated and Wild Relatives

GENE TRANSFER BETWEEN CONTIGUOUS CULTIVATED COTTON AND BETWEEN CULTIVATED COTTON AND WILD RELATIVES

Report to *Monsanto* Company

James McD. Stewart, PhD.

This discussion is limited to the potential of genetic material to move from cultivated cotton to a related wild relative or to a contiguous genotype of the same species within the geopolitical boundaries of the USA. First, the genetic potential for horizontal gene flow will be addressed. This will be followed by a discussion of the physical limitations to outcrossing. A brief comment on the potential of a cultivated cotton or wild relative containing Bt and NPT II genes becoming a weedy pest concludes this report.

For gene flow to occur via normal sexual transmission certain conditions must exist. 1) The two parents must be sexually compatible; 2) their periods of fecundity must coincide; 3) a suitable pollen vector must be present and capable of transferring pollen between the two parents; 4) resulting progeny must be fertile and ecologically fit for the environment in which they find themselves. All *Gossypium* species are self-fertile but can be cross-pollinated by certain insects. Wind transport of pollen is not a factor.

Gene Transfer to Wild Species

The criterion of sexual compatibility greatly limits the potential of gene flow from cultivated *Gossypium* in the geopolitical boundaries of the USA. No genera in the Gossypieae tribe occur naturally in the USA. Very wide hybridization between a *Gossypium* sp. and other genera is rare and has been reported only for *Abelmoschus esculentus* (Brown, 1947). In this instance cotton was the maternal parent and the one hybrid plant was depauperate and both male and female sterile. I have made numerous pollinations of hibiscus *(Hibiscus acetosella, H. syriacus),* okra *(Abelmoschus esculentus),* and *Alyogyne* spp. onto semigametic cotton. In many instances seed have been obtained, but in all cases the resulting plants have been cotton. Apparently parthenogenesis is occurring, a prospect that we intend to study more closely. I have made numerous attempts to cross cotton (semigametic *G. barbadense*) onto *Hibiscus* as the maternal parent without success. The available experience indicates that the potential for *Gossypium* to outcross with other malvaceous genera is extremely low to nil.

In the absence of intergeneric hybridization, the major issue to be considered is the probability that cultivated cotton species *(G. hirsutum* and *G. barbadense)* will hybridize with feral or wild species of *Gossypium.* This

potential exists in only three locations in the USA where *Gossypium* species occur naturally. These are 1) south Florida, 2) the Hawaii Islands, and 3) southern Arizona. In no instance has frequency data on outcrossing been taken.

The wild diploid, *G. thurberi,* occurs in the mountains of southern Arizona (Fryxell, 1979). Under controlled conditions this species can be made to hybridize with *G. hirsutum* when the latter is the female parent (Beasley, 1942; Gerstel, 1956; Gerstel and Phillips, 1958). I have made numerous attempts to make hybrids between *G. hirsutum* and a. *thurberi* with the latter as the maternal parent - all were unsuccessful. The possibility is not nil because several (7) other wild diploids have been hybridized as maternal parents including the closely related *G. trilobum* (Meyers,1973; Umbeck and Stewart, 1985; Stewart, unpublished). However, hybrids between *G. hirsutum (or G. barbadense)* and *G. thurberi* are triploid (3x=39) (Beasley, 1942) and completely male and female sterile. For fertility to be obtained the chromosome complement must be doubled to the hexaploid level, and this has been done experimentally (Beasley, 1942; Brown and Menzel, 1952; Gerstel, 1956; Gerstel and Phillips, 1958). No natural hexaploids of *Gossypium* exist in nature even though tetraploid and diploid species have coexisted in the Americas in excess of one million years (Wendel, 1989). To my knowledge no record exists of genetic movement from a higher ploidy genotype to a diploid *Gossypium* either in nature or by human manipulation. All recorded genetic movement involving diploids has been from diploids to higher ploidy lines.

The potential for genetic information to flow from a cultivated *Gossypium* species *to G. thurberi* is nil by all reasonable criteria. *G. thurberi* is restricted to the mountainous regions of southern Arizona and does not occur in the desert valleys where cotton is grown. *G. thurberi* blooms late in the season (Sept. - Oct.) when commercial cotton in the area is being harvested, so there is only minor overlap in blooming. Pollen transfer between the two species is highly unlikely, sexual compatibility is very low, and should any progeny ever occur, they would be sterile.

Feral *G. hirsutum occurs* in the strand areas of southern Florida (Everglades National Park) and the Florida Keys (Percival, 1987). The potential for genetic transfer to this feral cotton would not differ from the potential for transfer to other contiguous cultivated cottons should a transgenic line be grown in the vicinity. Cotton is not grown in southern or central Florida, so the potential for genetic transfer by natural means is extremely low. Direct human intervention by deliberate hybridization or by cultivation of transgenic plants as ornamentals in the area would increase the potential.

A wild tetraploid species, *G. tomentosum,* is endemic to some of the Hawaiian Islands (Stephens, 1964). All of the known tetraploid species of *Gossypium,* including *G. tomentosum,* have the 2(AD) genomic constituency and will hybridize with any of the other tetraploids (Beasley,

1940a,b). Apparently *G. tomentosum* is opportunistic and blooms whenever sufficient moisture is available (Stephens, 1964), so the potential for hybridization is not related to season. Hybrids (F_1) between *G. tomentosum* and *G. hirsutum* are vigorous in vegetative growth but, while fertile, are not particularly fruitful (Stewart, personal observations). Observations on subsequent generations have not been observed in terms of relative fitness for survival. Stephens (1964) reported the occurrence of what he considered hybrid swarms from *G. barbadense* x *G. tomentosum* hybridizations on the island of Oahu. He noted that the plants looked more like *G. barbadense* with some *G. tomentosum* introgression. Wendel (Iowa State University, unpublished) has grown several accessions of *G. tomentosum* under greenhouse conditions and examined these for morphological and isozymic diversity. He observed morphological variation which he thought represented introqression of *G. hirsutum.* He is of the opinion that his preliminary isozyme data supported the supposition but to a lesser degree than what morphological observations would have indicated (Wendel, per. comm.). Stephens (1964) considered the degree of diversity within *G. tomentosum* to be low, but in fact, a thorough documentation of the diversity does not exist. Thus, the question of the degree of interspecific introgression, if any, is an unanswered one.

My observations on a related wild/cultivated *Gossypium* interaction in NE Brazil is similar to that of Stephens on the Hawaiian species. In plots of Moco cotton (cultivated perennial *G. hirsutum* race 'Marie Galante') I commonly found plants with a few morphological features characteristic of *G. mustelinum.* I interpret this as gene flow from the wild species to the cultivated. In one instance a *G. mustelinum* plant was found growing in a field of Moco cotton. (Would you call this an invader or an escape from the wild?) The wild populations of *G. mustelinum* showed no morphological evidence of introgression from cultivated types. A third model can be found on the Galapagos Islands with G. *darwinii* and *G. barbadense* (Wendel and Percy, 1991). In this case the phylogenetic lineage is very close (species pair) and introgression apparently occurs in both directions.

Given the opportunity by proximity, concurrent flowering, and pollen vector, wild tetraploids, including *G. tomentosum,* will hybridize with cultivated cotton in both directions. Factors that influence the probability that a hybridization event will actually occur in Hawaii have been addressed by Monsanto in obtaining an experimental use permit (Montgomery, 1991). A major point of consideration is the proximity of the wild species to the transgenic cultivated type. Distance will exert the same barrier to interspecific cross-pollination as on intra-specific crossing. Available evidence indicates that *G. tomentosum* is restricted to the arid regions of Niihau, Oahu, Molokai, Maui, Lanai and Kahoolawe (Stephens, 1964). The use of one or more of these islands as a winter garden for seed increase of transgenic cotton would increase the potential for outcross to the wild species while cultivation on the other islands would pose no threat. Due regard for plot location relative to wild populations would need to be taken (if the transgenic material is deemed undesirable).

Gene Transfer to Cultivated Genotypes.

In as much as similar cotton genotypes are fully compatible, any pollen that is transferred has the potential to produce a hybrid seed. The degree of outcrossing in a production field is strongly dependent upon the geographic location of the field (Simpson, 1954), which means upon the crop ecology. The most important factors are the kinds and numbers of insect pollen vectors. Bumble bees *(Bombus* spp.) and honey bees *(Apis mellifera)* are the most significant (Theis, 1953; McGregor, 1959; Moffett and Stith, 1972; Simpson and Duncan, 1956) with the former being the most efficient pollinator. Typical outcrossing percentages for a number of locations in the cottonbelt are listed in Table 1. These are all old reports made under crop ecological conditions that may no longer exist. This is specifically addressed in the report of Meredith and Bridge (1973) whose results indicate that out-crossing has declined in the Mississippi Delta (from 28% reported by Simpson to 2% average over 11 locations with a range of 0.0% to 5.9%). This may be typical of many of the cotton growing areas where loss of insect habitat and heavy use of insecticides is the norm. On the other hand, if production of bioengineered cotton becomes wide-spread and insecticide use declines, bee populations may increase and raise the potential for out-crossing to previous levels.

Considerable work has been done on the degree of outcrossing between adjacent plants, rows and plots of cultivated cotton (Afzal & Rahn, 1950a,b; Green & Jones, 1953 ; Thies, 1953; and others summarized in Brown, 1938). Recently, both Monsanto (1990 report to APHIS on 7 locations) and Agracetus (Umbeck et al., 1992) used molecular techniques to determine outcrossing from transgenic cotton plots buffered by cotton. Both reports showed that no more than 6% outcrossing occurred on border rows and the percentage dropped rapidly in rows successively distant from the plot. These results adequately show that the containment strategies used under the experimental use license were adequate. The question of potential escape under wide-spread cultivation is not addressed by any of these data. Almost without question, the transgenic material can be expected to be transferred to other cultivated genotypes over time. Because of the perceived benefits of the Bt gene in worm resistance, surreptitious outcrossing to other cultivated cotton can be expected. This will be independent of distance, pollinators, etc. Only a strong legal stance by the proprietary developers will slow this process, and this ultimately will have no bearing. The basic question must be centered on the potential for Bt cotton to become a pest or contribute genes that will make a relative a pest.

Pest Potential of Bt Cotton.

