Monsanto

94-308-01P

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November 3, 1994

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: Bollgard™ Cotton Line 531
Monsanto #94-142

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 Bollgard™ Cotton Line 531. This petition requests a determination from APHIS that Bollgard™ Cotton Line 531 and any progenies derived from crosses between Bollgard™ Cotton Line 531 and traditional cotton varieties no longer be considered a regulated article under regulations in 7 CFR part 340. Bollgard™ Cotton Line 531 has been tested for 3 years at 21 locations. The copies of the final reports for this field trials are included in this petition.

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 (314-537-6385).

Sincerely,

Frank Serdy

Frank Serdy Ph.D.
Regulatory Affairs Director

DDE 11.4.94

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

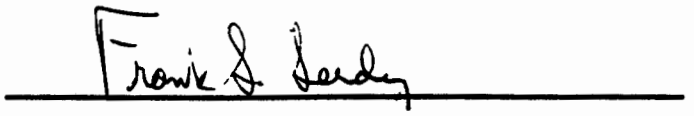
#2 11/4/94

Petition for Determination of Non-regulated Status:

Bollgard™ Cotton Line 531 (*Gossypium hirsutum* L.) with the gene from *Bacillus thuringiensis* subsp. *kurstaki*.

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:



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November 7, 1994
#94-142

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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 Bollgard™ Cotton Line 531 which expresses a form of the insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*). This petition requests a determination from APHIS that Bollgard™ Cotton Line 531 and any progeny derived from crosses between Bollgard™ Cotton Line 531 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 with approximately 13 million acres grown primarily in the tier of 15 southern states stretching from North Carolina to California. Lepidopteran insects (primary cotton bollworm, tobacco budworm and pink bollworm) are the main insect pest problem on these acres with approximately 80% of the planted acres infested, and approximately $180 M is spent annually on chemical insecticides for their control.

Bollgard™ Cotton Line 531 developed by Monsanto produces the insect control protein *B.t.k.* This protein is effective in controlling the cotton bollworm, tobacco budworm and pink bollworm. Microbial formulations containing these insecticidal proteins have been registered by EPA and commercially available for lepidopteran caterpillar control for nearly 30 years. Growers planting Bollgard™ Cotton Line 531 are not likely to require insecticide applications to control these destructive caterpillars. This substantial reduction in insecticide use will enhance the effectiveness of biological control and implementation of pest management strategies for other cotton insect pests.

The protein produced by Bollgard™ Cotton Line 531 is nearly identical in structure and activity to that found in nature and in commercial *B.t.k.* formulations registered with the EPA. Field experiments were conducted in 1991, 1992 and 1993 at 21 locations throughout the United States cotton growing region demonstrated that the Bollgard™ Cotton Line 531 is protected season long from feeding damage caused by these lepidopteran caterpillars. Beneficial insects are unaffected and may increase in number. In addition, these plants exhibit no plant pathogenic properties, are no more likely to become weeds than the non-modified parental cotton lines, are unlikely to increase the weediness potential for any other cultivated plants or native species and are equivalent compositionally to the parental cotton line.

The use of Bollgard™ Cotton Line 531 will have a more positive impact on the environment than the use of chemical insecticides to control lepidopteran caterpillars. The *B.t.k.* protein is ecologically benign, i.e., it breaks down rapidly in the soil, is safe to non-target organisms such as fish, birds and mammals and specifically controls many species of lepidopteran caterpillars on cotton. In addition, the risk of an uncontrolled introduction of this cotton into the environment through hybridization or out-crossing to a native species resulting in a new weed variety is virtually non-existent on the mainland of the United States, where all of the United States cotton production takes place.

Bollgard™ is a registered trademark of Monsanto Company, St. Louis Missouri.

The determination that Bollgard™ Cotton Line 531 and its progenies are no longer regulated articles and their subsequent commercialization will represent an efficacious and environmentally compatible addition to the existing options for cotton insect pest management. In addition, Bollgard™ Cotton Line 531 will provide significant benefits to growers, the general public and the environment, including:

1. A more reliable, economical and less labor intensive means to control lepidopteran insect pests.

2. Insect control without harming non-target species, including humans.

3. A means for growers to significantly reduce the amount of chemical insecticides now applied to the crop while maintaining comparable yields. Therefore, lepidopteran insect control can be achieved in a more environmentally compatible manner than is currently available.

4. A reduction in the manufacturing, shipment and storage of chemical insecticides used on cotton.

5. A reduction in the exposure to workers to the pesticide and pesticide spray solution.

6. A reduction in the number of empty pesticide containers and amount of pesticide spray solution that must be disposed of according to applicable environmental regulations.

7. An ideal fit with Integrated Pest Management Programs (IPM) and sustainable agricultural systems.

In conclusion, the consistent lepidoptera control offered by Bollgard™ Cotton Line 531 will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop for control of cotton bollworm, tobacco budworm and pink bollworm. As a result, they will be able to utilize a host of IPM practices that cannot presently be implemented because of the lack of options other than use of chemical insecticides to control these pests. An increase in the biological and cultural control of non-target cotton pests and a more judicious use of chemical insecticides will result in a positive impact on the environment, which will ultimately be advantageous to the grower and the public as well.

Therefore, the Agricultural Group of Monsanto requests a determination from APHIS that Bollgard™ Cotton Line 531 and any progenies derived from crosses between Bollgard™ Cotton Line 531 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.

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NOTE TO THE REVIEWER

Justification for the Use of the CryIA(c) Designation for the protein expressed in Bollgard™ Cotton Line 531.

The insecticidal protein produced in Bollgard™ Cotton Line 531 is classified as CryIA(c). This designation is appropriate even though the *B.t.k.* gene producing the protein is the result of fusing a 5' portion of a *cryIA(b)* gene and the majority of the *cryIA(c)* gene. The appropriate classification of the expressed *B.t.k.* protein is dictated by the protein produced and not the gene or the method by which the gene was constructed. The portion of the *cryIA(b)* gene used encodes an N-terminal amino acid sequence that is highly homologous to the N-terminal amino acid sequence encoded by the *cryIA(c)* gene. The protein produced contains only 6 amino acid differences in this region, resulting in only 6 amino acid changes for the entire 1178 amino acid *B.t.k.* protein that result from using the sequence of the *cryIA(b)* gene portion. Furthermore, these 6 changes occur in the N-terminal, highly conserved portion of the *B.t.k.* protein. These changes are not located in the hypervariable region, which has been shown to be responsible for determining the insecticidal activity of the *B.t.* proteins (Geiser, *et al.*, 1986). These amino acid changes did not, as expected, affect the insecticidal specificity of the resulting *B.t.k.* protein.

The protein encoded by the *B.t.k.* gene introduced into Bollgard™ Cotton Line 531 is identical in length (1178 amino acids) and 99.4% identical in amino acid sequence to the protein encoded by the *cryIA(c)* gene (Adang, *et al.*, 1985). The Cry nomenclature developed by Hofte and Whiteley (1989) categorizes the vast array of *B.t.* proteins in classes, rather than single, distinct proteins, based on their structural relatedness and insect toxicity spectra. The CryIA(c) protein reported by Adang *et al.* (1985) was the only member of the CryIA(c) class at the time of Hofte and Whiteley's (1989) publication. Since that report, four other CryIA(c) proteins have been reported (Dardenne, *et al.*, 1990; Von Tersch, *et al.*, 1991; M73248; M73249). For example, the CryIA(c) protein that was characterized by Von Tersch *et al.* (1991) contains seven amino acid differences (6 amino acid substitutions and one amino acid deletion) and was isolated from a different subspecies (*B.t.* subsp. *kenyae*) than the protein characterized by Adang *et al.* (1985). All five of these CryIA(c) proteins plus the CryIA(c) protein used to produce Bollgard™ Cotton Line 531 are ≥99 identical at the amino acid level. In contrast, the CryIA(b) proteins, which are the next most homologous class of *B.t.* proteins, show <90% amino acid identity to any of the members of the CryIA(c) class of *B.t.* proteins. These homologies clearly establish that all six of the CryIA(c) proteins (including the *B.t.* protein expressed in the Bollgard™ Cotton Line 531) are closely related and are all appropriately classified as CryIA(c) proteins. The CryIA(c) class designation for the protein expressed in Bollgard™ Cotton Line 531 is, therefore, accurate and consistent with the established nomenclature of Hofte and Whiteley (1989).

Therefore, it is appropriate to refer to the protein expressed in Bollgard™ Cotton Line 531 as a CryIA(c) protein and the gene expressing this protein as *cryIA(c)* gene.

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Abbreviations Used in this Petition for the Determination of Non-Regulated Status of Insect Resistant Cotton Line 531

aad	Gene for 3"(9)-O-aminoglycoside adenylyltransferase
AAD	3"(9)-O-aminoglycoside adenylyltransferase
APHIS	Animal Plant Health Inspection Service
ATP	Adenosine triphosphate
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
C	Centigrade
C312	Coker cotton variety 312
CFR	Code of Federal Regulations
cryIA(c)	Class I (Lepidoptera-specific) crystal protein gene
DNA	Deoxyribonucleic Acid
E35S	Promoter for *cryIA(c)* gene
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EUP	Experimental Use Permit
F	Fahrenheit
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
g	gram
GLP	Good Laboratory Practice
IPM	Integrated Pest 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
ppm	part per million
sp	species
T-DNA	Transfer-DNA
µg	microgram
USDA	United States Department of Agriculture
w/w	weight/weight

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Part I. Introduction

A. Rationale For Development of the Insect Resistant Cotton Plant

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. Lepidopteran insects are the main insect pest problem on these acres. During the growing season other insects (e.g., cotton boll weevil, lygus bugs, fleahoppers, spider mites, thrips, and aphids) are also present. The primary lepidopteran pests infesting cotton are cotton bollworm, tobacco budworm and pink bollworm. These insect pests infest approximately 80% of the planted acres and approximately $180 M is spent annually for chemical control.

Monsanto has developed a genetically modified cotton plant that controls many of the lepidopteran caterpillars which are serious pests in cotton production. This cotton plant, hereafter referred to as Bollgard™ Cotton Line 531, produces an insect control protein derived from the common soil bacterium *Bacillus thuringiensis* var. *kurstaki* (*B.t.k*). Microbial formulations containing these insecticidal proteins have been registered by the Environmental Protection Agency (EPA) and commercially available for lepidopteran caterpillar control for nearly 30 years. The protein produced by Bollgard™ Cotton Line 531 is nearly identical in structure and activity to that found in nature and in commercial *B.t.k.* formulations registered with the EPA. This protein is highly selective in controlling many lepidopteran caterpillars and is expressed at an effective level in plant tissue throughout the growing season. Field experiments were conducted in the years 1991, 1992 and 1993 at 21 locations throughout the United States cotton growing region under permits from the United States Department of Agriculture (USDA) (#90-347-01, 91-144-01, 91-347-02, 93-011-02 and 93-011-05), and an Experimental Use Permit granted by EPA (#524-EUP-73) (1992 and 1993). Results from these experiments have demonstrated that the Bollgard™ Cotton Line 531 is protected season long from feeding damage caused by many lepidopteran caterpillars. Beneficial insects are unaffected and may increase in number, providing predatory control of the lepidopteran pests. Growers planting Bollgard™ Cotton Line 531 are not likely to require insecticide applications to control these destructive caterpillars. This substantial reduction in insecticide use will enhance the effectiveness of biological control and implementation of pest management strategies for other cotton insect pests.

Safety studies summarized in this submission, as well as data generated by manufacturers of commercial *B.t.k.* products, have demonstrated that non-target animals such as fish, birds and mammals are unaffected by the *B.t.k.* protein. In addition, agronomic evaluations consisting of plant vigor, growth habit characteristics and general disease susceptibility, have shown Bollgard™ Cotton Line 531 to be equivalent to the parental Coker 312 cotton.

The commercialization of Bollgard™ Cotton Line 531 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 insect pest management. In addition, it will provide significant benefits to growers, the general public and the environment, including:

1. A more reliable, economical and less labor intensive means to control lepidopteran insect pests.

2. Insect control without harming non-target species, including humans.

3. A means for growers to significantly reduce the amount of chemical insecticides now applied to the crop while maintaining comparable yields. Therefore, lepidopteran insect control can be achieved in a more environmentally compatible manner than is currently available.

4. A reduction in the manufacturing, shipment and storage of chemical insecticides used on cotton.

5. A reduction in the exposure to workers to the pesticide and pesticide spray solution.

6. A reduction in the number of empty pesticide containers and amount of pesticide spray solution that must be disposed of according to applicable environmental regulations.

7. An ideal fit with Integrated Pest Management Programs (IPM) and sustainable agricultural systems.

B. Benefits of Insect Resistant Cotton

1. Summary

Lepidopteran insects are the main pest problem on most of the 13 million acres of cotton produced in the United States. During the growing season other insects (e.g., cotton boll weevil, lygus bugs, fleahoppers, spider mites, thrips, and aphids) are also present. The primary lepidopteran pests infesting cotton are cotton bollworm, tobacco budworm and pink bollworm. These insect pests infest approximately 80% of the planted acres with approximately $180 M spent annually for chemical insecticides for their control. These insect resistant cotton plants are expected to replace a significant part of the chemical insecticides now applied to control lepidopteran insect pests.

There are additional reasons why these insect resistant cotton plants have advantages over cotton plants which must be sprayed with insecticides to control lepidopteran pests, including:

a. Chemical insecticides are costly and sometimes unreliable under intended use conditions. New chemical insecticides are expensive to develop and register and as a result must be sold at ever increasing prices so that the developer can recover these costs. The effectiveness of these chemicals can also be negatively influenced by environmental conditions. Rain following application, for example, reduces the length of control, and a dense canopy of foliage reduces penetration and effectiveness. Areas of the field that do not receive the spray will be damaged by insects. All of these conditions result in increased production costs and potentially lower yields for the grower.

b. Many chemical insecticides have the potential to cause environmental damage if not used as labelled.

c. Insect resistant cotton plants provide an ideal fit with existing IPM and sustainable agricultural programs. Essentially all cotton produced in the United States is grown under IPM programs. By reducing the use of non-selective insecticides, insect resistant cotton plants will enhance the effectiveness of these programs, due to the presence of increased numbers of beneficial insects and other predators. Natural pest defense systems are compatible with the goals of sustainable agriculture production systems.

d. Applicator and field worker exposure to chemical insecticides will be reduced.

e. Many insects have or are developing resistance to the available chemical insecticides. This resistance requires farmers to apply chemicals at higher rates and/or more frequently, with the prospect of eventually not being able to use them at all.

f. Bollgard™ Cotton Line 531 will likely have reduced levels of aflatoxin in the seed compared to non-modified cotton

The following are summaries of the Agronomic and Economic Benefits of Bollgard™ Cotton Line 531 as prepared by Luttrell *et al.* (1993) and Spurlock (1993) respectively. Copies of these full papers are found in Appendices I and II, respectively.

2. Agronomic Benefits

Cotton production in the United States is highly mechanized and dependent upon maximum utilization of new technology to remain competitive in a worldwide market. Pest problems, particularly insects, and environmental constraints, such as inadequate temperature and moisture, are major limiting factors to optimum cotton production. Most cotton production regions of the United States rely on extension specialists and crop consultants to design and implement effective IPM programs. Insect control decisions are largely based on routine field monitoring by agricultural consultants, extension personnel and growers. The intensity of monitoring varies among locations and is associated with production capabilities, potential insect damage and availability of consultants (Luttrell 1994). Numerous advances in IPM technology (Frisbie and Adkisson 1985, Frisbie *et al.* 1989) have

encouraged a systems approach to insect management in United States cotton where insect control decisions are integrated into an overall crop production and management scheme. Perhaps the best example of this is the wide acceptance of early-maturing varieties and short-season cotton production systems first recommended in Texas. The Texas system of short-season cotton production (Walker *et al.* 1978) has been widely adopted across United States cotton and is recommended by agronomists and entomologists because it optimizes the production of valuable fiber and encourages the "avoidance" of damaging late-season populations of insects.

Although advances in IPM technologies have fostered improved cotton insect management systems, insect control is still largely based on the use of chemical insecticides (Herzog *et al.* 1993). Estimates of insect control costs and losses (Head 1991, 1992, 1993) averaged for the 1990's indicate that United States cotton growers apply an average of 4.86 applications of insecticide to 11.8 million acres of cotton and spend more than $400 million each year for control of cotton insects. This represents a large portion of total insecticide use in the United States. Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests (Luttrell 1994). The need for alternative insect control measures is becoming more critical to profitable cotton production in the United States. Environmental concerns limit the availability of existing insecticide chemistry and increase the developmental costs of new chemistry. Because of the high costs of developing and registering new insecticide chemistry, availability has declined over the past few years.

Bollgard™ Cotton Line 531 offers unique, innovative alternatives to traditional chemical control measures. Although alternative insect control tactics are often cited as major components of cotton IPM and research is continuously pursuing improved management methods (Frisbie *et al.* 1989), few alternative insect control methods are of sufficient efficacy to replace chemical control methods. Other methods, such as biological control, host plant resistance and cultural control, provide suppression of pest populations without disrupting natural control, but generally lack the high efficacy and curative action of conventional insecticides. Bollgard™ Cotton Line 531 is the first major exception to this historical trend.

Bollgard™ Cotton Line 531 offers new mechanisms to produce and deliver a highly effective insecticide to target pests (i.e. production by cells of the crop plant rather than industrial facilities and application by spray equipment). The technology couples the environmental advantages of host plant resistance with the efficacy of an effective biological insecticide. Since the insecticidal activity is expressed throughout the plant for the entire season, improved control of some pest species over that provided by conventional insecticides is likely. Current technology which depends on foliar application of insecticides cannot dependably deposit insecticides to some regions of the plant canopy infested by pest species. This is especially true of pests that burrow and feed inside plant tissue (e.g. pink bollworms). Because Bollgard™ Cotton Line 531 expresses the *B.t.k.* protein that only has activity against certain Lepidoptera (moths and butterflies) and must be ingested to kill the pest, the technology offers selective activity against susceptible lepidopteran pest complexes without directly disrupting pest suppression by natural enemies, such as parasites and predators.

3. Economic Benefits

Pesticide regulation has become more restrictive in the United States resulting in the ban or severe restriction of the use of particular insecticides. Economic studies have been conducted to examine the likely impacts from such restrictive pesticide regulations. Taylor *et al.* (1991) developed a regional model and concluded that agricultural income in the South would be negatively impacted by more restrictive pesticide regulations. Richardson *et al.* (1991) 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. It is possible that genetically modified plants which are designed to control insects without the use of insecticide sprays will be able to offset some of the negative impacts from increased pesticide regulations.

Bollgard™ Cotton Line 531 is designed to sufficiently control infestations of lepidoptera, eliminating the need to control these pests with conventional insecticide applications. Revenue-related factors such as lint yields and quality characteristics are expected to be similar under both conventional cotton and Bollgard™ Cotton Line 531 production systems. However, per-acre production costs of Bollgard™ Cotton Line 531 are expected to be lowered due to the reduction in insecticide use with the substitution of Bollgard™ Cotton Line 531 seed for conventional cotton seed. Growers who adopt Bollgard™ Cotton Line 531 will simply substitute this seed for conventional cotton seed and certain types of insecticides. Thus, the added cost of the Bollgard™ Cotton Line 531 seed must be compared with the savings obtained from replacing conventional seed and some insecticides.

Due to the diverse and complex interactions throughout the agricultural sector and related sectors of the economy, it is difficult (if not impossible) to predict future magnitudes of key variables with a high degree of accuracy. However, it is possible to speculate on the direction of change in these variables. For instance, pesticide regulations in the United States will likely become more restrictive over time. Reductions in insecticide use without Bollgard™ Cotton Line 531 will cause cotton yields to decline, farm profits to decline and acres devoted to cotton production to decline, especially in those regions where insecticide use is an integral production practice. A scenario which allows for the introduction of Bollgard™ Cotton Line 531 results in a very different forecast. Reductions in insecticide 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 adoption by large farms over that of 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 Bollgard™ Cotton Line 531 is not expected to be related to farm size; i.e., small and large farms will have the same per-acre costs and benefits from the adoption of this improved cotton and, thus, will likely have equal adoption rates.

In summary, the introduction of Bollgard™ Cotton Line 531 will have significant positive impacts on the profitability of some farmers and agribusinesses. It will allow cotton growers to eliminate some conventional insecticide applications and thus reduce pesticide expenses. Based on available cost and acreage data and assumptions concerning the portion of current cotton acres that would be converted to Bollgard™ Cotton Line 531, it is estimated that cotton producers could save over $77 million per year on insect control costs.

C. Regulatory Approvals

Before commercializing the Bollgard™ Cotton Line 531, Monsanto will obtain the following regulatory approvals:

1. This determination from USDA/APHIS that Bollgard™ Cotton Line 531, and all progenies derived from crosses between Bollgard™ Cotton Line 531 and other cotton cultivars, is no longer a regulated article according to 7CFR §340.6.

2. Regulatory approval from the EPA of the *B.t.k.* insecticidal protein as expressed in Bollgard™ Cotton Line 531 under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). This petition was submitted on February 15, 1994 (Petition #4F4331).

3. An exemption from the requirement of a tolerance for the *B.t.k.* insecticidal protein under sections 408 of the Federal Food Drug and Cosmetic Act (FFDCA) from the EPA and Food and Drug Administration (FDA). The petition for the exemption from the requirement of a tolerance for the *B.t.k.* protein was submitted to EPA on February 15, 1994 (Petition #4F4331).

The EPA has exempted the NPTII protein and the genetic material necessary for the production of the protein from the requirement of a tolerance in or on all agricultural commodities when used as a plant-pesticide inert ingredient (EPA 1994). FDA has approved the request from Calgene Inc. to amend the food additive regulations to provide for the safe use of NPTII as a processing aid in the development of new varieties of tomato, oilseed rape and cotton (Calgene, Inc., 1993, FDA 1994). No additional regulatory approvals are planned for the NPTII protein.

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

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 Coker and McNair cultivars account for most of the production in Georgia and the Carolinas.

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. Adaption 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.

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.

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).

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).

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.

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 virtually nonexistent 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

Bollgard™ Cotton Line 531, contains the following 3 genes inserted via genetic engineering techniques:

- The *cryIA(c)* gene which encodes for an insecticidal protein, *B.t.k.* HD-73, derived from the common soil microbe *Bacillus thuringiensis* variety *kurstaki* (*B.t.k.*).

- The *nptII* gene which encodes the selectable marker enzyme neomycin phosphotransferase II (NPTII), was needed to identify transformed cells that contained the *B.t.k.* protein. It served no other purpose and has no pesticidal properties.

- The *aad* gene which encodes the bacterial selectable marker enzyme 3"(9)-O-aminoglycoside adenylyltransferase (AAD), allowed for the selection of bacteria containing the PV-GHBK04 plasmid on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter and the encoded protein is not detected in Bollgard™ Cotton Line 531.

Bollgard™ Cotton Line 531 was produced using the *Agrobacterium tumefaciens* transformation system. This system and the related genes are described below.

A. Characteristics of the Non-transformed Cultivar

Bollgard™ Cotton Line 531 was developed by transforming the parental cotton cultivar Coker 312 (*Gossypium hirsutum* L.). This cotton was released by the Coker Pedigree Seed Company in 1974, and the variety is currently owned by the SeedCo Corporation of Lubbock, Texas. This is an older cotton variety, and little to none is being grown today. Therefore, Monsanto does not intend to introduce the Bollgard™ Cotton Line 531 variety, but will allow our seed company partners to transfer the trait into 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. Therefore, the Bollgard™ Cotton Line 531 generated with a Coker 312 background is acceptable from an agronomic perspective for testing purposes.

B. *Agrobacterium* Vectors and Transformation

Generally when using *Agrobacterium* vectors, only the T-DNA is transferred and integrated into the plant genome (Zambryski, 1992). It is generally 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.

The transformation vector contains well-characterized DNA segments required for selection and replication of the plasmid vector in bacteria and transfer of the T-DNA into plant cells. The plant expression vector was assembled and then transformed into *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 pMP9ORK (Koncz and Schell, 1986), in a chloramphenicol resistant derivative of the *Agrobacterium tumefaciens* strain A208. The disarmed pMP9ORK Ti plasmid does not carry the T-DNA phytohormone genes and is no longer considered a threat as a plant pest (Huttner, *et al.*, 1992). The pMP9ORK 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 T-DNA vector is transferred to the plant cells via the *vir* functions encoded by the disarmed pMP9ORK Ti plasmid (Klee, *et al.*, 1983 and Stachel and Nester, 1986). The Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium*.

The T-DNA, which includes the *cryIA(c)*, *nptII* and *aad* genes, was transferred into the genome of individual cotton cells thereby allowing selection on kanamycin. After a few days, the residual *Agrobacterium* cells were killed using different antibiotics. Procedures for *Agrobacterium* transformation of cotton hypocotyl sections were performed with modifications as described by Umbeck *et al.* (1987). Plants were regenerated with modifications of those as described by Trolinder and Goodin (1987). Subsequently, the cotton tissues were treated to stimulate regeneration of transgenic cells into shoots and ultimately plantlets were grown in soil and assayed for insect resistance.

C. Plant Expression vector - PV-GHBK04

The plasmid vector, PV-GHBK04, is an 11.4 Kb single border binary transformation vector (Figure III-1). It contains 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) integrated into the plant genomic DNA. The host for all DNA cloning and vector construction was *E. coli* MM-294, a derivative of the common laboratory *E. coli* K-12 strain. The PV-GHBK04 vector is composed of several genetic components; the sizes listed here include non-functional DNA needed for cloning events. Table III-1 summarizes and references all the genetic components of PV-GHBK04. The 0.70 Kb *oriV* fragment from the RK2 plasmid (Stalker, *et al.*, 1981) provides the origin of replication for maintenance in *Agrobacterium tumefaciens* and is

fused to the 3.0 Kb *Sal*I to *Pvu*I segment of pBR322 which contains the origin of replication for maintenance in *E. coli* (*ori322*) and the *bom* site for the conjugational transfer into *Agrobacterium tumefaciens* (Boliver, *et al.*, 1977 and Sutcliffe, 1978). This was fused to a 0.09 Kb DNA fragment from the pTiT37 plasmid which contains the nopaline-type T-DNA right border (Depicker, *et al.*, 1982 and Bevan, *et al.*, 1983). The remaining portion of plasmid DNA consists of two chimeric genes (genes with signals for plant expression) that encode the *B.t.k.* HD-73 and NPTII proteins and a bacterial selectable marker gene (*aad*) under the control of a bacterial promoter.

The chimeric gene responsible for the efficacious control of Lepidoptera (E35S/*cryIA(c)*/7S 3') consists of the enhanced 35S promoter (Kay *et al.*, 1987; Odell *et al.*, 1985), the *cryIA(c)* gene which encodes the *B.t.k.* HD-73 protein and the non-translated region of the soybean alpha subunit of the beta-conglycinin gene which provides the mRNA polyadenylation signals (Schuler, *et al.*, 1982) referred to as 7S 3' terminator sequence. This is fused to the 0.93 Kb fragment containing the *aad* gene, isolated from transposon Tn7, which encodes a protein that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985). Downstream of the *aad* gene is the chimeric gene for selection on kanamycin (35S/*nptII*/NOS 3') which consists of the cauliflower mosaic virus 35S promoter, the neomycin phosphotransferase type II (*nptII*) gene and the non-translated region of the 3' region of the nopaline synthase gene referred to as NOS 3' (Rogers *et al.*, 1985).

D. Inserted Genes

1. The *cryIA(c)* gene

The *cryIA(c)* gene contained within PV-GHBK04 was constructed by combining the first 1398 nucleotides of the *cryIA(b)* gene (corresponding to amino acids 1 to 466) (Fischhoff *et al.*, 1987) with nucleotides number 1399 to 3534 of the *cryIA(c)* gene (corresponding to amino acids 467 to 1178) (Adang *et al.*, 1985). With the exception of 6 amino acid differences, the *cryIA(b)* region is identical to the analogous region of the *B.t.k.* HD-73 protein encoded by the *cryIA(c)* gene as described by Adang *et al.* (1985). The *cryIA(c)* portion of the gene encodes a protein that is identical to the CryIA(c) protein present in nature (Adang *et al.*, 1985) with the exception of one amino acid at position 766. The protein found in nature contains a leucine at amino acid 766 and the *cryIA(c)* gene within PV-GHBK04 encodes a serine at position 766. The discrepancy was unintentional and occurred during the genetic design of the gene for plant expression. Since the *B.t.k.* HD-73 protein produced in Bollgard™ Cotton Line 531 yields an insecticidally active trypsin-resistant core product of approximately 600 amino acids in size, the amino acid at position 766 will be lost in the insecticidally inactive fragment upon exposure to trypsin (or the proteases within the insect gut) and, therefore, will not affect the host range of the active N-terminal portion of the protein (Bietlot, 1989).

Both regions of the *B.t.k.* HD-73 gene were genetically improved for increased plant expression using a strategy comparable to that described by Perlak *et al.*, 1990 and 1991. Since the *B.t.k.* HD-73 protein present in Bollgard™ Cotton Line 531 contains the hypervariable region of the CryIA(c) protein, which has been shown to be responsible for insecticidal specificity (Geiser *et al.*, 1986), the gene in PV-GHBK04 is

referred to as a *cryIA(c)* gene. The *cryIA(c)* gene contained within PV-GHBK04 encodes a near-nature identical *B.t.k.* HD-73 protein as described by Adang *et al.* (1985) with the encoded protein produced in Bollgard™ Cotton Line 531 being 99.4% identical to the naturally occurring *B.t.k.* HD-73 protein.

The *cryIA(c)* gene sequence, as introduced in Bollgard™ Cotton Line 531, is shown in Figure III-2. The corresponding amino acid sequence is shown in Figure III-3.

2. The *nptII* Marker Gene

The *nptII* gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). The NPTII enzyme uses ATP to phosphorylate neomycin and the related kanamycin, thereby inactivating these aminoglycoside antibiotics and preventing them from killing the cells producing NPTII. The coding sequence for the *nptII* gene is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982). The sole purpose of inserting the *nptII* gene into cotton cells with the *cryIA(c)* gene is to have an effective method of selecting cells that contain the insecticidal gene. In general, the frequency of cells that are transformed is often as low as 1 in 10,000 or 1 in 100,000 of the cells treated (Fraley *et. al.*, 1983). Therefore, to facilitate this process, a selectable marker gene, *nptII*, and selective agent, kanamycin, are used. Consequently, cells selected for plant generation that contain the *cryIA(c)* gene also contain the *nptII* gene.

The *nptII* gene sequence, as introduced into Coker 312 to produce Bollgard™ Cotton Line 531 is shown in Figure III-4. The corresponding amino acid sequence is shown in Figure III-5.

3. The *aad* Bacterial Marker Gene

The *aad* gene was isolated from transposon Tn7 (Fling, *et al.*, 1985) and is under the control of its own bacterial promoter which provided a selectable marker for genetic manipulations in the bacterial hosts. The *aad* gene encodes the enzyme 3"(9)-O-aminoglycoside adenylyltransferase (AAD) which allows for the selection of bacteria containing the PV-GHBK04 plasmid on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter and its lack of detectable expression was confirmed by an ELISA developed for the AAD protein (Monsanto Report, MSL No. 13275). The *aad* gene sequence, as introduced into Coker 312 to produce Bollgard™ Cotton Line 531 is shown in Figure III-6. The corresponding amino acid sequence is shown in Figure III-7.