For anyone familiar with the cottons of the world, this does not merit consideration. All wild and feral relatives of cotton are tropical, woody, perennial shrubs other than a few herbaceous perennials in NW Australia. With the exception of *G. thurberi* discussed above and *G. sturtianum* in Australia, these cannot naturally exist even in the milder temperate

regions. In most instances the distribution of these species is determined by soil and climatic conditions rather than insect pressure. As perennials the plants are not particularly programmed to produce seed each year. In fact, they tend to drop fruit in response to stress. It is unlikely that Bt would impact survival either way. The only species that approaches the designation of pest is the arborescent *G. aridum* in parts of central western Mexico where it grows in fence rows much like sassafras in parts of the US.

In those areas of the USA where feral or wild cottons occur (south Florida, Hawaii) the problem is not potential proliferation of plants but loss of the germplasm resource. In this respect, introgression of additional pest resistance (Bt) might be viewed favorably. Ultimately if Bt should be transferred to a wild population of a tetraploid, and this was considered undesirable, the size of the plants, their perennial growth habit, their restricted habitat, and their low natural fecundity (say relative to something like Johnsongrass) would make control exceptionally easy.

Table 1. Typical early reports of out-crossing in cotton.

Location	Percentage	Reference
SE Missouri	14	Sappenfield, 1963
Tennessee	47	Simpson & Duncan, 1956
Central Texas	10	Simpson, 1954
Southeast	39	Simpson, 1954
College Station, TX	24 - 48	Richmond, 1962
	6.6	Simpson, 1954
Mississippi Delta	28	Simpson, 1954
	2	Meredith & Bridge, 1973

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Appendix IV

EPA EFGWB Data Evaluation Record

DATA EVALUATION RECORD

Biological Fate: Transgenic cotton plants containing a *Bacillus thuringiensis* delta-endotoxin and an NPTII marker enzyme (Monsanto Company; EPA File Symbol 524-EUP-TG)

REVIEWED BY:

Leo R. LaSota, Ph.D
Biologist
EFGWB/EFED

Signature: L. R. LaSota
Date: JAN 24 1992

APPROVED BY:

Paul Mastradone, Ph.D.
Chief, Section 1
EFGWB/EFED

Signature: Paul J Mastradone
Date: JAN 24 1992

CONCLUSIONS:

I. Based on the data submitted and a review of the scientific literature, EFGWB concludes that the protocols for this EUP present no unreasonable risk of unplanned pesticide production through expression of the *Bt* delta-endotoxin or NPTII marker enzyme genes in wild relatives of the transformed cotton, *Gossypium hirsutum* L. Only two wild species of *Gossypium* occur in the United States: *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seeman (Brown and Ware, 1958; Fryxell, 1979; Munro, 1987). The former has been described by Kearney and Peebles (1951):

> *Gossypium thurberi* Todaro (*Thurberia thespesiodes* Gray). Graham, Gila, Pinal, Maricopa, Cochise, Santa Cruz, and Pima counties, reported also from the Bradshaw Mountains (Yavapai County), 2,500 to 5,000 (rarely 7,000) feet, rather common on rocky slopes and sides of canyons, late summer and autumn. Southern Arizona and northern Mexico.
>
> A handsome shrub, known in Sonora as algodoncillo (little cotton), reaching a height of 4.2 m. (14 feet). Petals normally spotless, but plants with faint crimson basal spots are not rare. The plant is interesting because a subspecies of the cotton boll weevil breeds in the capsules. The form of this insect of which *G. thurberi* is the normal host also occasionally attacks nearby cultivated cotton, consequently the United States Department of Agriculture endeavored at one time to eradicate the plant where it grew near areas of cotton cultivation. (p. 553)

The Casa Grande, Maricopa and Yuma, Arizona sites for this EUP are in desert valleys which provide distance and habitat isolation from populations of *G. thurberi*. Notwithstanding, any gene exhange betweeen plants of *G. hirsutum* and *G. thurberi* would result in triploid (3x=39), sterile plants because *G. hirsutum* is an allotetraploid (4x=52) and *G. thurberi* is a diploid (2x=26). Under

controlled conditions, hybrids have been produced when *G. thurberi* served as the paternal parent; allohexaploids have not been reported in the wild (Stewart, 1991).

The range for Hawaiian cotton, *G. tomentosum* has been described by Degener (1946):

> **LOCAL RANGE:** Found on the larger islands as well as on Nihau and Kahoolawe. It grows on arid, rocky or clay plains not far from the sea. On the larger islands, it is hence found chiefly on the dry, leeward side. On Oahu it is common near Koko Crater, and grows scattered between Honouliuli and Makus Valley. On Molokai it is extremely common on the southwestern end; elsewhere it is rare except near Kamalo. Specimens growing near Kaunakakai, according to Hillebrand, differ from the typical. On Maui the species may be found far from the sea in one of the valleys south of Wailuku. According to Watt ("Cotton Plants of the World" 71. 1907) "In the British Museum there is a specimen with very small leaves, entire or three-lobed, which bears the remark that it is '*G. parvifolium* Nutt. MS.' It certainly is nothing more than a variety, but it is worthy of separate mention. It would appear to have been collected at Owhyhee (Hawaii). A specimen in the Kew Herbarium from the Molokai Island has the three leaves very much narrower than is customary and is thus probably also this variety of the species." From our present knowledge of all these plants, it still seems best to treat them as a single species.
>
> **EXTRA RANGE:** Endemic to the Hawaiian Islands but cited erroneously in the Fiji Islands as well. The closest relatives of this species are native to the Galapagos Islands and to Australia. (n.p.).

A later assessment by Stephens (1964) indicated the probable geographic range for *G. tomentosum* as being limited to the six islands of Kahoolawe, Lanai, Maui, Molokai, Nihau and Oahu (See Appendix 1). The only Hawaiian site requested for this EUP is for the seed increase nursery on the island of Kauai. Two surveys by Montgomery (1990, 1991) found no *G. tomentosum* growing-or reported growing-in the wild on Kauai; cultivated plants of *G. tomentosum* were reported as growing in a private garden 10 miles from the test site. Naturalized plants of sea island cotton (pulpulu haole, *G. barbadense* L.) growing within 0.5 miles of the test have been destroyed.

Upland, Hawaiian and sea island cotton are all interfertile tetraploids (Beasley, J.O. 1940a,b, 1942). It is noted that the tropical climate of Hawaii, which permits a true perennial habit for all three *Gossypium* species, poses a monitoring concern already experienced near the test site: "To reduce seed production and dispersal it [a plant of *G. barbadense* within the survey area] "had been chopped down in July, 1990 by this writer [Montgomery, 1991], but it has quickly regrown, and was flowering prolifically from Dec. to early March, 1991." Introgression has been claimed for

what Stephens (1964) considered hybrid swarms of *G. barbadense* x *G. tomentosum*. The possibility of the capture and expression of the *Bt* protein and NPTII enzyme by either species can be prevented by restricting pollen movement from the test site, denying unauthorized personnel access, destroying all propagules (seed, vegetative plant parts) not used for further study and monitoring for volunteers and suckers following harvest (See Recommendations below).

II. Based on the data submitted and a review of the scientific literature, EFGWB concludes the protocols for this EUP present no unreasonable risk of unplanned pesticide production through expression of the *Bt* delta-endotoxin or NPTII marker enzyme genes in feral populations of *G. hirsutum* or *G. barbadense* in the continental United States. The inability of plants or seeds of either of these species to survive freezing temperatures restricts their persistence as perennials or recurrent annuals to tropical areas. Feral populations of *G. barbadense* exist in parts of southern Florida (Percival, 1987), but feral populations of neither this species nor *G. hirsutum* have been reported near any of the continental test sites subject to this EUP.

III. Based on the data submitted and a review of the scientific literature, EFGWB concludes that expression of the *Bt* delta-endotoxin or NPTII marker enzyme genes in cultivated cotton grown for the EUP will neither create nor agrravate weedy or agressive characteristics. Acquisition of the *Bt* delta-endotoxin would confer selective advantage (specific insect resistance) to cultivated cotton, but would not modify the hardiness, habit (shrub), reproductive (not asexually propagated), cultural (host to other pests not controlled by *Bt*) and other limits which have prevented either upland or sea island cotton from becoming aggressive or weedy despite their long cultivation in the cotton-growing regions of the continental United States.

IV. Based on the data submitted and a review of the scientific literature, EFGWB concludes that the containment strategy of a minimum of 24 buffer rows of nontransgenic cotton, or an isolation distance of at least of 0.25 miles from any other cotton, will minimize, but not eliminate, the capture and expression of the *Bt* and NPTII genes by cultivated cotton growing near the test sites. Outcrossing rates of 3% or less are expected in cotton adjacent to the last (24th) border row or in cotton isolated by a distance of 0.25 miles.

With this EUP request, the applicant has submitted the results of a 1990 study on the use of border rows for containment of transgenic pollen. (See Reported Results: Table 1) EFGWB concludes that the data submitted with this study do not support the outcrossing rates expressed in the tables because samples were pooled from different locations on plants and different positions within rows. The sampling procedure did include these parameters but subsequent pooling before seed selection means data presented

do not reflect either developmental or spatial variabilities in outcrossing potential.

The 1990 study was conducted in conjunction with other tests of trangenic and nontransgenic cotton plants at the same sites and was not designed solely to determine outcrossing rates. There was not a uniform distribution of single-line transgenic plants in all quadrants of the experimental plots. Some border rows were perpendicular to the transgenic plants; other were parallel. Kind and number of alternate pollen sources varied with site. Nor can data from seven 1990 sites be assumed to reflect the expected variability at 24 sites during the 1992-93 field tests where new locations, field designs, contiguous crops, and pollinator densities will interact with unpredictable weather conditions.

Notwithstanding the predictive limitations of the 1990 Monsanto outcrossing study, EFGWB concludes that an expected outcrossing rate of 3% or less with either 24 border rows or a 0.25 mile buffer to other cotton is consistent with known information concerning the effectiveness of buffer rows in reducing outcrossing in cotton (see below), the foraging behavior of bee pollinators (Kareiva et al, 1991), and the use of isolation distance to limit, but not eliminate, gene flow (Association of Official Seed Certifying Agencies, 1971; Green and Jones, 1953).

Species in the genus *Gossypium* are self-compatible (Fryxell, 1979) with the timing of anther dehiscence and stigma receptivity for *G. hirsutum* being synchronous (homogamy). The amount of cross-pollination or "natural crossing" (McGregor, 1976) that occurs has been attributable to many factors including:

1. The species and number of insect pollinators present (Thies, 1953);
2. Sugar concentration and composition of floral nectaries (Moffett et al, 1975);
3. Location with respect to alternate nectar sources, such as summer-flowering tamarisk (Moffett and Stith, 1972).
4. "Flowering habits of the varieties grown, by the abundance of unlike pollen, by location of the fields in relation to insect habitats,... by distance between unlike topography and barrier crops, and by other environmental, climatic and biotic factors" (Simpson, 1954).

Insect pollinators, primarily bumblebees (*Bombus* spp) and honey bees (*Apis mellifera* L.), are the agents for pollen dispersal in the cotton growing regions of the United States; wind is not considered a vector (Thies, 1953). Buffer rows have been shown to provide effective traps for the outflow of pollen. Simpson and Duncan (1956) have explained the dilution effect of such rows as follows:

> Assuming that a pollen-free bumblebee enters a cotton field at random, its first flower visitation will provide an initial load. Since the bumblebee's search for food is quite

systematic, its flights after entering the field are short, usually to the next visible flower. Maximum transfer of pollen would logically occur at the first stop after picking up an initial load. Pollen distribution from a focal center is essentially a 'put and take' procedure. Every step away from the focal point results in the loss of some fraction of the pollen acquired at the initial stop. And also, every step becomes a new focal point for further distribution. (p. 307)

Using foliar color differences to detect outcrossing events, Simpson and Duncan recorded a drop from over 40% to approximately 3% in outcrossing through 75 feet of cotton buffer (See Appendices 2-4). Their experimental design resulted in a decrease with distance in the area that was sampled to determine outcrossing. Competition between self-pollination and three different sources for cross-pollination confound the interpretation of the effects of distance and trapping on pollen dispersion.

Green and Jones (1953) examined all progeny (over 100,000) from an experiment comparing the effects of distance and buffer rows on outcrossing (Appendices 5-6). Buffer rows were more effective than distance in reducing hybrid production; outcrossing decreased from 19.5% to 1% through 33 feet (2 rods of buffer); the decline was to only 4.7% across a cotton-free zone of the same distance. Unequal or missing samples and the possible contribution of edge effects complicate the interpretation of this data.

In other cotton outcrossing experiments, where sample sizes are small and population variability is high, the significance of the results is diminished. For example, Meredith and Bridge (1973) state in the "Abstract" of their study of "Natural Crossing of Cotton (*Gossypium hirsutum* L.) in the Delta of Mississippi":

> The glandless trait was used to study the amount of natural crossing in cotton (*Gossypium hirsutum* L.) in the Delta of Mississippi. We sampled 102 hills of glandless cotton planted in fields of glanded cotton at 11 locations in 1972. Natural crossing varied from 0.0 to 5.9% and averaged 2.0%. There was only 0.2% natural crossing in the five Central Delta locations. These results indicated that in the Central Delta of Mississippi, cotton is essentially a self-pollinated crop. (p. 552)

The sources for the analysis of variance in this experiment were locations (10 degrees of freedom [df]), rows within location (7df), location + rows (17df) and hills within rows (84df). "The coefficient of variability for hills within a row was 295% The ranges [of outcrossing] were from 0 to 41.1% ...for all hills." (p. 552)

Summary data from different locations representing several years of outcrossing experiments may suggest trends, but this measure can also mask variability. Sappenfield (1963) provides a mean of the means for six years data on natural crossing of upland

cotton in Missouri indicating that the "average amount of natural crossing for the 6-year period over the general production area was only moderate and estimated at 13.6%." The range for one year (1958) was from 1.0% for Bragg City to 32.2% for Diehlstadt. In 1959 the Diehlstadt rate was 4.4%; in 1961 it was 23.0% (See Appendix 7). Thus not only is there substantial variability in natural outcrossing from site to site, but from year to year at the same site as well.

Other variables that must be considered in evaluating "natural" outcrossing data for cotton include the plant materials being tested. Prior to the development of recombinant DNA technology, morphological differences, such as glanded versus glandless and red-leaf versus green leaf, or progeny counts from male sterile lines, provided ways to detect outcrossing events. Morphological markers may bias outcrossing rates by affecting pollinator preference. In the case of male sterile plants, all progeny result from outcrossing because there is no self-pollination.

In summary, based on the data submitted and a review of the scientific literature, EFGWB concludes that maximum outcrossing rates in cotton are site specific and that buffer rows are effective in reducing these rates. The reduction curve is asymptotic, with the most rapid decline in outcrossing occurring in the rows closest to the foreign pollen source. A rate of 3% for a minimum of 24 buffer rows is consistent with that reported in earlier studies--and within the 95% confidence limits of Monsanto's own data for Boissier City. Serdy. 1991c, 1992.

RECOMMENDATIONS:

I. EFGWB recommends that all sites except the seed increase nursery in Hawaii be surrounded by either a mimimum of 24 rows of non-transgenic *Gossypium hirsutum* or be isolated from any other cotton by at least 0.25 miles.

II. EFGWB recommends that in addition to the four rows of nontransgenic cotton surrounding the Hawaii seed increase field, the following additional measures be taken to prevent the removal of propagules from the test site or the expression of the transgenic pesticides in perennial cotton:

A. Guarantee through physical barriers (fencing) and/or other security measures that the test site will be limited to authorized personnel only.

B. Extend the monitoring period at the test site for volunteers or suckers to five months following harvest; destroy all suckers or volunteers.

C. Resurvey the area within 0.5 miles of the test site following harvest for any feral plants of *Gossypium* spp; destroy any found.

MATERIALS AND METHODS:

Monsanto Outcrossing Experiment: Buffer Rows and Cotton

Purpose: To determine levels of outcrossing as affected by buffer rows; included in field tests of transgenic cotton plants containing the delta-endotoxin from *Bacillus thuringiensis*

Year conducted: 1990

Sites(7): Boissier, Brawley, Casa Grande, College Station Halfway, Maricopa and Starkville; fields adjacent to College Station and Brawley were also surveyed (no sampling information given) for outcrossing

Genotypes: Segregating and homozygous lines from five independent transgenic plants of Coker 312 carrying Monsanto construct pMON 5377; nontransgenic controls

Procedures:

The experiment will be surrounded by 24 border rows to provide a trap for all outgoing pollen carried by insects and wind. The line used for the border rows will be glandless cotton. Since the gene for glandless is recessive to the gene for glands (carried by the transgenic cotton), out-cross events can be identified by glands on the seed embryos. At the end of the season, samples will be collected from the border cotton by harvesting a boll every 10', alternating among the bottom, middle, and top of the plants harvested. These samples will be collected around the field on every other row starting with the row closest to the transgenic cotton. This scheme will provide a total of 12 samples per test. These samples will be sent to Monsanto's laboratory in Chesterfield, MO so they can be evaluated for outcrossing events. The plants that exhibit glands will be used to confirm that the border rows were effective in maintaining the gene within the confines of the experimental area.

As it turned out, we were not able to rely solely on the marker to determine the rate of outcrossing since seed of the glandless line used as border was contaminated with some seed with the gene for glanding. Therefore, another assay was used to determine which glanded seed harvested out of the border area were actually due to an outcrossing event with *Bt* cotton. An ELISA assay developed at Monsanto is used routinely to identify seed/plants that are expressing the *Bt* protein. The assay is specific to the *Bt* protein and very sensitive to small quantities of the protein.

Therefore, the samples were randomly collected from every other border row surrounding the field. No attempt was made to keep the seed from the different locations on the plant separate. The 150 seeds were randomly selected from the seed collected at each distance.

REPORTED RESULTS:

Table 1

Percent outcrossing at varying distances from the *Bt* cotton observed at six [seven] test sites [and at three adjacent fields].

	Location										
Approximate distance from test (ft)	A %	B %	S.D.+	C %	S.D.	D %	E %	S.D.	F %	S.D.	G %
3.3	0.0*	0.0		3.3	1.5	0.0	4.7	1.7	2.0	1.1	0.0
9.9	0.0	0.0		2.0	1.1	0.0	0.0		3.3	1.5	0.0
16.7	0.0	0.0		0.7	0.7	0.0	0.0		0.0	0.0	0.0
23.3	0.0	0.0		0.0		0.0	0.0		0.7	0.7	0.0
30.0	0.0	1.3	0.9	0.0		0.0	0.0		0.0		0.0
36.7	0.0	0.0		0.0		0.0	2.0	1.1	2.0	1.1	0.0
43.3	0.0	0.0		0.7	0.7	0.0	0.0		1.3	0.9	0.0
50.0	0.0	0.0		0.0		0.0	0.0		0.0		0.0
56.7	0.0	0.0		0.0		0.0	0.0		0.0		0.0
63.0	0.0	0.7		0.0		0.0	0.0		0.7	0.7	0.0
70.0	0.0	0.0		0.0		0.0	0.7	0.7	0.0		0.0
76.7	0.0	0.0		0.0		0.0	0.7	0.7	0.0		0.0
H	0.0	0.0									
I		0.0									
J		0.0									

A. College Station
B. Halfway
C. Brawley
D. Maricopa
E. Bossier City
F. Starkville
G. Casa Grande
H. Adjacent Field 1
I. Adjacent Field 2
J. Adjacent Field 3

*Values represent the percent seed harvest at a given distance expressing the *Bt* protein in ELISA assay.

+Standard deviations were calculated where a positive event was observed using the binomial distribution (Snedecor and Cochran, 1967, Iowa State Univ. Press. p. 207-209.)