4. Description of a Genetic Element Contained in PV-GHBK04 but Absent from Bollgard™ Cotton Line 531

The *ori322* region is present on the plasmid PV-GHBK04, but was not transferred and hence not present in the genome of Bollgard™ Cotton Line 531. The *ori322* region is a 1.8 Kb segment of pBR322 (contained on a 3.0 Kb *Sal*I to *Pvu*I fragment) which provides the origin of replication for maintenance of the PV-GHBK04 plasmid in *E. coli* and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells

(Bolivar *et al.*, 1977; Sutcliffe, 1978) and is located upstream of the *oriV* segment on PV-GHBK04. The absence of this genetic element in Bollgard™ Cotton Line 531 was demonstrated by Southern blot analyses, described in Part III E(1)(a).

E. Genetic Analysis

1. Insert number, copy number and insert integrity

As described in Part III-B, the Bollgard™ Cotton Line 531 was generated by *Agrobacterium tumefaciens* mediated transformation with the plasmid PV-GHBK04. DNA analyses were performed to characterize the inserted T-DNA in terms of insert number (number of integration events), copy number (number of T-DNA copies at a particular genetic locus) and insert integrity (gene size, composition and linkage). The characterization was performed by Southern blot analysis (Southern, 1975) on genomic DNA isolated from the leaf tissue of the control (Coker 312) and Bollgard™ Cotton Line 531 cotton plants.

a. Insert Integrity and Copy Number

SspI results: There are two *Ssp*I sites within PV-GHBK04; one is near the right border and the second is approximately 7.4 Kb downstream of the first, Figure III-1. Digestion with *Ssp*I was predicted to release a 7.4 Kb fragment along with a border fragment containing the *oriV* region and a second non-detectable border fragment (containing less than 100 bp of the plasmid DNA, which is typically not detected in these analyses) released near the right border. Figure III-8A, lane 7 shows that upon digestion of the DNA from Bollgard™ Cotton Line 531 with *Ssp*I, three fragments of approximate sizes 7.4, 1.7 and 0.7 Kb hybridized to the entire plasmid PV-GHBK04 probe.

The 7.4 Kb fragment hybridized to the *cryIA(c)* and *nptII* probes establishing that an intact fragment containing these two genes integrated into the cotton genome (Figures III-9A and III-10A, lane 7). The 0.7 Kb fragment did not hybridize to either the *cryIA(c)* or *nptII* probes (Figures III-9A and III-10A, lane 7) but did hybridize to the *oriV* probe (Figure III-11A, lane 7). The summation of the three fragment sizes, 7.4, 0.1 (from the *Ssp*I site to the right border) and 0.7 Kb, from the *Ssp*I digestion, established that the T-DNA insertion event from this copy can be no larger than approximately 8.2 Kb in size. This T-DNA, therefore, maximally contains the *cryIA(c)*, *nptII* and *aad* genes and part or all of the *oriV* region. Based on its maximum size, it does not contain the *ori322* region and this is supported by the *Hin*dIII in combination with *Eco*RI digestion results, described below.

The 1.7 Kb fragment, released by the *Ssp*I digestion, hybridized to the *cryIA(c)* gene probe, (Figure III-9A, lane 7) but not the *nptII* probe, (Figure III-10A, lane 7). These data indicated that a second (smaller) T-DNA integrated into the cotton genome. Since the origin of transfer is typically initiated from the right border (Zambryski, 1992), the second copy is presumed to contain the 7S 3' termination sequence (0.45 Kb in size) and maximally 1300 bp of the 3' portion of the *cryIA(c)* gene (1.7 minus 0.45 Kb). The 1.3 Kb 3' region of the *cryIA(c)* gene is the non-insecticidally active portion of the gene (Geiser, *et al.*, 1986) and, therefore, cannot encode an

insecticidally active protein. The sizes of the fragments generated from the DNA isolated from Bollgard™ Cotton Line 531 and cleaved with *Ssp*I are schematically illustrated in Figures III-8B through III-11B.

EcoRI/HindIII results: There is one site each for the restriction enzymes *Hin*dIII and *Eco*RI within PV-GHBK04. The *Eco*RI site is approximately 500 bp downstream of the right border and the *Hin*dIII site is approximately 4.2 Kb downstream of the *Eco*RI site (Figure III-1). If a single copy of PV-GHBK04 had integrated into the cotton genome, digestion with the combination of both enzymes would be expected to release a 4.2 Kb fragment containing the *cryIA(c)* gene, a border fragment containing the *nptII* gene (and part or all of the *oriV* region) and an undetectable border fragment (released nearest the right border and containing less than 450 bp of the plasmid DNA, which is typically not detected in these analyses). As shown in Figure III-8A, lane 10, three fragments were released: the 4.2 Kb fragment which hybridized to the *cryIA(c)* gene probe (Figure III-9A, lane 10), a 3.6 Kb border fragment which hybridized to the *nptII* and *oriV* probes (Figures III-10A and III-11A, lanes 10, respectively) and a third band of approximately 1.3 Kb which also hybridized to the *cryIA(c)* gene probe (Figure III-9A, lane 10). The fragment at approximately 7.8 Kb is considered to be a result of a partial digestion of the DNA since it is the approximate summed size of the two other fragments released and it hybridized to all four probes shown in Figures III-8A through III-11A, lane 10. The partial fragment is marked with an asterisk in the figures. The border fragment that hybridized with the *nptII* gene probe is approximately 3.6 Kb in length and, therefore, demonstrates (in combination with the *Ssp*I results) that this border contains no more than the *nptII* gene, the *oriV* region (0.62 Kb in size) and no more than 200-400 bp downstream of the *oriV* region. The size of this border fragment (3.6 Kb) also established that the *ori322* region did not integrate into the cotton genome since it is too small to have included the *ori322* region which is upstream of the *oriV* region.

The approximately 1.3 Kb fragment that hybridized with the *cryIA(c)* probe confirmed the integration of a second, smaller copy of PV-GHBK04 within the genome. The presence of an approximately 1.0 Kb fragment (containing two copies of the 7S 3' region) would have indicated that the two copies of T-DNA had inserted in an head-to-head arrangement (right border to right border). Since this fragment was not observed in the *Hin*dIII/*Eco*RI digestion, it was concluded that the two copies integrated in a head-to-tail arrangement as shown in the schematics. Based on the size of the 1.3 Kb T-DNA insert containing the partial *cryIA(c)* gene, no more than 1300 bp (maximum) of the 3' end of the *cryIA(c)* gene could have integrated (initiated from the right border) into the cotton genome. These results, in combination with the *Ssp*I results, demonstrate that an intact *cryIA(c)* gene inserted into the cotton genome (contained within the 4.2 Kb fragment released with *Hin*dIII in combination with *Eco*RI) and that a second, small region of T-DNA also integrated into the cotton plant genome (contained within the 1.3 Kb fragment released with *Hin*dIII in combination with *Eco*RI). Since the size of the *Eco*RI fragment is approximately 1.3 Kb, this is the maximum amount of the 3' region of the *cryIA(c)* gene contained within the second copy of T-DNA.

Additionally, these results, in combination with the *Ssp*I results described above and the *Hin*dIII results described below, demonstrate that the two T-DNA copies must be located in close proximity to each other and that an *Eco*RI site must be present between the two T-DNA copies since the smaller T-DNA copy (the 1.3 Kb fragment released with *Eco*RI in combination with *Hin*dIII) is approximately 450 bp smaller than the fragment released with *Ssp*I alone (1.7 Kb). The sizes of the fragments generated from the *Hin*dIII/*Eco*RI digestion are schematically illustrated in Figures III-8B through III-11B.

2. Insert Number

HindIII results: To obtain information on the number of T-DNA inserts transferred into the cotton genome, the isolated genomic DNA was cut with the restriction endonuclease *Hin*dIII. For a single copy and single insertion event, the *Hin*dIII restriction enzyme was expected to yield two fragments each joined to the plant genomic DNA referred to as border fragments. Two fragments of approximate sizes 4.0 and 8.5 Kb were generated, Figure III-8A, lane 9. The 4.0 Kb fragment hybridized to the *nptII* and *oriV* probes, Figures III-10A and III-11A, lane 9, while the 8.5 Kb fragment hybridized only to the *cryIA(c)* probe, Figure III-9A, lane 9 thereby identifying each of the border fragments. From the two digestion results above (*Ssp*I and *Hin*dIII in combination with *Eco*RI), it was demonstrated that two T-DNA inserts integrated into the cotton genome to produce Bollgard™ Cotton Line 531. The *Hin*dIII digestion results establish that the second, partial T-DNA copy must be upstream of the approximately 8.2 Kb T-DNA copy since the *Hin*dIII digestion released only a single fragment that hybridized to the *cryIA(c)* gene, Figure III-9A, lane 9. If the second, partial copy had inserted on a separate chromosome or downstream of the 8.2 Kb T-DNA copy, then a separate *Hin*dIII fragment containing the *cryIA(c)* gene would have been generated. Therefore the two copies are tightly linked (with no *Hin*dIII site between them) and the 8.5 Kb HindIII fragment contains a full and partial copy of the *cryIA(c)* gene on a single fragment. The sizes of the fragments generated from the *Hin*dIII digestion is schematically illustrated in Figures III-8B through III-11B.

Further evidence that the two T-DNA inserts are tightly linked was provided from the analysis of commercial lines that were crossed with Bollgard™ Cotton Line 531. Comparison of eight different progenies from two commercial lines, with three generations of back-crossing, demonstrated that the smaller T-DNA insert existed in all progenies (data not included). This confirms that the two T-DNA inserts are linked.

In summary, genetic analyses demonstrated that two T-DNA copies inserted in a head-to-tail arrangement into the cotton genome to produce Bollgard™ Cotton Line 531. One T-DNA insert, of approximately 8.2 Kb in size, contains a full length *cryIA(c)* gene and an *nptII* gene (without the *ori322* region) and the second insert, of approximately 1.7 Kb maximum size, contains a 3' portion of the *cryIA(c)* gene that cannot be insecticidally active since it does not contain the insecticidally active 5' region of the *cryIA(c)* gene. The two inserts were shown to be linked and this is supported by segregation data from commercial backcrossed lines.

3. Segregation

Segregation data for R1 plants (progeny of the initial transformant, which is referred to as R0) and the progeny of the R1 plants is presented in Table III-2. These results are consistent with a tightly linked two-insertion event containing one active copy of the *cryIA(c)* gene.

4. Stability of Gene Transfer

The stability of the *cryIA(c)* gene has been demonstrated over four generations of backcrossed derivatives of Bollgard™ Cotton Line 531 in several elite cultivar lines (Table III-3). The Chi square test for the BC3F1, BC3F2 and BC3F3 segregates were not different than expected. The Chi square test for the BC3F2 progeny test (expected segregation of 1 homozygote:2 heterozygotes) was significant at P=0.05 but not at P=0.01. By definition, we would expect a deviation of this magnitude from the expected ratio in approximately 5% of the cases. Thus, this result is most likely due to random sampling. Additionally, the R1 (Table III-2) progeny results were as expected. In fact, the results were a perfect fit.

In summary, the data from the genetic analysis of the Bollgard™ Cotton Line 531 demonstrate that a single active copy and an inactive partial copy of the *cryIA(c)* gene was introduced into genomic cotton DNA inserted at two tightly linked sites and that the integrity of this insertion was maintained during the transfer. The gene for *cryIA(c)* segregated in a manner consistent with two tightly linked insertion events and was stably transferred with crossing. The selfed data from the crosses further demonstrated the stability of transfer from generation to generation.

F. Description of the Expressed Proteins

1. *Bacillus thuringiensis* Crystal Proteins

a. Biochemistry

Bacillus thuringiensis is a crystalliferous spore-forming gram-positive bacterium that has been used commercially over the last 30 years to control insect pests. These microbes are found naturally in soil worldwide. Numerous different strains have been identified, characterized and used commercially. Several strains have been extensively studied and have been shown to be insecticidally active against selected insect pests as summarized below:

B. thuringiensis subsp. *israelensis* strains are active against Dipteran insects (mosquitoes and black flies);

B. thuringiensis subsp. *san diego* and *tenebrionis* strains are active against Coleoptera (potato beetle, elm leaf beetle);

B. thuringiensis subsp. *kurstaki*, *sotto* and *aizawai* strains are all active against Lepidoptera (tomato hornworm, gypsy moth, cabbage looper, tobacco budworm, cotton bollworm, etc.).

The protein produced in Bollgard™ Cotton Line 531, (*cryIA(c)*), is >99.4% identical to the protein produced by the *B.t.k.* HD-73 bacterial strain. This strain controls insect pests by the production of crystalline insecticidal proteins known as delta-endotoxins. These proteins are produced as the bacterium enters the sporulation phase and can account for approximately one-third of the weight of the bacterial cell. To be active against the target insect, the protein must be ingested. In the insect gut, the protein binds to specific receptors on the insect mid-gut, inserts into the membrane and forms ion-specific pores. These events disrupt the digestive processes and cause the death of the insect. Strains of *B. thuringiensis* have been used commercially to control selected insect pests. Commercial quantities of these microbes are prepared in large-scale cultures in which the bacteria are allowed to sporulate. The spores and proteins are then formulated for application to plants.

Two classes of insecticidal proteins (delta-endotoxins) are produced upon sporulation by *B.t.k.* strains. These are termed P1 and P2 proteins based on relative molecular weights. The *B.t.k.* HD-73 (CryIA(c)) protein falls in the P1 class. The P1 proteins range in molecular weight from 130,000 to 140,000 daltons and are comprised of 1100 to 1200 amino acids The P2 proteins are typically significantly smaller in size than the P1 proteins. The most well studied P2 proteins are 71 Kda in size and are comprised of 633 amino acids (Widner and Whitely, 1989). The P1 proteins can be divided into an amino terminal and a carboxy terminal domain. The amino acid sequences of the carboxy terminal domain have been conserved (Thorne *et al.*, 1986; Jaquet *et al.*, 1987) across bacterial strains and contain a number of cysteine residues which form intramolecular bonds that are important in the formation of the protein crystal structure. The carboxy terminal domain is not essential for insect toxicity; it can be cleaved from the protein molecule without affecting the activity of the remaining protein towards insects (Adang *et al.*, 1987; Thorne *et al.*, 1986).

The amino terminal end of the P1 protein retains the insecticidal activity (Fischhoff et. al., 1987). Comparison of the amino acid sequence for various P1 proteins from several *B. thuringiensis* strains reveals considerable differences (22% homology in amino acid content for the *B.t. kurstaki* and *tenebrionis* subspecies) which account for the selectivity in activity against various insect orders.

b. Mode-of-Action

As stated previously, *B.t.k.* proteins must be ingested by the insect to exert insecticidal activity. The protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH (Bulla *et al.*, 1977); however, the pH of the larval insect gut is alkaline which favors solubilization of the protein crystal. The solubilized protein is subsequently activated by proteases in the insect gut. These proteases cleave the carboxy terminal domain from the rest of the protein (Chroma and Kaplan, 1990) as well as approximately 28 amino acids from the amino terminal end of the protein (Bietlot *et al.*, 1989). The activated protein, which consists of approximately 600 amino acids, diffuses through the

peritrophic membrane of the insect to the midgut epithelium. There, it binds to specific high affinity receptors on the surface of the midgut epithelium of target insects (Wolfersberger *et al.*, 1986; Hofmann *et al.*, 1988; Hofmann *et al.*, 1988a; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990). Non-target insects, mammals, birds and fish do not possess such receptors. Pores are formed in the membrane leading to leakage of intracellular contents (e.g. K+) into the gut lumen and water into the epithelial gut cells (Sacchi, *et al.*, 1986; Knowles *et al.*, 1989). The larval gut epithelial cells swell due to osmotic pressure and lyse. The gut becomes paralyzed as a consequence of changes in electrolytes and pH in the gut causing the larval insect to quit eating and die.

c. Evidence for "Species-Selectivity"

The protein delta-endotoxins produced by the various subspecies of *B. thuringiensis*, although related, exhibit differences in the amino acid sequence for the amino terminal domain of the proteins. These differences account, in part, for their selective action against certain insect pests. More importantly, non-target insects lack receptors for the proteins on the surface of their gut cells. This has practical application in assessing the safety of *B. thuringiensis* protein delta endotoxins towards other non-target organisms such as fish, birds and mammals. No receptors for these proteins have been identified on intestinal cells of mammals such as rats and rabbits (Sacchi *et al.* 1986; Hoffman *et al.* 1988; Van Mellaert *et al.* 1988). This explains the absence of toxicity for the protein delta-endotoxins of *B. thuringiensis* subspecies such as *kurstaki* to non-target organisms. The *B.t.k.* HD-73 protein expressed in Bollgard™ Cotton Line 531 shows a strict host-range specificity for lepidopteran insects and has no deleterious effects on non-target organisms (see Part V(J)).

d. Human Food Safety Considerations

There are no receptors for the protein delta-endotoxins of *B. thuringiensis* subspecies on the surface of mammalian intestinal cells; therefore, humans are not susceptible to these proteins. This has been confirmed in numerous safety studies carried out in laboratory animals which are traditionally experimental surrogates for humans. The results of some of these studies have been published in scientific reviews (Ignoffo,1973; Shadduck *et al.*, 1983; Siegel and Shadduck, 1990). Results of unpublished safety studies generated by registrants of *B. thuringiensis* commercial preparations have also been summarized in a recently issued EPA Registration Standard for *Bt* Formulations (EPA, 1988). In published reviews and the EPA document, studies are referenced where large doses (5000 mg/kg) of *B. thuringiensis* formulations were administered as single or multiple oral doses (up to 2 years) to different laboratory animals, with no adverse effects. Avian and aquatic organisms have also been fed *B. thuringiensis* formulations, with no adverse effects. A typical formulation is composed of *Bt* spores and *Bt* protein endotoxin, the latter compromising up to one-third of the weight of the spores. While target insects are susceptible to oral doses of *B.t.k.* proteins (µg per gram of body weight), there was no evidence of any toxic effects observed in non-target laboratory mammals, fish or birds given the equivalent of up to 10^6 µg of protein per gram of body weight. No

deleterious effects were observed on non-target insects at doses over 100 fold higher than needed to control target insects (EPA 1988). In addition to the lack of receptors for the *B.t.k.* proteins, the absence of adverse effects in non-target animals is further supported by the poor solubility and stability of the *B.t.k.* proteins in the acid milieu of the stomach. The acid conditions in the stomach and the presence of bile acids denature the *B.t.k.* proteins facilitating their rapid degradation by pepsin. *In vitro* enzymatically activated delta-endotoxins are also non-toxic when administered orally to laboratory animals (Nishitsutsuji-Uwo *et al.* 1980). Even if activated *B.t.k.* protein toxins could enter the mammalian gastrointestinal tract, there are no receptors on the surface of gastrointestinal tissues to permit binding of the protein toxin to the cell surface. These scientific considerations support the history of safe use of *B. thuringiensis* preparations. Based on the available scientific data, EPA and other regulatory scientists worldwide have determined that use of registered *B. thuringiensis* products pose no significant risks to human health or non-target organisms.

e. Lack of Exposure to Fish and Wildlife

As reported in the EPA Registration Standard for *Bacillus thuringiensis*, the naturally occurring *B.t.* proteins have been demonstrated to be virtually non-toxic to fish, avian species, mammals and other non-targets. Furthermore, 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 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 species should not feed on the large lint covered seed. In addition, the seed is not expected to enter aquatic habitats; therefore, fish should not be exposed.

Since the naturally occurring *B.t.k.* proteins have been demonstrated to be virtually non-toxic to fish, avian species, non-target insects, mammals and other non-target species and exposure to these species is not expected due their feeding preferences, no adverse effects are expected to wildlife from the commercialization of these plants.

Finally, no endangered or threatened lepidopteran insects, as listed in 50CFR 17.11 and 17.12, feed on cotton plants.

2. Biochemistry of the Neomycin Phosphotransferase II

The Neomycin Phosphotransferase II protein (NPTII), which has no insecticidal effect, is ubiquitous in the environment and found in microbes present on food and within the human digestive system (Flavell *et al.* 1992; Calgene, Inc., 1993). This protein has also been used as a selectable marker for animal and human cell transformation and for human gene therapy experiments (Culver *et al.*, 1991; Brenner *et al.*, 1993). The safety of NPTII and other selectable markers are addressed in recent reviews by Flavell *et al.* (1992) and Nap *et al.* (1992), and in two separate papers by Monsanto Scientists; Fuchs *et al.* (1993a) and Fuchs *et al.* (1993b). FDA has recently approved the request from Calgene Inc. to amend the food additive regulations to provide for the safe use of NPTII as a processing aid in the

development of new varieties of tomato, oilseed rape and cotton (Calgene, Inc., 1993, FDA 1994). In addition, the EPA has exempted the NPTII protein and the genetic material necessary for the production of the protein from the requirement of a tolerance in or on all agricultural commodities when used as a plant-pesticide inert ingredient (EPA 1994).

These reviews and the approvals by the FDA and EPA support the safety of NPTII protein for use as a selectable marker in crops grown for human and animal consumption.

Conclusions

- The *Agrobacterium tumefaciens* transformation system utilized in the modification of this insect resistant cotton is well understood and has been utilized for many years in the modification of many dicotyledonous plants. The system is dis-armed and cannot transmit the crown gall disease.

- This transformation system stably inserts the genes into the chromosome of the plant cell.

- All of the elements of the plasmid vector PV-GHBK04, which was utilized in the modification of Bollgard™ Cotton Line 531, are well characterized and understood. The function of each element is known and the genes have been cloned so they have no potential to transfer any plant pest characteristics to the host organism.

- The *cryIA(c)*, *nptII* and *aad* genes present in the PV-GHBK04 plasmid vector have been completely sequenced.

- Two T-DNA inserts integrated in close proximity, in a head-to-tail arrangement, into the cotton genome to produce Bollgard™ Cotton Line 531. The *cryIA(c)* gene segregated in a manner consistent with a single active copy of the gene and was stably transferred with crossing.

- The amino acid sequences for the *B.t.k.* and NPTII proteins as present in Bollgard™ Cotton Line 531 have been elucidated based on nucleotide sequence.

- The *B.t.k.* protein produced in Bollgard™ Cotton Line 531, (CryIA(c)), is >99.4% identical to the protein produced by the *B.t.k.* HD-73 bacterial strain. To be active against the target insect, the protein must be ingested. In the insect gut, the protein binds to specific receptors on the insect mid-gut, inserts into the membrane and forms ion-specific pores. These events disrupt the digestive processes and cause the death of the insect.

- Strains of *B. thuringiensis* have been used commercially, for nearly 30 years, to control selected insect pests.

- The CryIA(c) protein produced in Bollgard™ Cotton Line 531 is considered non-toxic to non-target insects, birds, fish and mammals. These species lack receptors for the proteins on the surface of their gut cells.

- The NPTII enzyme expressed in Bollgard™ Cotton Line 531 functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation. It has no pesticidal activity and is not known to be toxic to any species.

- The *aad* gene, present in Bollgard™ Cotton Line 531, was used as a selectable marker for genetic manipulations in the bacterial hosts prior to plant transformation. The gene is under the control of its own bacterial promoter, and the AAD protein was not detected in Bollgard™ Cotton Line 531.

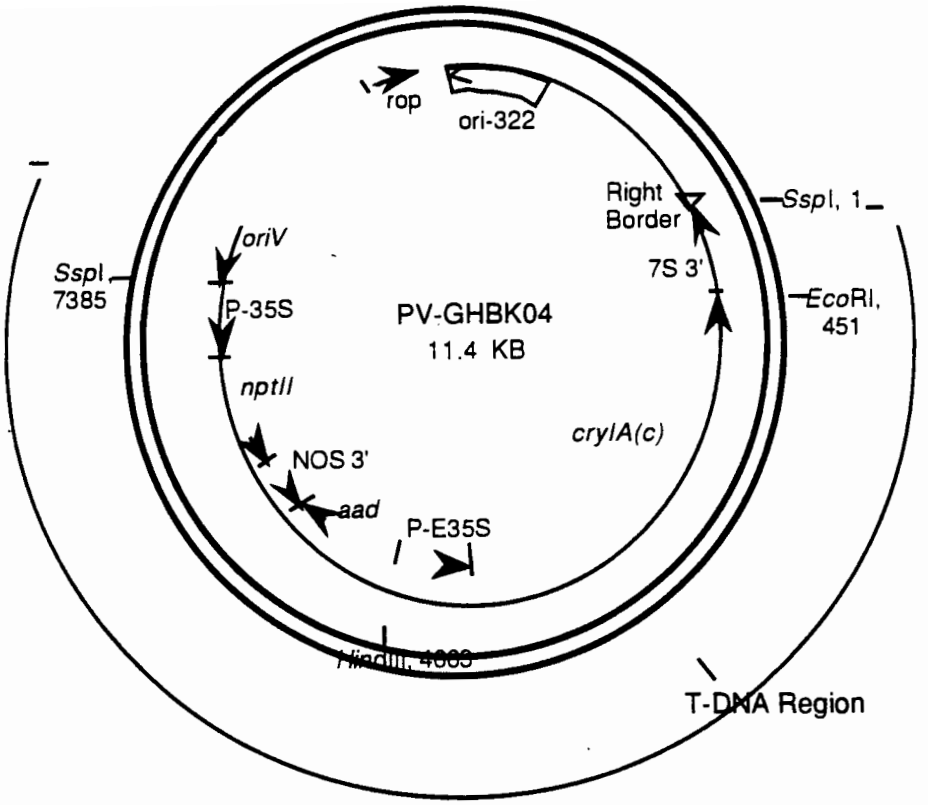

Figure III-1. Plasmid map of the 11.4 Kb binary vector PV-GHBK04 used to produce Bollgard™ Cotton Line 531. Restriction sites and their locations in bp, utilized during Southern analyses are shown. The T-DNA region is marked and the right border is denoted by an open triangle.

Figure III-2. Nucleotide sequence of the *B.t.k.* HD-73 protein encoded by *cryIA(c)* in Bollgard™ Cotton Line 531 plants containing the PV-GHBK04 vector. It is composed of the first 1-1398 nucleotides (1-466 amino acids) of *cryIA(b)* and 1399-3534 nucleotides (467-1178 amino acids) of *cryIA(c)*.

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Figure III-3. Amino acid sequences for the *B.t.k.* HD-73 full length protein which is present in Bollgard™ Cotton Line 531 plants containing the PV-GHBK04 vector.

```
   1  MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51  VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101  EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151  QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201  GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251  DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301  NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351  VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401  YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451  PMFSWIHRSA EFNNIIASDS ITQIPAVKGN FLFNGSVISG PGFTGGDLVR
 501  LNSSGNNIQN RGYIEVPIHF PSTSTRYRVR VRYASVTPIH LNVNWGNSSI
 551  FSNTVPATAT SLDNLQSSDF GYFESANAFT SSLGNIVGVR NFSGTAGVII
 601  DRFEFIPVTA TLEAEYNLER AQKAVNALFT STNQLGLKTN VTDYHIDQVS
 651  NLVTYLSDEF CLDEKRELSE KVKHAKRLSD ERNLLQDSNF KDINRQPERG
 701  WGGSTGITIQ GGDDVFKENY VTLSGTFDEC YPTYLYQKID ESKLKAFTRY
 751  QLRGYIEDSQ DLEIYSIRYN AKHETVNVPG TGSLWPLSAQ SPIGKCGEPN
 801  RCAPHLEWNP DLDCSCRDGE KCAHHSHHFS LDIDVGCTDL NEDLGVWVIF
 851  KIKTQDGHAR LGNLEFLEEK PLVGEALARV KRAEKKWRDK REKLEWETNI
 901  VYKEAKESVD ALFVNSQYDQ LQADTNIAMI HAADKRVHSI REAYLPELSV
 951  IPGVNAAIFE ELEGRIFTAF SLYDARNVIK NGDFNNGLSC WNVKGHVDVE
1001  EQNNQRSVLV VPEWEAEVSQ EVRVCPGRGY ILRVTAYKEG YGEGCVTIHE
1051  IENNTDELKF SNCVEEEIYP NNTVTCNDYT VNQEEYGGAY TSRNRGYNEA
1101  PSVPADYASV YEEKSYTDGR RENPCEFNRG YRDYTPLPVG YVTKELEYFP
1151  ETDKVWIEIG ETEGTFIVDS VELLLMEE
```

Figure III-4. Nucleotide sequence for the neomycin phosphotransferase II (*nptII*) gene present in Bollgard™ Cotton Line 531 plants containing the PV-GHBK04 vector.

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Figure III-5. Amino acid sequence for neomycin phosphotransferase II (NPTII) protein present in the cotton plants containing the PV-GHBK04 vector.

```
  1  MIEQDGLHAG SPAAWVERLF GYDWAQQTIG CSDAAVFRLS AQGRPVLFVK
 51  TDLSGALNEL QDEAARLSWL ATTGVPCAAV LDVVTEAGRD WLLLGEVPGQ
101  DLLSSHLAPA EKVSIMADAM RRLHTLDPAT CPFDHQAKHR IERARTRMEA
151  GLVDQDDLDE EHQGLAPAEL FARLKARMPD GEDLVVTHGD ACLPNIMVEN
201  GRFSGFIDCG RLGVADRYQD IALATRDIAE ELGGEWADRF LVLYGIAAPD
251  SQRIAFYRLL DEFF
```

Figure III-6. Nucleotide sequence for the aminoglycoside adenylyltransferase (*aad*) gene present in Bollgard™ Cotton Line 531 plants containing the PV-GHBK04 vector.