Serdy, F. 1991b, 1992. [Chart derived from both documents: Casa Grande does not appear in document 1991b; standard deviations are misaligned for 3 entries in document 1991a]

APPENDICES:

Appendix 1

Figure 1: Geographic Range of *Gossypium tomentosum* in the Hawaiian Islands

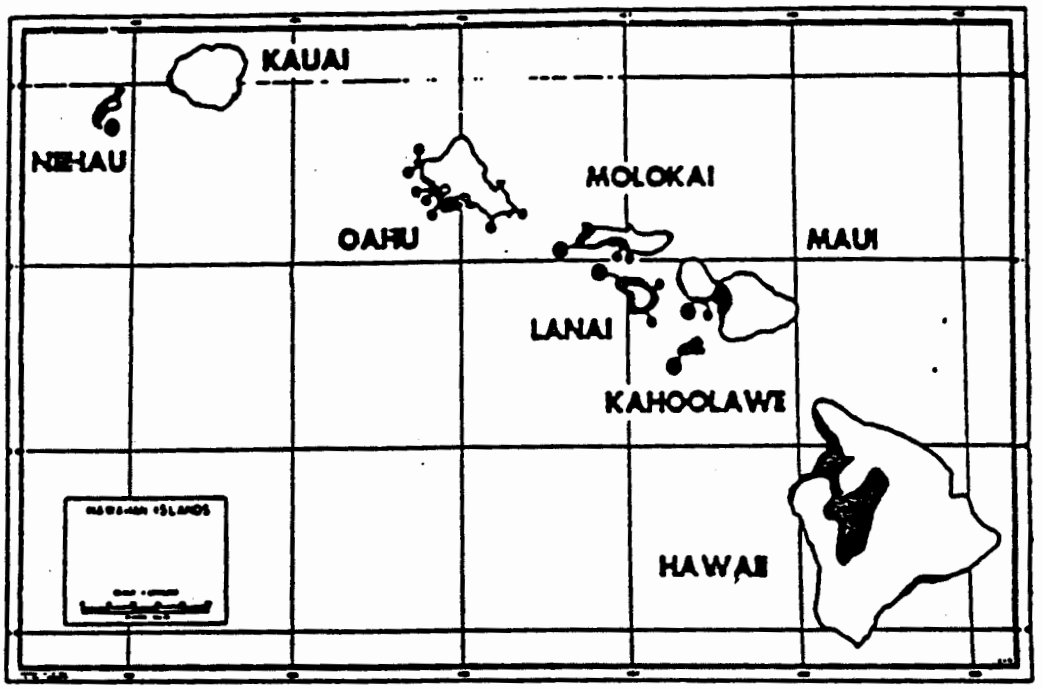

Figure 1. Geographic range of *Gossypium tomentosum* Nutt. in the Hawaiian Islands (1963). Solid circles indicate collection sites; those enclosed in rings represent sites of former collections unchecked during the present study. The open circle indicates site of hybrid populations. Shaded areas correspond to regions with an average rainfall of 20 inches or less. Stephens, S.G. 1964. p.387

Appendix 2

Cotton Pollen Dispersal By Insects: Field Layout

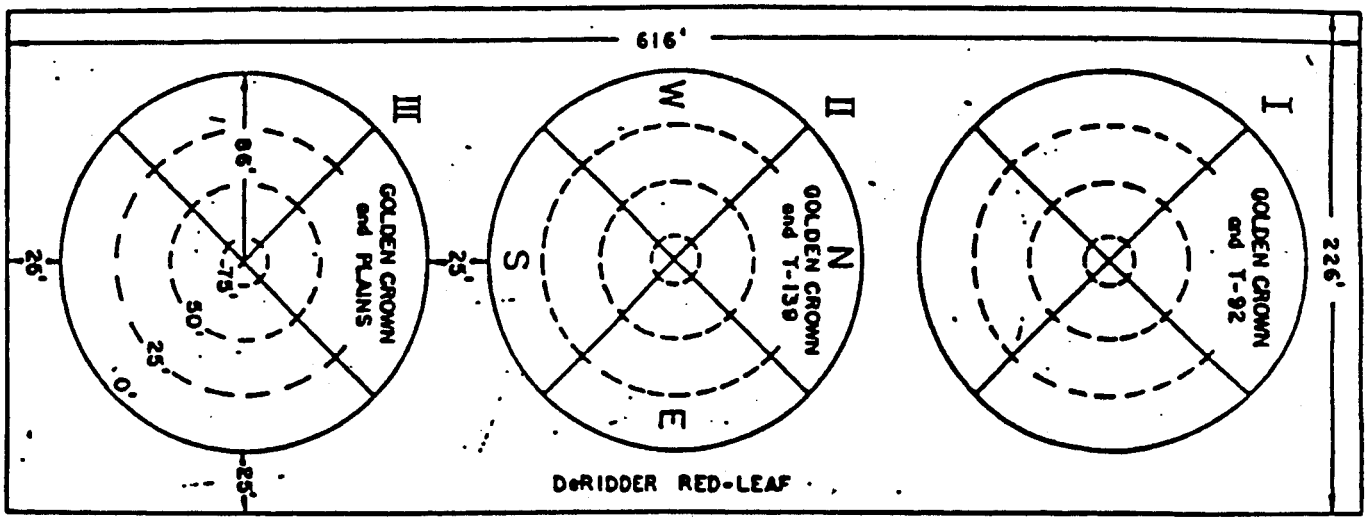

Figure 1.-Diagram of field lay-out of natural crossing experiment. The circles were planted in alternate rows of Golden Crown and green-leaf varieties. The area outside the circles was planted with DeRidder, a red-leaf cotton. Simpson, D.M. and E.N. Duncan, 1956. p. (306)

Appendix 3

Cotton Pollen Dispersal by Insect: Table 1

Table 1.-Natural crossing between green-leaf varieties and Golden Crown planted in alternate rows within circles surrounded by DeRidder red-leaf. [Averages only cited]

Circles	Natural crossing percentage at sampling point indicated			
	0	25	50	75
	T-92 X Golden Crown			
I	29.4	41.2	43.4	45.1
	T-139 X Golden Crown			
II	35.8	38.0	42.8	38.6
	Plains X Golden Crown			
III	32.4	41.3	45.9	44.7

Simpson, D.M. and E.N. Duncan, 1956. p. (307)

Appendix 4

Cotton Pollen Dispersal by Insects: Table 2

Table 2.-Natural crossing between DeRidder red-leaf and other varieties at specified isolation distances. [Averages only cited]

Circles	Natural crossing percentage at designated isolation distance (feet)			
	0	25	50	75
	DeRidder X T-92			
I	24.1	3.9	1.9	2.5
	DeRidder X Golden Crown			
	25.2	4.1	1.6	2.7
	DeRidder X T-139			
II	31.6	5.4	3.0	3.4
	DeRidder X Golden Crown			
	22.1	3.8	2.0	2.7
	DeRidder X Plains			
III	27.2	4.5	2.5	2.6
	DeRidder X Golden Crown			
	25.4	3.9	2.9	2.5

Simpson, D.M. and E.N. Duncan, 1956. (p 307)

Appendix 5

Isolation of Cotton for Seed Increase: Field Layout

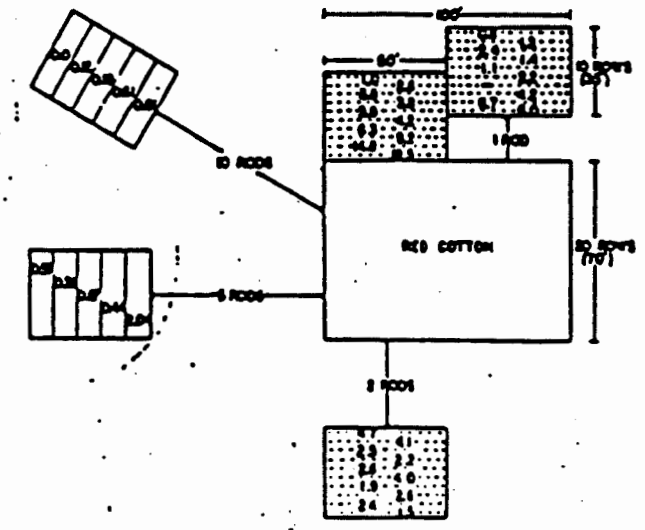

Figure 1.-Arrangement of the blocks of red and green cotton grown in 1951 near Lake Carl Blackwell, Okla. The five smaller blocks were planted to normal green cotton. Percentages of hybrids resulting from natural crossing are indicated for each row in the blocks at 0, 1, and 2 rods, and for 10 foot sections of the blocks at 5 and 10 rods. Green, J.M. and M.D. Jones. 1953. (p. 367)

Appendix 6

Isolation of Cotton for Seed Increase: Table 1

Table 1.- Total numbers of plants counted and percentages of hybrids observed in the progeny of green plants grown at the indicated distances from a block of red cotton.

Row in Block	Distance in Rods from Red Cotton 0 Total	0 %Hybrids	1 Total	1 %Hybrids	2 total	2 %Hybrids
1	4583	19.48	3313	5.98	1311	4.73
2	4160	14.83	3371	6.73	1146	4.10
3	5030	9.22	496	4.23	3368	2.50
4	2805	6.31	----	----*	3569	2.21
5	7462	4.21	930	2.15	1474	2.64
6	5369	3.75	7823	1.11	753	3.98
7	3185	3.80	2538	1.42	1711	1.93
8	1904	3.83	1270	2.36	1081	2.59
9	377	2.62	7884	1.23	1523	2.36
10	96	1.04	3538	0.82	2064	1.50
Totals	28284	6.95	31163	2.39	17990	2.61

Table 1 (cont.).- Total numbers of plants counted and percentages of hybrids observed in the progeny of green plants grown at the indicatded distances from a block of red cotton.

Row in Block	Distance in Rods from Red Cotton 5 Total	5 %Hybrids	10 Total	10 %Hybrids
1	1317	0.61	1325	0.60
2	837	0.96	427	0.47
3	1275	1.49	1202	0.08
4	824	2.30	856	0.00
5	1397	0.72	1115	0.27
6	1093	1.45	954	0.00
7	647	0.15	549	0.55
8	1289	0.54	1021	0.29
9	1797	1.00	1506	0.07
10	2241	0.67	731	0.27
Totals	14302	0.86	9686	0.24

Green, J.M. and M.D. Jones. 1953. (p. 367)

Appendix 7

Natural Crossing in Upland Cotton In Southeast Missouri: Table 1

Table 1-Estimates of natural crossing in Upland cotton in southeast Missouri, 1956-61.

Location	Percent natural crossing						
	1956	1957	1958	1959	1960	1961	Mean
Sikeston	7.4	15.9	5.3	5.9	5.5*		8.0
Dorena		28.9	12.8	6.6			16.1
Malden		24.5	25.5*	7.5*			19.2
Bucoda		9.1	7.2				8.2
Diehlstadt			32.2*	4.4*		23.0*	19.9
Bell City			17.1				17.1
Bragg City			1.0*	13.9			7.5
Portageville					7.7	7.4	7.6
Dry Bayou						20.6	-
Mean		19.6	14.4	7.7	6.6	17.0	13.9

*Irrigated

Sappenfield, W.P. 1963. p. (566)

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Appendix V

Permit Final Reports

1992 *RR* COTTON FIELD RELEASE
(USDA PERMIT#91-347-01)
FINAL REPORT

Robert E. Buehler
Monsanto Co.

The purpose of this field release was to test cotton genetically-modified to contain gene(s) which confer Roundup tolerance. The cotton was grown at one site.

Sites and cooperators

Loxley Alabama site

Mr. Dane Williamson
Monsanto Research Farm
25920 Experiment Farm Rd.
Loxley, AL 36551
(205)964-6236

Genotypes:

This field release included the following genotypes:

- Derivatives of Coker 312 homozygous and segregating for PV-GHGT02, PV-GHGT03, PV-GHGT05, PV-GHGT06, PV-GHGT07, and PV-GHGT08.
- Coker 312 controls.