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Figure III-7. Amino acid sequence for the aminoglycoside adenylyltransferase (*aad*) gene present in Bollgard™ Cotton Line 531 plants containing the PV-GHBK04 vector.

```
  1  MREAVIAEVS TQLSEVVGVI ERHLEPTLLA VHLYGSAVDG GLKPHSDIDL
 51  LVTVTVRLDE TTRRALINDL LETSASPGES EILRAVEVTI VVHDDIIPWR
101  YPAKRELQFG EWQRNDILAG IFEPATIDID LAILLTKARE HSVALVGPAA
151  EELFDPVPEQ DLFEALNETL TLWNSPPDWA GDERNVVLTL SRIWYSAVTG
201  KIAPKDVAAD WAMERLPAQY QPVILEARQA YLGQEDRLAS RADQLEEFVH
251  YVKGEITKVV GK
```

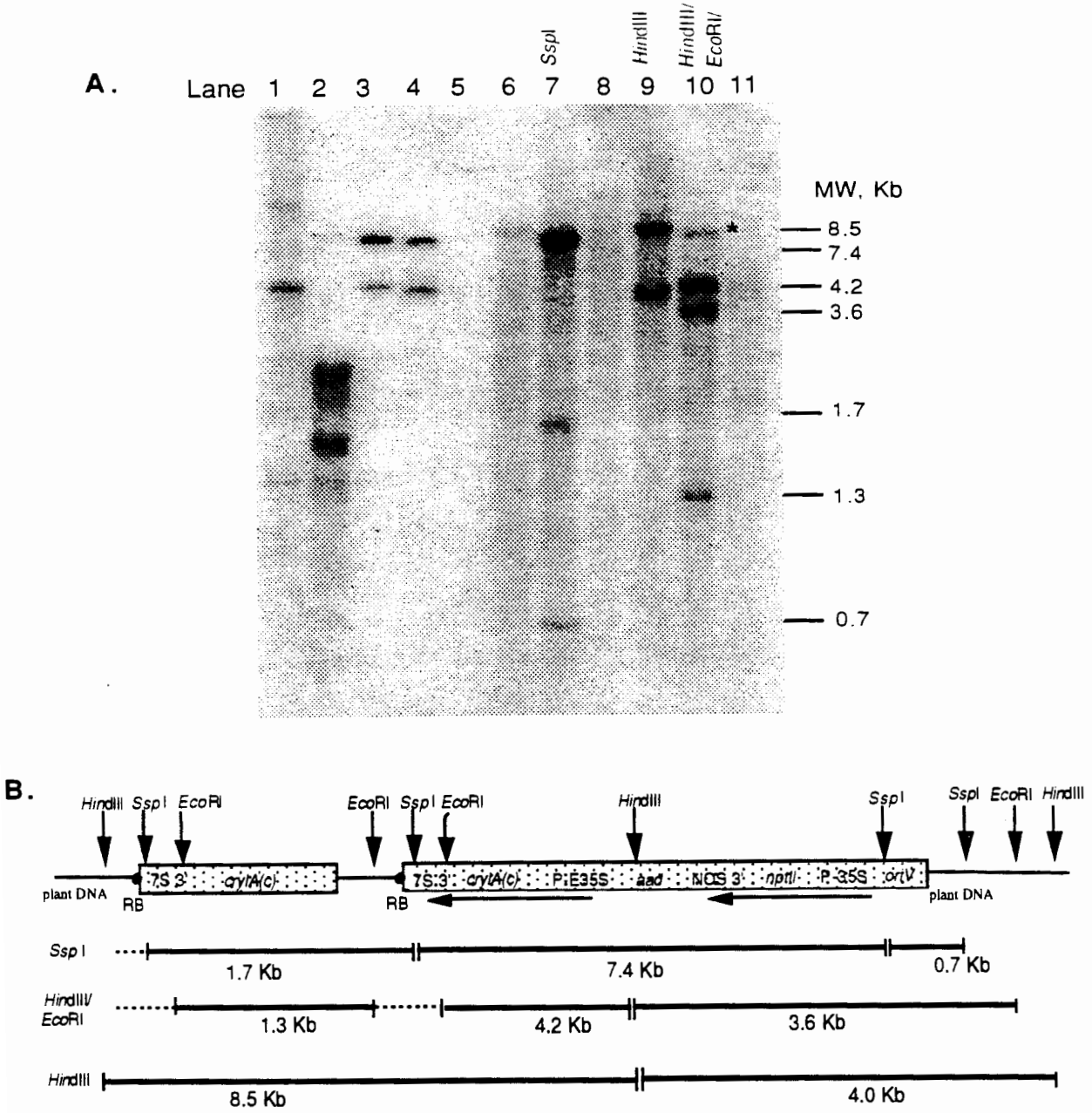

Figure III-8. Southern blot analysis using PV-GHBK04 as the probe. **A.** Southern blot analysis of DNA from line 531 using the entire plasmid, PB-GHBK04, as a probe. Lanes 1 and 2 are molecular weight standards and lanes 3 and 4 are plasmid PV-GHBK04 cleaved with the restriction enzymes *Hin*dIII and *Eco*RI (which produced expected size fragments of 7.2 and 4.2 Kb) and *Ssp*I and *Eco*RI (which produced expected size fragments of 6.9 and 4.1 Kb), respectively. Lane 5 was left empty. Lanes 6, 8 and 11 are approximately 10 micrograms of DNA from control C312 cleaved with the restriction enzymes *Ssp*I, *Hin*dIII and *Hin*dIII in combination with *Eco*RI, respectively. Lanes 7, 9 and 10 are approximately 10 micrograms of DNA cleaved with the restriction enzymes *Ssp*I, *Hin*dIII and *Hin*dIII in combination with *Eco*RI, respectively. *Indicates a partial digestion. **B.** A schematic illustration of the Southern blot results from Figures 2 through 5 indicating the orientation of the two T-DNA copies in line 531 (not to scale). The dotted region within the box illustrates the location of the probe homology. The vertical arrows denote the locations of the restriction sites within the T-DNAs and the dashed lines indicate nondetected fragments. All border fragment sizes are estimates. The right border is denoted by RB and is shown for orientation purposes (*i.e.*, an intact border sequence is not implied).

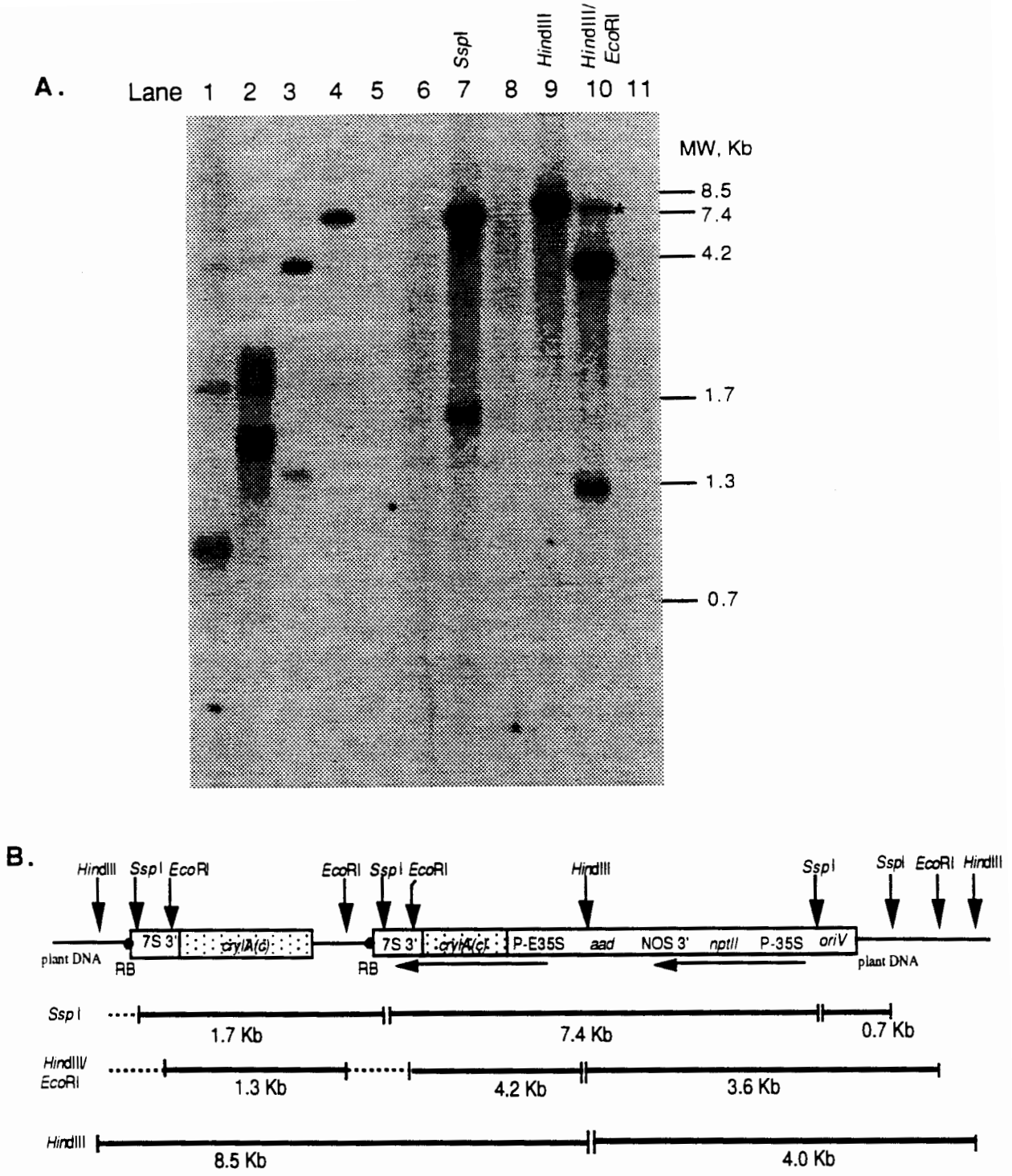

Figure III-9. Southern blot analysis using *cryIA(c)* as the probe. A. The same Southern blot from Figure 2, the plasmid probe removed, and reprobed with the *cryIA(c)* gene. Lane designations are the same as in Figure III-8. *Indicates a partial digestion. **B.** Schematic illustration of the T-DNA insertion events in line 531. The dotted region within the box indicates the location of the probe homology. All other designations are as in Figure III-8B.

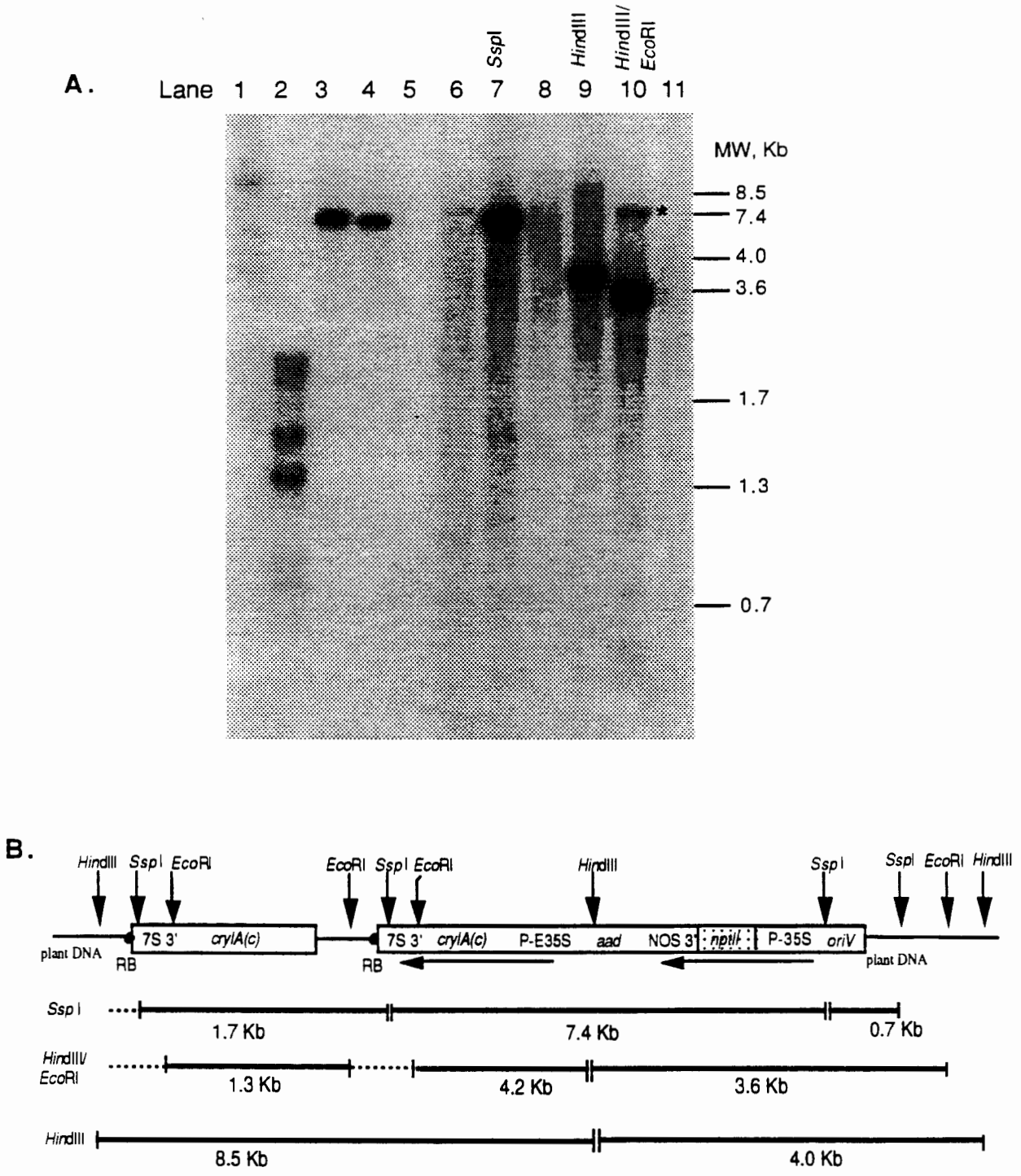

Figure III-10. Southern blot analysis using *nptII* as the probe. A. The same Southern blot from Figure III-8, the plasmid probe removed, and reprobed with the *nptII* probe. Lane designations are the same as in Figure III-8. *Indicates a partial digestion. **B.** Schematic illustration of the T-DNA insertion events in line 531. The dotted region within the box indicates the location of the probe homology. All other designations are as in Figure III-8B.

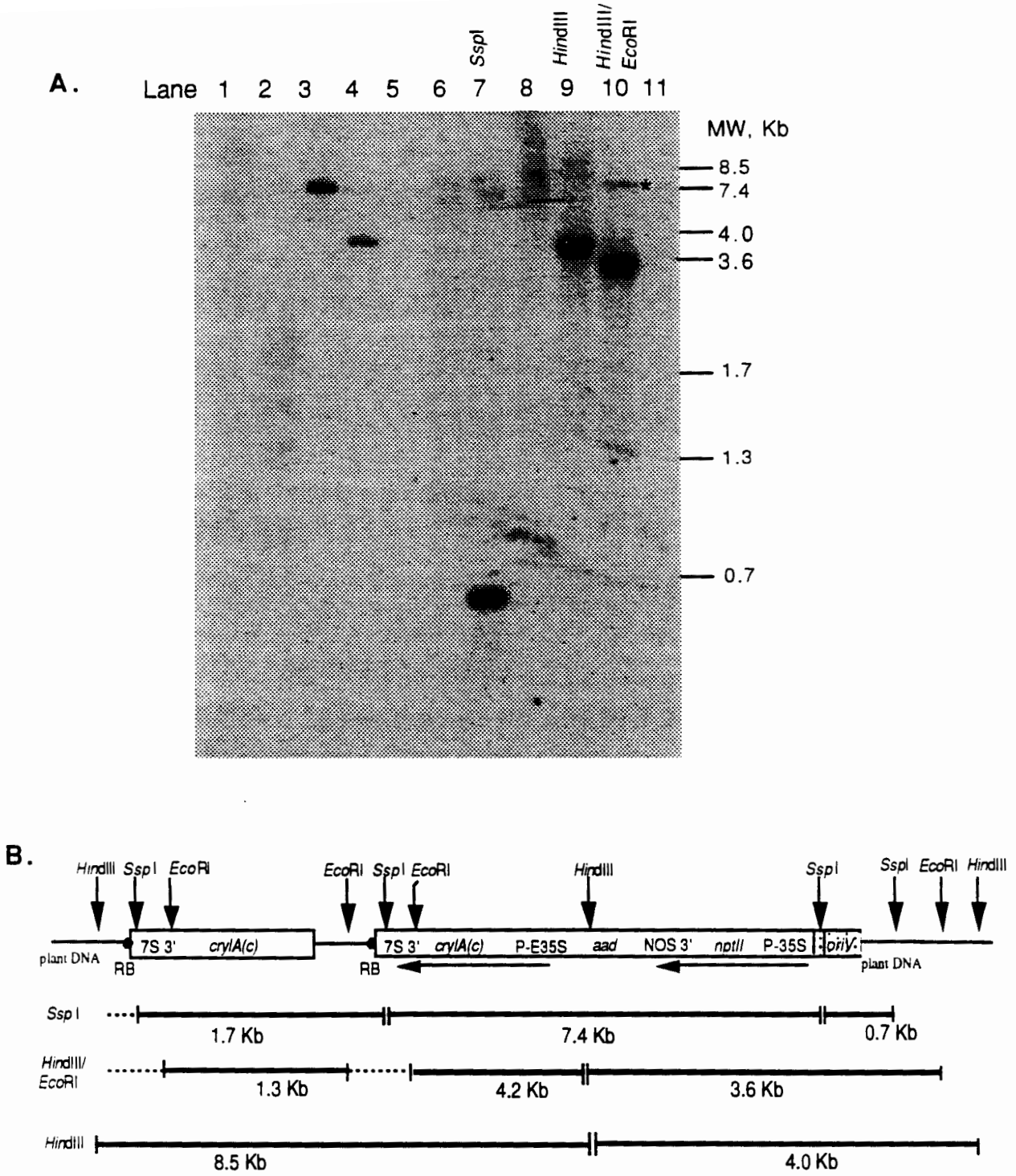

Figure III-11. Southern blot analysis using *oriV* as the probe. A. The same Southern blot from Figure III-8, the plasmid probe removed, and reprobed with an *oriV* probe. Lane designations are the same as in Figure III-8. *Indicates a partial digestion. **B.** Schematic illustration of the T-DNA insertion events in line 531. The dotted region within the box indicates the location of the probe homology. All other designations are as in Figure III-8B.

Table III-1. Summary of DNA Components in PV-GHBK04.

Genetic Element	Size, Kb*	Function
right border (RB)	0.09	A DNA fragment from the pTiT37 plasmid containing the 24 bp border nopaline-type T-DNA right border used to initiate the T-DNA transfer (RB) from *Agrobacterium tumefaciens* to the plant genome (Depicker *et al.*, 1982, and Bevan *et al.*, 1983).
P-E35S	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1987).
cryIA(c)	3.5	The gene which confers insect resistance. The modified gene encodes an amino acid sequence that is 99.4% identical to the *cryIA(c)* gene as described by Adang *et. al* (1985)
7S 3'	0.43	A 3' non-translated region of the soybean alpha subunit of the beta-conglycinin gene that provides the mRNA polyadenylation signals (Schuler *et al.*, 1982).
aad	0.79	The gene for the enzyme 3"(9)-O-aminoglycoside adenylyltransferase that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985).
P-35S	0.32	The 35S promoter region of the cauliflower mosaic virus (CaMV) (Gardner *et al.*, 1981; Sanders *et al.*, 1987).
nptII	0.79	The gene isolated from Tn5 (Beck *et al.*, 1982) which encodes for neomycin phosphotransferase type II. Expression of this gene in plant cells confers resistance to kanamycin and serves as a selectable marker for transformation (Fraley *et al.*, 1983).
NOS 3'	0.26	A 3' non-translated region of the nopaline synthase gene which functions to terminate transcription and direct polyadenylation of the *nptII* mRNA (Depicker *et al.*, 1982; Bevan *et al.*, 1983).
oriV	0.62	Origin of replication for ABI *Agrobacterium* derived from the broad-host range plasmid RK2 (Stalker *et al.*, 1981).
ori322/rop	1.8	A segment of pBR322 which provides the origin of replication for maintenance of the PV-GHBK04 plasmid in *E. coli,* the replication of primer *(rop)* region and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells (Bolivar *et al.*, 1977; Sutcliffe, 1978).

*Sizes given are the actual size of the genetic elements and do not include DNA border sequences, necessary for cloning purposes, unless otherwise indicated.

Table III-2. Segregation data and analysis of progeny of Bollgard™ Cotton Line 531.

		Single insert		Double insert	
	actual	expected (3:1)	Chi square value	expected (15:1)	Chi square value
R1 plants	67:20	65:22	0.24*	82:5	47.7+
R1 progeny¶	7: 14	7: 14	0 *	- -	- -

* not significant at P = 0.05 (Chi Square value= 3.84).
+ significant at P = 0.05 (Chi square value= 3.84).
¶ data expressed as R1 homozygotes: R1 heterozygotes.

Table III-3. Segregation data for backcross (BC) derivatives of Bollgard™ Cotton Line 531 with elite cultivar (EC) varieties. Values are in ratios of plants that are positive or negative for the *B.t.k.* HD-73 protein as determined by ELISA.

	actual	expected	Chi square value
BC3 F1 segregation (expect 1:1) BC3 F1 EC1,2,3	146:119	132.5:132.5	2.76*
BC3 F2 segregation (expect 3:1) BC3 F2 EC1,2	142:56	148.5:49.5	1.13*
BC3 F2 progeny test (expect 1 homozygote :2 heterozygote) BC3 F2 EC1,2	34:104	46:92	4.7+
BC3 F2 progeny test (segregation of heterozygotes, expect 3:1) BC3 F2 EC1,2	950:330	960:320	0.41*

* not significant at P = 0.05
+ significant at P = 0.05, but not at P = 0.01

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Part IV. Results of Field Trials

A. Field Test Permits and Locations

Bollgard™ Cotton Line 531 has been field tested in 1991, 1992 and 1993 at 21 locations throughout the mainland United States and Hawaii.

The following are the sites at which this testing was conducted.

USDA Permit #90-347-01

Location - Starkville, MS

USDA Permit #91-144-01

Location - Kauai, HI

USDA Permit #91-347-02

Locations - Loxley, AL
Maricopa, AZ
Tifton, GA
Bossier City, LA
Starkville, MS
College Station, TX

USDA Permit #93-011-02

Locations - Jamesville, NC
Richlands, NC
Rocky Mount, NC

USDA Permit #93-011-05

Locations - Loxley, AL
Wabbaseka, AR
Maricopa, AZ
Yuma, AZ
Tifton, GA
Bossier City, LA
Scott, MS
Starkville, MS
Halfway, TX
Sinton, TX

The final reports for these USDA permitted studies are found in Appendix V of this Determination.

At all of these sites the following information was collected:

Weediness Characteristics.
Differences in morphology, plant growth characteristics and crop development.
Susceptibility of Bollgard™ Cotton Line 531 to attack by non-target insects.
Susceptibility of Bollgard™ Cotton Line 531 to disease infection.
Monitoring for volunteers.

B. Plant growth and general observations

Bollgard™ Cotton Line 531 was compared to the non-transformed parental line Coker 312 at each location with the exception of the breeding sites at Wabbaseka, AR and Scott, MS. At selected locations, the yield and control of target insects were measured. 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 Bollgard™ Cotton Line 531 and the Coker 312 control.

No significant differences in weediness or survival characteristics were noted between Bollgard™ Cotton Line 531 and the Coker 312 control (Appendix V). All locations reported similar emergence of Bollgard™ Cotton Line 531 compared to Coker 312 except for Tifton, GA in 1993. At this location, there was better emergence and seedling vigor for transgenic plants compared to the non-transgenic control. Differences were not significant according to the researcher. Results showing increased vigor were noted in laboratory germination assays comparing Bollgard™ Cotton Line 531 versus Coker 312 produced in North Carolina (Buehler, unpublished results). In this test, cold temperature vigor was greater for Bollgard™ Cotton Line 531 compared to Coker 312. The increased vigor noted in the laboratory tests, however, was not transferred to the field since none of the sites reported a significant increase in vigor.

At Wabbaseka in 1993, some dormancy was noted in transgenic lines harvested in the greenhouse immediately prior to planting. This is a frequent occurrence for cotton seed produced in the greenhouse and is not associated with the introduced gene(s).

In addition to monitoring for weediness, morphological observations were also recorded at the field sites. No significant morphological, growth or developmental differences were observed for Bollgard™ Cotton Line 531 in the field (Appendix V). These included; germination, morphology, time to flowering and fruiting, boll formation, boll development and yield (if insect damage was controlled in the Coker 312 control).

In the 1993 field tests, some transgenic lines other than Bollgard™ Cotton Line 531 differed from Coker 312 in certain agronomic characteristics such as time to flowering and maturity (Appendix V). Such differences were not observed for Bollgard™ Cotton Line 531. The lines for which these differences were observed will not be pursued commercially if the yield or agronomic performance is negatively impacted. Final reports for 1993 contained more detailed, line specific information than previous reports. Since reports prior to 1993 did not contain line specific information, observations of differences between transgenic lines and Coker 312 cannot be attributed to particular lines.

In addition to the field observations discussed above, some additional analyses were performed. Analyses of lint from cotton grown in Starkville, MS in 1993 showed no differences between Bollgard™ Cotton Line 531 and Coker 312 for micronaire, length, strength, elongation, or lint % (Jenkins, unpublished results, Appendix VI). Boll size of Bollgard™ Cotton Line 531 was smaller than Coker 312 at this location. However, yield data from this location and other locations establishes that this characteristic does not negatively impact yield (Section IV. C.). Additionally, several leading cotton

geneticists and breeders concluded that the smaller boll size is not detrimental since yields of Bollgard™ Cotton Line 531 are equivalent to or better than Coker 312 (see attached letters in Appendix VI).

No differences in susceptibility to non-target insects were noted between Bollgard™ Cotton Line 531 and Coker 312 at any location (Appendix V). Specific notations were made for similar responses of the Bollgard™ Cotton Line 531 and Coker 312 to the following pests: sweet potato whitefly, armyworm, leaf miners, Lygus bugs, aphids, boll weevils, and European corn borer.

Similarly, no differences in susceptibility to diseases were noted between Bollgard™ Cotton Line 531 and Coker 312 at any location (Appendix V). Specific notations were made only for similar response to *Rhizoctonia.* Additionally, the plants were monitored for symptoms of infection by *Agrobacterium.* No symptoms were noted at any location.

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 volunteers were observed in the fall at some locations where harvest was early. No volunteers were reported to be present in the spring. These observations from the field monitoring show that there is no difference in the over-wintering ability between the Bollgard™ Cotton Line 531 and Coker 312.

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 only during mild and dry winters can cotton seed be expected to over-winter and germinate the following spring.

Based on results of the field monitoring program, there were no significant differences between Bollgard™ Cotton Line 531 and Coker 312. The differences observed in boll size are common between cotton varieties and do not cause any concern in the commercialization of the crop. Furthermore, this does not impart any special adaptive, competitive or survival characteristics to Bollgard™ Cotton Line 531. Finally, no new variety expressing the *B.t.k.* protein will be commercialized unless it meets all morphological, yield and quality characteristics of cotton varieties produced in the United States.

C. Efficacy of Bollgard™ Cotton Line 531 - Summary of 1991, 1992 and 1993 Results

1. Field Trials

Bollgard™ Cotton Line 531 was tested in the field during the 1991, 1992, and 1993 growing seasons. Due to limited seed supply in 1991, only one location (Starkville MS) was planted. The 1992 study was conducted at six locations across the cotton belt: Tifton GA, Loxley AL, Starkville MS, Bossier City LA, College Station TX, and Maricopa AZ. Each location evaluated Bollgard™ Cotton Line 531, Coker 312 control and a commercially adapted variety.

Evaluation of Bollgard™ Cotton Line 531 was repeated at thirteen locations in 1993: Loxley, AL, Wabbaseka, AR, Maricopa, AZ, Yuma, AZ, Tifton, GA, Bossier City, LA, Scott, MS, Starkville, MS, Jamesville, NC, Richlands, NC, Rocky Mount, NC, Halfway, TX and Sinton, TX. Different sites utilized different protocols. In general, protocols were similar except for the Wabbaseka, AR and Scott, MS locations which were breeding nurseries.

Data collection was initiated after damage of the unsprayed, non-transgenic control(s) exceeded 5% and was continued on a weekly basis until the damage dropped below this level. For locations infested by *Heliothis spp.*, insect control was evaluated by counting the economically damaged squares and/or bolls on randomly selected squares and/or bolls from each plot. For the location infested by *Pectinophora gossypiella* (AZ), the following data was collected: % rosetted blooms, # of surviving larvae/boll, % damaged seed, and # of diapausing larvae/boll. Yield was also determined at each site.

2. Results

Bollgard™ Cotton Line 531 provided excellent square and boll protection in the 1991 Mississippi trial (Table IV-1). This location was artificially infested with *Heliothis virescens* in addition to the naturally occurring infestation. *Heliothis* pressure was intense as indicated by the high levels of square and boll damage in the unsprayed Coker 312 plots; season average for square damage was 39% and for boll damage was 20%. Square damage in Bollgard™ Cotton Line 531 unsprayed was less than 5% throughout the growing season and was equivalent to the sprayed Coker 312 and the sprayed Bollgard™ Cotton Line 531. Similarly, boll damage was equivalent in Bollgard™ Cotton Line 531 sprayed, Bollgard™ Cotton Line 531 unsprayed, and Coker 312 sprayed plots for the first three evaluation dates. Boll damage in the Bollgard™ Cotton Line 531 unsprayed was significantly less than the unsprayed Coker 312 on 8/21, but was greater in the unsprayed Bollgard™ Cotton Line 531 compared to the sprayed Bollgard™ Cotton Line 531 and sprayed Coker 312.

Heliothis infestations were extremely low in 1992 at Tifton GA. Throughout the season, Bollgard™ Cotton Line 531 provided effective control of this low infestation and also protected yield (Table IV-2). Damage from *Heliothis spp.* was very high in Alabama (Table IV-2), particularly in late July and early August. Throughout the season, unsprayed Bollgard™ Cotton Line 531 provided equal or better *Heliothis* control than the sprayed Coker 312. Unsprayed Bollgard™ Cotton Line 531 provided the highest

yield of any of these four treatments. The 1992 trial in Mississippi was artificially infested with *Heliothis virescens,* just as it was in 1991. As a result, insect damage was high (Table IV-2). Yield was reduced by approximately 81% in the unsprayed Coker relative to the other treatments and was equivalent in the Bollgard™ Cotton Line 531 sprayed, Bollgard™ Cotton Line 531 unsprayed, and Coker 312 sprayed plots.

Square damage became increasingly worse throughout the season in Louisiana, steadily increasing throughout the 1992 growing season in the unsprayed Coker 312 plots (data not shown). Likewise, square damage also increased in the sprayed Coker 312 plots throughout the season indicating that the pyrethroids were increasingly ineffective at controlling *Heliothis.* This ineffective control by the pyrethroids is also reflected in the decreased yield of the sprayed Coker 312 relative to the unsprayed Bollgard™ Cotton Line 531. Similarly, Bollgard™ Cotton Line 531 provided excellent protection from *Heliothis* damage in Texas (Table IV-2). Throughout the season, square and boll protection afforded by Bollgard™ Cotton Line 531 equalled or exceeded the lepidopteran control afforded by the weekly applications of an insecticide.

1992 season averages of square damage for all locations indicate that Bollgard™ Cotton Line 531 provided excellent control of lepidopteran pests (Table IV-2). For all locations, the season average of square protection provided by Bollgard™ Cotton Line 531 was greater than or equal to the square protection provided by weekly applications of lepidopteran insecticides. This line also provided excellent boll protection throughout the growing season and this square and boll protection was reflected in the yield data.

The results of the 1993 trials confirmed the results obtained in 1991 and 1992. Bollgard™ Cotton Line 531 unsprayed consistently gave comparable or better insect control than the parental Coker 312 sprayed or unsprayed (Table IV-3) and with one exception consistently out-yielded the Coker 312 sprayed line (Table IV-4).

3. Summary

Bollgard™ Cotton Line 531 provided excellent control of *Heliothis spp.* and *Pectinophora* at all locations in 1991, 1992, and 1993. At all sites, insect control with Bollgard™ Cotton Line 531 was greater than or equal to the sprayed Coker 312 treatments. At most locations, Bollgard™ Cotton Line 531 provided essentially complete control of lepidopteran damage throughout the growing season (Tables IV-1, IV-2, IV-3). At these sites, control was at least equivalent, and often superior to, the control provided by weekly applications of insecticides. Yield was also consistently equal to or greater than the Coker 312 control (Tables IV-2 and IV-4).

The only location exhibiting significant damage in the Bollgard™ Cotton Line 531 was Alabama. At this site, square damage >10% occurred for three observations (7/21, 7/27/ and 8/3, mean damage = 17%). This location had extremely high levels of infestation; the mean square damage for this period in the unsprayed controls was 70%. Insecticide treatments were not effective at controlling this infestation (29% mean square damage through this period). Thus, square damage was almost 2 fold greater in the sprayed non-transgenic control compared to the unsprayed Bollgard™ Cotton Line 531. The square damage observed in Alabama did not translate into yield loss; the highest yielding treatment was the unsprayed Bollgard™ Cotton Line 531. This indicates

that the observed damage was not biologically significant. Since *B.t.k.* is a stomach toxin, some feeding must occur by the pests in order to ingest enough *B.t.k.* protein to be lethal. The damage which occurred in Alabama was concluded to be slight feeding damage which must always occur with these lines to deliver a lethal dose of *B.t.k.* protein to the insects.

These data demonstrate that Bollgard™ Cotton Line 531 provides excellent control from damage by lepidopteran pests and is generally superior to the currently available commercial standards.

Table IV-1. Summary of Insect Damage in Mississippi for Bollgard™ Cotton Line 531 and Coker 312 control in 1991. Results are presented for both sprayed and unsprayed plots.

% damaged squares*

	date of observation					
Line	7/10	7/17	7/24	7/31	8/7	8/14
531 unspr.	3 b	3 b	0 b	0 b	1 b	1 b
531 spr.	2 b	0 b	0 b	0 b	1 b	2 b
C312 unspr.	27 a	42 a	50 a	37 a	44 a	36 a
C312 spr.	3 b	2 b	0 b	2 b	0 b	3 b

% damaged bolls*

	date of observation			
Line	7/31	8/7	8/14	8/21
531 unspr.	3 b	1 b	2 b	11 b
531 spr.	0 b	0 b	1 b	0 c
C312 unspr.	15 a	18 a	28 a	20 a
C312 spr.	0 b	1 b	1 b	1 c

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

* 20 squares or bolls were sampled from the center 2 rows of each 4 row plot. Trial was replicated 6 times. Values are averages of all replications for each date, (6 x 20 = 120).

Table IV-2. 1992 Season Averages of Insect Damage and Yield across locations for Bollgard™ Cotton Line 531 and non-transgenic Coker 312. Results are presented for both sprayed and unsprayed plots.

% damaged squares*

Line	Location GA	AL	MS	LA	TX	AZ
531 unspr.	2	10	4	1	0	0
531 spr.	1	6	0	2	0	1
C312 unspr.	9	42	40	30	26	9
C312 spr.	5	21	4	14	11	5

% damaged bolls*

Line	location GA	AL	MS	LA	TX	AZ
531 unspr.	1	0	2	3	1	0
531 spr.	0	3	1	1	0	0
C312 unspr.	1	7	17	24	14	30
C312 spr.	0	1	2	11	7	42

Yield (# seed cotton/A**)

	GA	AL	MS	LA	TX	AZ
531 unspr.	3289 a	3877 a	2029 a	2633 ab	6135 a	565
531 spr.	3249 a	3339 b	2140 a	2796 a	6889 a	429
C312 unspr.	2667 b	2214 c	394 b	865 c	2916 b	431
C312 spr.	3035 ab	3332 b	2069 a	2380 b	3878 b	335

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

* 20 squares or bolls were sampled from the center 2 rows of each 4 row plot. Trial was replicated 6 times. Values are season averages for 5 dates (5 x 20 x 6 = 600) at GA and LA and 6 dates (6 x 20 x 6 = 720) at AL, MS and TX. For AZ, 100 squares were measured for each plot at one date, (1 x 20 x 6 = 600).

* * Yields taken from the center 2 rows of the 4 row plots 30 feet in length averaged over 6 replications.

Table IV-3. 1993 Summary Data (% Damaged Fruiting Sites*)

Site	Coker 312 w/o lep trt	Coker 312 w/ lep trt	Bollgard™ Cotton Line 531 w/o lep trt
Louisiana	8	5	1
Mississippi	22	7	5
Texas	8	5	0
North Carolina (Edgecomb Co.)	40	3	7
North Carolina (Martin Co.)	25	8	1
North Carolina (Onslow Co.)	23	8	1
Average	21	6	2

* 25 squares or bolls were sampled from each plot. Trial was replicated 6 times. Values are season averages for 5 dates (5 x 25 x 6 = 750) at LA and TX and 6 dates (6 x 25 x 6 = 900) at MS. For NC Martin Co., 3 dates (3 x 25 x 6 = 450) and Edgecomb and Onslow Counties were averages for 5 dates (5 x 25 x 6 = 750).

Table IV-4. 1993 Summary Data Yield (Pounds of Seed Cotton or Lint per Acre*)

Site	Coker 312 w/o lep trt	Coker 312 w/ lep trt	Bollgard™ Cotton Line 531 w/o lep trt
Louisiana	1880	2116	2211
Mississippi	1101	2127	2167
Texas	355	1097	2029
North Carolina (Edgecomb Co.)	894	2799	2501
North Carolina (Martin Co.)	1264	2012	2271
North Carolina (Onslow Co.)	1626	2317	2648
Average	1187	2078	2305

* Yields taken from center 2 rows of the 4 row plots 30 feet in length averaged over 6 replications.

Reference

Hughes, H. D. and E.R. Nelson, 1957. "Crop Production, Principles and Practices". The MacMillian Company, New York

Part V. Detailed Description of the Phenotype of Bollgard™ Cotton Line 531

INTRODUCTION

Data and information supplied in this Petition for Determination of Non-Regulated Status demonstrate that Bollgard™ Cotton Line 531 is substantially equivalent to the non-modified cotton line, Coker 312 (*Gossypium hirsutum*), except for the inserted genetic sequences, the expressed proteins [*B.t.k.* CryIA(c) protein and neomycin phosphotransferase II (NPTII) enzyme], and the ability of the plant to resist damage from Lepidopteran insects. The information supplied in this section and referenced from other sections of this petition will demonstrate that the modified, Bollgard™ Cotton Line 531 is 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, safety of the inserted proteins and cottonseed products, and the environmental characteristics.

A variety of studies were conducted to characterize the unique traits of the modified cotton line and to establish that Bollgard™ Cotton Line 531 is substantially equivalent to the parental cotton line, C312. The inserted genetic material and insecticidal efficacy of Bollgard™ Cotton Line 531 were described in the previous sections (Part III and IV). The following characteristics of Bollgard™ Cotton Line 531 are described in this section:

- expression of the *B.t.k.* and NPTII proteins,
- the comparison of Bollgard™ Cotton Line 531 and line C312 on the basis of composition and quality of the cottonseed and processed cottonseed products,
- comparison of the natural toxicants of the seed and vegetative tissues,
- safety assessment of the *B.t.k.* HD-73 protein to non-target insects,
- demonstration of the wholesomeness of cottonseed food/feed products,
- the environmental fate of the *B.t.k.* HD-73 protein,
- the disease susceptibility of Bollgard™ Cotton Line 531 versus line C312, and
- the potential for out-crossing and weediness.

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 VII. The following sections summarize these investigations.

A. Expression of the Introduced Genes in Tissues from Bollgard™ Cotton Line 531

As described in Part III, Bollgard™ Cotton Line 531 has been modified to express a protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 [CryIA(c)] (abbreviated as *B.t.k.* HD-73) which has insecticidal activity against lepidopteran insect pests (Hofte and Whiteley, 1989; Perlak *et al.*, 1990; Perlak *et al.*, 1991; MacIntosh *et al.*, 1990). In addition to the *B.t.k.* HD-73 gene, a gene encoding the NPTII protein is present as a result of its use as a selectable marker during the development of the insect resistant cotton plants. A second selectable marker gene encoding aminoglycoside adenylyltransferase (AAD) is present in Bollgard™ Cotton Line 531 as a result of its use in selection for the microbial systems used for the genetic engineering process. The *aad* gene is controlled by a bacterial promoter; therefore, the protein was not expected to be expressed in the cotton leaf or seed tissue from Bollgard™ Cotton Line 531. The control line, C312 is the parental variety from which Bollgard™ Cotton Line 531 was generated and does not contain the genes encoding the *B.t.k.* HD-73, NPTII or AAD proteins.

Levels of the expressed proteins (*B.t.k.* HD-73, NPTII, and potentially AAD) were evaluated in young leaf (3-6 week plantlets) and seed tissues collected from six field locations during the 1992 growing season using Enzyme Linked Immuno-Sorbent Assay (ELISA) (Harlow and Lane, 1988) and western blot (Matsudaira, 1987) methods. The six field sites were as follows: Starkville, Mississippi; Bossier City, Louisiana; College Station, Texas; Tifton, Georgia; Maricopa, Arizona; and Loxley, Alabama. In addition, at one field site (Starkville, Mississippi), young leaf tissue was collected at 3 time points throughout the season after the initial sampling and whole, mature cotton plants were collected just prior to defoliation and harvest to establish the consistency of expression throughout the season and to estimate the amount of *B.t.k.* HD-73 and NPTII protein that might enter the environment at the end of the growing season. The expression of the *B.t.k.* HD-73 protein was also evaluated in nectar and pollen collected from Bollgard™ Cotton Line 531 (plants grown in the greenhouse) to provide information on the degree of non-target insect exposure to the insecticidal protein via pollen and nectar produced by the modified cotton line.

1. **Young Leaf and Seed** - These data show that the *B.t.k.* HD-73 and NPTII proteins were expressed at extremely low and relatively consistent levels in leaf and seed from Bollgard™ Cotton Line 531 across all six sites (Tables V-1, V-2, and V-3). Bollgard™ Cotton Line 531 contained less than 2 µg/gram fresh weight of *B.t.k.* HD-73 and less than 4 µg/gram fresh weight of NPTII in leaf and seed tissue, respectively, with levels varying only two to three fold across the six field sites. *B.t.k.* HD-73 protein levels varied less than three fold in young leaf tissue over the growing season with the highest level observed late in the season at the one field site evaluated (Table V-4). These results establish minimal variability in the expression of the *B.t.k.* HD-73 protein when the insect resistant cotton plants were grown at different geographical locations and different environmental conditions.

As predicted, no AAD was detected in leaf or seed tissue from Bollgard™ Cotton Line 531. The sensitivity of the ELISA for the AAD protein was approximately 0.008 µg AAD/gram fresh weight of leaf and 0.005 µg AAD/gram fresh weight of seed.

As expected, no *B.t.k.* HD-73 or NPTII protein was detected in leaf tissue from the Coker 312 control. During the analysis of the cottonseed from several field sites, *B.t.k.* HD-73 and NPTII proteins were detected in the seed of the Coker 312 control. After additional investigation, this was documented and shown to result from limited out-crossing between the Bollgard™ Cotton Line 531 and the Coker 312 control. The level of out-crossing ranged from 0% (at Texas) to 15% (at Louisiana and Alabama). These levels of out-crossing are well within the previously established ranges of out-crossing for both commercial cotton varieties (Afzal and Khan, 1950; Green and Jones, 1953; Theis, 1953; Simpson and Duncan, 1956) and other genetically engineered cotton varieties (Umbeck *et al.*, 1992; Kareiva and Morris, 1992).

These levels of out-crossing did not significantly impact the expression analysis for the Bollgard™ Cotton Line 531 as the expression values for the *B.t.k.* HD-73 and NPTII protein for Texas (which showed no out-crossing) were not significantly different and definitely no higher (as would be expected if the out-crossing from C312 into the insect resistant lines decreased the overall expression of these proteins in seed from Bollgard™ Cotton Line 531) than the values at the other sites in which out-crossing was observed. It was also concluded that the out-crossing did not significantly impact the quality and toxicant data or the quail feeding study (See paragraph G. Effects on Non-Target Organisms in this Part of this Petition of Determination of Non-Regulated Status).

2. Whole Plant - Levels of *B.t.k.* HD-73 and NPTII proteins in whole plant tissue were much lower, on a fresh weight basis, than in young leaf tissue. The *B.t.k.* and NPTII proteins were present at approximately 0.044 µg/gram fresh weight and 0.57 µg/gram fresh weight (respectively) in mature Bollgard™ Cotton Line 531 (Table V-5). These values were obtained by estimating the levels of these two proteins in the leaf, stem, root and boll (excluding seed and fiber) fractions of the insect resistant cotton Bollgard™ Cotton Line 531. *B.t.k.* HD-73 protein was not detected in the leaves, stems and roots of the mature cotton plants. Low levels of this protein was detected in the boll (minus the lint and seed) and was used to estimate the total amount of protein present in the plant and on a per acre basis. The NPTII protein was detected in the leaves, stems, roots and bolls of the mature cotton plants, with 0.566, 0.957, 0.011 and 0.314 µg/g fresh weight tissue detected respectively. Approximately 1.44 and 19.14 grams/acre of *B.t.k.* HD-73 and NPTII protein, respectively, were estimated to enter the soil environment by incorporating the insect resistant cotton plants from Bollgard™ Cotton Line 531 into the soil after harvest (assuming 60,000 plants per acre) (Table V-5). A soil degradation study was performed that confirmed the rapid degradation of the *B.t.k.* HD-73 protein expressed in the insect resistant cotton plants in the soil (See paragraph M, Possible Impact on the Environment in this Part of this Petition of Determination of Non-Regulated Status).

3. Nectar and Pollen - The level of *B.t.k* HD-73 protein measured in pollen (11.5 ng/g fresh weight of pollen) and nectar (<1.6 ng/g fresh weight of nectar) were extremely low (Table V-6). The *B.t.k.* HD-73 protein level was just above the detection limit of the assay for pollen (8.0 ng/g fresh weight of pollen) in one experiment and below the detection limit in a second experiment. *B.t.k.* HD-73 levels were below the limit of detection for nectar (1.6 ng/g fresh weight of nectar). The expression level of the *B.t.k.* HD-73 protein in the pollen and nectar of Bollgard™ Cotton Line 531 was

obtained to serve as a basis of exposure of beneficial (non-target) insects to the *B.t.k.* HD-73 protein expressed in these plants (See paragraph K, Lack of Effects on Non-Target Organisms in this Part of this Petition of Determination of Non-Regulated Status).

B. Composition, Quality, and Toxicant Analyses of the Cottonseed from Bollgard™ Cotton Line 531

Field grown cottonseed from Bollgard™ Cotton Line 531 and the control cotton line, C312 were shown to be compositionally equivalent based upon analysis of the major cottonseed components (protein, lipid, moisture, ash, carbohydrate, calories), the fatty acid profile and the levels of important toxicants (gossypol, cylcopropenoid fatty acids and aflatoxin). The levels of the major components (protein, oil, carbohydrate, moisture, ash and calories) in the cottonseed from Bollgard™ Cotton Line 531 and the parental control were comparable. There were no differences in any of these components for seed collected from the six field sites (Table V-7). There were no significant differences in the total lipids between cottonseed from Bollgard™ Cotton Line 531 and the C312 control (Table V-8). Minor, but statistically significant differences were observed between Bollgard™ Cotton Line 531 and the C312 control for three of the eleven individual fatty acids (Table V-9). However, these all fell within the published ranges for commercial cotton varieties, and therefore, represent the inherent variability within cotton varieties and are not attributed to the insertion of the genes for insect resistance.

No statistically significant differences were observed in gossypol levels at any of the six locations between the Bollgard™ Cotton Line 531 and the C312 control (Table V-10). Gossypol is a biologically active terpenoid substance that is present in discrete glands in various plant tissues, including the seed (Abou-Donia, 1976). The gossypol levels for both lines fell well within the ranges previously reported for cotton varieties (Pons *et al.*, 1958; Abou-Donia, 1976) and the variability across locations was consistent with previously reported data (Altman *et al.*, 1989; Berardi and Goldblatt, 1980).

Levels of the toxicant, cyclopropenoid fatty acids (dihydrosterculic, sterculic and malvalic), for cottonseed from the six field sites showed no statistically significant differences between seed from Bollgard™ Cotton Line 531 and the C312 control (Table V-10).

The four primary aflatoxins commonly found in cottonseed were undetectable at a sensitivity of 1 part per billion for the Bollgard™ Cotton Line 531 at all six sites and for the C312 control at five of the six sites (Table V-10). The sample of the C312 control seed grown at the Arizona field site showed relatively high aflatoxin contamination. Cottonseed produced in Arizona (and regions in which pink bollworm is a significant insect pest) typically have high levels of aflatoxin due to the boll damage caused by the pink bollworm (McMeans, *et al.*, 1976, Ashworth, *et al.*, 1971). Often the levels are sufficiently high that the seed cannot be used for animal feed. This insect pest enters the seed, uses the embryo and endosperm of the seed for food and a site for laying eggs. As the insect exits the seed, it leaves even larger holes, which are sites for infection by the *Aspergillus flavus* fungus which causes aflatoxin contamination.

Bollgard™ Cotton Line 531 expresses the *B.t.k.* protein, which is effective in controlling the pink bollworm, that caused damage only at the Arizona site. The dramatic reduction in aflatoxin level between Bollgard™ Cotton Line 531 and the control line grown in Arizona is additional evidence of the effective control of the target insect by the insect resistant cotton plants.

Compositional data showed that the Bollgard™ Cotton Line 531 and the C312 control are comparable for all characteristics except for the aflatoxin data from Arizona, which established an additional benefit that insect resistant cotton will have on feed safety of cottonseed from Bollgard™ Cotton Line 531.

C. Cottonseed Processing

The quality of the processed cottonseed products from Bollgard™ Cotton Line 531 were shown to be equivalent to the control line. Seed cotton from four of the six field sites (Mississippi, Louisiana, Texas and Georgia) were ginned and pooled (by line) across all four sites as a source of seed for processing. The composite cottonseed sample was processed at the Food Protein Research & Development Center at Texas A&M University into defatted/ toasted meal and refined oil which are the primary cottonseed products used for animal feed (except cattle, which consume whole seed) and human food.

When compared for yield of processed fraction relative to starting material (linters, linter motes, delinted seed, hulls, kernels, toasted meal, crude oil and refined oil), the yields were comparable for both the Bollgard™ Cotton Line 531 and the C312 control and similar to the means and ranges previously reported for processed cottonseed fractions from other cotton cultivars (Table V-11).

There were no meaningful differences in the levels of total and free gossypol in the raw cottonseed kernels, toasted meal and refined oil from both Bollgard™ Cotton Line 531 and C312 (Table V-12). Reduction of 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, 1990). Under the 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 the amount of free gossypol was reduced to trace levels in the toasted meal from both lines. Total gossypol levels were reduced by approximately 18% in the toasted meal for both lines.

The total protein content of the toasted meal and refined oil fractions from both the Bollgard™ Cotton Line 531 and control, C312 line were shown to be consistent with commercial quality products. Cottonseed meal used as a feed additive (protein concentrate) for livestock feed is typically prepared at $\geq$ 41% total protein. The toasted meal fractions from both the Bollgard™ Cotton Line 531 and control lines contained > 40% total protein by weight. As expected, protein was not detected (sensitivity of 1.3 parts per million) in the refined oil fractions from either line.

Finally, processing cottonseed from Bollgard™ Cotton Line 531 dramatically decreased the amount of biologically active *B.t.k.* HD-73 and NPTII proteins in the toasted meal. The level of these proteins in the toasted meal were reduced by more than 97.1% and 96.7%, respectively, versus the levels detected in raw cottonseed meal. No *B.t.k.* HD-73 or NPTII protein was detected by functional assays specific for each protein. Likewise, by western blot analyses, neither protein was detected in the processed meal, even in the denatured state. Therefore, processed cottonseed meal does not represent a source of significant exposure to either the *B.t.k.* HD-73 or NPTII protein.

These data establish that cottonseed from Bollgard™ Cotton Line 531 processes comparably to cottonseed from the C312 control and that the level of the important toxicant, gossypol, is comparable for both lines. Therefore, insertion of the genes to provide insect resistance did not alter the processing characteristics of the cottonseed or the quality of two major cottonseed products, toasted meal and refined oil.

D. Allelochemical Levels in Vegetative Tissues

Cotton contains allelochemicals, in addition to gossypol, that may be involved in pest control (Hedin, *et al.*, 1983; Hedin, *et al.*, 1988; Hedin, *et al.*, 1991). 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 analyzed from samples obtained from the 1992 and 1993 field tests in Starkville, Mississippi. As expected, no meaningful differences in the levels of gossypol, flavonoids, tannins and anthocyanins were detected between Bollgard™ Cotton Line 531 and the non-modified C312 control and the levels of these allelochemicals were representative for *G. hirsutum* lines. The complete reports of the 1992 and 1993 analyses are found in Appendix VIII. Since a variety of insect resistant lines and other modified cotton lines were evaluated in these analyses, the specific results for Bollgard™ Cotton Line 531 and control line, C312 are summarized in Table V-13.

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 Bollgard™ Cotton Line 531 possesses no disease or pest susceptibilities different than the parental non-transformed cotton.

In addition, a study was conducted in the 1992 field test in Mississippi to compare the incidence of cotton boll rot (*Anthracnose* boll rot) between the control cotton line, C312 and two modified, insect Resistant cotton lines, including Bollgard™ Cotton Line 531. There were no significant differences in the frequency of boll rot between the insect resistant cotton lines and the parental control, line C312. The data from this study are summarized in Appendix IX.

F. Plant Pest Risk

In all field and green house trials, Bollgard™ Cotton Line 531 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 the Bollgard™ Cotton Line 531 to express any plant disease (See part III). Bollgard™ Cotton Line 531 does 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 Bollgard™ Cotton Line 531 to the parental C312 showed no differences (see section B, above). Therefore, it is concluded that the Bollgard™ Cotton Line 531 does 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 the Bollgard™ Cotton Line 531 or any *Gossypium* species crossing with Bollgard™ Cotton Line 531 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 *B.t.k.* 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 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. Ultimately, if *B.t.k.* should be transferred to a wild population of a tetraploid, and if this was considered undesirable, the size of the plants, their perennial growth habit, their restricted habitat and their low natural fecundity would make control exceptionally easy (Stewart, 1992; Appendix III).

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 better able to withstand damage from foliar eating insects. This insect resistance would not be 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 *B.t.k.* and *nptII* genes. Expression of the gene products (*B.t.k.* HD-73 and NPTII proteins) in the modified cotton plant would not change any of the above listed attributes.

H. Germination and Vigor Results for Bollgard™ Cotton Line 531 and Coker 312

Field germination studies comparing Coker 312 and Bollgard™ Cotton Line 531 have not been conducted to date. For most studies, the seed for Bollgard™ Cotton Line 531 and Coker 312 were not produced in the same location, thus making it impossible to make such a comparison. For example, the seed for Bollgard™ Cotton Line 531 for most field

studies was grown in a winter nursery while the Coker 312 seed was purchased from SeedCo Co. in Lubbock, TX. Data is available from a laboratory study and two field locations which utilized seed from the Bollgard™ Cotton Line 531 and the Coker 312 produced at the same location. These data support the conclusion that no significant differences exist between the germination rates of Bollgard™ Cotton Line 531 and Coker 312.

Laboratory Germination Study

Bollgard™ Cotton Line 531 and Coker 312 were tested in multiple locations in 1993. Seed from one of the sites in North Carolina was harvested and then processed at Monsanto for use in the 1994 trials. After ginning and delinting, the "finished" seed from both Bollgard™ Cotton Line 531 and Coker 312 was tested to determine germination rates in both warm and cold temperatures. The standard germination test was done in germination paper, stored in the greenhouse (approx. 90° F), and read after 4 days. The cold test was also done in germination paper, stored at 19° C, and read 7 days later. The tests were replicated 4 times with 20 seeds per replication.

Results for the two lines in the warm test were not significantly different (Table V-14). However, Bollgard™ Cotton Line 531 had a significantly higher percent germination than Coker 312 in the cold test (83% and 60%, respectively). Similarly, the number of seedlings with a radicle greater than 1 inch was higher for Bollgard™ Cotton Line 531 compared to Coker 312 (75% and 41%, respectively). These results suggest that under certain conditions (i.e. cold temperatures) Bollgard™ Cotton Line 531 may be more vigorous than Coker 312.

These differences under laboratory conditions have not been observed in the field when the seed is planted under normal agronomic and environmental conditions. Therefore, the differences may be due to the condition of the seed in the lots used for the germination studies. This seed was produced in North Carolina which is not a typical location for seed production due to adverse weather as the crop matures. The quality of this seed was not up to the standards one would expect from seed produced in more typical production areas, such as Arizona, which tends to be of excellent quality. Also, the Coker 312 seed used in this germination study exhibited signs of insect damage whereas the Bollgard™ Cotton Line 531 seed did not. The Bollgard™ Cotton Line 531 plants were more efficacious in terms of *Heliothis* control than the insecticide sprays and it is likely that more bolls were damaged by late season infestations of *Heliothis*. The insect damage would then provide an entry point for pathogens into the Coker 312 seed. This may be the reason that the Coker seedlings were stunted and more susceptible to disease than the Bollgard™ Cotton Line 531.

To assess germination under field conditions, stand counts were taken at two of the field sites: West Sinton, TX and Bossier City, LA. At West Sinton, stand counts were taken in each of the middle two rows of all 12 replications, for a total of 24 replications. The stand counts for the Coker 312 and Bollgard™ Cotton Line 531 did not differ significantly (Table V-15). Similarly, at Bossier City, stand counts were taken in four of the replications (Table V-16). Again, there was no difference in the stand counts between Bollgard™ Cotton Line 531 and Coker 312.

The field results described above support the conclusion that no meaningful differences exist in the germination or survival rates of Bollgard™ Cotton Line 531 and Coker 312. Additionally, no cooperator has reported a difference in the overall growth and development of Bollgard™ Cotton Line 531 compared to Coker 312. The differences noted in the laboratory assays were not observed in the field.

I. Out-Crossing Potential

The potential for pollen transfer from cotton to other species and for Bollgard™ Cotton Line 531 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. Bollgard™ Cotton Line 531 (*Gossypium hirsutum*) is 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 Bollgard™ Cotton Line 531 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 Bollgard™ Cotton Line 531 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 *B.t.k.* 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 the *B.t.k.* cotton in 1990. One part of these studies was to study the out-crossing potential of these cotton plants. Sites where these tests were located were:

Casa Grande, Arizona
Maricopa, Arizona
Bossier City, Louisiana
Starkville, Mississippi
Brawley, California
College Station, Texas
Lubbock, Texas

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 Table V-17.

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 Texas sites.

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. Non-target Insects

There is extensive information about microbial preparations of *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) containing the *B.t.k.* proteins, including the CryIA(c) protein (*B.t.k.* HD-73). The literature has established that the *B.t.k.* proteins are:

- extremely selective for the lepidopteran insects (MacIntosh *et al.*, 1990; Klausner, 1984; Aronson *et al.*, 1986; Dulmage, 1981; Whitely and Schnepf, 1986),
- bind specifically to receptors on the mid-gut of lepidopteran insects (Wolfersberger *et al.* 1986; Hofmann *et al.* 1988a; Hofmann *et al.* 1988b; Van Rie, *et al.* 1989; Van Rie, *et al.* 1990), and
- have no deleterious effect on beneficial/non-target insects, including predators and parasitoids of lepidopteran insect pests or honeybee (*Apis mellifera*) (Flexner *et al.*, 1986; Krieg and Langenbruch, 1981; Cantwell et al., 1972; EPA, 1988; Vinson, 1989; Melin and Cozzi, 1989).

The chapters by Vinson (1989) and Melin and Cozzi (1989) provide comprehensive reviews of the extensive literature that has established the safety of the *B.t.k.* microbes and encoded proteins to an array of beneficial insects. To compliment these chapters, Monsanto conducted a study to compare the *B.t.k.* protein expressed in Bollgard™ Cotton Line 531 with commercially available microbial pesticides containing *B.t.* The conclusion reached from the results of this study were that the protein expressed by Bollgard™ Cotton Line 531 was similar in molecular weight and immunological reactivity to one or more proteins contained in the commercial *B.t.* products Dipel® and Thuricide®. Thus the literature demonstrating the safety of these insecticides to non-target organisms is useful in predicting the safety of the *B.t.k.* HD-73 protein expressed in Bollgard™ Cotton Line 531. The complete report of this study is found in Appendix X.

To confirm the specificity of the *B.t.k.* HD-73 protein expressed in Bollgard™ Cotton Line 531, a study was completed to evaluate the insecticidal activity of the full-length *B.t.k.* HD-73 protein and the activated, trypsin-resistant core of the protein versus ten species from five different orders of insects, including Lepidoptera. Of the ten species tested, only the four species of Lepidoptera were sensitive to both forms of the *B.t.k.* HD-73 protein. These data confirm the insecticidal specificity of the protein expressed by Bollgard™ Cotton Line 531 for insect species in the Order Lepidoptera.

In addition, separate studies were undertaken to assess the potential toxicity of *B.t.k.* HD-73 protein to other non-target insects:

- parasitic Hymenoptera (*Nasonia vitripennis*), a beneficial parasite of the housefly (*Musca domestica*),
- the larva and adult honey bee (*Apis mellifera* L.), a beneficial insect pollinator,
- ladybird beetles (*Hippodamia convergens*), a beneficial predaceous insect which feeds on aphids and other plant bugs commonly found on stems and foliage of weeds and cultivated plants, and
- green lacewing larvae (*Chrysopa carnea*), a beneficial predaceous insect commonly found on cotton and other cultivated crops.

In each study, the maximum nominal *B.t.k.* HD-73 protein (full-length) concentration tested (20 ppm) was greater than 1600 times the maximum *B.t.k.* HD-73 protein expression level in pollen (0.012 ppm) and nectar (<0.002 ppm) of the Bollgard™ Cotton Line 531. These studies established that the LC50 for the *B.t.k.* HD-73 protein is greater than 20 ppm versus all the species tested. Therefore, the "no observed effect level" was 20 ppm.

2. Non-Target Birds and Fish

A study was conducted to assess the wholesomeness of insect resistant 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 historical data demonstrating safety of *B.t.* proteins to fish 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 *B.t.k.* proteins have been demonstrated to be virtually non-toxic to fish, avian species, non-target insects, mammals and other non-target 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

Based upon the results of these studies, the host range of toxicity of the *B.t.k.* HD-73 protein as produced in the Bollgard™ Cotton Line 531 is comparable to the proteins produced in nature by the *Bacillus thuringiensis* variety *kurstaki* soil microorganism. This protein is accepted by EPA as being non-toxic to all non-target organisms (EPA, 1988).

L. Impact on Endangered Species

No endangered or threatened lepidopteran insects, as listed in 50CFR 17.11 and 17.12, feed on cotton plants.

M. Possible Impact on the Environment

Persistence in the environment following harvest - The *B.t.k.* HD-73 protein in Bollgard™ Cotton Line 531 is present in the plant tissue remaining in the field after harvest of the lint and seed. This cotton plant residue is typically tilled into the soil. The environmental fate of *B.t.k.* HD-73 protein in soil was determined by measuring the rate at which the bioactivity of the *B.t.k.* HD-73 protein dissipates when added to soil as the purified protein and as a component of insect resistant cotton tissue.

Two test substances were used in this study: 1) *B.t.k.* HD-73 that was purified from *E. coli*, characterized and shown to be equivalent to the *B.t.k.* HD-73 protein expressed in insect resistant cotton plants, and 2) lyophilized cotton tissue powder prepared from

field-grown Bollgard™ Cotton Line 931 plants. Bollgard™ Cotton Line 931 expresses the same *B.t.k.* HD-73 protein as Bollgard™ Cotton Line 531. Bollgard™ Cotton Line 931 was used in this study due to its higher expression of the *B.t.k.* HD-73 protein. *B.t.k.* HD-73 purified protein was added to soil at the rates of 0.3, 0.8 and 1.5 µg/ g dry wt soil; Bollgard™ Cotton Line 931 tissue powder was added at 0.01, 0.03 and 0.05 g/ g dry wt soil. These samples were incubated in soil (Dupo silt loam) at approximately 24°C for up to 54 days at a relatively constant soil moisture level. Aqueous soil suspensions was prepared from incubated soil samples, incorporated into artificial insect diet and presented to tobacco budworm *Heliothis virescens* (TBW) larvae. Half-lives were calculated using the equation for first-order rate of dissipation. Recovery of *B.t.k.* HD-73 protein TBW activity was assessed for both test substances at all rates evaluated.