Distribution of Lines for Evaluations:

USDA # 91-347-01		
Testing Site		Lines Evaluated[1]
Loxley, AL		1085, 1120, 1698, 886, 1360, 1420, 1421, 1445, 1513

[1] Vector #	Lines
PV-GHGT06	1698
PV-GHGT07	1360, 1421, 1445
PV-GHGT03	886
PV-GHGT05	1085, 1120
PV-GHGT02	1513
PV-GHGT08	1420

Schedule of major operations:

May	Seed were packaged according to the protocol and shipped from the Monsanto research center in Chesterfield, Missouri to the cooperator via overnight delivery service. All the seed arrived safely and were stored in accordance with the conditions described in the permit.
May	Seed planted.
November	Harvest and shipment of seed samples back to Monsanto.
post-harvest	After completion of the test at each site, the seed cotton not shipped to Monsanto was spread in the field. The entire field was disked. The area was observed for volunteer plants and all volunteer plants were destroyed by hand weeding, cultivation, or with chemical sprays.

Summary of Observations

Plant growth and general observations:
The transgenic plants did deviate slightly from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to the *RR* protein, level of expression, etc. There are several explanations for that variation including random selection out of the genetic variation in the cultivar, genetic alteration due to the transformation/tissue culture process, or changes in cotton gene functioning due to the introduced gene. Observations suggest that the engineered plants were within the range of variation expected based on these sources of variation.

The plants were regularly monitored for *Agrobacterium* infection symptoms. None could be found.

Responses to specific issues:
1) Horizontal movement:
The *RR* plots were surrounded by 24 border rows (~80') of non-transgenic cotton. This cotton served as a sink for pollen carried by insect from the test area. Based on previous data, it is unlikely that pollen from the *RR* plants was carried outside of the test area.

2) Changes in survival characteristics:
There was no evidence of changes in the survival characteristics of the transgenic cotton plants.

3) Expression level of the genes:
All lines behaved as expected when treated with Roundup i.e. the plants were tolerant to Roundup. Thus, the expression level was as expected.

4) Stability and inheritance of the new genes:
No unusual inheritance patterns were observed.

5) Published data:
At this point, there is no published data from these experiments.

Specific Location Evaluations

Loxley, Alabama

Planted - May, 1992
Harvested - November, 1992

Monitoring for Plant Growth Characteristics

No differences in plant vigor, leaf morphology plant height and other characteristics were observed among the lines.

Field Monitoring for Insect Susceptibility

No differences between transformed and non-transformed plants in terms of insect susceptibility were observed.

Field Monitoring for Disease Susceptibility

No differences between transformed and non-transformed plants in terms of disease susceptibility were noted.

1993 Glyphosate Tolerant Cotton Field Release (USDA Permit # 93-012-02)(Mons # 93-003) Final Report May 15, 1994

Eric M. Johnson, Ph.D.
Monsanto Agricultural Group

The purpose of this field release was to test cotton genetically-modified to be tolerant to glyphosate herbicide for weed control efficacy. One site was planted under this permit with a single cooperator.

Site Location and Cooperator

Lewiston North Carolina Site

[CBI DELETED]

Line Distribution

A single line was evaluated, Line 1698.

Genotypes:

This field release included the following genotypes:
- Derivatives of Coker 312 homozygous for gene constructs PV-GHGT06,
- Coker 312 controls

Schedule of major operations:

May - Seed were labelled and packaged at the Monsanto Life Sciences Research Center in Chesterfield, Missouri according to the Standard Permit Conditions for the Introduction of a Regulated Article (7-CFR 340.3(f)). Delivery to cooperator was via a overnight delivery service. All seed arrived safely and was immediately planted.

May - Seed were planted in the field. Any remaining seed was either buried within the test plot.

Aug - Trial was established strictly for weed control evaluation purposes and thus destroyed prior to mature seed formation.

post-harvest - After completion of the test, the entire filed was disked. The area was observed in the fall for volunteer plants. Continued monitoring for volunteer cotton plants within the test and buffer areas will be ongoing through the 1994 cropping. All volunteer plants observed will be destroyed by hand weeding, cultivation, or with chemical sprays.

Plant growth and general observations:

The only difference observed between the transformed and non-transformed cotton was slightly better germination in the transformed cotton. This is presumed to be a function of seed production and handling rather than an effect of the transformation process.

The plants were regularly monitored for *Agrobacterium* infection symptoms. None were observed.

Responses to specific issues:

1. Horizontal movement: The glyphosate tolerant plot was surrounded by 24 border rows (~80 feet) of non-transgenic cotton. This cotton served as a filter for pollen collected by insects from the test area, excluding it from movement to areas outside the test and buffer area. Based on previous data collected for transgenic cotton plants, it is unlikely that pollen from the glyphosate tolerant plants was carried outside the test area.

2. Changes in survival characters: There was no evidence of changes in the survival characteristics of the transgenic cotton plants excepts for the planned tolerance to glyphosate herbicide.

3. Expression level of the genes: The expression of the tolerance gene was measured through susceptibility to applications of glyphosate herbicide. Vegetative tolerance was excellent for the line evaluated.

4. Stability and inheritance of the new genes: No unusual patterns were observed.

5. Published data: At this time, there are no published data from these experiments.

Individual Site Information

Lewiston North Carolina Site: A single experiment evaluating weed efficacy of glyphosate treated plots was established at this location.

Planted: 21 May, 1993
Destroyed: 20 August, 1993

Field Monitoring for Weediness Characteristics: The transformed cotton exhibited slightly better germination when compared to the non-transformed cotton. Six days after planting, 100% of the transformed cotton had germinated and was in the cotyledon stage. The non-transformed cotton was 90-95& germinated. Twelve days after planting, all cotton uniformly germinated and no differences could be discerned. Observation was made on 2 June, 1993.

Number of Days from planting to flowering (75% of plants have initiated flowers): No differences were observed between the transformed and non-transformed plants. Observation was made on 19 July, 1993.

Number of flowers or bolls per plant. No difference were observed between the transformed and non-transformed plants. Observation was made on 11 August 1993.

Monitoring for Plant Growth Characteristics: The trial was observed on 27 May, 2, 15, 28 June, 6, 12, 19, 25 July, 11, and 18 August, 1993. No differences in vigor, bushiness, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility: The trial was observed on 27 May, 2, 15, 28 June, 6, 12, 19, 25 July, 11, and 18 August, 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants

Field Monitoring for Disease Susceptibility: The trial was observed on 27 May, 2, 15, 28 June, 6, 12, 19, 25 July, 11, and 18 August, 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

1993 Glyphosate Tolerant Cotton Field Release (USDA Permit # 93-012-03)(Mons # 92-156R) Final Report May 15, 1994

Eric M. Johnson, Ph.D.
Monsanto Agricultural Group

The purpose of this field release was to test cotton genetically-modified to be tolerant to glyphosate herbicide for yield and weed control efficacy, generate field tissue samples under GLP for laboratory studies, and for breeding nurseries. Initial plans were for planting the transformed cotton at seventeen sites by seventeen different cooperators as listed below. However, two locations were not planted.

Site Locations and Cooperators

Belle Mina Alabama Site [CBI DELETED]	**Buttonwillow California Site** [CBI DELETED]
Loxley Alabama Site [CBI DELETED]	**Shafter California Site** [CBI DELETED]
Maricopa Arizona Site [CBI DELETED]	**Tifton Georgia Site #1** [CBI DELETED]
Wabbesaka Arkansas Site [CBI DELETED]	**Tifton Georgia Site #2** [CBI DELETED]

Site Locations and Cooperators

Bossier City Louisiana Site [CBI DELETED]	**Aiken Texas Site** [CBI DELETED]
St. Joseph Louisiana Site [CBI DELETED]	**College Station Texas Site** [CBI DELETED]
Scott Mississippi Site [CBI DELETED]	**West Sinton, Texas Site** [CBI DELETED]
Starkville Mississippi Site [CBI DELETED]	**Lubbock Texas Site** [CBI DELETED]
Stoneville Mississippi Site [CBI DELETED]	

Line Distribution

Test Location	Lines Evaluated
Bell Mina AL Site	1445, 1698, 1421
Loxley AL Site	1445, 1698, 1421, 1367, 2060, 1964, 1950
Maricopa AZ Site	1445, 1698, 1421
Wabbesaka AR Site	1445, 1698, 1421, 1367
Buttonwillow CA Site	Not Planted
Shafter CA Site	1445, 1698, 1367
Tifton GA Site #1	1445, 1698, 1367
Tifton GA Site #2	Not Planted
Bossier City LA Site	1445, 1698, 1421
St. Joseph LA Site	1698
Scott MS Site	1445, 1698, 1421
Starkville MS Site	1445, 1698, 1421
Stoneville MS Site	1445, 1698
Aiken TX Site	1445, 1698, 1421, 1367
College Station TX Site	1445, 1698
Corpus Christi TX Site	1445, 1698, 1421
Lubbock TX Site	1445, 1698

Genotypes:

This field release included the following genotypes:

Derivatives of Coker 312 homozygous for gene constructs PV-GHGT01, PV-GHGT02, PV-GHGT03, PV-GHGT04, PV-GHGT05, PV-GHGT06, PV-GHGT07, and PV-GHGT08,

Coker 312 controls

Schedule of major operations:

May - Jun — Seed were labelled and packaged at the Monsanto Life Sciences Research Center in Chesterfield, Missouri according to the Standard Permit Conditions for the Introduction of a Regulated Article (7-CFR 340.3(f)). Delivery to cooperators was via a overnight delivery service. All seed arrived safely and were either immediately planted or stored separated from other cotton seeds in a secure area until planting.

May - Jun — Seed were planted in the field. Any remaining seed was either buried within the test plot or returned to the Monsanto Life Science Research Center in Chesterfield, Missouri.

Aug — Trials established strictly for weed control evaluation purposes were destroyed.

Sep-Dec Trials established for GLP sample collection, breeding nursery or for yield evaluation purposes were harvested.

post-harvest After completion of the test at each site, the seed cotton not shipped to Monsanto was spread across the test area. The entire filed was disked. The area was observed in the fall for volunteer plants. Continued monitoring for volunteer cotton plants within the test and buffer areas will be ongoing through the 1994 cropping. All volunteer plants observed will be destroyed by hand weeding, cultivation, or with chemical sprays.