Purified *E. coli B.t.k.* HD-73 protein TBW bioactivity dissipates with an estimated half-life of 9.3 to 20.2 days, depending on the dose. *B.t.k.* HD-73 protein TBW bioactivity, added to soil at 0.01g tissue powder per g dry wt soil as a component of Bollgard™ Cotton Line 931 tissue, dissipates with an estimated half-life of 41 days. Recovery of *B.t.k.* HD-73 protein TBW bioactivity was high when added to soil as the purified protein and as a component of lyophilized cotton tissue powder.

The results of this study suggest that the *B.t.k.* HD-73 protein will degrade readily (estimated half-life of 41 days), when added to soil as a component of post-harvest insect resistant cotton plants. The measured half-life of the purified *B.t.k.* protein in soil is comparable to that measured for the microbial *B.t.k.* preparations (West, 1984; Pruett *et al.*, 1980).

Other potential effects that could conceivably be associated with the commercialization of Bollgard™ Cotton Line 531 were evaluated. A review of all available information including extensive field test results, safety studies and independent scientific research indicates that the commercial use of Bollgard™ Cotton Line 531 will not result in any adverse effects to the environment. In fact, it is likely that commercialization will have a positive impact on the environment by promoting integrated pest management practices and reduced reliance on traditional chemical insecticides.

N. Summary

1. Expression of the Inserted Genes

Bollgard™ Cotton Line 531 has been modified by the insertion of the PV-GHBK04 plasmid which contains the gene imparting the insect resistance trait. Bollgard™ Cotton Line 531 expresses two new proteins, the insecticidally active *B.t.k.* HD-73 protein and the selectable marker, NPTII protein. The *B.t.k.* HD-73 and NPTII proteins were expressed at extremely low and relatively consistent levels in Bollgard™ Cotton Line 531 across all six field sites. Bollgard™ Cotton Line 531 contained less than 2 µg/gram fresh weight of *B.t.k.* HD-73 and less than 4 µg/gram fresh weight of NPTII in leaf and seed tissue, respectively, with levels varying only two to three fold across the six field sites. *B.t.k.* HD-73 protein levels varied less than three fold in young leaf tissue over the growing season. The levels of *B.t.k.* HD-

73 and NPTII proteins in mature, whole plant tissues were much lower, on a fresh weight basis, than in leaf tissue. It was estimated that approximately 1.44 and 19.14 grams/acre of *B.t.k.* HD-73 and NPTII protein, respectively, would enter the soil environment by incorporating the plants from Bollgard™ Cotton Line 531 into the soil after harvest. *B.t.k.* HD-73 protein in nectar (<1.6 ng/g fresh weight of nectar) and pollen (11.5 ng/g fresh weight of pollen) derived from Bollgard™ Cotton Line 531 was extremely low; at or near the level of detection (8.0 ng/g fresh weight of pollen and 1.6 ng/g fresh weight of nectar).

A second selectable marker gene encoding aminoglycoside adenylyltransferase (AAD) is present in the Bollgard™ Cotton Line 531; 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 Bollgard™ Cotton Line 531.

2. Composition, Quality, and Processing of the Seed

The cottonseed and processed cottonseed products from Bollgard™ Cotton Line 531 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, oil, 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 the two lines, except that seed from Bollgard™ Cotton Line 531 showed undetectable aflatoxin levels for all sites whereas seed from the C312 control line showed significant amount of aflatoxin contamination at the Arizona site. Pink bollworm was controlled by the insect resistant plants (unique to the Arizona site), which indirectly lead to the reduction in aflatoxin contamination. Reduction in aflatoxin levels provides an important safety benefit for cottonseed produced by the insect resistant plants which is used for animal feed.

Cottonseed from Bollgard™ Cotton Line 531 processed comparably to the C312 control, with comparable reductions in the levels of gossypol in the processed meal prepared from both lines. No gossypol was observed in refined cottonseed oil. Both *B.t.k.* HD-73 and NPTII proteins were reduced to non-detectable levels in processed cottonseed meal.

3. Plant Pest Risk

Bollgard™ Cotton Line 531 does not pose any different plant pest risk to other plants and the environment than non-transformed cotton varieties.

In all field and green house trials, Bollgard™ Cotton Line 531 plants were repeatedly inspected for any signs of *Agrobacterium* infection and other disease symptoms, and none were found. Bollgard™ Cotton Line 531 possesses 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

insect resistance 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

Bollgard™ Cotton Line 531 and the expressed proteins have no adverse effect on non-target organisms or the environment.

A series of safety studies were conducted with the purified, active ingredient in Bollgard™ Cotton Line 531 (*B.t.k.* HD-73 protein) on several non-target beneficial insects. No toxicity was observed at a level representing approximately 1600 times the maximum *B.t.k.* HD-73 protein expression level in pollen and nectar in the Bollgard™ Cotton Line 531.

An additional 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 historical data demonstrating safety of *B.t.* proteins to fish and the unlikely event of exposure, a study with cottonseed in fish was not considered necessary.

No endangered or threatened lepidopteran insects, as listed in 50 CFR 17.11 and 17.12, feed on cotton plants.

The *B.t.k.* HD-73 protein was shown to degrade readily when added to soil as purified protein or as tissue from insect resistant cotton plants. The rate of degradation was similar to the degradation rates reported for commercial microbial pesticides containing *B.t.k.* protein.

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. In fact, the use of Bollgard™ Cotton Line 531 will have a more positive impact on the environment than the use of chemical insecticides to control lepidopteran caterpillars. The *B.t.k.* protein is ecologically benign, i.e. it breaks down rapidly in the soil, is safe to nontarget organisms such as fish, birds and mammals and specifically controls many species of lepidopteran caterpillars on cotton. In addition, the risk of an uncontrolled introduction of this cotton into the environment through hybridization or out-crossing to a native species resulting in a new weed variety is non-existent on the mainland of the United States.

The consistent Lepidoptera insect control offered by Bollgard™ Cotton Line 531 will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop for control of cotton bollworm, tobacco budworm and pink bollworm. As a result, they will be able to utilize many IPM practices that cannot presently be implemented because of the lack of options other than use of chemical insecticides to control these pests. An increase in the biological and cultural control of non-target cotton pests and a more judicious use of chemical insecticides will result in a positive impact on the environment, which will ultimately be advantageous to the grower and the public as well.

Therefore, it is concluded that the Bollgard™ Cotton Line 531 does not pose any different plant pest risk to other plants and the environment than is now caused by non-transformed cotton varieties.

Table V-1. Mean Expression of the *B.t.k.* HD-73 and NPTII Proteins Across Sites (Bollgard™ Cotton Line 531, 1992 Field Trials).

Tissue	*B.t.k.* HD-73 µg/g fwt*		*B.t.k.* HD-73 Range**	NPTII µg/g fwt*		NPTII Range
Leaf	1.562	(0.148)†	1.18 - 1.94	3.145	(0.269)	2.46 - 3.84
Seed	0.857	(0.180)	0.40 - 1.32	2.451	(0.185)	1.97 - 2.93

* Mean expression level across all field test locations. N=36, 6 samples per each of six sites.

* * The 95% confidence interval for the mean expression levels across field locations expressed as µg/g fresh/frozen weight of tissue (fwt).

† Numbers in parenthesis are the standard error for the mean expression level across all field locations.

Table V-2. Expression of the *B.t.k.* HD-73 and NPTII Proteins in Leaf Tissue from the Bollgard™ Cotton Line 531 (1992 Field Trials).

Site	*B.t.k.* HD-73 µg/g fwt*	*B.t.k.* HD-73 %CV**	NPTII µg/g fwt*	NPTII %CV
Mississippi	1.402	53	3.405	13
Louisiana	1.834	28	2.951	37
Texas	2.037	30	3.980	23
Georgia	1.727	78	2.298	34
Arizona†	1.269	40	3.677	18
Alabama	1.101	27	2.561	7.3
Overall Mean	1.562	50††	3.145	30††

* Mean value among plots in the same location (N = 6 samples per site).

* * Variability among plots in the same location, expressed as % coefficient of variation (%CV).

† Two replicates yielded non-detectable results and were excluded from statistical analyses

†† Variability among all plots in different locations, expressed as %CV.

Table V-3. Expression of the *B.t.k.* HD-73 and NPTII Proteins in Cottonseed from the Bollgard™ Cotton Line 531 (1992 Field Trials).

Site	*B.t.k.* HD-73 µg/g fwt*	%CV**	NPTII µg/g fwt*	%CV
Mississippi	0.524	32	3.156	16
Louisiana	0.529	32	2.601	18
Texas	0.490	24	2.234	11
Georgia	0.983	27	2.343	14
Arizona	1.616	12	1.790	16
Alabama	1.001	16	2.580	17
Overall Mean	0.857	55†	2.451	24†

* Mean value among plots in the same location (N = 6 samples per site).
* * Variability among plots in the same location, expressed as % coefficient of variation (%CV).
† Variability among all plots in different locations, expressed as %CV.

Table V-4. Expression of the *B.t.k.* HD-73 and NPTII Proteins in Young Leaf Tissue of Bollgard™ Cotton Line 531 throughout the Growing Season (1992 Field Trials).

Date of Sampling	*B.t.k.* HD-73 µg/g Fresh Weight §	Range % Recovery of HD-73 Spike †	% Efficiency of Extraction
6/1/92	1.40 (0.74)*	82 - 106 (12) **	80.0 (3.7)††
7/8/92	1.49 (0.59)	63 - 107 (24)	not done
8/10/92	3.55 (1.14)	56 - 105 (24)	not done
9/14/92	1.30 (0.69)	53 - 117 (32)	76.1 (6.0)

§ Mean expression level from analysis of six leaf samples at each time point.
† Range of recovery from buffer (no matrix) was 80.0 to 112.9% for spike levels ranging from 25 to 100 ng/ml. Higher recovery is seen for lower level of spike.
* Numbers in parenthesis represent the standard deviation (n=6 for each time point)
* * Numbers in parenthesis represent the standard deviation for mean percent recovery over 3 spikes, n=6 (duplicates at each level).
†† Mean extraction efficiency for three samples, with standard deviation of the mean in parenthesis.

Table V-5. Expression of the *B.t.k.* HD-73 and NPTII Proteins in Mature Cotton Plants (Bollgard™ Cotton Line 531, 1992 Field Trial*).

Tissue	*B.t.k.* HD-73** µg/g fwt**	*B.t.k.* HD-73** µg/plant	NPTII** µg/g fwt**	NPTII** µg/plant
Mature Cotton Plants	0.044	24.5	0.57	318.8

* One plant from each of 3 reps (N=3), was collected from the Starkville, MS site, per line.

* * these values represent the mean level of expression minus the control background present (due to assay matrix effects) in the control line, C312, and corrected for extraction efficiency and recovery of spike.

Table V-6. Assessment of the *B.t.k.* HD-73 protein levels in cotton nectar and pollen§.

Sample Type	Cotton Line	*B.t.k.* HD-73 (ng/g fwt)	Total Protein (mg/g fwt)
Pollen	C312	<8.0*	7.0 (1.5)†
	Line 531	11.5* <8.0**	12.6 (0.26)
Nectar	C312	<1.6*	
	Line 531	<1.6* <1.6**	<0.06 (0.00)

§ Pollen was collected on a single day from flowers selected from 19 plants. All pollen collected that day was pooled into a single sample. This single pooled sample was sampled three times and evaluated for *B.t.k.* Nectar was collected from all open flowers between 9/18 and 10/26/92; all nectar collected for each day was pooled (14 separate nectar samples were collected on 14 separate days). Reps were prepared by pooling separate days into a single sample, but each pool represents unique samples. Therefore, three true reps of nectar were evaluated for *B.t.k.*

* Pollen and nectar samples were stored for approximately nine months at approximately -80°C before being analyzed. The data for pollen and nectar from Bollgard™ Cotton Line 531 samples represent the mean from three replicates. The data for pollen and nectar from C312 samples represent a single determination.

* * Pollen and nectar samples were stored for approximately two weeks at approximately -80°C before being analyzed. The data for pollen from Bollgard™ Cotton Line 531 represents a single determination. The data for nectar from Bollgard™ Cotton Line 531 represents the mean value of two replicates.

† Protein levels for pollen and nectar represent the mean and standard deviation () from three replicate samples

Table V-7. Proximate Analysis of Cottonseed from Bollgard™ Cotton Line 531 and Control Line, C312, Grown under Field Conditions[1,2]

Component	Coker 312	Bollgard™ Cotton Line 531
Protein	22.7 (2.6)	22.8 (2.1)
Fat (Oil)	19.7 (2.1)	20.8 (2.5)
Carbohydrates	38.5 (1.9)	39.1 (2.3)
Ash	3.8 (0.4)	3.9 (0.4)
Moisture	15.4 (5.1)	13.5 (3.9)
Calories	422.0 (29.2)	434.5 (26.0)

[1] Components are expressed as g/100 g except for calories, which are expressed as calories/100 g.

[2] The values for all components represent the mean across all six field locations (1 sample per site, N=6). The numbers in parentheses indicate the standard deviation of the mean. Means were compared using the paired t-test described in Table V-1.

Table V-8. The Level of Lipids in Cottonseed from Bollgard™ Cotton Line 531 and Coker 312 Grown under Field Conditions[1]

	Site Number						
Line Number	701 MS	702 LA	703 TX	704 GA	705 AZ	706 AL	mean[2]
C312	35.4	39.0	37.1	42.6	38.0	43.2	39.21
Line 531	36.0	45.3	40.7	41.5	34.1	42.1	39.97

[1] Lipid levels were determined for cottonseed grown in study 92-01-36-07, experiments 92-427-701 through 91-427-706 and are expressed as the percent of total lipid compared to the lyophilized dry weight of the cotton meal. Sites 701 through 706 refer to: Starkville, MS; Bossier City, LA; College Station, TX; Tifton, GA; Maricopa, AZ; and Loxley, AL, respectively.

[2] Mean percent lipid across all sites (1 sample per site, N=6). Comparison of the means were performed by pairing values within each site; the value for C312 was subtracted from the value for Bollgard™ Cotton Line 531. A one sample t-test was then completed on the resulting differences for each analyte using a 0.05 level of significance.

Table V-9. Levels of Major Fatty Acids in Seed from Bollgard™ Cotton Line 531 and Control Line C312 Grown under Field Conditions[1]

Fatty Acid	Normal Range/Mean	Line No.	Site Number/Location 701 MS	702 LA	703 TX	704 GA	705 AZ	706 AL	mean[2]
14:0	(0.64-1.30)[5]	312	0.9	0.9	1.0	0.8	1.1	0.7	0.90
		531	0.7	0.8	0.8	0.8	0.9	0.6	0.77*
16:0	(22.18-27.76)[5]	312	20.9	24.0	25.7	23.9	27.9	21.7	24.01
		531	22.7	25.5	23.8	25.9	26.6	23.4	24.65
16:1	(0.56-0.82)[4]	312	0.6	0.6	0.7	0.6	0.7	0.5	0.62
		531	0.5	0.6	0.6	0.5	0.7	0.5	0.57
17:0		312	0.1	0.2	0.1	0.1	0.2	0.2	0.15
		531	0.2	0.3	0.1	0.2	0.2	0.2	0.20
18:0	(2.14-3.23)[5]	312	2.0	2.2	2.2	2.3	2.6	2.2	2.25
		531	2.4	2.7	2.5	2.6	2.7	2.6	2.58*
18:1	(13.95-21.16)[5]	312	15.0	15.0	16.6	15.1	16.7	14.9	15.54
		531	16.4	17.1	17.1	15.0	18.4	16.6	16.77*
18:2	(45.84-57.83)[5]	312	55.0	52.0	49.8	53.0	45.0	53.2	51.33
		531	52.4	46.4	50.9	44.1	46.4	51.4	48.60
18:3	(0.23)[5]	312	0.2	0.2	0.1	0.2	0.2	0.2	0.18
		531	0.2	0.2	0.2	0.2	0.2	0.2	0.20
20:0	(0.41)[5]	312	0.2	0.2	0.2	0.2	0.3	0.2	0.22
		531	0.2	0.3	0.2	0.2	0.3	0.2	0.23
22:0		312	0.1	0.1	0.1	0.2	0.1	0.1	0.12
		531	0.1	0.2	0.1	0.1	0.2	0.1	0.13
24:0	(0.18)[5]	312	0.1	nd[3]	nd	0.1	nd	nd	0.03
		531	nd	nd	nd	0.1	nd	nd	0.02

1 Values are expressed as % of the total lipid; see Table 1 for field test sites.

2 1 sample per site, N=6. Means indicated by an asterisk were found to be different from the control line (C312) at a 0.05 level of significance using the paired t-test procedure described in Table V-1.

3 nd = not detected

4 Cherry, J.P., and Leffler, H.R. Seed. In *Cotton:* (1984) Kohel, R.J., and Lewis, C.F., Eds., Amer. Soc. Agron.: Madison, WI. Chapter 13, pp 512-558.

5 Cherry, J.P. (1983), Cottonseed Oil. JAOCS 60: 312-319.

Table V-10. The Level of Toxicants in Cottonseed from Bollgard™ Cotton Line 531 and Coker 312 Grown under Field Conditions[1]

Toxicant	Normal Range	Line No.	Site Number/Location 701 MS	702 LA	703 TX	704 GA	705 AZ	706 AL	Mean[6]
Gossypol[2]	(0.39 - 1.7)[2]	312	1.33	1.46	1.25	1.44	1.13	1.39	1.33
		531	1.21	1.22	1.21	1.43	1.09	1.49	1.28
C-19[3]	(0.2 - 0.8)[4]	312	0.3	0.3	0.2	0.3	0.3	0.2	0.27
		531	0.3	0.3	0.2	0.2	0.2	0.2	0.23
Sterculic[3]	(0.3 - 0.7)[4]	312	0.5	0.5	0.5	0.5	0.8	0.7	0.58
	(0.3 - 0.5)[7]	531	0.5	0.9	0.6	1.5	0.5	0.5	0.75
Malvalic[3]	(< 0.1 - 1.9)[4]	312	0.4	0.1	0.3	0.5	0.3	0.2	0.30
	(0.7 - 1.5)[7]	531	0.5	0.1	0.4	0.3	0.2	0.4	0.32
Aflatoxin[5]									
B1		312	nd	nd	nd	nd	92.7	nd	n/a
		531	nd	nd	nd	nd	nd	nd	n/a
B2		312	nd	nd	nd	nd	4.4	nd	n/a
		531	nd	nd	nd	nd	nd	nd	n/a
G1		312	nd	nd	nd	nd	nd	nd	n/a
		531	nd	nd	nd	nd	nd	nd	n/a
G2		312	nd	nd	nd	nd	nd	nd	n/a
		531	nd	nd	nd	nd	nd	nd	n/a

1 Toxicant levels were determined for Cottonseed grown in study 92-01-36-07, experiments 92-427-701 through 91-427-706 and are expressed as percent of seed by weight; sites 701 through 706 refer to: Starkville, MS; Bossier City, LA; College Station, TX; Tifton, GA; Maricopa, AZ; and Loxley, AL, respectively.

2 Expressed as percent of seed on a dry weight basis. Range reported in Berardi and Goldblatt, 1980.

3 C-19 = dihydrosterculic acid. Levels of C-19, sterculic and malvalic acids are reported as percent of total lipids in the seed, on a dry weight basis

4 Wood, R., 1986b.

5 Reported as parts per billion (ppb); nd = not detected (less than 1 ppb) ; n/a = mean not appropriate.

6 1 sample per site, N=6. Means were compared using the paired t-test described in Table V-1.

7 Phelps *et al.*, 1965.

Table V-11. Yield Fractions from Processing Cottonseed.

Process Fraction§	Yield (lbs) Line C312	Yield (lbs) Line 531	% Yield Line C312	% Yield Line 531	% Yield Across Cultivars	
Fuzzy Cottonseed	45.1	50.9	n/a*	n/a*		
Delinted Cottonseed	36.4	41.9	80.7†	82.3†		
Hulls	9.7	11.7	26.7††	27.9††	25.5[2]	
Linters	5.3	7.5	11.8†	14.7†	9.9-12.4[1]	8.4[2]
Kernels**	24.3	29.8	53.9†	58.6†	43.5-53.4[1]	46.0[2]
Crude Oil	4.93	6.30	13.5††	15.0††	16.3[2]	
Refined Oil**	4.12	6.17	11.3††	14.7††		
Toasted Meal**	12.0	15.4	33.0††	36.8††		

§ Based on 1 sample per line bulked across all sites
1 Cherry and Leffler, 1984
2 Cottonseed and its Products, 1989.
* n/a = not applicable, yields for % fuzzy seed in seed-cotton not calculated.
* * Free and total gossypol were measured in these fractions.
† Percent weight of fuzzy cottonseed.
†† Percent weight of delinted seed.

Table V-12. Summary of Gossypol Levels in Kernel, Toasted Meal and Refined Cottonseed Oil.

Fraction[1]	% Free Gossypol* Line C312	% Free Gossypol* Line 531	% Total Gossypol Line C312	% Total Gossypol Line 531
Kernels**	1.18	1.10	1.47	1.34
Toasted Meal	trace †	0.004	1.20	1.11
Refined Oil	ND††	ND	ND§	ND§

1 Based on 1 sample per line bulked across all sites
* based upon dry weight sample.
* * prior to processing.
† detectable, but not quantifiable.
†† not detected at 0.000008% or 8 parts per million on a weight basis.
§ not detected, values not different than average blank, lowest standard for oil would translate detection limit to <0.04% total gossypol per weight of oil used for analysis.

Table V-13. Allelochemical Levels in Vegetative Tissues from Bollgard™ Cotton Line 531 and Control Line, C312.*

A. 1992 Field Season†

Line	Tissue	Gossypol	Anthocyanin	Flavonoid	Tannin
531	Square	0.202	0.083	0.427	9.755
C312	Square	0.223	0.081	0.390	9.519
531	Leaf	0.123	0.203	0.875	13.36
C312	Leaf	0.144	0.220	0.822	13.60

B. 1993 Field Season†

Line	Tissue	Gossypol	Anthocyanin	Flavonoid	Tannin
531	Square	0.291	0.12	0.38	12.25
C312	Square	0.294	0.11	0.39	14.89
531	Leaf	0.120	0.31	0.72	11.85
C312	Leaf	0.143	0.34	0.80	17.11

* Reported as percent of dry weight of tissue.

† Mean value reported from six samples taken from replicated plots for each line.

Table V-14. Germination and vigor results for Bollgard™ Cotton Line 531 and Coker 312 grown in North Carolina in 1993.

Line	% germ. (std.)	% germ. (cold)	% w/ radicle > 1 in (cold)	% diseased (std)
C 312	70 a	60 b	41 b	16 a
Line 531	84 a	83 a	75 a	10 a

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

Table V-15. Stand Counts* at West Sinton, Texas

Line	Avg. # plants/30 ft
Coker 312	137.5 a
Bollgard™ Cotton Line 531	139.7 a

Means followed by the same letter do not significantly differ (Duncan's MRT, P=0.05).
Planted on 5/17/93 at a seeding rate of 5 seeds/foot
* Plants per 30 foot of row

Table V-16. Stand Counts* at Bossier City, Louisiana

Line	Avg. # plants/30 ft
Coker 312	107 a
Bollgard™ Cotton Line 531	109 a

Means followed by the same letter do not significantly differ (Duncan's MRT, P=0.05).
Planted on 5/12/93 at a seeding rate of 4.5 seeds/foot, counts taken on 6/30
* Plants per 30 foot of row

Table V-17. Percent Outcrossing at varying distances from the *B.t.k.* cotton observed at six sites in 1990.

Approximate distance from test (ft)	Location: College Station % *	Halfway %	Halfway S.D.†	Brawley %	Brawley S.D.	Maricopa %	Bossier City %	Bossier City S.D.	Starkville %	Starkville S.D.
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. Current Cotton Agronomic Practices and the Impact of Insect Resistant Cotton on Cotton Pest Management

Luttrell *et al.* reviews the current agronomic practices for cotton production and the potential impact of insect resistant cotton on cotton pest management. The following is a summary of this review, which can be found in Appendix I.

Cotton production in the United States is highly mechanized and dependent upon maximum utilization of new technology to remain competitive in a worldwide market. Pest problems, particularly insects, and environmental constraints, such as inadequate temperature and moisture, are major limiting factors to optimum cotton production. Most cotton production regions of the United States rely on extension specialists and crop consultants to design and implement effective IPM programs. Insect control decisions are largely based on routine field monitoring by agricultural consultants, extension personnel and growers. The intensity of monitoring varies among locations and is associated with production capabilities, potential insect damage and availability of consultants (Luttrell 1994). Numerous advances in IPM technology (Frisbie and Adkisson 1985, Frisbie *et al.* 1989) have encouraged a systems approach to insect management in United States cotton where insect control decisions are integrated into an overall crop production and management scheme. Perhaps the best example of this is the wide acceptance of early-maturing varieties and short-season cotton production systems first recommended in Texas. The Texas system of short-season cotton production (Walker *et al.* 1978) has been widely adopted across United States cotton and is recommended by agronomists and entomologists because it optimizes the production of valuable fiber and encourages the "avoidance" of damaging late-season populations of insects.

Although advances in IPM technologies have fostered improved cotton insect management systems, insect control is still largely based on the use of chemical insecticides, which include all classes of chemical insecticides such as pyrethroids, organophosphates, carbamates, etc. (Herzog *et al.* 1993). Estimates of insect control costs and losses (Head 1991, 1992, 1993) averaged for the 1990's indicate that United States cotton growers apply an average of 4.86 applications of insecticide to 11.8 million acres of cotton and spend more than $400 million each year for control of cotton insects. This represents a large portion of total insecticide use in the United States. Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests (Luttrell 1994). The need for alternative insect control measures is becoming more critical to profitable cotton production in the United States. Environmental concerns limit the availability of existing insecticide chemistry and increase the developmental costs of new chemistry. Because of the high costs of developing and registering new insecticide chemistry, availability has declined over the past few years.

Bollgard™ Cotton Line 531 offers unique, innovative alternatives to traditional chemical control measures. Although alternative insect control tactics are often cited as major components of cotton IPM and research is continuously pursuing improved management methods (Frisbie *et al.* 1989), few alternative insect control methods are of sufficient efficacy to replace chemical control methods. Other methods, such as biological control, host plant resistance and cultural control, provide suppression of pest populations without disrupting natural control, but generally lack the high efficacy and curative action of conventional insecticides. Bollgard™ Cotton Line 531 is the first major exception to this historical trend.

Bollgard™ Cotton Line 531 offers new mechanisms to produce and deliver a highly effective insecticide to target pests (i.e. production by cells of the crop plant rather than industrial facilities and application by spray equipment). The technology couples the environmental advantages of host plant resistance with the efficacy of an effective biological insecticide. Since the insecticidal activity is expressed throughout the plant for the entire season, improved control of some pest species over that provided by conventional insecticides is likely. Current technology which depends on foliar application of insecticides cannot dependably deposit insecticides to some regions of the plant canopy infested by pest species. This is especially true of pests that burrow and feed inside plant tissue (e.g. pink bollworms). Because Bollgard™ Cotton Line 531 expresses the *B.t.k.* protein that only has activity against certain Lepidoptera and must be ingested to kill the pest, the technology offers selective activity against susceptible lepidopteran pest complexes without directly disrupting pest suppression by natural enemies, such as parasites and predators.

B. Development of Pest and Resistance Management Strategies for Insect Resistant Cotton

Some organisms are resistant to single or multiple pesticides in use today. It has not been established whether this resistance is because the organism has adapted metabolically to be able to tolerate the effects of the pesticide, or that a small segment of the population was naturally resistant and dominate as the numbers of the susceptible members have been reduced. Regardless of how resistance is obtained, it is a potentially serious problem with some pests.

Some insect resistance to the *B.t.k.* insect control protein has been reported in the past 5 years. Examples of insects for which resistance has been reported are the Indianmeal moth (*Plodia interpunctella*), almond moth (*Caudra cautella*) and the diamondback moth (*Plutella xylostella*). There are also some examples of insecticides such as the organophosphates for which little resistance has been reported. In fact some of these chemical insecticides are able to control the same insects at the same dosages as when they were commercialized over 30 years ago.

It is currently not possible to accurately predict whether resistance will occur by an insect to an insecticide. Therefore, is important that every insecticide commercialized be used in a manner and as a part of an overall pest control program so as to maximize its usefulness. Monsanto is developing a pest control strategy aimed at reducing the probability of resistance becoming a problem. This strategy is included in

Appendix XI of this Petition for Determination of non-Regulated Status. This will be offered to growers choosing this cottonseed. We believe by implementing these strategies, the development of resistance (if it occurs at all), can be managed to maximize the usefulness of this modified cotton.

To achieve the benefits described above, it is important that insect resistant cotton be implemented and managed properly. In this respect, these plants are no different than any other crop protection product that has been used over the last century. It is clear from the knowledge gained over that time, that to successfully maximize the long-term use of insect resistant cotton, two interconnected management components are required. First, is the development of integrated pest management techniques that allow the farmer to optimize the utility of these plants for cotton pest control. In essence, this is the development of a total insect management package that will be centered around insect resistant cotton. Second, to maximize the durability of this cotton, is the development and implementation of strategies targeted to prevent the development of insect resistance to the insect control protein produced by the plants.

For the last several years, extensive consultations have been held with the leading cotton pest and resistance management researchers to develop a program to maximize the use and durability of insect resistant cotton. Laboratory and field studies designed in collaboration with these experts from academia and extension are in progress and are providing the data needed for developing this management program. These studies are examining the impact of insect resistant cotton on populations of beneficial and pest insects endemic to the crop, the impact on the use of conventional insecticides for controlling non-target pests, the establishment of the baseline susceptibility of our insect targets to the *B.t.k.* insect control protein, and the impact of mixtures of resistant and non-resistant plants on yield loss.

Monsanto scientists have worked for several years on laboratory and field studies of insect resistance, and with outside collaborators nearly every suggestion made for resistance management in insect resistant cotton is being examined. These strategies, developed in consultation with an expert advisory panel, take into account existing research and an understanding of cotton production and agronomic practices. They include:

1) High dose expression of the *B.t.k.* insect control protein in cotton to control caterpillars heterozygous for resistance alleles.

2) Refugia as hosts for sensitive insects provided through non-insect resistant cotton.

3) Monitoring of insect populations for susceptibility to the *B.t.k.* insect control protein.

4) Agronomic practices that minimize insect exposure to the *B.t.k.* insect control protein.

5) Development of novel lepidopteran control proteins with a distinct mode of action from the *B.t.k.* insect control protein.

Those pest and resistance management strategies best suited for use in cotton production and with the potential for delaying or preventing the development of resistance will be recommended. In addition, an extensive effort has been initiated to educate cotton growers as to the most effective ways to integrate insect resistant cotton within their current production practices. This cooperative effort between growers, academia, extension, seed company partners and Monsanto will help ensure that the benefits of insect resistant cotton are fully realized and sustained.

C. 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 the Bollgard™ Cotton Line 531 plants 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 the *B.t.k.* insect control protein and the Bollgard™ Cotton Line 531 plant.

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

D. Potential for the Bollgard™ Cotton Line 531 to Become a Weed

Bollgard™ Cotton Line 531 is not expected to have any different weedy characteristics than other cotton grown in the United States. A detailed discussion of the potential for Bollgard™ Cotton Line 531 to become a weed is addressed in Part V paragraph G, of this Petition for Determination of Non-Regulated Status.