Plant growth and general observations:

The transgenic plants deviated slightly from the agronomic standard, Coker 312. The variation is random in its expression with no correlation to the level of tolerance to glyphosate herbicide. There are several explanations for that variation including different production practices in seed production, random selection out of the genetic variation in the cultivar, genetic alteration due to the transformation/tissue culture process, or changes in cotton gene functioning due to the introduced gene. Observations suggest that the engineered plants were within the range of variation expected based on these sources of variation.

The plants were regularly monitored for *Agrobacterium* infection symptoms. None were observed.

Responses to specific issues:

1. Horizontal movement: The glyphosate tolerant plots were surrounded by 24 border rows (~80 feet) of non-transgenic cotton. This cotton served as a filter for pollen collected by insects from the test area, excluding it from movement to areas outside the test and buffer area. Based on previous data collected for transgenic cotton plants, it is unlikely that pollen from the glyphosate tolerant plants was carried outside the test area.
2. Changes in survival characters: There was no evidence of changes in the survival characteristics of the transgenic cotton plants excepts for the planned tolerance to glyphosate herbicide.
3. Expression level of the genes: The expression of the tolerance gene was measured through susceptibility to applications of glyphosate herbicide. Vegetative tolerance was excellent for all of the lines evaluated. Lines which achieved tolerance vía an engineered enhanced metabolism route exhibited transient chlorosis in some leaves. Reproductive tolerance was good. This tolerance was lower when application of glyphosate was timed close to flower formation. Reduced fertility was observed in these flowers.
4. Stability and inheritance of the new genes: No unusual patterns were observed.
5. Published data: At this time, there are no published data from these experiments.

Individual Site Information

Belle Mina Alabama Site:

A single experiment evaluating yield potential of glyphosate treated plants was established at this location.

Planted: 3 May, 1993
Harvested: 5 October, 1993

Field Monitoring for Weediness Characteristics:

Line 1421 appeared to have a lower germination rate than the other lines. Fifty plants were observed for each line tested on 26 May, 1993. For line 1421, final stand was `20% lower than in the other stands.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were observed between the transformed and non-transformed plants. Observation was made on 50 plants for each line tested on 7 July, 1993.

Number of flowers or bolls per plant.

Fifty plants of each line tested were observed on 20 July, 1993. Differences in boll set and final maturity were observed in the trial. These differences were not observed between transformed and non-transformed lines when glyphosate herbicide was not applied. Differences were associated with rate and application timing of the herbicide within each line.

Monitoring for Plant Growth Characteristics:

The trial was observed on 5 May, 10, 22, 30 June, 7, 20 July, 11 August, and 7, 24 September 1993. No differences in vigor, bushiness, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

The trial was observed on 5 May, 10, 22, 30 June, 7, 20 July, 11 August, and 7, 24 September 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants.

Field Monitoring for Disease Susceptibility:

The trial was observed on 5 May, 10, 22, 30 June, 7, 20 July, 11 August, and 7, 24 September 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of Agrobacterium infection was specifically sought but was not observed.

Loxley Alabama Site

Four separate experiments were planned for this site (GLP Sample Collection, Gene Performance, Yield, and Weed Efficacy). The Weed Efficacy trial was not planted.

Planted: 26 May and 1,2 June, 1993
Harvested: 20, and 26 October, 1993

Field Monitoring for Weediness Characteristics:

Line 1964 appeared to have a lower vigor than the other lines in the gene performance trial. Twenty plants were observed for each line tested on 20, 21 or 26 June, 1993.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were observed between the transformed and non-transformed plants. Observation was made on 25 plants for each line tested on 19 or 22 July, 1993.

Number of flowers or bolls per plant:

Twenty five plants of each line tested were observed on 7 September, 1993. Differences in boll set and final maturity were not observed in the trial.

Monitoring for Plant Growth Characteristics:

The trial was observed on 28 June, 26 July, 23 August, 20 September, and 11 October 1993. No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

The trial was observed on 28 June, 26 July, 23 August, 20 September, and 11 October 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants.

Field Monitoring for Disease Susceptibility:

The trial was observed on 28 June, 26 July, 23 August, 20 September, and 11 October 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Maricopa Arizona Site:

A single experiment for GLP sample collection was established at this location.

Planted: 18 May, 1993
Harvested: 28 October, 1993

Field Monitoring for Weediness Characteristics:

Germination was similar for the control, Coke 312 non transformed, and the test lines. The trial was observed on 30 May 1993 and germination estimated at ~95%.

Number of Days from planting to flowering (~75% of plants have initiated flowers):

No differences were observed between the transformed and non-transformed plants. Observation was made each replicate for each line tested on 28 June, 1993.

Number of flowers or bolls per plant:

All replicates of each line evaluated were observed on 20 July, 1993. Small variations were observed between the different lines but no general or outstanding trends in these differences were noted.

Monitoring for Plant Growth Characteristics:

The trial was observed on 5 May, 10, 22, 30 June, 7, 20 July, 11 August, and 7, 24 September 1993. No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

The trial was observed on 5 May, 10, 22, 30 June, 7, 20 July, 11 August, and 7, 24 September 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants.

Field Monitoring for Disease Susceptibility:

The trial was observed on 5 May, 10, 22, 30 June, 7, 20 July, 11 August, and 7, 24 September 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Wabbesaka Arkansas Site:

A single yield experiment was conducted at this site.

Planted: 15 May 1993
Harvested: October through December, 1993

Field Monitoring for Weediness Characteristics:

Normal germination's was observed on 26 May, 1993 with all transgenic lines evaluated. Those seed which were most recently harvested (produced in winter nursery) emerged slower than older seed.

Number of Days from planting to flowering (75% of plants have initiated flowers):

Differences observed between transformed and non-transformed plants were very small and represented the expected normal genetic variation. Observation was may on 20 July, 1993.

Number of flowers or bolls per plant:

Differences observed between transformed and non-transformed plants were very small and represented the expected normal genetic variation. Observation was may on 26 August, 1993.

Monitoring for Plant Growth Characteristics:

The trial was observed on 17 June, 20 July, 17 August, 21 September, and 13 October 1993. No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

The trial was observed on 17 June, 20 July, 17 August, 21 September, and 13 October 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants. Normal infestations of thrips, lepidopteran pests, and boll weevils were noted at various times during the growing season.

Field Monitoring for Disease Susceptibility:

The trial was observed on 17 June, 20 July, 17 August, 21 September, and 13 October 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Normal infection of seedling diseases and verticillium wilt were noted. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Shafter California Site:

A single breeding nursery was established at this site.

Planted: 24 May 1993
Harvested: 5 November, 1993

Field Monitoring for Weediness Characteristics:

Due to very late plantings of cotton for this area, it was difficult to compare growth habits. However, no unusual characteristics were observed.

Number of Days from planting to flowering (75% of plants have initiated flowers):

Due to very late plantings of cotton for this area, it was difficult to compare growth habits. However, no unusual characteristics were observed.

Number of flowers or bolls per plant:

Due to very late plantings of cotton for this area, it was difficult to compare growth habits. However, no unusual characteristics were observed.

Monitoring for Plant Growth Characteristics:

Due to very late plantings of cotton for this area, it was difficult to compare growth habits. However, no unusual characteristics were observed. Observation was made on 1 June, 1993.

Field Monitoring for Insect Susceptibility:

The trial was observed on 1 June, and 1 October 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants. Very light insect pressure was present throughout the year.

Field Monitoring for Disease Susceptibility:

The trial was observed on 1 June, and 1 October 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. The field had very little disease incidence. Evidence of Agrobacterium infection was specifically sought but was not observed.

Tifton Georgia Site #1:

A single yield experiment was conducted at this site.

Planted: 6 May 1993
Harvested: 17 November, 1993

Field Monitoring for Weediness Characteristics:

Line 1421 emerged erratically and a poor stand was established. Seedling vigor appeared low for this line. Observation was made on 20, May, 1993.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were noted in this trial.

Number of flowers or bolls per plant:

No differences were noted in this trial.

Monitoring for Plant Growth Characteristics:

The trial was observed on 20 May, 18 June, 8, 29 July, 19 August, 16 September, and 8 October 1993. No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

The trial was observed on 20 May, 18 June, 8, 29 July, 19 August, 16 September, and 8 October 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants.

Field Monitoring for Disease Susceptibility:

The trial was observed on 20 May, 18 June, 8, 29 July, 19 August, 16 September, and 8 October 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Bossier City Louisiana Site:

A single GLP Sample Collection plot was conducted at this site.

Planted: 18 May 1993
Harvested: 14 October, 1993

Field Monitoring for Weediness Characteristics:

Line 1421 had poor seedling emergence and reduced vigor as compared to the other transformed lines and Coker 312. These observations were made on 27 May, and 30 June, 1993.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were noted in this trial on 22 July, 1993.

Number of flowers or bolls per plant:

No differences were noted in this trial on 3 August, 1993.

Monitoring for Plant Growth Characteristics:

The trial was observed on 18 June, 14 July, 11 August, and 14 September 1993. No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

The trial was observed on 18 June, 14 July, 11 August, and 14 September 1993. Transformed plants did not have a higher incidence of insect infestation than non-transformed plants.

Field Monitoring for Disease Susceptibility:

The trial was observed on 18 June, 14 July, 11 August, and 14 September 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

St. Joseph Louisiana Site:

A single weed efficacy experiment was conducted at this site.

Planted: 14 May 1993
Harvested: 6 August, 1993

Field Monitoring for Weediness Characteristics:

No differences in seedling emergence or vigor were noted between the transformed plants and the non-transformed plants.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were observed between the non-transformed plants and the transformed cotton plants. Plots were observed on 1 July, 1993.

Number of flowers or bolls per plant:

No differences were observed between the non-transformed plants and the transformed cotton plants. Twenty plants per plot were observed on 21 July, 1993.

Monitoring for Plant Growth Characteristics:

Plots were observed on 8, 16, 23 June, 1, 8, 14, 21 July, and 4 August No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

Plots were observed on 8, 16, 23 June, 1, 8, 14, 21 July, and 4 August 1993. No differences in susceptibility to insects were noted between the transformed and non-transformed cotton plants.

Field Monitoring for Disease Susceptibility:

Plots were observed on 8, 16, 23 June, 1, 8, 14, 21 July, and 4 August 1993. No differences in disease susceptibility were noted between the transformed and non-transformed cotton plants.

Starkville Mississippi Site:

A single GLP Sample Collection plot was conducted at this site.

Planted: 14-18 May 1993
Harvested: 27 September, 1993

Field Monitoring for Weediness Characteristics:

No differences observed season long.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were noted in this trial.

Number of flowers or bolls per plant:

Transformed lines appeared to have less fruit and fewer open bolls. Researcher noted that the number may have been the same as the Coker 312 but the yield appeared less.

Monitoring for Plant Growth Characteristics:

No differences in vigor, business, leaf morphology, plant height or other general plant characteristics were observed between the transformed and non-transformed plants.

Field Monitoring for Insect Susceptibility:

Transformed plants did not have a higher incidence of insect infestation than non-transformed plants.

Field Monitoring for Disease Susceptibility:

Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Stoneville Mississippi Site:

A single yield experiment was conducted at this site.

Planted: 17 May 1993
Harvested: 12 and 13 October 1993

Field Monitoring for Weediness Characteristics:

No differences in seedling emergence or vigor were noted.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were noted in this trial.

Number of flowers or bolls per plant:

Plant mapping data indicated less fruiting when glyphosate herbicide was applied at the time of flowering. No differences in fruiting were observed between non-sprayed transformed plants and non-sprayed Coker 312 plants.

Monitoring for Plant Growth Characteristics:

The trial was observed on 6, 14, 18 June; 6, and 26 July, 1993. A developmental lag and terminal inhibition was observed. This was determined to be the result of Zorial and/or thrips injury. Subsequently, the transformed cotton was observed to be shorter than the conventional Coker 312.

Field Monitoring for Insect Susceptibility:

The trial was observed on 10, 18 June, 6, and 26 July 1993. A higher incidence of thrips was noted in the transformed plants compared with non-transformed plants in the border area on the 18 June observation.

Field Monitoring for Disease Susceptibility:

The trial was observed on 6, 14, 18 June; 6, and 26 July, 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Aiken Texas Site:

A breeding nursery was established at this site.

Planted: 2 June 1993
Harvested: 1 October through 30 November 1993

Field monitoring for Weediness Characteristics:

No differences in seedling emergence or vigor were noted.

Number of days from planting to flowering (75% of plants have initiated flowers):

No differences were noted between the transformed and non-transformed cotton plants in this trial. However, male sterility was observed in all material sprayed with glyphosate. This sterility gradually disappeared as the season progressed.

Number of flowers or bolls per plant:

No differences were noted between the transformed and non-transformed cotton plants in this trial.

Monitoring for Plant Growth Characteristics:

Throughout the growing season, variable degrees of male sterility were noted. Leaf defoliation was more severe in transformed plants by November, 1993.

Field Monitoring for Insect Susceptibility:

No differences were noted in this trial.

Field Monitoring for Disease Susceptibility:

No differences were noted in this trial. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

College Station Texas Site:

A single yield experiment was conducted at this site.

Planted: 13 May 1993
Harvested: 23 September 1993

Field Monitoring for Weediness Characteristics:

No differences in seedling emergence or vigor were noted.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were noted in this trial.

Number of flowers or bolls per plant: No differences were noted.

Monitoring for Plant Growth Characteristics:

Fifty to eighty plants were observed on 21 May, 8, 14, 24 June; 1, 16, 26 July, 2, 23 August, and 8 September 1993. No differences in general appearance and growth between the transformed and non-transformed plants were noted.

Field Monitoring for Insect Susceptibility:

The trial was observed on 21 May, 3, 17, 29 June, 16 July, 11, 23, and 30 August, 1993. Twenty plants were examined on each observation date. No differences in insect susceptibility were noted between transformed and non-transformed plants.

Field Monitoring for Disease Susceptibility:

The trial was observed on 21 May, 3, 29 June, 16 July, and 11 August, 1993 Twenty plants were examined on each observation date. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

West Sinton, Texas Site:

A single yield experiment was conducted at this site.

Planted: 17 May 1993
Harvested: 15 and 16 September 1993

Field Monitoring for Weediness Characteristics:

No differences in seedling emergence or vigor were noted.

Number of Days from planting to flowering (75% of plants have initiated flowers):

Lines 1445 and 1698 were later blooming than Line 1421 and Coker 312. Observation was made on July 15, 1993.

Number of flowers or bolls per plant: No differences were noted.

Monitoring for Plant Growth Characteristics:

The trial was observed on 8 June; 2, 29 July, and 26 August 1993. The transformed lines were observed to be slightly more vigorous at the later observations and Line 1421 appeared to open bolls earlier than Coker 312.

Field Monitoring for Insect Susceptibility:

The trial was observed on 8 June; 2, 29 July, and 26 August 1993 No differences in insect susceptibility were noted between transformed and non-transformed plants. Normal infestations of fleahoppers, boll weevil, tobacco budworm and cotton bollworm were observed.

Field Monitoring for Disease Susceptibility:

The trial was observed on 8 June, 2, 29 July, and 26 August, 1993 Transformed plants did not have a higher incidence of disease than non-transformed plants. Natural infestation of bacterial blight and Ascochyta were observed after an extended period of rainy weather. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

Lubbock Texas Site:

Separate yield and weed efficacy experiments were conducted at this site.

Planted: 22 May 1993
Harvested: 14 October 1993

Field Monitoring for Weediness Characteristics:

No differences in seedling emergence or vigor were noted.

Number of Days from planting to flowering (75% of plants have initiated flowers):

No differences were noted in this trial.

Number of flowers or bolls per plant:

No differences were noted.

Monitoring for Plant Growth Characteristics:

The site was observed on 10, 29 June; 14, 30 July, 19 August, 10, 28 September, and 11 October 1993. No differences in plant growth or appearance were noted in the trials.

Field Monitoring for Insect Susceptibility:

The site was observed on 10, 29 June; 14, 30 July, 19 August, 10, 28 September, and 11 October 1993. No differences in insect susceptibility were noted between transformed and non-transformed plants.

Field Monitoring for Disease Susceptibility:

The site was observed on 10, 29 June; 14, 30 July, 19 August, 10, 28 September, and 11 October 1993. Transformed plants did not have a higher incidence of disease than non-transformed plants. Evidence of *Agrobacterium* infection was specifically sought but was not observed.

1993-1994 *RR* COTTON FIELD RELEASES
(USDA PERMIT#93-210-02)
FINAL REPORT

Robert E. Buehler
Monsanto Co.

The purpose of these field releases was to increase seed for further testing of cotton genetically-modified to contain gene(s) which confer Roundup tolerance. The cotton was grown at one site.

Sites and cooperators

Santa Isabel site

[CBI DELETED]

Santa Isabel, Puerto Rico

Genotypes:
These field releases included the following genotypes:

- Derivatives of Coker 312 homozygous and segregating for PV-GHGT06 and PV-GHGT07
- Coker 312 controls.

Distribution of Lines for Evaluations:

USDA # 93-223-02N		
Testing Site		Lines Evaluated[1]
Santa Isabel, PR		1367, 1421, 1445, 1698

[1]

Vector #	Lines
PV-GHGT06	1698
PV-GHGT07	1367, 1421, 1445

Schedule of major operations:

Sept.	Seed were packaged according to the protocol and shipped from the Jacob Hartz Seed Co. in Stuttgart, Arkansas to the cooperator via overnight delivery service. All the seed arrived safely and were stored in accordance with the conditions described in the permit.
October	Seed planted.
April	Harvest and shipment of seed samples back to Jacob Hartz Seed Company.
post-harvest	After completion of the test at each site, the seed cotton not shipped to Hartz was spread in the field. The entire field was disked. The area was observed for volunteer plants and all volunteer plants were destroyed by hand weeding, cultivation, or with chemical sprays.

Summary of Observations

Plant growth and general observations:
The transgenic plants did deviate slightly from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to the *RR* protein, level of expression, etc. There are several explanations for that variation including random selection out of the genetic variation in the cultivar, genetic alteration due to the transformation/tissue culture process, or changes in cotton gene functioning due to the introduced gene. Observations suggest that the engineered plants were within the range of variation expected based on these sources of variation.

The plants were regularly monitored for *Agrobacterium* infection symptoms. None could be found.

Responses to specific issues:
1) Horizontal movement:
The *RR* plots were surrounded by 24 border rows (~80') of non-transgenic cotton. This cotton served as a sink for pollen carried by insect from the test area. Based on the previous data, it is unlikely that pollen from the *RR* plants was carried outside of the test area.

2) Changes in survival characteristics:
There was no evidence of changes in the survival characteristics of the transgenic cotton plants.

3) Expression level of the genes:
These plants were not treated with Roundup. Thus, conclusions cannot be drawn regarding gene expression.

4) Stability and inheritance of the new genes:
No unusual inheritance patterns were observed.

5) Published data:
At this point, there is no published data from these experiments.

Specific Location Evaluations

Santa Isabel, Puerto Rico

Planted - October, 1993
Harvested - April, 1994

Monitoring for Plant Growth Characteristics

Multiple observations of the plots were taken throughout the growing season with no differences in plant vigor, leaf morphology plant height and other characteristics observed.

Field Monitoring for Insect Susceptibility

Multiple observations of the plots were taken throughout the growing season with no differences observed between transformed and non-transformed plants in terms of insect susceptibility.

Field Monitoring for Disease Susceptibility

Multiple observations of the plots were taken throughout the growing season with no differences observed between transformed and non-transformed plants in terms of disease susceptibility.

1993-1994 *RR* COTTON FIELD RELEASES
(USDA PERMIT#93-223-02N)
FINAL REPORT

Robert E. Buehler
Monsanto Co.

The purpose of this field release was to increase seed for further testing of cotton genetically-modified to contain gene(s) which confer Roundup tolerance. The cotton was grown at one site.

Sites and cooperators

Santa Isabel site

[CBI DELETED]

Santa Isabel, Puerto Rico

Genotypes:
This field release included the following genotypes:

- Derivatives of Coker 312 homozygous and segregating for PV-GHGT06 and PV-GHGT07
- Coker 312 controls.

Distribution of Lines for Evaluations:

USDA # 93-223-02N		
Testing Site		Lines Evaluated[1]
Santa Isabel, PR		1367, 1421, 1445, 1698

[1]

Vector #	Lines
PV-GHGT06	1698
PV-GHGT07	1367, 1421, 1445

177

Schedule of major operations:

Sept.	Seed were packaged according to the protocol and shipped from the Monsanto research center in Chesterfield, Missouri to the cooperators via overnight delivery service. All the seed arrived safely and were stored in accordance with the conditions described in the permit.
October	Seed planted.
April	Harvest and shipment of seed samples back to Monsanto.
post-harvest	After completion of the test at each site, the seed cotton not shipped to Monsanto was spread in the field. The entire field was disked. The area was observed for volunteer plants and all volunteer plants were destroyed by hand weeding, cultivation, or with chemical sprays.