E. Increased Numbers of Beneficial Insects

Aside from the benefit of a decrease in the use of chemical insecticides an additional benefit has been identified, that being an increase in the numbers of beneficial insects present in the cotton fields.

The worst enemies of most insects are predatory insects. These predators feed on other insects thus providing a "natural" level of control. Most chemical insecticides used in cotton are fairly general in the range of insects controlled, and therefore, most insects including the beneficial predators are controlled. Over the period of a growing season their numbers can be depleted to the point that control of pests by the predators is essentially non-existent. Since the *B.t.k.* insect control protein is very specific in its range of control, an increase in the numbers of beneficial insects has been observed in the field and are expected to supplement the control of the cotton insect pests. This increased presence of beneficials will likely reduce the need for insecticide applications targeted to control of cotton pests not susceptible to the *B.t.k.* insect control protein.

Conclusion

None of the environmental consequences identified are of a nature as to justify that Bollgard™ Cotton Line 531 should not be commercialized. Bollgard™ Cotton Line 531 is not expected to become a weed 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 *B.t.k.* protein and the Bollgard™ Cotton Line 531 cotton plants. The positive consequences of reduced pesticide use, increases in the numbers of beneficial insects, the substantial equivalence of Bollgard™ Cotton Line 531 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.

Finally, the potential for susceptible cotton insect pests to develop resistance to the *B.t.k.* protein has been considered and resistance management options developed. When one considers the benefits that this cotton will provide to the grower, the public and the environment, (the decreased use of chemical insecticides), it is justified to proceed in this careful manner versus the alternative of not allowing Bollgard™ Cotton Line 531 to be commercialized.

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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 Bollgard™ Cotton Line 531 developed using the plasmid vector PV-GHBK04. Therefore, on the basis of the substantial potential benefits to the farmer, the environment, and the significantly reduced risk to public health, Monsanto requests that Bollgard™ Cotton Line 531 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 insect resistant cotton.

Appendix I

Agronomic Benefits of Insect Resistant Cotton

Impact of Transgenic Cotton Expressing Endotoxin Proteins from Bacillus thuringiensis on Cotton Insect Management in the USA

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Background

Transgenic cotton expressing delta endotoxin proteins of *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*-cotton) represents one of the first implementable products of plant genetic engineering for production agriculture (Gasser and Fraley 1989, Meeusen and Warren 1989, Vaech *et al.* 1987). Development of *B.t.k.*-cotton has progressed from initial insertion of *B.t.k.* genes into cotton plants in 1987 (Umbeck *et al.* 1987) and 1988 (Deaton 1991, Perlak *et al.* 1990) to the current state of a commercial insect-control product with confirmed high levels of efficacy (Bartlett 1993, Benedict *et al.* 1991, 1992, 1993, Buehler 1993, Deaton 1991, Gannaway *et al.* 1991, Jenkins *et al.*. 1991, 1992, 1993, Micinski and Caldwell 1991, Williamson and Deaton 1991, Wilson and Flint 1991, Wilson *et al.* 1992, 1993). Early field tests in 1989 of initial B.t.k.-cottons developed by Agracetus (Middleton, Wis.) indicated low levels of protein expression in the plants and low levels of insect control (Benedict *et al.* 1992, Jenkins *et al.* 1990, Umbeck *et al.* 1990). Improved expression of the insect control protein genes as a result of coding sequence modifications by Monsanto (St. Louis, Missouri) scientists (Amstrong *et al.* 1990, Deaton 1991, Perlak *et al.* 1991) resulted in transgenic cottons with higher levels of insect control (Benedict *et al.* 1993, Jenkins *et al.* 1993, Wilson *et al.* 1992). Mortality rates of tobacco budworm, one of the most important insects pests of cotton, exposed to these improved *B.t.k.*-cottons (Benedict *et al.* 1992, 1993, DeSpain *et al.* 1993) were as high as those expected from efficacious chemical insecticides [i.e. greater than 85% mortality (Luttrell *et al.* 1987, Roush and Luttrell 1989)] and much higher that those obtained with conventional spray applications of *B. thuringiensis* var. *kurstaki* [i.e. less than 60% mortality (Luttrell *et al.* 1982)].

Cotton production in the USA is highly mechanized and dependent upon maximum utilization of new technology to remain competitive in a worldwide market. Pest problems, particularly insects, and environmental constraints, particularly adequate temperature and moisture, are major limiting factors to optimum cotton production.

Most cotton production regions of the USA rely on extension specialists and crop consultants to design and implement effective integrated pest management (IPM) programs. Insect control decisions are largely based on routine field monitoring by agricultural consultants, extension personnel, and growers. The intensity of monitoring varies among locations and is associated with production capabilities, potential insect damage, and availability of consultants (Luttrell 1994). Numerous advances in IPM technology (Frisbie and Adkisson 1985, Frisbie *et al.* 1989) have encouraged a systems approach to insect management in USA cotton where insect control decisions are integrated into an overall crop production and management scheme. Perhaps the best example of this is the wide acceptance of early-maturing varieties and short-season cotton production systems first recommended in Texas. The Texas system of short-season cotton production (Walker *et al.* 1978) has been widely adopted across USA cotton and is recommended by agronomists and entomologists because it optimizes the production of valuable fruit and encourages the "avoidance" of damaging late-season populations of insects.

Although advances in IPM technologies have fostered improved insect management systems in USA cotton, insect control is still largely based on the use of chemical insecticides (Herzog *et al.* 1993). Estimates of insect control costs and losses (Head 1991, 1992, 1993) averaged for the 1990's indicate that USA cotton growers apply an average of 4.86 applications of insecticide to 11.8 million acres of cotton and spend more than $400 million each year for control of cotton insects. This represents a large portion of total insecticide use in the USA. Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests (Luttrell 1994). The need for alternative insect control measures is becoming more critical to profitable cotton production in the USA. Environmental concerns are limiting the availability of existing insecticide chemistry and increasing the developmental costs of new chemistry. Because of the high costs of developing and registering new insecticide chemistry, availability of new insecticide chemistry has declined over the past few years.

Transgenic cotton plants expressing insecticidal proteins offer unique, innovative alternatives to traditional chemical control measures. Although alternative insect control tactics are often cited as major components of cotton IPM and research is continuously pursuing improved management methods (Frisbie *et al.* 1989), few alternative insect control methods are of sufficient efficacy to replace chemical control methods. Other methods, such as biological control, host plant resistance, and cultural control, provide suppression of pest populations without disrupting natural control, but generally lack the high efficacy and curative action of conventional insecticides. *B.t.k.* cotton is perhaps the first major exception to this historical trend.

Transgenic cotton offers new mechanisms to produce and deliver an insecticide to target pests (i.e. production by cells of the crop plant rather than industrial facilities and application by spray equipment). The technology actually couples the environmental advantages of host plant resistance with the efficacy of an effective conventional insecticide. Since the insecticidal activity is expressed throughout the plant for the entire season, improved control of some pest species over that provided by conventional insecticides is likely. Current technology which depends on foliar application of insecticides cannot dependably deposit insecticides to some regions of the plant canopy

infested by pest species. This is especially true of pests that burrow and feed inside plant tissue. Because *B.t.k.*-cotton expresses insecticidal proteins that only have activity against certain Lepidoptera (moths and caterpillar insects) and must be fed upon to kill the pest, the technology offers selective activity against susceptible lepidopteran pest complexes without directly disrupting pest suppression by natural enemies, such as parasites and predators.

The accomplishments of molecular biology and genetic engineering over the past 10 years have created an abundance of social and economic questions relative to transgenic plants. The unique characteristics of this new technology provide, perhaps, the best historical opportunity to reduce the inputs of conventional insecticides (most of which are nerve poisons) and still maintain optimum protection of cotton from economically damaging pest populations. Growers are anxious to obtain this new technology because of the demonstrated high levels of insect control afforded by *B.t.k.*-cotton. Other factors that contribute to the heightened interest in *B.t.k.*-cotton are recurring problems with insecticide resistant pests of cotton (Elzen *et al.* 1992), outbreaks of secondary and new pests, and increased societal demands for long-term, environmentally safe and biologically rational methods of pest control.

Questions about the environmental safety of transgenic plants have dominated much of the interest in the technology. There also has been a great deal of interest focused on the potential development of pest populations resistant to the *B. thuringiensis* endotoxins and recommended deployment strategies to manage resistance (McGaughey and Whalon 1992). These are important issues that must be considered in deployment strategies for *B.t.k.*-cotton. However, it is equally important to recognize that *B.t.k.*-cotton offers a truly efficacious, environmentally safe alternative to conventional insecticidal control.

This report examines the potential impacts of *B.t.k.*-cotton on current cotton IPM programs and speculates what future opportunities may develop as implementation and improvement of the technology advances. These projections are based on current knowledge of a new technology which is less than 5 years of age and an appreciation of the importance of managing insect resistance to insecticides. Because cotton production and associated IPM programs vary across the different geographic regions of cotton production in the USA, regional perspectives of the possible role of *B.t.k.*-cotton in IPM programs are included. Estimates of the crop loss and control costs associated with cotton insect pests were developed from 1990, 1991, and 1992 data published by the Beltwide Cotton Conference (Head 1990, 1991, 1992). These estimates were used throughout the report as a standardized reference to the level of economic damage and control costs involved. Percent crop loss refers to amount of crop damage suffered in the presence of control measures. Total economic loss includes control costs and crop loss. All data in Tables 1, 2, and 3 were derived by averaging 1990-1992 annual estimates and arranged relative to the amount of crop loss and insecticide use due to: (a) tobacco budworm-bollworm complex (*Heliothis-Helicoverpa* complex) alone, (b) all Lepidoptera (includes tobacco budworm-bollworm complex), and (c) all insects (includes all Lepidoptera). Current research indicates that *B.t.k.*-cotton will provide a high level of control of the tobacco budworm-bollworm complex and pink bollworm. Less experimental data are available on the effects of *B.t.k.*-cotton on other Lepidoptera. Comparing estimates for tobacco budworm-bollworm complex and all Lepidoptera should provide a realistic range of possible estimates for the value of *B.t.k.*-cotton. Since

B.t.k.-cotton only affects Lepidoptera, the impact of non-lepidopteran pests on cotton production (i.e. those not directly affected by *B.t.k.*-cotton) can be estimated by subtracting crop loss and control cost data for all Lepidoptera from that reported for all insects (Tables 1, 2, 3).

Potential Impact of *B.t.k.* Cotton on Cotton Pest Management

Pimental *et al.* (1989) suggested that genetic engineering would improve crop yields and improve the efficiency of crop production. In an early review of the potential effects of genetically engineered crops on insect control, Meeusen and Warren (1989) listed several potential advantages and disadvantages (or uncertainties) of the new technology from an agricultural industry perspective. The advantages envisioned were:

1. growers would be less dependant on favorable weather conditions for application of insecticides because insecticidal activity would be continuously expressed and not altered by inclement weather,

2. lower locations of plant canopies (or locations inside tissues) where insecticide sprays cannot be deposited dependably would be protected from insect damage because the insecticidal toxins could be expressed constitutively (i.e. in all tissues and cells) throughout the plant,

3. the need to scout crops would be reduced because of the continuous expression of insecticidal activity,

4. the costs of spraying crops would be eliminated or greatly reduced,

5. the cost of developing a commercial insect-resistant crop line (genetically engineered) would be less than that of developing a new chemical insecticide (currently at $60 to $100 million),

6. spray drift and groundwater contamination would be reduced because the active materials are produced directly in the crop tissue,

7. adverse effects on non-target organisms should be reduced because the only organisms able to receive a dose of the active material would be those feeding on the crop,

8. monitoring of crops for safety for human consumption should be easier since the insecticidal protein expression would be known in advance of harvest and the need for expensive toxicological and residue tests would be eliminated or reduced.

Disadvantages envisioned by Meeusen and Warren (1989) included the likely selection for pest populations resistant to the insecticidal toxins and uncertainties over regulatory and patent procedures and policies. Some of these issues have been resolved or are in the process of being resolved through private- and public-sector sponsored research.

Based on our current knowledge and perspectives as public supported entomologists, we believe that *B.t.k.*-cotton offers unique opportunities to improve existing IPM programs on cotton. Most of the opportunities are associated with the potential of *B.t.k.*-cotton to reduce the use of conventional insecticides. Reduced insecticide use will provide expanded opportunities for non-insecticidal control measures previously limited by the ecological disruptive nature of broad-spectrum, conventional insecticides.

Reduced Insecticide Use

The most obvious and direct effect of *B.t.k.*-cotton on existing cotton pest management is the likely reduction in use of chemical insecticides for control of susceptible lepidopterous pests, especially the tobacco budworm (*Heliothis virescens*) and the bollworm (*Helicoverpa zea*). Species of Lepidoptera vary in their inherent susceptibility to *B.t.k.* proteins (Hofte and Whiteley 1989, Krieg and Langenbruch 1981, MacIntosh *et al.* 1990). The tobacco budworm is more susceptible than the bollworm to the endotoxin proteins. However, initial field tests (Benedict *et al.* 1991, 1993, Jenkins *et al.* 1992) suggest that *B.t.k.*-cotton will provide a high level of field control of both pest species. Nationwide, the tobacco budworm-bollworm complex accounts for 39.6% of all acre applications (acre application = 1 application of an insecticide on 1 acre) of insecticide in cotton (Table 2). The percent of total insecticide applications directed at the tobacco budworm-bollworm complex is 58.2, 37.2, 30.3, and 3.6%, respectively, for the Southeast, Mid-south, Southwest and West regions of cotton production in the USA. Considerable variation exists among (Table 1) and within (Table 3) different cotton production regions in the intensity of insecticide use for tobacco budworm-bollworm; however, eliminating insecticide use for this pest complex would be a major economic and ecological accomplishment for USA cotton production. During the past 3 years, USA cotton growers have annually spent an average of $180 million for control of this pest complex on cotton. Although *B.t.k.*-cotton may not eliminate all of the insecticide applied to control tobacco budworm-bollworm on all of the cotton acreage in the USA, current research indicates that the technology possesses efficacy necessary to have a major impact on insecticide use directed at tobacco budworm-bollworm. Actual use in the production system will be influenced by marketing policies and alternatives.

Potential effects of *B.t.k.*-cotton on species of Lepidoptera other than the tobacco budworm-bollworm complex are less defined. Armyworms (*Spodoptera* spp.) are more tolerant to endotoxin proteins than the tobacco budworm and the bollworm (Jenkins *et al.* 1992), and the level of control expected from *B.t.k.*-cotton is questionable at this time. However, large plot field studies have suggested that damage from the beet armyworm (*S. exigua*) will be reduced in *B.t.k.*-cotton (Luttrell personal observation., Wilson *et al.* 1992). Control of these less susceptible species may be higher than that suggested from results of laboratory assays because the continuous expression of insecticidal activity in the transgenic plants will insure continuous contact with the toxin. A cumulative toxic effect is likely. Several other pest species, particularly the pink bollworm (*Pectinophora gossypiella*), are very susceptible to endotoxin (Graves and Watson 1970), and *B.t.k.*-cotton offers an excellent opportunity to reduce insecticide use for these species. If one assumes that *B.t.k.*-cotton will effectively eliminate insecticide applications for all lepidopterous pests of cotton, insecticide use could be reduced by more than 45% (Table 2) and the total costs of controlling cotton

insects could be reduced by approximately 50% (Table 2). However, the effects of *B.t.k.*-cotton on many species of Lepidoptera are not experimentally tested, and these savings do not include the price of *B.t.k.*-cotton seed. Projecting benefits of *B.t.k.*-cotton on the basis of eliminating the crop loss and insecticide costs of all Lepidoptera is likely an over-estimate of the actual benefits of *B.t.k.*-cotton. However, the vast majority of control costs and crop loss due to lepidopterous pest attacking cotton are associated with species that are very susceptible to *B.t.k.*-cotton (tobacco budworm, bollworm, pink bollworm). Most of the insecticide directed against lepidopterous pests in the Mid-south and Southwest is targeted at the tobacco budworm-bollworm complex. In the Southeast, more insecticide is used for control of other lepidopterous pests, especially the beet armyworm, European corn borer (*Ostrinia nubilalis*), and soybean looper (*Pseudoplusia includens*), but tobacco budworm-bollworm is the primary target of control measures. Most of the insecticide directed against lepidopterous pests of cotton in the West is targeted at pink bollworm. Because pink bollworm is extremely susceptible to endotoxin proteins (Graves and Watson 1970, Bartlett 1993), *B.t.k.*-cotton offers a unique opportunity to reduce insecticide use in the West for pink bollworm and improve the marginal efficacy of current chemical control methods. Eliminating use of insecticide for lepidopterous pests of cotton in the entire USA would result in a savings of $206 million in insecticide costs to the cotton industry (Table 2).

Expanded Opportunities for Biological Control

Field surveys indicate that the number of arthropod species associated with the cotton may range from a few hundred to more than a thousand (Hearne and Fitt 1992). Most of these species are predators and parasites of the phytophagous species, and most of the crop damage can usually be explained by the presence of 5 to 10 pest species. Damaging populations of arthropod pests are often associated with insecticide use. Broad spectrum insecticides disrupt the ecological interrelationships among the numerous pest arthropods and their natural enemies, and often result in a rapid increase in pest densities when natural enemies are eliminated. Reductions in insecticide use due to planting *B.t.k.*-cotton would enhance natural control and provide a better opportunity for augmentative approaches to biological control which have historically been limited in cotton because of the disruptive nature of insecticides (King and Coleman 1989). The extent of expanded opportunities is difficult to estimate because *B.t.k.*-cotton will not eliminate the need for insecticides against non-lepidopterous pests of cotton. Some pest species have been historically controlled by applications directed at lepidopterous pests. If the applications directed at the lepidopterous pests are removed, additional applications may be required to suppress these previously unrecognized pest problems. Conversely, some pest species have reached pest status because insecticide applications directed at lepidopterous pests disrupted natural control agents. Reductions in applications for control of Lepidoptera would likely result in reduced need to control some insecticide induced pests. Although the extent of expanded opportunities for biological control is unknown, *B.t.k.*-cotton certainly represents one of the most realistic opportunities in the history of cotton IPM to enhance biological control of cotton insects.

Improved Control of Some Pest Species

Some lepidopterous pests of cotton, such as the pink bollworm and fall armyworm (*S. frugiperda*), possess behavioral characteristics which allow them to avoid contact with insecticide deposits on upper portions of the plant canopy. Insecticide sprays cannot dependably deliver insecticide deposits to lower portions of the plant canopy. Since *B.t.k.*-cotton expresses endotoxin proteins in all plant tissues, pest species which are commonly located in plant canopy levels shielded from insecticide deposits or within fruiting structures will not be able to escape contact with the insecticidal toxins.

Foliar applications of *B. thuringiensis* have historically resulted in variable levels of control of cotton insects (Phillips *et al.* 1979). This variable performance in efficacy has been associated with the need for the target insect pests to ingest spray deposits of *B. thuringiensis* on the upper plant canopy. These foliar sprays have limited residual activity, and fruit-feeding insects such as the tobacco budworm and bollworm typically feed in plant locations that receive reduced deposits of insecticide. Precise timing of treatments relative to larval development and location in the plant canopy is critical to obtain adequate control.

The continuous expression of insecticidal activity by *B.t.k.* plants should eliminate management decisions and risks associated with accurate timing of insecticide treatments for pests susceptible to *B.t.k.*-cotton. Routine crop and insect monitoring will continue to be an important component of cotton IPM programs because of the variation in susceptibility of different lepidopteran pests to endotoxin proteins (MacIntosh *et al.* 1990) and the presence of numerous non-lepidopterous pests in USA cotton. Changes in some pest management procedures will be necessary because lepidopteran insects must feed on the plant to receive a toxic dose of insecticide and current management techniques rely to some extent on detection of insect eggs to trigger control action. Ring *et al.* (1993a) describe changes that may be required in treatment threshold recommendations.

Environmentally Safe Mode of Action and Delivery System

The insecticidal proteins of *B.t.k.*-cotton are derived from one of the most studied and environmentally-safe biological insecticides, *B. thuringiensis* var. *kurstaki* (Burgess 1981, Burgess and Hussey 1971, Heimpel 1967). This bacterium is a common soil-borne pathogen of insects that produces a proteinaceous crystalline structure during sporulation. Use of *B. thuringiensis* as a microbial insecticide spans more than 100 years, and commercial products have been registered for use on a wide range of USA crops since the early 1960's. This environmentally-safe, microbial insecticide is used for control of lepidopterous pests in many environments ranging from home vegetable gardens to area-wide spraying of national forests.

Insecticidal activity of the bacteria is associated with the crystalline structure which must be consumed by an insect and activated in the insects midgut to become insecticidal. The insecticidal toxins or protein subunits of the intact crystal (endotoxins) are activated through the action of proteolytic enzymes on the crystalline structure in the insects midgut. These proteinaceous subunits bind to receptors on the midgut lining of the insect and create ruptures or pores in the midgut epithelial cells. As a result of this

action, the contents of the insects gut and the insects hemolymph (blood) are no longer separated. The insect generally dies of gut paralysis, although septicemia may occur when an insect ingests an intact bacterial cell with spore and crystal. *B.t.k.*-cotton mimics the gut paralysis mode of action.

Considerable variation exists in the range of activity of different varieties and isolates of *B. thuringiensis* (Burgess 1981, Hofte and Whiteley 1989). The insecticidal activity of *B.t.k.*-cotton is derived from the insecticidal activity of *B. thuringiensis* var. *kurstaki* which is only toxic to lepidopteran insects. Other varieties or subspecies of *B. thuringiensis* exhibit activity against Coleoptera (beetles) and Diptera (flies).

Because insecticidal activity of *B.t.k.*-cotton is derived from the environmentally safe *B. thuringiensis* var. *kurstaki*, *B.t.k.*-cotton offers a unique mechanism to deliver an alternative insecticidal action (gut paralysis) to a limited range of insect pests (lepidopterans). Most conventional insecticides used in cotton are nerve poisons that potentially affect a wide range of target and non-target organisms. *B.t.k.*-cotton will only affect the lepidopteran insects that feed on the plant tissue, and it will only kill insects with appropriate binding sites in their midgut. Non-target exposure of insects belonging to other orders or other animals is eliminated or greatly reduced as compared to that associated with conventional insecticides.

The mode of action of *B.t.k.*-cotton also offers an efficacious alternative to the nerve poisons. Alternating and mixing insecticidal modes of action is an important component of some resistance management strategies. Conventional formulations of *B. thuringiensis* also offer an alternative mode of action, but their efficacy against fruit-feeding insects of cotton is limited (Phillips *et al.* 1979).

Since *B.t.k.*-cotton will deliver the toxic agent to the target insect by producing an insecticidal protein within the plant tissue that serves as a food source for the insect, non-target exposure to the toxic agent is greatly reduced. Application costs should also be reduced, and the need for manufacturing, shipping, storing, and handling costs of traditional chemical insecticides should be eliminated or reduced. This improved safety to farm workers and reduced exposure of non-target organisms should be viewed as a major advantage of transgenic technology.

Unique Opportunities for Population Regulation of Some Pests

Although the effects of *B.t.k.*-cotton on population growth of target pests are difficult to estimate and will ultimately be influenced by many biological and ecological factors (e.g. number of other plant species attacked by the pest, dispersal or migration range of species involved, extent of farmer adoption of *B.t.k.*-cotton, methods of *B.t.k.*-cotton deployment, host range of species involved, insertion of *B.t.k.* genes in other crops, etc.), the high levels of pest mortality observed in recent experiments suggests that *B.t.k.*-cotton could have a major impact on population growth of lepidopterous pest species susceptible to endotoxin, especially tobacco budworm and pink bollworm. Autocidal and some augmentative biological control methods of insect control are typically targeted at population suppression or eradication of pest species (King and Coleman 1989, Laster *et al.* 1988). The success of these projects often depends on creating a high ratio of released insects to native insects. If *B.t.k.*-cotton were planted

over a high portion of the cotton acreage in a given area, it is possible that populations of some lepidopterous pests, particularly the very susceptible tobacco budworm, could be dramatically reduced. While these populations were at extremely low levels, autocidal and biological control programs would have a unique opportunity to create high ratios of released insects to naturally-occurring insects. It is important to note that resistance management strategies require refugia (i.e. refuge locations where the pest's food plants do not contain *B.t.k.* genes and susceptible insects can survive) (Fischoff 1992, McGaughey and Whalon 1992). Therefore, it would not necessarily be advantageous to plant a high fraction of the total cotton acreage in an area to *B.t.k.*-cotton. Given that the high efficacy of *B.t.k.*-cotton may provide a unique opportunity for the release of autocidal or biological control agents, further examination and experimentation of these issues are warranted.

Relationships with Other Control Measures

B.t.k.-cotton is exceptionally compatible with many other ecologically sound methods of insect control such as biological control and host plant resistance. Research is actively investigating the pyramiding of traditional host plant resistance traits (plant secondary chemistries and morphologies) with the transgenic expression of endotoxin proteins (Benedict *et al.* 1993, Sachs *et al.* 1993). Certain secondary chemistries, such as increased concentrations of terpenes and tannins, have been utilized in some cottons to reduce injury from the tobacco budworm-bollworm complex. Traditional host plant resistance mechanisms to suppress plant damage from the tobacco budworm-bollworm complex, cabbage looper, and pink bollworm have low ability to suppress damage and kill pests (in the range of 20 to 60% larval mortality) compared to *B.t.k.*-cotton (90 to 100% larval mortality) (Benedict *et al.* 1976, 1985, Wilson *et al.* 1992, Zummo *et al.* 1983). Preliminary results show that pyramiding the traditional host plant resistance mechanisms with *B.t.k.*-cotton increases plant resistance to bollworm (Sachs *et al.* 1993). *B.t.k.*-cotton can be viewed as the first successful example of an antibiosis mechanism of host plant resistance in cotton. Because host plant resistance mechanisms are inherent to the plant's genome and they begin their pest defenses at plant emergence (Benedict *et al.* 1988), they are the foundation of all other IPM opportunities.

Preliminary field studies conducted by Monsanto and Mississippi State University suggest that *B.t.k.*-cotton does not exhibit a direct, negative impact on the major predators and parasites in cotton (Stone, unpublished data, Monsanto; Luttrell, unpublished data, Mississippi State University). Current preliminary research in large-plot experiments does suggest that densities of natural enemies, particularly some predators, are affected indirectly by the density of pest species present (Luttrell, unpublished data). Because densities of predators and parasites respond to the densities of the pest (i.e. prey or host species), a decrease in densities of parasites and predators is expected as densities of pest species decline. Additional experimentation in large plots is necessary to confirm these initial, but anticipated observations.

Community Ecology and Managing Insect Resistance to *B.t.k* Cotton

In many USA cotton production regions, cotton is grown in close proximity, a mosaic, with corn, soybean, sorghum, vegetables, and other crops. Polyphagous insect species often utilize several crops and population growth of a pest on one crop is often dependent

upon management actions in another crop. The seasonal buildup of massive populations of sweet potato whitefly (*Bemisia tabaci*) as a result of favorable cropping sequences of multiple crops in a given geographic region (Watson *et al.* 1992) illustrates the importance of crop and community ecology in insect management. These relationships are often poorly understood and warrant additional research.

Community ecology issues associated with managing the development of insect populations resistance to endotoxin proteins are of particular interest. Some insect species like soybean looper occur in soybean and cotton. The soybean looper is effectively controlled in soybean with foliar applications of *B. thuringiensis* and has limited pest status in cotton. However, soybean looper only reaches pest status on soybean in areas where soybean is grown in close proximity to cotton, presumably because the female moths utilize cotton as a source of nectar with significant increases in fecundity (Burleigh 1972). *B.t.k.*-cotton could provide a mechanism to suppress population growth of soybean looper. It could also provide a source of selection for resistant genotypes that would decrease the effective life of foliar applications of *B. thuringiensis* on soybean. Deployment strategies for *B.t.k.*-cotton that include refugia as a component of resistance management for tobacco budworm should also limit the selection for endotoxin resistance in the soybean looper. The extent of soybean looper reproduction in cotton is unknown, and although the moths use cotton as a source of nectar, most oviposition probably occurs in soybean. This example is presented to illustrate the importance of community ecology to effective management of polyphagous insect pests.

The development of transgenic corn expressing endotoxin proteins of *B. thuringiensis* would also create several social and biological questions relative to management of insect resistance to the endotoxins in the numerous species of polyphagous insects (European corn borer, fall armyworm, cotton bollworm) inhabiting both crops within the same cropping region. As with the soybean looper, the suppressive action of transgenic plants can enhance the selection for resistant genotypes (Gould 1988), but it can also provide a highly effective population suppression mechanism. These ecological relationships need additional examination. It is important to note that the impact of insect control activities on multiple cropping systems within an area is not unique to *B.t.k.*-cotton. The same concerns should be expressed for all insecticides targeted at polyphagous insect species on most agronomic crops.

The potential impact of pest resistance on the long-term utility of *B.t.k.*-cotton is a significant issue (McGaughey and Whalon 1992). Any insect control method that provides high levels of control is likely to provide significant selection for the evolution of resistant genotypes. The value of *B.t.k.*- cotton for managing crop pest injury is high and warrants protection from resistance problems. Private (Fischoff 1992) and public sector (McGaughey and Whalon 1992) scientists are addressing these issues. It is important to note that the continuous, constant expression of insecticidal activity by *B.t.k.* plants may make *B.t.k.* cotton an ideal theoretical technology for resistance management. Some problems with the development of insecticide- resistant pest populations are associated with the decaying of the active ingredient on the plant and thus the selective killing of susceptible genotypes at low doses. The continuous, high-dose expression of insecticidal activity by *B.t.k.*-cotton would avoid the influence of insecticidal decay on selection for resistance in susceptible pest populations.

Relationships with Boll Weevil Control and Management of Other Insect Pests

The ability of *B.t.k.*-cotton to protect the plant from insect injury without disrupting natural control of insects compliments the goals of several contemporary programs designed to eliminate or manage the boll weevil (*Anthonomus grandis grandis*) in USA cotton. USDA entomologists in the Rio Grande Valley of Texas are developing a biological control program to control boll weevils that is dependent upon *B.t.k.*-cotton becoming available to control the tobacco budworm-bollworm complex rather than insecticides (Summy *et al.* 1993). The program is using a small wasp that attacks and kills the boll weevil larva. The wasp is very susceptible to insecticides used to control the tobacco budworm-bollworm complex. Combining *B.t.k.*-cotton with this program would control three major pests of cotton (boll weevil, tobacco budworm, bollworm) without conventional insecticides. The reduction in insecticide usage would be increased over that estimated for deployment of *B.t.k.*-cotton alone. The ideal addition to this program would be a plant bug resistant cotton variety to eliminate almost all insecticide usage. Plant bug resistant varieties are grown in several areas of Texas (Masud *et al.* 1990, Ring *et al.* 1993b).

The Boll Weevil Eradication Project has successfully removed boll weevil as a major pest of cotton in much of the Southeast. The program will expand into the Mid-south and Southwest in the near future. Successful removal of the boll weevil as a pest of cotton would further reduce the need for insecticide applications and expand opportunities for non-insecticidal control measures in conjunction with *B.t.k.*-cotton. Most of the insecticide applications made to cotton in the Mid-south and Southwest are targeted at two pests, the boll weevil and the tobacco budworm-bollworm complex. The boll weevil is, perhaps, the most important key pest of cotton because its presence in a management system triggers control actions early in the season. The early season applications often reduce densities of parasites and predators and set the stage for subsequent insect pest outbreaks. Removal of the boll weevil coupled with an ecological sound method of managing the tobacco budworm-bollworm complex would offer opportunities for management of cotton insects not previously possible.

Although *B.t.k.*-cotton and boll weevil eradication will have a major impact on insecticide use in cotton, they will not eliminate the need for insecticides. The sucking pests of cotton (mirids, aphid, whiteflies, thrips, etc.) will not be directly affected by the *B.t.k.*-cotton insecticidal proteins, nor will the eradication project eliminate the need for control of sucking pests. In fact, some pests previously suppressed by chemical insecticides directed at tobacco budworm-bollworm and/or boll weevil may emerge as being more important. In the Southeast, this happened when boll weevil sprays were eliminated by the eradication effort and stinkbugs became more common as a pest of cotton (Barbour *et al.* 1988). *B.t.k.*-cotton will not eliminate the need for crop monitoring and management by professional scouts. Trained professionals must be available to note the changes in the pest complex and implement appropriate plant protection measures. They must also be relied upon to integrate *B.t.k.*-cotton into an overall insect management and crop production scheme.

Implications of *B.t.k.* Cotton Introduction on Current IPM Programs in Different Geographic Regions of the USA

Based on the Beltwide Cotton Conference estimates (Head 1990, 1991, 1992) summarized in Tables 1, 2, and 3, and assuming that *B.t.k.*-cotton would effectively remove the crop loss and control costs of all lepidopterous pests of cotton, introduction of *B.t.k.*-cotton on all USA cotton acreage would reduce total losses to cotton arthropod pests by 42.6% or $312 million. However, this estimate assumes that *B.t.k.*-cotton is highly active on all lepidopteran pests of cotton. It is not equally effective on all lepidopteran pests and additional research is necessary to measure the crop protection provided by *B.t.k.*-cotton against lepidopteran pests less susceptible to endotoxin proteins (e.g. armyworm species). If the assumptions regarding effectiveness were reduced to the savings associated only with tobacco budworm-bollworm, a conservative assumption based on confirmed efficacy of *B.t.k.* cotton in field experiments, total annual losses would be reduced 35.3% or $258 million.

The benefits of *B.t.k.*-cotton vary with the production capabilities and pest spectrums of the different geographic regions of cotton production in the USA. Considering the acreage involved and average annual costs of control plus crop loss, the total annual cost of all lepidopterous pests (including tobacco budworm-bollworm) to cotton producers in the Southeast, Mid-south, Southwest, and West is $70.78 million, $150.19 million, $56.25 million, and $34.75 million (Table 2), respectively. Similar total costs for the tobacco budworm-bollworm complex alone are $54.08 million, $145.83 million, $55.05 million, and $3.64 million, respectively, for the Southeast, Mid-south, Southwest, and West (Table 2). These data illustrate the importance of the tobacco budworm-bollworm complex in the Southeast, Mid-south and Southwest, and the importance of pink bollworm in the West.

Opportunities for expanding cotton IPM with the introduction of *B.t.k.*-cotton vary among and within (Table 3) each production region. The following provides a prospectus of the potential impact of *B.t.k.*-cotton on regional cotton IPM programs in the Southeast, Mid-south, Southwest, and West.

Southeast

The Southeast region of the USA Cotton Belt is one of the areas that has a high potential for benefiting from the utilization of transgenic *B.t.k.*- cotton. This area historically has the highest populations of tobacco budworm-bollworm complex extending over a longer period of time than anywhere else in the Cotton Belt. As a result the Southeast receives high inputs of insecticide.

During the last few years, the average crop loss to the tobacco budworm-bollworm complex in the Southeast ranged from 1.3% in South Alabama to a high of 6.8% in North Carolina (Table 3). This represents the preponderance of all loss from lepidopterous insect pests. Insecticide use on cotton in the Southeast, likewise, is targeted primarily at the tobacco budworm-bollworm complex with a low of 1.4 applications per season in Virginia to 5.7 applications in Florida, compared to 1.4 to 6.0 for all lepidopterous pests in the same regions, respectively. These data are an average of 1990 - 1992 estimates and may be somewhat low in comparison to historical averages. Prior to the

Boll Weevil Eradication Program, applications in the Southeast ranged from about 4 in the areas of lowest infestations to as many as 12 in the more heavily infested areas. A substantial portion of the reduction in insecticide use can be directly attributed to the lack of disruption of the natural enemy complex historically associated with insecticidal control of the boll weevil.

Cotton acreage in the Southeast is increasing rapidly as a result of the success of the Boll Weevil Eradication Program. Growers have found that the crop can be grown with a wider margin of profit than many alternative crops. This is due largely to a reduction in insecticide input for boll weevil. However, high inputs of insecticide are still required for control of the tobacco budworm-bollworm complex. If the impact of this pest complex could be dramatically reduced or eliminated with the use of *B.t.k.*-cotton, additional acreage might be placed into cotton production in the Southeast.

The indirect impact of *B.t.k.*-cotton on secondary, non-lepidopterous pests may also allow for a reduction in insecticide applications. The natural enemy complex for aphids, *Lygus* spp. and whiteflies are typically destroyed by insecticide applications targeted at other pests. If these disruptive applications are reduced the natural enemy complex may be allowed to regulate secondary pest populations. Biological control is a recognized important component of cotton aphid control in most of the Southeast with fewer insecticide applications being required for their control than in other regions (Head 1990, 1991, 1992). The benefits of reduced insecticide use can be extended to management of other pests. In 1992, fields in south Georgia that had not been treated with insecticide were found to have a high rate of parasitization (up to 90%) in sweet potato and bandedwinged whitefly populations. Fields that had been treated with insecticide did not benefit from the whitefly parasitoids, and populations of the pest reached high densities with subsequent crop loss (Herzog, unpublished data).

The development of resistance to endotoxin is an obvious concern once commercialization and widespread utilization of *B.t.k.*- cotton has occurred. The Southeast may not be as likely to be affected from this problem as other areas of the Cotton Belt. Historically, resistance to insecticide classes have shown up in other areas much earlier and at a much higher magnitude than in the Southeast. It is believed that the reason for this is because of the tremendous diversity in agricultural enterprises that may be found in the Southeast. There are numerous crops that are grown that do not have the intensity of insecticide inputs associated with cotton. These crops provide refugia for untreated tobacco budworm-bollworm populations and thus delay resistance buildup.

Mid-south

The Mid-south is one of the major targets for marketing of *B.t.k.*-cotton because of the large acreage typically treated several times annually with insecticide for control of tobacco budworm-bollworm. This pest complex costs Mid-south growers ca. $145 million each year in insecticide costs and crop loss. The intensity of the pest pressure varies within the region. The % crop loss due to tobacco budworm-bollworm complex ranges from 0.74% in Tennessee to 4.98% in Louisiana. Tennessee growers apply an average of 0.74 applications of insecticide per acre for tobacco budworm-bollworm control. Louisiana growers apply an average of 4.8 applications per acre.

Other than the tobacco budworm-bollworm complex, Mid-south cotton insect problems are dominated by the presence of boll weevil, a complex of mirids (particularly the tarnished plant bug, *Lygus lineolaris*), thrips, and aphids. Introduction of *B.t.k.*-cotton is likely to reduce total insecticide inputs in the Mid-south, but the extent of the reduction is unclear. Cotton will continue to need infurrow insecticides for thrips control. Foliar applications of insecticide will also be required for boll weevil and mirids during the early season. Introduction of *B.t.k.*-cotton may reduce the need for some insecticide applications against aphids because they are largely an insecticide-induced pest problem. Traditional applications of insecticide for tobacco budworm-bollworm also provide some suppression of other pests, particularly mirids in the Mid-south. Removal of the insecticide treatments for tobacco budworm-bollworm would likely result in an increase in applications for some other pests, probably tarnished plant bug. Based on current observations and limited data, it appears that *B.t.k.*-cotton may result in a reduction of 2-4 insecticide applications per year across most of the cotton in the Mid-south depending upon the development of secondary pest problems.

Recent problems with insecticide resistant populations of tobacco budworm and cotton aphids are threatening profitable cotton production in some areas of the Mid-south (Luttrell 1994). Growers in the Mississippi Delta spent more than $100 per acre during 1992 for cotton insect control. Because of these problems, Mid-south growers will readily adopt *B.t.k.*-cotton when it is commercially available, and insecticide use should decline as the technology is deployed.

Interestingly, the planned commercial release of *B.t.k.*-cotton coincides with the westward movement of the Boll Weevil Eradication Program into the Mid-south. The vast majority of all insecticide use on Mid-south cotton is targeted at the tobacco budworm-bollworm complex and the boll weevil. Simultaneous introduction of *B.t.k.*-cotton and implementation of boll weevil eradication efforts offers a historical opportunity to dramatically reduce the use of conventional insecticides on Mid-south cotton. Mirid pests, especially the tarnished plant bug, are likely to emerge as the most important key pests of cotton. Research should be initiated immediately to develop appropriate management strategies for the sucking pests of cotton assuming insecticide applications for tobacco budworm-bollworm and boll weevil will be dramatically reduced.

Southwest

The Southwest cotton production region of New Mexico, Oklahoma and Texas harvested cotton from an average of 5.2 million acres per year during the 1990-1992 period (Table 1). The Southwest has yields ranging from less than one-half bale per acre on arid dryland cotton, to three bales per acre on irrigated river bottom land. The tobacco budworm-bollworm complex costs Southwest growers ca. $55 million each year in insecticide costs and crop loss (Table 2). The % crop loss in Texas due to the tobacco budworm-bollworm complex ranges from 0.67 in Texas District 10 to 7.96 in Texas District 13. District 13 is a high input irrigated area known as the Winter Garden because of its winter production of numerous vegetable crops. It is a green island surrounded by arid desert and brushland. District 13 also has the highest number of insecticide applications, 6.17, for the tobacco budworm-bollworm complex whereas Texas District 1 has the lowest, 0.43. The intensity of injury for this complex varies across the region and between years.

In New Mexico and Oklahoma the % crop loss due to the tobacco budworm-bollworm complex ranges from 3.14 to 2.00, respectively. New Mexico growers apply an average of 0.63 applications of insecticide per acre and Oklahoma growers apply 1.10 applications per acre for tobacco budworm-bollworm control. Most of the % crop loss and number of applications for all Lepidoptera in the Southwest region are due to the tobacco budworm-bollworm complex. However, in some Texas Districts beet armyworms and fall armyworms occur occasionally in densities requiring treatment. In New Mexico and Texas District 1 the pink bollworm is a frequent pest requiring insecticidal control.

The other primary pests of Texas and Oklahoma cotton are the boll weevil and a complex of mirids (tarnished plant bug, lygus bugs, and cotton fleahopper) that vary in severity across the region. Some areas such as the High Plains of Texas, Districts 1, 2, and 3, and New Mexico are free of boll weevil infestations or experience only infrequent sub-economic infestations.

Introduction of *B.t.k.*-cotton should reduce insecticide use dramatically in some areas particularly where boll weevils are not pests such as New Mexico and Texas Districts 1, 2, and 3. Throughout areas of Texas two different insecticides (pyrethroids for tobacco budworm-bollworm and organophosphorus insecticides for boll weevil) are applied simultaneously to control the tobacco budworm-bollworm complex and boll weevils. However in other areas growers use pyrethroids or methyl parathion (an organophosphorus insecticide) alone to simultaneously control both pests. In those areas where two insecticides are used, it is expected that with the introduction of *B.t.k.*-cottons a reduction in insecticide usage and a savings in dollars will occur.

In recent years insecticide-resistant tobacco budworm (particularly to pyrethroids) have been reported in several Texas Districts where insecticide usage is high, such as District 13. Moreover where multiple late season applications of pyrethroids are used frequently, outbreaks of spider mites or aphids occur which may require additional insecticide applications. Introduction of *B.t.k.*-cotton could relieve developing pyrethroid resistance problems, reduce secondary outbreaks of spider mites and aphids, and reduce insecticide applications in many cotton producing areas of the Southwest. Across the Southwest region reductions could range from 0 to 8 applications with an estimated average reduction of 1 application on 5.2 million acres per year. However the extent of the reduction is unclear.

West

Cotton pest control strategies in the desert Southwest (Arizona and Southern California) changed dramatically in 1966. This resulted from the spread of the pink bollworm, *Pectinophora gossypiella* (Saunders) across the entire cotton-producing area of Arizona and southern California. Once established in these areas the pink bollworm required routine scheduled insecticide applications in order to maintain it below economic thresholds. Initially growers were applying up to 20 or more insecticide applications in order to control this insect alone. Once research established sound economic thresholds and more precision in timing of applications, this number was reduced to a more reasonable level. The numbers vary from area to area, generally associated with elevation, e.g., in eastern Arizona the average number is 4-5, in central Arizona 6-8, and western Arizona and southern California 9-12 applications are generally required.

Were it not for the need to control pink bollworm (excluding the present problem with the sweet potato whitefly) much less insecticidal control would be needed, and then, on a non-scheduled basis.

A related impact of the pink bollworm in the desert Southwest has been the continual threat to the vast acreage of cotton in the San Joaquin Valley of California. High infestations in the Imperial Valley provide the source for wind movement of moths into the San Joaquin Valley. This initiated an annual multi-million-dollar, sterile-moth release program in the San Joaquin Valley in an effort to prevent establishment of the pink bollworm there.

Indirect costs associated with the pink bollworm problem have been those resulting from secondary pest outbreaks following scheduled applications for this key pests.

The biology and seasonal history of the pink bollworm make it ideally suited for management with *B.t.k.*-cotton. The pink bollworm over-winters as diapausing larvae in the cotton field where they were produced. Spring moth emergence occurs over an extended period of time with a large proportion of the moths emerging and dying prior to the production of susceptible cotton fruit (squares). Therefore, the key to the initiation of a new years infestation is the coincidence of susceptible fruit and last-emerging moths. This coupled with high winter mortality, results in a fairly low population level to start the new infestation. A high level of mortality during this first generation would probably preclude the subsequent development of populations to damaging levels for the rest of the season.

Without the need for scheduled applications of insecticides for pink bollworm control, a great deal of flexibility would be possible with the remainder of the pest complex in western cotton. For example, biological and cultural control methods could be used for management of other pests such as lygus bugs, *Heliothis* spp., beet armyworm and cotton leafperforator.

There are indications of low-level resident populations of pink bollworm in the southern end of the San Joaquin Valley. If this persists and spreads, scheduled applications of foliar sprays will be required in order to prevent serious loss. This would almost surely result in serious wide-spread outbreaks of spider mites and certain other Lepidoptera such as bollworm and cabbage looper.

By eliminating the pink bollworm as an in-season pest requiring scheduled insecticide applications, a number of benefits would ensue which would re-establish western cotton production as a profitable enterprise. In addition to the overall improvement in the management of all other pests, and more economically at that, yields would probably move upward towards the levels obtained in pre-pink bollworm days.

Summary and Overall Prospectus of *B.t.k.* Cotton

Results of published experiments with Monsanto's *B.t.k.* cottons indicate that these transgenic cottons exhibit a high level of efficacy against tobacco budworm and pink bollworm, and that bollworm is effectively controlled in field environments by these cottons. We believe that *B.t.k.*-cotton offers opportunities for improved pest control, and that the technology will be actively sought by growers for control of the lepidopterous pests of cotton susceptible to endotoxin proteins. Control of other lepidopterous pests, such as armyworm species which are less susceptible to endotoxin proteins, is possible but sufficient data are not available to suggest that *B.t.k.*-cotton will eliminate insecticide treatments for these more tolerant pests.

Transgenic technology provides an innovative, unique mechanism to deliver an insecticide selectively to target pest species. This selective activity will not disrupt populations of beneficial insects as is the case with traditional control measures that use broad-spectrum, nerve-toxin insecticides. Opportunities for expanded use of biological control should develop as the intensity of insecticide use is reduced with expanded implementation of *B.t.k.*-cotton.

Major economic and management advantages of *B.t.k.*-cotton are associated with its potential to reduce the use of traditional insecticides and increase yields of USA cotton. Reduced insecticide use with *B.t.k.*-cotton is likely, but the extent of reduction is difficult to predict because of the dynamic, interrelationships among cotton pest and beneficial arthropods. If *B.t.k.*-cotton effectively reduced all insecticide inputs for lepidopterous pests of cotton in the USA without altering control inputs for other pests, cotton growers would save $312 million in control costs and crop damage. If the reduction in insecticide use and crop loss is limited to that due to the tobacco budworm-bollworm complex, growers would still save $258 million. These estimates do not consider the added cost of *B.t.k.*-cotton seed to the farmer.

The development of *B.t.k.*-cotton provides a unique opportunity to manage lepidopterous pests of cotton with a highly efficacious, environmentally safe control measure. The technology couples the efficacy of an effective insecticide with the environmental advantages of host plant resistance. This technology should serve as the foundation for historical improvements in cotton IPM programs over the next 5 to 10 years.

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Table 1. Production and insect control characteristics of different geographic regions of cotton production in the USA.

Characteristic	Geographic Region Southeast	Mid-south	Southwest	West
Acres Harvested X 1000	1408	3794	5208	1456
Yield in Bales Per Acre	1.30	1.50	0.77	2.53
% Crop Loss to *Heliothis* and *Helicoverpa*	3.4	2.7	1.4	0.1
% Crop Loss to Lepidoptera	4.5	2.7	1.4	1.0
% Crop Loss to All Insects	6.8	6.7	5.2	6.0
No. Insecticide Applic./Acre for *Heliothis* and *Helicoverpa*	3.5	3.0	0.8	0.2
No. Insecticide Applic./Acre for Lepidoptera	4.1	3.1	0.8	1.6
No. Insecticide Applic./Acre for All Insects	6.6	7.4	2.5	3.9
$ Spent/Acre for Control of *Heliothis* and *Helicoverpa*	24.14	25.45	7.16	1.71
$ Spent/Acre for Control of Lepidoptera	31.16	26.60	7.39	15.89
$ Spent/Acre for Control of All Insects	49.42	48.04	18.59	49.44

* Calculated as an average of annual estimates published by the Beltwide Cotton Conferences (Head 1990, 1991, 1992).

* * Estimates of % crop loss, number of insecticide applications per acre, and $ spent per acre for Lepidoptera include similar estimates for *Heliothis* and *Helicoverpa* (tobacco budworm and bollworm). Estimates for all insects include those for Lepidoptera.

Table 2. Total annual expenditures for cotton insect control in different geographic regions of the USA (average of 1990-1992 data).

	Total Annual Estimated Amount X 1,000,000				
	Southeast	Mid-south	Southwest	West	Entire USA
Estimates for Heliothis-Helicoverpa Complex					
No. of Acre Applications	4.93	11.27	4.17	0.06	20.42
$ Expended for Control Costs	33.98	96.56	37.29	2.49	170.32
$ Crop Loss Above Control Costs	20.10	49.27	17.76	1.15	88.29
$ Total Loss	54.08	145.83	55.05	3.64	258.61
Estimates for All Lepidoptera					
No. of Acre Applications	5.74	11.61	4.32	2.27	23.94
$ Expended for Control Costs	43.87	100.92	38.49	23.14	206.42
$ Crop Loss Above Control Costs	26.91	49.27	17.76	11.61	105.56
$ Total Loss	70.78	150.19	56.25	34.75	311.98
Estimates for All Insects					
No. of Acre Applications	9.32	28.23	11.18	9.83	51.55
$ Expended for Control Costs	69.58	182.26	96.82	71.98	420.65
$ Crop Loss Above Control Costs	41.67	127.51	68.63	73.36	311.16
$ Total Loss	111.25	309.77	165.45	145.34	731.81

* Calculated from annual estimates published by the Beltwide Cotton Conference (Head 1990, 1991, 1992). Dollar values for yield loss assumed that each bale weighed 480 pounds and that cotton was valued at $0.65 per pound. Seed values were not included.

* * Estimates for All Lepidoptera include estimates for *Heliothis-Helicoverpa* (tobacco budworm-bollworm). Estimates for All Insects include estimates for All Lepidoptera.

Table 3. Cotton crop losses and control expenditures for *Heliothis-Helicoverpa* (HEL), all Lepidoptera (LEP), and all arthropod pests (ALL) within major geographic production regions of the USA.*

	% Crop Loss Due To			No. Insecticide Applic./Acre For			$ Spent/Acre For Control Of		
	HEL	LEP**	ALL**	HEL	LEP	ALL	HEL	LEP	ALL
SOUTHEAST REGION									
Alabama-Central	1.67	3.07	7.60	3.67	4.00	9.80	18.25	9.42	43.34
Alabama-North	2.23	2.32	9.84	1.80	1.80	5.57	9.55	9.55	17.39
Alabama-South	1.30	3.42	5.90	4.30	5.03	7.77	21.60	33.80	40.30
Florida	5.37	6.01	6.22	5.73	6.00	6.57	48.19	51.88	57.91
Georgia	2.58	4.68	5.34	4.43	5.93	9.07	28.85	43.80	83.29
North Carolina	6.84	7.45	8.00	2.97	3.03	3.93	27.62	29.71	41.95
South Carolina	2.34	3.02	4.76	3.77	4.13	5.57	25.92	30.92	39.53
Virginia	5.56	5.56	6.23	1.40	1.40	2.07	16.22	16.22	22.87
MID-SOUTH REGION									
Arkansas-North	1.15	1.15	2.27	1.15	2.20	5.17	17.88	17.88	36.09
Arkansas-South	2.65	2.65	4.21	2.65	4.43	7.27	41.42	41.78	60.69
Louisiana	4.98	4.99	8.33	4.80	4.98	10.20	38.52	38.88	58.35
Mississippi-Delta	3.50	3.57	6.61	3.50	4.33	10.16	38.35	42.47	72.43
Mississippi-Hill	1.85	1.98	7.18	1.85	2.27	9.33	14.93	16.66	45.90
Missouri	1.44	1.44	8.87	1.44	0.23	2.37	1.97	1.97	22.79
Tennessee	0.74	0.74	10.19	0.74	0.33	2.93	1.97	1.97	13.92
SOUTHWEST REGION									
New Mexico	3.14	3.89	11.21	0.63	0.83	2.30	6.42	8.20	20.92
Oklahoma	2.00	2.00	4.36	1.10	1.10	3.53	10.95	10.95	25.59
Texas-Dist. 1	3.14	3.14	5.23	0.43	0.43	1.47	4.20	4.20	9.02
Texas-Dist. 2	0.98	0.98	4.60	0.80	0.80	1.97	7.79	7.79	16.30
Texas-Dist. 3	1.25	1.26	5.00	0.50	0.53	1.77	4.63	5.03	12.05
Texas-Dist. 4	1.50	1.50	6.41	0.50	0.50	3.33	3.10	3.10	14.15
Texas-Dist. 5&9	1.73	1.73	8.45	0.93	0.93	3.97	10.06	10.07	31.61
Texas-Dist. 6	1.44	1.86	5.47	0.60	0.80	1.93	4.50	5.80	15.59
Texas-Dist. 7	4.29	4.29	11.21	1.37	1.37	4.17	10.27	10.27	26.60
Texas-Dist. 8	5.71	5.71	23.83	1.13	1.13	7.03	11.40	11.40	40.88
Texas-Dist. 10	0.67	0.67	1.53	1.97	1.97	3.03	12.59	12.59	18.22
Texas-Dist. 11	1.36	1.36	4.62	1.10	1.10	5.40	7.18	7.18	19.31
Texas-Dist. 12	1.48	1.59	7.01	1.06	1.17	5.00	9.37	10.47	43.29
Texas-Dist. 13	7.96	7.96	29.22	6.17	6.17	20.30	63.71	63.71	149.03
Texas-Dist. 14	1.12	1.23	6.30	0.63	0.63	4.83	4.92	4.92	27.31
WEST REGION									
Arizona	0.11	2.23	6.33	0.43	4.57	9.70	4.33	45.77	113.94
California	0.13	0.43	5.93	0.03	0.20	1.37	0.47	2.30	21.63

* Calculated from annual estimates published by the Beltwide Cotton Conference (Head 1990, 1991, 1992).

** Estimates for LEP include estimates for HEL. Estimates for ALL include estimates for LEP.

Appendix II

Economic Impacts of *B.t.k.* Insect Resistant Cotton

Economic Impacts of *B.t.k.* Insect Resistant Cotton

Dr. S. Spurlock
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Abstract

The introduction of genetically engineered plants which are designed to control insects without the use of chemicals will have significant impacts on the profitability of some farmers and agribusinesses. *B.t.k.* cotton, created to control Lepidoptera infestations, will allow cotton growers to eliminate some conventional insecticide applications, and thus reduce pesticide expenses. Based on available cost and acreage data and assumptions concerning the portion of current cotton acres that would be converted to *B.t.k.* cotton, it is estimated that cotton producers could save over $77 million per year on insect control costs by adopting *B.t.k.* cotton. As the *B.t.k.* cotton seed market develops and grows during the adoption period, the demand for conventional cotton seed and some insecticides will decrease.

Introduction

In recent years, public concern about the use of some agricultural chemicals has increased in the United States. Frequently, legal action was taken to force the EPA to ban or severely restrict the use of particular pesticides. Economic studies have been conducted to examine the likely impacts from such restrictive pesticide regulations. Taylor *et al.* (1991) developed a regional model and concluded that agricultural income in the South would be negatively impacted by more restrictive pesticide regulations. Richardson *et al.* (1991) 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. It is possible that genetically engineered plants which are designed to control insects without the use of chemicals will be able to offset some of the negative impacts from increased pesticide regulations.

B.t.k. cotton is designed to control Lepidoptera infestations, eliminating the need to control these pests with conventional insecticide applications. Revenue-related factors such as lint yields and quality characteristics are expected to be similar under both conventional and *B.t.k.* cotton production systems. However, per-acre production costs of *B.t.k.* cotton are expected to be impacted due to the reduction in insecticide use and the substitution of *B.t.k.* cotton seed for conventional cotton seed. Growers who adopt *B.t.k.* cotton will simply substitute *B.t.k.* cotton seed for conventional cotton seed and certain types of insecticides. Thus, the added cost of the *B.t.k.* cotton seed must be compared with the savings obtained from eliminating conventional seed and some insecticides.

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, it is possible to speculate on the direction of change in these variables. For instance, pesticide regulations in the U.S. will likely become more restrictive over time. Reductions in insecticide use without

B.t.k. insect resistant cotton will cause cotton yields to decline, farm profits to decline, and acres devoted to cotton production to decline, especially in those regions where insecticide use is an integral production practice. A scenario which allows for the introduction of *B.t.k.* cotton results in a very different forecast. Reductions in insecticide 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 adoption by large farms over that of 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 *B.t.k.* cotton is not expected to be related to farm size; i.e., small and large farms will have the same per-acre costs and benefits from the adoption of *B.t.k.* cotton, and thus will likely have equal adoption rates.

Economic Impacts

The introduction of *B.t.k.* insect resistant cotton will provide cotton growers with a choice of either maintaining or altering their current production practices. Each cotton grower will need to evaluate the profit potential of *B.t.k.* cotton relative to that of conventional cotton. Due to different Lepidopteran insect population pressures across the country, it is expected that some growers will be able to increase profits by adopting *B.t.k.* cotton, while 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 *B.t.k.* seed, conventional seed, and some insecticides will shift over time as the *B.t.k.* 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; i.e, to allow the market to achieve a new equilibrium position. Directional impacts on price and quantity from shifts in supply and demand may be summarized as follows:

Type of Shift	Impact on Price	Impact on Quantity
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 *B.t.k.* cotton seed (used for planting) market will exhibit growth during the first few years after introduction. Participants will gather information during this early stage of the adoption period. There will be much uncertainty in supply and demand, generating an environment in which price discovery will evolve over time. As the *B.t.k.* cotton seed market matures over time, a more stable supply-demand relationship should develop.

Cotton growers who decide to adopt *B.t.k.* cotton will replace conventional cotton seed with *B.t.k.* cotton seed. Seed companies will retain some of the *B.t.k.* cotton seed produced with the current year's *B.t.k.* cotton crop and make it available to growers for production of the next year's *B.t.k.* cotton crop. Thus, the supply of *B.t.k.* cotton seed is expected to increase during the first few years. As the *B.t.k.* 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 *B.t.k.* cotton.

Growers who use *B.t.k.* cotton seed will be able to reduce their applications of chemical insecticides that are used to control Lepidoptera infestations. Thus, a decrease in the demand for these types of insecticides will occur, causing a decrease in both the quantity and price of certain insecticides. In some regions of the country, a common practice is for cotton growers to hire custom applicators (either ground rigs or aerial sprays) to apply some insecticides and other chemicals. Therefore, in conjunction with the decline in insecticide use, there will also be a decrease in the demand for custom applicators in these regions.

Cotton insect scouts and consultants are often hired by cotton growers to help make management decisions throughout the growing season. It is expected that growers who adopt *B.t.k.* cotton will still utilize scouts and consultants for various kinds of insect problems. Therefore, the impact of the introduction of *B.t.k.* cotton on scouts and consultants is expected to be minor.

The economic impacts on cotton growers who adopt *B.t.k.* cotton could be significant. Elimination of certain pesticides will reduce a grower's insecticide cost and application cost. However, *B.t.k.* 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 *B.t.k.* cotton seed, the profits from *B.t.k.* cotton production must be greater than the profits from conventional cotton production. Thus, to assure adoption of *B.t.k.* cotton, the increased expense of *B.t.k.* cotton seed must be more than offset by the savings from reduced insecticide use. Supply and demand relationships in related markets will adjust over time until an equilibrium position exists between *B.t.k.* cotton and conventional cotton. It is expected that growers who adopt *B.t.k.* cotton will exhibit an increase in profitability.

In some regions of the country, cotton production is unprofitable due to high insecticide costs, and thus acreage is not allocated to cotton in these regions. The introduction of *B.t.k.* cotton, which will have lower insecticide costs, could allow cotton production to become profitable in these regions, allowing the acreage devoted to cotton production to increase. If an increase in cotton acreage and thus the supply of cotton occurs, the price of cotton should decrease, leading to lower wholesale and retail prices of cotton-related products.

Insect Control Cost Reductions

An estimate of the insect control cost reductions due to adoption of *B.t.k.* cotton provides an indication of the potential benefits that cotton producers may expect to achieve. The United States was divided into four regions based on differing insect problems and control practices. The regions were defined as follows: 1) Southeast - Alabama, Georgia, Florida, South Carolina, and North Carolina; 2) Delta - Louisiana, Mississippi, and Arkansas; 3) Coastal Bend of Texas - Districts 10 through 14; 4) West - Arizona. Insects considered were bollworms and budworms in all four regions and leaf perforators and pink bollworms in the West. Although *B.t.k.* cotton may provide some level of control on other insects, the economic impacts would be small relative to the impacts on these major pests.

Results presented here were derived from data compiled by Head for the years 1990-1992. The per-acre costs of controlling major susceptible Lepidopteran insects for each region are presented in Table 1. The acres of cotton that were harvested are reported in Table 2. The estimates of the reduction in insect control costs due to the introduction and adoption of *B.t.k.* cotton are presented in Table 3.

Insect control cost per acre varied across regions and years (Table 1). The three-year average ranged from a low of $10.05 per acre in the Coastal Bend of Texas to a high of $42.97 per acre in the West. Variation in cost per acre from year to year is expected due to the fluctuations in insect populations. Cotton acres also varied somewhat over years (Table 2). The three-year average is considered to be representative of the average cotton acreage over the near future.