Summary of Observations

Plant growth and general observations:
The transgenic plants did deviate slightly from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to the *RR* protein, level of expression, etc. There are several explanations for that variation including random selection out of the genetic variation in the cultivar, genetic alteration due to the transformation/tissue culture process, or changes in cotton gene functioning due to the introduced gene. Observations suggest that the engineered plants were within the range of variation expected based on these sources of variation.

The plants were regularly monitored for *Agrobacterium* infection symptoms. None could be found.

Responses to specific issues:

1) Horizontal movement:
The *Btk* plots were surrounded by 24 border rows (~80') of non-transgenic cotton. This cotton served as a sink for pollen carried by insect from the test area. Based on the previous data, it is unlikely that pollen from the *RR* plants was carried outside of the test area.

2) Changes in survival characteristics:
There was no evidence of changes in the survival characteristics of the transgenic cotton plants.

3) Expression level of the genes:
These plants were not treated with Roundup. Thus, conclusions cannot be drawn regarding gene expression.

4) Stability and inheritance of the new genes:
No unusual inheritance patterns were observed.

5) Published data:
At this point, there is no published data from these experiments.

Specific Location Evaluations

Santa Isabel, Puerto Rico

Planted - October, 1993
Harvested - April, 1994

Monitoring for Plant Growth Characteristics

Multiple observations of the plots were taken throughout the growing season with no differences in plant vigor, leaf morphology plant height and other characteristics observed.

Field Monitoring for Insect Susceptibility

Multiple observations of the plots were taken throughout the growing season with no differences observed between transformed and non-transformed plants in terms of insect susceptibility.

Field Monitoring for Disease Susceptibility

Multiple observations of the plots were taken throughout the growing season with no differences observed between transformed and non-transformed plants in terms of disease susceptibility.

Appendix VI

Summary of the Methods Utilized to Conduct the Protein Extraction, Analysis and Quantitation, Compositional Analysis, Cottonseed Processing, Preparation of Seeds for Gossypol and Fatty Acid Analyses, Moisture Determination, Gossypol Levels and Quantitation of Fatty Acid Levels

Summary of analytical methods

ELISAs, western blots, and enzymatic activity assays were performed by Monsanto personnel. Quality analyses consisting of proximate, amino acid, and aflatoxin analyses on the seed and tocopherols in oil were performed at Hazleton Wisconsin, Incorporated (HWI). Toxicant analyses consisting of gossypol and cyclopropenoid fatty acids were performed Texas A&M University (TAMU) and USDA-ARS. Proximate analysis of the full fat flour was performed at Ralston Analytical Laboratories. All transfers of samples were conducted with sample transfer forms to document chain-of-custody. Methods were validated in compliance with GLP.

1. CP4 EPSPS ELISA: A double antibody indirect enzyme-linked immunosorbent assay was developed and validated for the detection of CP4 EPSPS in cotton leaf and seed tissue (Taylor, 1994). ELISA validation involved determining the assay precision, accuracy, and threshold of detection for the protein of interest in seed and leaf matrices. The ELISA utilized two antibodies from two different animal species raised against the non-denatured CP4 EPSPS protein. The double antibody sandwich was detected by with donkey anti-rabbit alkaline phosphatase conjugate followed by development with *para*-nitrophenyl phosphate. The optical density of the resulting yellow product was measured with a spectrophotometric plate reader. Quantitation of the sample CP4 EPSPS concentration was accomplished by extrapolation from the logistic curve fit of the standard curve using CP4 EPSPS purified from *Escherichia coli*. The standard extraction buffer (TBA) used was 100 mM Tris-HCl, pH 7.8, 100 mM sodium borate, 5 mM magnesium chloride, 0.05% v/v Tween 20, and 0.2% sodium ascorbate.

2. NPTII ELISA: A double antibody direct ELISA was developed and validated for the detection of NPTII in cotton leaf and seed tissues (Ebert, 1994). ELISA validation involved determining the assay precision, accuracy, and threshold of detection for the protein of interest in seed and leaf matrices. The ELISA utilized polyclonal antibodies raised in rabbits to trap NPTII. The initial complex was captured by a second antibody conjugated to horseradish peroxidase (HRP). Development of HRP with substrate produced a blue product. The reaction was stopped with 0.5 M phosphoric acid. The optical density of the resulting yellow product was measured with a spectrophotometric plate reader. Quantitation of the sample NPTII concentration was accomplished by extrapolation from the logistic curve fit of the standard curve using NPTII purified from *E. coli*. TBA extraction buffer was the standard extraction buffer used for sample preparation.

3. CP4 EPSPS enzymatic assay: The procedure utilized for determining the amount of functionally active EPSPS entailed the use of an HPLC with a radioactivity detector, which has been previously described (Padgette, 1988; 1987). Labeled substrate ^{14}C-phosphoenolpyruvate (^{14}C-PEP) is converted to ^{14}C-5-enolpyrvyl-shikimate-3-phosphate (^{14}C-EPSP) in the presence of

shikimate-3-phosphate (S3P) by EPSPS, and the resultant ^{14}C-EPSP is detected using HPLC and radioactive flow detection. The final reagent concentrations in the assay were 50 mM Hepes, 0.1 mM ammonium molybdate, 5 mM potassium fluoride, 1 mM ^{14}C-PEP, and 2 mM S3P, pH 7.0. Reactions were run at approximately 25°C. The reactions were quenched after 2 to 5 minutes with an equal volume of 9:1 ethanol: 0.1 M acetic acid pH 4.5. The samples were then centrifuged and chromatographed by HPLC anion exchange using 0.16 M potassium phosphate, pH 6.5, as the mobile phase. Turnover of ^{14}C-PEP to ^{14}C-EPSP was determined by peak integration. For EPSPS, 1 unit (U) is defined to be 1 µmol EPSP produced/min. at approximately 25°C, under the assay conditions described.

4. Western blot analysis for CP4 EPSPS and NPTII: Detection of low levels of CP4 EPSPS and NPTII from a variety of samples was accomplished by separating protein samples on polyacrylamide gels and electrophoretically transferring the proteins onto polyvinylidene fluoride (PVDF) membrane. For the visualization of CP4 EPSPS or NPTII, specific antibodies were hybridized to the blots, then reacted with either ^{125}I-Protein A or ^{125}I-Protein G. Results were quantitated by imaging the blot directly using luminescence detection system and quantitative image analysis (BioMolecular Dynamics PhosphorImager).

5. BioRad Protein Assays for extracts: Protein levels in TBA extracts used for ELISA and western blot analyses were determined using the BioRad protein assay in a 96-well plate format (Nida, 1992).

6. Total kjeldahl nitrogen-protein: Nitrogenous compounds in the sample were reduced, in the presence of boiling sulfuric acid, catalyzed by a potassium sulfate/titanium dioxide/cupric sulfate mixture, to form ammonium sulfate. The resultant solution was cooled, diluted, and made alkaline with a sodium hydroxide-thiosulfate solution. Ammonia was liberated and distilled into a known amount of standard acid. The distillate was titrated, and nitrogen or protein (N x 6.25) was calculated from the known amount of reacting acid. Seed samples were analyzed at Hazleton Wisconsin, Inc based on published procedures (Bradstreet, 1965; Kalthoff and Sandell, 1948; AOAC, 1990a).

7. Moisture: The full fat flour samples were placed in Similarly, the seed samples were placed in a vacuum oven at HWI and USDA-ARS at 100°C and dried to a constant weight (approximately 5 hours) (AOAC, 1990b).

8. Ash: Volatile organic matter is driven off when the sample is ignited at 550°C in a muffle or electric furnace. The residue was quantitated gravimetrically and calculated to determine percent ash. Using a 3 g sample, the lowest confidence level of this method is 0.2%. This method has been previously published (AOAC, 1984).

9. Carbohydrates: Carbohydrates were calculated by difference using the fresh weight-derived data and the following equation (USDA, 1975):

% carbohydrates = 100% - (% protein + % fat + % ash + % moisture)

10. Calories: Calories were calculated using the Atwater factors with the fresh weight-derived data and the following equation (USDA, 1975b):

calories (kcal/100g) = (4 * % protein) + (9 * % fat) + (4 * % carbohydrates)

11. Fat-Soxhlet Extraction: The sample of seed tissue is dried to remove excess moisture, extracted with pentane, dried to remove pentane, and weighed to determine the amount of fat removed (AOAC, 1990c, d).

12. Amino Acid Composition: Samples were hydrolyzed with hydrochloric acid, adjusted to pH 2.2. The individual amino acids are quantitated using an automated amino acid analyzer. This assay was based on previously published references (AOAC, 1990e).

13. Aflatoxin: The levels of aflatoxin B_1, B_2, G_1, and G_2 were determined in seed samples from each site. The sample was wetted with dilute hydrochloric acid and extracted with chloroform. A portion of the extract is applied to a silica gel column. Aflatoxin were eluted with methylene chloride/acetone and concentrated with a rotary evaporator. The extracts were then separated by high performance liquid chromatography and compared to a known standard (JAOAC, 1988a,b,c).

14. Alpha-tocopherol: Oil samples were saponified to release the tocopherols, which were then extracted with organic solvent, followed by quantitation on an HPLC silica column using fluorescence detection (Cort, 1983; Speek et al., 1985; McMurray et al., 1980).

15. Gossypol: Samples were extracted with an aniline extraction buffer. To determine percent free gossypol, extracts were separated by high performance liquid chromatography. Total percent gossypol was analyzed by monitoring absorbance at 440 nm. The methods have been previously published (Stipanovic et al., 1988; Pons et al., 1958; AOCS, 1989).

16. Fatty Acid Profile: Lipids were extracted using a double Bligh and Dyer (1959) procedure, as recently described (Wood, 1991). Lipid was extracted from samples using a chloroform/methanol solvent. The dry weight of the sample and weight of the extracted lipid were used to calculate the total percentage lipid in the sample. Approximately 2 mg of total lipid were saponified to obtain free fatty acids by a mild alkaline hydrolysis procedure (Wood, 1986). The free fatty acids were converted quantitatively to phenacyl derivatives according to the procedure of Wood and Lee (1983).

The phenacyl derivatives were analyzed by high performance liquid chromatography (Wood, 1986a, 1986b). The absorption data for each peak were collected directly from the UV monitor and were integrated for percent of total peak area using an IBM model 900 laboratory computer.

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