If it is assumed that *B.t.k.* cotton will be used on one-half of all cotton acres in a region, then the potential reduction in insect control costs for a region may be determined. Based on available cost and acreage data and assumptions concerning the portion of acres using *B.t.k.* cotton, it is estimated that cotton producers could save over $77 million per year (on average) on insect control costs by adopting *B.t.k.* cotton. The Delta region could expect an impact of over $46 million. Some portion of the cost savings will have to be used to offset the expected higher seed cost. Also, it is expected that some other insect control practices could change with the adoption of *B.t.k.* cotton. The economic impacts (whether positive or negative) of these changes are expected to be relatively small compared to the cost reductions presented in Table 3.

Conclusions

The adoption rate of *B.t.k.* insect resistant cotton will be influenced by economic factors. Cotton growers will evaluate the profit potential of *B.t.k.* cotton relative to that of conventional cotton. Due to varying Lepidopteran insect populations in different regions of the country, some growers will be able to increase profits by adopting *B.t.k.* cotton, while 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 *B.t.k.* cotton seed market grows, it is expected that the markets for conventional cotton seed and some insecticides will exhibit a decline in demand.

Table 1. Insect control cost per acre by region and year[1]

Region	1990	1991	1992	avg.
	----------($/acre)----------			
Southeast	24.63	26.38	21.89	24.30
Delta	26.48	19.53	48.69	31.57
Coastal Bend	7.67	6.51	15.96	10.05
West	78.50	37.00	13.40	42.97

[1] Insects are the bollworm, budworm, leaf perforator, and pink bollworm.

Table 2. Cotton acres harvested by region and year

Region	1990	1991	1992	avg.
	----------(thousand acres)----------			
Southeast	1,127.5	1,546.0	1,534.0	1,402.5
Delta	2,702.6	2,966.4	3,185.0	2,951.3
Coastal Bend	787.3	1,051.1	787.3	875.2
West	460.0	450.0	410.0	440.0

Table 3. Insect control cost savings by region[1]

Region	*B.t.k.* Acres	Insect Control Cost	Reduction in Cost
	thousand	$/acre	million $
Southeast	701.3	24.30	17.04
Delta	1,475.7	31.57	46.59
Coastal Bend	437.6	10.05	4.40
West	220.0	42.97	9.45
Total	2,834.5	27.33	77.48

[1] Assuming that one-half of all cotton acres are converted to *B.t.k.* cotton.

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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)

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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].

Approximate distance from test (ft)	Location A %	B %	B S.D.+	C %	C S.D.	D %	E %	E S.D.	F %	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

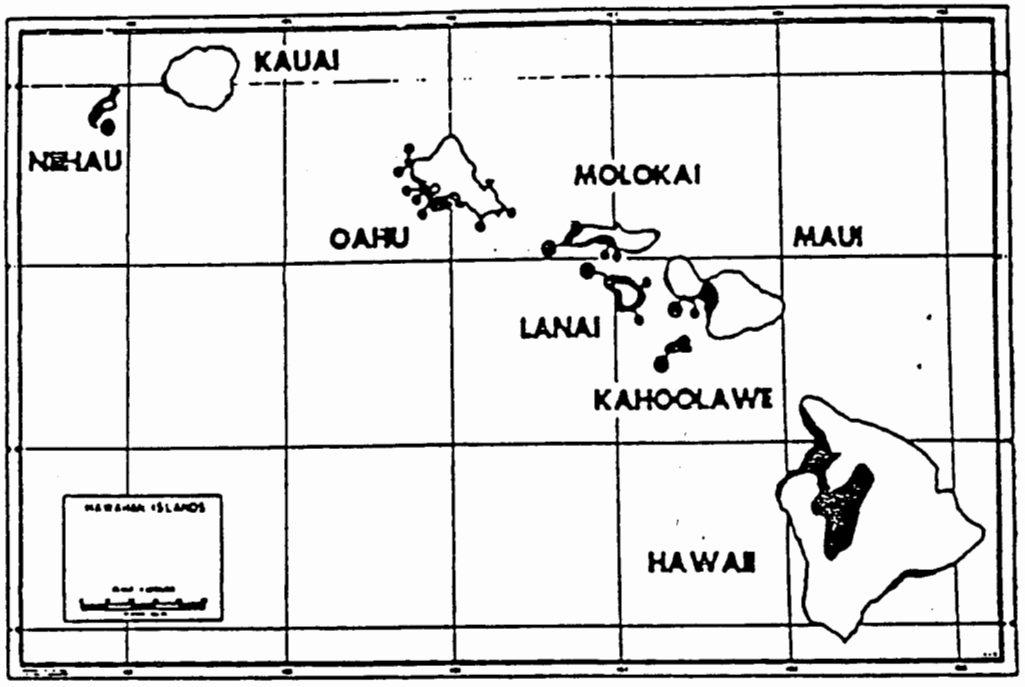

Figure 1. Geographic range of *Gossypium tomentosum* Nutt. in the Hawaiian Islands (1963). Solid circles indicate collection sites; those enclosed in rings represent sites of former collections unchecked during the present study. The open circle indicates site of hybrid populations. Shaded areas correspond 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

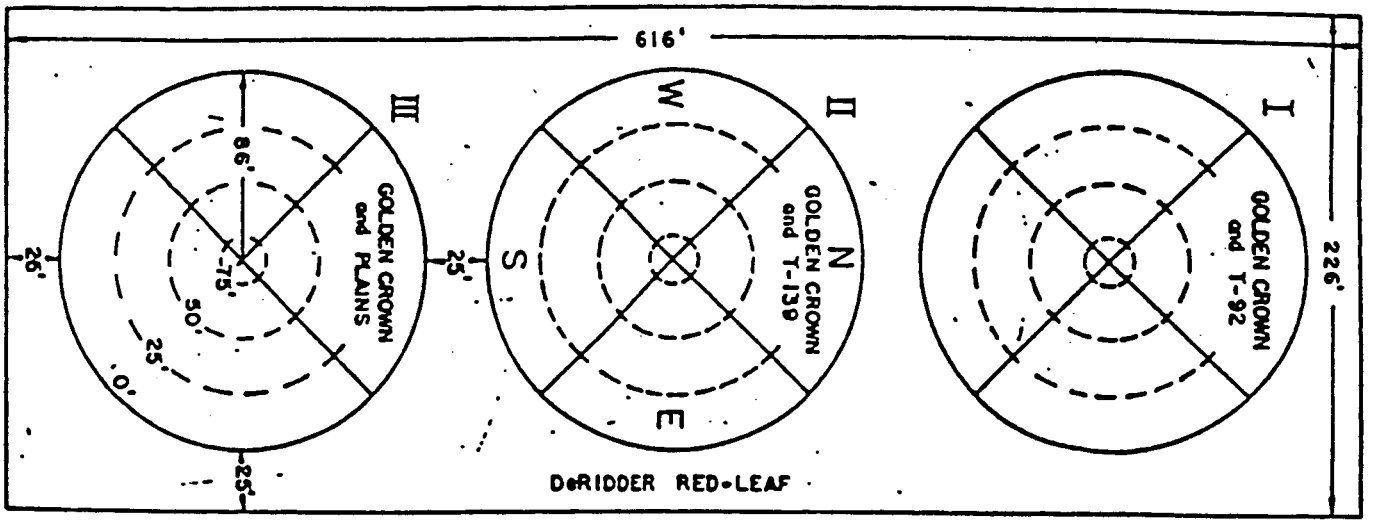

Figure 1.-Diagram of field lay-out of natural crossing experiment. The circles were planted in alternate rows of Golden Crown and green-leaf varieties. The area outside the circles was planted with DeRidder, a red-leaf cotton. Simpson, D.M. and E.N. Duncan, 1956. p. (306)

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

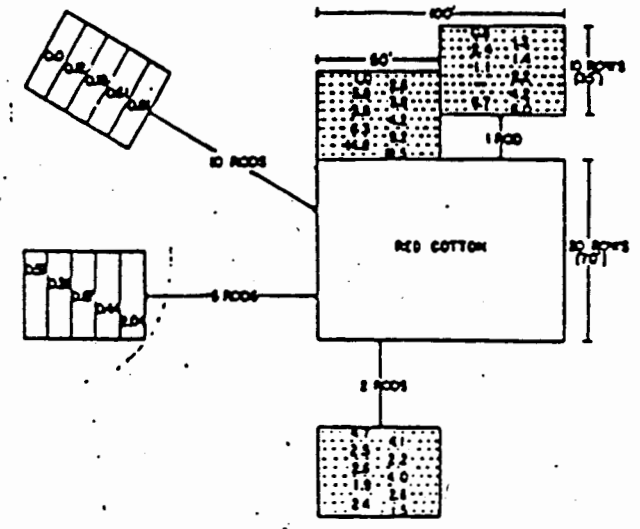

Figure 1.-Arrangement of the blocks of red and green cotton grown in 1951 near Lake Carl Blackwell, Okla. The five smaller blocks were planted to normal green cotton. Percentages of hybrids resulting from natural crossing are indicated for each row in the blocks at 0, 1, and 2 rods, and for 10 foot sections 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		1		2	
	Total	%Hybrids	Total	%Hybrids	total	%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		10	
	Total	%Hybrids	Total	%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

1991 *B.t.k.* COTTON FIELD RELEASES (USDA PERMIT #90-347-01) FINAL REPORT

W. Randy Deaton, Ph.D.
Monsanto Co.

Amended April 18, 1994

The purpose of this field release was to test the insect control in cotton genetically-modified to contain the gene from *Bacillus thuringiensis* (*B.t.k.*) that encodes its insect control protein. The cotton was tested at six sites by six different cooperators (listed below).

Sites and cooperators	Cotton Lines Tested
Loxley, AL site	65, 81, 247, 249
[CBI DELETED]	
Maricopa, AZ	65, 81, 247, 249
[CBI DELETED]	
Bossier City, LA site	65, 81, 247, 249
[CBI DELETED]	
Starkville, MS site	65, 81, 247, 249, 531
[CBI DELETED]	

College Station, TX site 65, 81, 247, 249

[CBI DELETED

]

Halfway, TX site 65, 81, 247, 249

[CBI DELETED

]

Genotypes:

This field release included the following genotypes:

- Segregating and homozygous lines from independent transgenic plants of Coker 312 carrying the following vectors:

VECTOR #	LINES
PV-GHBK01	Line 65, 81
PV-GHBK02	Line 247, 249
PV-GHBK04	Line 531, 757, 931

- Non-transgenic controls.

Schedule of major operations:

April-May	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.
April-May	Seed planted
Sept.-Dec.	Harvest and shipment of seed cotton 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 two months following termination for any volunteer cotton, which was destroyed by either hand weeding or additional cultivation.

Plant growth and general observations:

The transgenic plants did deviate from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to *B.t.k.* 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:

At the Loxley, AL site, no border rows were used since no commercial cotton was planted within 1/4 mile of the test. At the other five sites, the *B.t.k.* 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 years' data, it is safe to assume that little or no pollen from the *B.t.k.* 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:

Only one line was evaluated for gene expression levels. However, that line probably represents the level of expression in the other lines tested. In the leaf, the mean expression across all sites was 13.3µg *B.t.k.* protein/g fresh weight with a standard deviation of 3.8µg/g. The range was 8.3-17.9µg/g fresh weight. In the seed, the mean expression across all sites was 8.5µg *B.t.k.* protein/g fresh weight with a standard deviation of 2.3µg/g. The range was 6.0-11.9µg/g fresh weight.

Ultimately, the expression of the *B.t.k.* gene was measured through insect control. Excellent insect control was observed at all sites with several different insects including cotton bollworm, tobacco budworm, fall armyworm, and cotton leaf perforator.

4) Stability and inheritance of the new genes:

No unusual inheritance patterns were observed when the material used in these tests were originally evaluated in our greenhouses.

5) Published data:

At this point, there is no published data from these experiments.

1991/92 KAUAI, HAWAII TRANSGENIC COTTON FIELD RELEASE

(USDA PERMIT #91-144-01)(Mons # 91-048 PS 64)

FINAL REPORT

W. Randy Deaton, Ph.D.
Monsanto Co.

Amended April 19, 1994

The purpose of this field release was primarily to increase seed of cotton genetically-modified to contain the gene from *Bacillus thuringiensis* (*B.t.k.*) that encodes its insect control protein. Some crosses were made between the transgenic lines and breeding lines. The field release was managed on the site by [CBI DELETED] of Northrup King Company, [CBI DELETED] HI, 96796.

Genotypes:

This field release included the following genotypes:

- Segregating and homozygous lines from independent transgenic plants of Coker 312 carrying the following vectors:

VECTOR #	LINES
PV-GHBK01	Line 81
PV-GHBK02	Line 247, 249
PV-GHBK04	Line 531, 629, 660, 931
PV-GHBK05	Line 1015

- Non-transgenic controls and breeding material.

Schedule of major operations:

Sept. 27, 1991	Seed were transported from the Monsanto research center in Chesterfield, Missouri to the Northrup King experimental farm in Kauai, Hawaii. The seed were shipped via an overnight carrier in containers as specified in the protocol. The seed arrived safely in Kauai and were stored at the Northrup King facility, in accordance with the conditions described in the permit.
October 4, 1992	First planting
October 18, 1991	Second shipment of seed
Oct. 25-26, 1991	Second planting

March 3-4, 1992	The bolls that were open were harvested for the first sample. As described in the permit application, the seed cotton was transported from the field site to the Northrup King facility. The seed cotton was place on dryer for at least two days. The seed cotton was treated with phosphine for five days as required by APHIS prior to shipping back to the Monsanto research facility.
March 12, 1992	First shipment of seed back to Monsanto
March 14-16, 1992	The bolls that were open were harvested for the second sample. The seed cotton was handled in the same manner as described above for the first harvest.
March 17, 1992	Second shipment of seed back to Monsanto
March 31- April 3, 1992	The bolls that were open were harvested for the third sample. The seed cotton was handled in the same manner as described above for the first harvest.
April 1, 1992	Third shipment of seed back to Monsanto
April 7-8, 1992	The bolls that were open were harvested for the fourth sample. The seed cotton was handled in the same manner as described above for the first harvest.
April 10, 1992	Fourth shipment of seed back to Monsanto
April 21-24, 1992	The bolls that were open were harvested for the fifth sample. The seed cotton was handled in the same manner as described above for the first harvest.
April 30, 1992	Fifth shipment of seed back to Monsanto
April, 1992- April, 1993	The plants in the field were pulled up and left to dry in the field. After drying, the plants were burned. The field was then disked. The area was observed for one year following termination for any volunteer cotton, which was destroyed by either hand weeding or additional cultivation.

Plant growth and general observations:

Except for some somaclonal variation in the transgenic plots due to mutations induced during the tissue culture process, both transgenic and non-transgenic plants grew normally during the course of the experiment.

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

Responses to specific issues:

1) Horizontal movement:

As required in the protocol, the planting was surrounded by 4 border rows of non-transgenic cotton to serve as a sink and dilution factor for pollen carried by insects. In the previous two years, we have conducted extensive surveys of the area for potential recipients of pollen from the transgenic plants. None have been identified within 1/2 mile of the site. Last year, we tested seed from the one ferrel plant identified at a 1/2 mile distance, and no *B.t.k.* gene expression was observed. Based on these previous observations, it is unlikely that any horizontal movement occurred in this field test.

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:

Since the sole purpose of this field release was to increase seed quantities, no expression data were collected nor was any attempt made to assess insect control.

4) Stability and inheritance of the new genes:

Gene inheritance and stability behaved as expected in the seed obtained from these increases.

5) Published data:

At this point, there is no published data by Monsanto for this specific test.

1992 *Bt* COTTON FIELD RELEASES (USDA PERMIT #91-347-02) FINAL REPORT

W. Randy Deaton, Ph.D.
Monsanto Co.

The purpose of this field release was to test and breed cotton genetically-modified to contain the gene from *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) that encodes its insect control protein. The cotton was tested at 17 sites by 16 different cooperators (listed below).

Sites and cooperators	Cotton Lines Tested
Loxley, AL site	249, 531
[CBI DELETED	
]	
Casa Grande, AZ site	081, 249
[CBI DELETED	
]	
Maricopa, AZ site	249
[CBI DELETED	
]	
Maricopa, AZ site	249, 531, 626, 660
[CBI DELETED	
]	

Yuma, AZ site 081

[CBI DELETED

]

Wabbesaka, AR site 081, 249

[CBI DELETED

]

Shafter, CA site 081, 249

[CBI DELETED

]

Tifton, GA site 249, 531, 660, 931

[CBI DELETED

]

Bossier City, LA site 249, 531

[CBI DELETED

]

St. Joseph, LA site 081

[CBI DELETED

]

Chatham, MS site 081

[CBI DELETED

]

Morgan City, MS site 081

[CBI DELETED

]

Scott, MS site 081, 249

[CBI DELETED

]

Starkville, MS site 249, 531, 626, 660, 931

[CBI DELETED

]

College Station, TX site 249, 531, 629, 660

[CBI DELETED

]

Corpus Christi, TX site 081

[CBI DELETED

]

Tivoli, TX site 081, 249

[CBI DELETED

]

Genotypes:

This field release included the following genotypes:

- Segregating and homozygous cotton lines as follows:

VECTOR #	LINES
PV-GHBK01	081
PV-GHBK02	249
PV-GHBK04	531, 626, 660, 931

- Non-transgenic controls.

Schedule of major operations:

April-May	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.
April-May	Seed planted
Sept.-Dec.	Harvest and shipment of seed cotton 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 twelve months following termination for any volunteer cotton, which was destroyed by either hand weeding or additional cultivation.

Plant growth and general observations:

The transgenic plants did deviate from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to *B.t.k.* 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 *B.t.k.* 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 *B.t.k.* 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:

The expression of the *B.t.k.* gene was measured through insect control. Excellent insect control was observed at all sites with several different insects including cotton bollworm, tobacco budworm, fall armyworm, and cotton leaf perforator.

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.

1993 *Bt* COTTON FIELD RELEASES (USDA PERMIT#93-011-02) FINAL REPORT

Eric M. Johnson
Monsanto Co.

The purpose of this field release was to test cotton genetically-modified to contain the gene from *Bacillus thuringiensis (Bt)* that encodes its insect control protein. The cotton was tested at three sites by three different cooperators (listed below).

Sites and cooperators

Richlands, NC site

[CBI DELETED

]

Rocky Mount, NC site

[CBI DELETED

]

Jamesville, NC site

[CBI DELETED

]

Genotypes:
This field release included the following genotypes:

- Derivatives of Coker 312 homozygous for PV-GHBK04
- Coker 312 controls.

Distribution of Lines for Evaluations:

USDA # 93-011-02		
Testing Site		Line Evaluated[1]
Richlands, North Carolina		531
Rocky Mount, North Carolina		531
Jamesville, North Carolina		531

[1] Vector # — Lines
PV-GHBK04 — Line 531

Schedule of major operations:

May	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.
May	Seed planted
October	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 in the fall for volunteer plants. continued monitoring for volunteers will continue until the end of the 1994 cropping cycle in this area. All volunteer plants observed will be destroyed by hand weeding, cultivation, or with chemical sprays.

Summary of Observations

Plant growth and general observations:
The transgenic plants did deviate from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to *Bt* 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 *Btk* 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:
The expression of the *Btk* gene was measured through insect control. Excellent insect control was observed at all sites with several different insects including cotton bollworm, tobacco budworm, and European corn borer.

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

Richlands, NC site

Planted - May 17, 1993
Harvested - October 14, 1993

Field Monitoring for Weediness Characteristics

Germination was reported as follows. Observation made on 30 foot of row on May 25, 1993:

Line 531 Untreated - 85% emergence
Line 531 Treated - 75% emergence
Coker 312 Untreated - 82%
Coker 312 Treated - 85%

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

No differences were observed between the transformed and non-transformed.

Number of flowers or bolls per plant

In the protected *Btk* versus the non-protected *Btk* plots, the total fruit was statistically similar, though numerically favoring Line 531. Observation made on 800 plants on June 8, 1993.

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

Approximately 400 bolls, including transformed and non-transformed, were observed on August 11, August 17, August 25 and September 1. No differences were observed between the transformed and non-transformed plants in the incidence of European Corn Borer infestation of the plants.

Field Monitoring for Disease Susceptibility

On May 25, approximately 1440 plants were observed for differences in susceptibility to plant diseases. Approximately 2% of all plants were determined to be infected with *Rhizoctionia* stem rot. No differences in infection were observed between the transformed and non-transformed plants.

Rocky Mount, NC site

Planted - May 17, 1993
Harvested - October 18, 1993

Field Monitoring for Weediness Characteristics

Germination of the *Btk* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on 10 foot of row on June 1, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on 800 plants on June 19, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on 800 plants on June 19, 1993.

Monitoring for Plant Growth Characteristics

Approximately 800 plants, including transformed and non-transformed, were observed on June 24, June 30, July 8, July 14, July 22, July 29, August 5, August 12, August 19, August 27 and September 10. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

Approximately 800 plants, including transformed and non-transformed, were observed on June 24, June 30, July 8, July 14, July 22, July 29, August 5, August 12, August 19, August 27 and September 10. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

On June 1, 10 foot of row was inspected, on June 17, all of the plots were visually inspected and approximately 800 plants, including transformed and non-transformed, were observed on June 24, June 30, July 8, July 14, July 22, July 29, August 5, August 12, August 19, August 27 and September 10. No differences in the susceptibility of the plants to diseases were observed.

Jamesville, NC site

Planted - May 17, 1993
Harvested - October 20, 1993

Field Monitoring for Weediness Characteristics

Germination of the *Btk* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on 10 foot of row on June 1, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on 800 plants on June 14, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on 800 plants on June 14, 1993.

Monitoring for Plant Growth Characteristics

Approximately 800 plants, including transformed and non-transformed, were observed on June 23, June 29, July 7, July 14, July 21, July 27, August 2, August 10, August 18 and August 23. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

Approximately 800 plants, including transformed and non-transformed, were observed on June 23, June 29, July 7, July 14, July 21, July 27, August 2, August 10, August 18 and August 23. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

On June 1, 10 foot of row was inspected, on June 16, all of the plots were visually inspected and approximately 800 plants, including transformed and non-transformed, were observed on June 23, June 29, July 7, July 14, July 21, July 27, August 2, August 10, August 18 and August 23. No differences in the susceptibility of the plants to diseases were observed.

1993 *Bt* COTTON FIELD RELEASES
(USDA PERMIT#93-011-05)
FINAL REPORT
April 11, 1994

Eric M. Johnson
Monsanto Co.

The purpose of this field release was to test cotton genetically-modified to contain the gene from *Bacillus thuringiensis* var. *kurstaki(B.t.k.)* that encodes its insect control protein. The cotton was tested at twenty one sites by twenty two different cooperators (listed below).

Sites and cooperators	Cotton Lines Tested
Loxley Alabama site	531, 757, 931, 1076, 1172, 1195
[CBI DELETED]	
Prattville Alabama site	1076
[CBI DELETED]	
Casa Grande Arizona site	Not Planted
[CBI DELETED]	
Maricopa Arizona site #1	531, 1076
[CBI DELETED]	
Maricopa Arizona site #2	531, 757. 1076, 1172
[CBI DELETED]	

Sites and cooperators	Cotton Lines Tested
[CBI DELETED	531, 1076
]	
Wabbaseka Arkansas site	531, 757, 931, 1076, 1172, 1578, 1626, 1849, 1888, 2020
[CBI DELETED	
]	
Wilmot Arkansas site	1076
[CBI DELETED	
]	
Shafter California site	757, 931, 1076, 1172
[CBI DELETED	
]	
Tifton Georgia site	531, 757, 1076, 1172, 1578, 1849, 2020
[CBI DELETED	
]	
Bossier City Louisiana site	531, 757, 931, 1076, 1172
[CBI DELETED	
]	

Sites and cooperators	Cotton Lines Tested
St. Joseph Louisiana site	1076
[CBI DELETED]	
Chatham Mississippi site	1076
[CBI DELETED]	
Morgan City Mississippi site	081, 757
[CBI DELETED]	
Scott Mississippi site	531, 757, 931, 1076, 1172, 1578 1626, 1849, 1888, 2020
[CBI DELETED]	
Starkville Mississippi site #1	531, 757, 931, 1076, 1172, 1195 1578, 1849, 1888, 2020
[CBI DELETED]	
Starkville Mississippi site #2	757
[CBI DELETED]	

Sites and cooperators	Cotton Lines Tested
Florence South Carolina site	1076
[CBI DELETED]	
Grand Junction Tennessee site	1076
[CBI DELETED]	
Corpus Christi Texas site	1076
[CBI DELETED]	
Halfway Texas site	531
[CBI DELETED]	
Sinton Texas site	531, 757, 931, 1076, 1172, 1195
[CBI DELETED]	

Genotypes:

This field release included the following genotypes:

- Derivatives of Coker 312 homozygous for PV-GHBK01,PV-GHBK02,PV-GHBK03, PV-GHBK04 and PV-GHBK07
- Coker 312 controls.

Schedule of major operations:

May-Jun	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
May-Jun	Seed planted
Aug-Dec	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 in the fall for volunteer plants. continued monitoring for volunteers will continue until the end of the 1994 cropping cycle in this area. All volunteer plants observed will be destroyed by hand weeding, cultivation, or with chemical sprays.

Plant growth and general observations:

The transgenic plants did deviate from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to *B.t.k.* 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 *B.t.k.* 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 *B.t.k.* 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:
The expression of the *B.t.k.* gene was measured through insect control. Excellent insect control was observed at all sites with several different insects including cotton bollworm, tobacco budworm, and European corn borer.

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.USDA#93-011-02

Individual Site Information

Loxley, AL site

Planted - June 2, 1993
Harvested - October 26, 1993

Field Monitoring for Weediness Characteristics
Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on 20 plants on June 28, 1993

Number of days from planting to flowering (75% of plants have initiated)
No differences were observed between the transformed and non-transformed. Observation made on 25 plants on June 19, 1993.

Number of flowers or bolls per plant
No differences were observed between the transformed and non-transformed. Observation made on 25 plants on September 7, 1993.

Monitoring for Plant Growth Characteristics
The trial was observed on June 28, July 26, August 23, September 20 and October 11. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

The trial was observed on June 28, July 26, August 23, September 20 and October 11. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

The trial was observed on June 28, July 26, August 23, September 20 and October 11. No differences in the susceptibility of the plants to diseases were observed.

Prattville, AL site

Planted - May 17, 1993
Harvested - October 15 and October 25, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on May 27, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 13, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on August 2, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 21, July 9, July 20, July 30, August 9, August 20, August 31 and September 10. No differences in plant vigor, leaf morphology plant height and other characteristics were observed. On , September 15 and September 17 it was reported that the plants seemed to be shedding their leaves prematurely. At first this was suspected to be due to the heavy boll load, as the leaf loss was more pronounced in the *B.t.k.* plots. Later laboratory analyses of the plants revealed that the leaf loss was due to a potassium deficiency.

Field Monitoring for Insect Susceptibility

Approximately 300 plants within the trial were observed on June 21, July 9, July 20, July 30, August 9, August 20, August 31, September 10, September 15 and September 17. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 21, July 9, July 20, July 30, August 9, August 20, August 31, September 10, September 15 and September 17. No differences in the susceptibility of the plants to diseases were observed.

Maricopa, Az site

Planted - May 18, 1993
Harvested -December 6 - 8, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on June 7, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 15, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on September 16, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 7, June 30, July 15, August 3, September 16, October 5, November 2 and December 7. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on May 26, June 7, June 30, July 15, July 29, August 3, August 13, September 16, October 5 and November 2. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted that both the transgenic and non-transgenic cotton plants were equally susceptible to the sweetpotato whitefly. This is expected as the *B.t.k.* protein does not have activity against the sweet potato whitefly.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 30, July 15, August 3, September 16, October 5, November 2 and December 7. No differences in the susceptibility of the plants to diseases were observed.

Yuma, Az site (Field 1)

Planted - May 18, 1993
Harvested -November 22, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on May 24, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Monitoring for Plant Growth Characteristics

These observations were not recorded.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on May 20, May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13 and July 16. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted on June 23 that both the transgenic and non-transgenic cotton plants were equally infested with Armyworm, leaf hoppers, miners and Lygus. This is not surprising as the *B.t.k.* protein present in these plants is not considered active against these pests.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on May 20, May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13 and July 16. No differences in the susceptibility of the plants to diseases were observed.

Yuma, Az site (Field 2)

Planted - May 18, 1993
Harvested -November 22, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on June 7, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13, July 16 and July 20. No differences in plant vigor, leaf morphology plant height and other characteristics, other than expected varietal differences were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on May 24, May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13, and July 16. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted on June 23 that both the transgenic and non-transgenic cotton plants were equally infested with Armyworm, leaf hoppers, miners and Lygus. On July 7, all plants were observed as having a high infestation of SPWF. This is not surprising as the *B.t.k.* protein present in these plants is not considered active against these pests.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on May 24, May 28, June 7, June 11, June 23, June 30, July 7, July 8 and July 20. No differences in the susceptibility of the plants to diseases were observed.

Wabbaseka, AR site (Breeding Nursery)

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

Field Monitoring for Weediness Characteristics

Some dormancy was observed in the transgenic seeds that had been harvested immediately before planting. This is normal because cottonseed has a dormant factor which breaks down over time. Transgenic seeds harvested 6 months earlier emerged at the same time as the control (May 26, 1993).

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

Some plants of line 931 were later in flowering than the non-transgenic parent line (July 20 1993).

Number of flowers or bolls per plant

Boll set on the transgenic lines was better due to insect control (August 26, 1993).

Monitoring for Plant Growth Characteristics

The following observations were made:

June 17 - all looked similar
July 20 - Some of the line 931 plants were later in blooming.
August 16 - there is variation in boll size and maturity but this is probably due to genetic variation which is much greater in the transgenic versus the non-transgenic.
September 20 - Variation exists in plant height, maturity and boll size but is no more than expected in segregating populations. 931 and 1172 appear to be later maturing than 1075 and 757.
October 13 - transgenic lines had much more genetic variation because of the early generation populations.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 17, July 20, August 16, September 20 and October 13. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted on July 20 and August 16 that aphids and boll weevils were present throughout the plot. This is not surprising as the *B.t.k.* protein present in these plants is not considered active against these pests. On October 13 it was noted that the non-transgenic plants showed greater boll damage than the non-transgenic plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 17, July 20, August 16, September 20 and October 13. No differences in the susceptibility of the plants to diseases were observed.

Wabbaseka, AR site (Breeding Nursery)

Planted - May 19, 1993
Harvested -not harvested, destroyed September 9, 1993

Field Monitoring for Weediness Characteristics

No significant differences in plant emergence was observed on May 25.

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

The time from planting to flowering was 63 to 70 days for all plants.

Number of flowers or bolls per plant

All plants were reported to have similar fruiting with the transgenic plants having longer peduncles.

Monitoring for Plant Growth Characteristics

All plants were observed on June 24, July 2, July 21, July 28, July 30 and August 3. It was reported that some difference in general appearance such as long peduncles and perhaps a slightly different growth rate in the transgenics.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on July 2, July 8, July 9, July 14, July 19, July 26, July 27, August 2, August 6 and August 10. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Approximately 50 plants within the trial were observed on June 4, July 8, July 14, July 19, July 26, August 2 and August 10. No differences in the susceptibility of the plants to diseases were observed.

Shafter, CA site

Planted - May 24, 1993
Harvested -November 11, 1993

Field Monitoring for Weediness Characteristics

Due to the very late planting of the transgenic cotton, it was very difficult to compare growth habits. However, no unusual characteristics were observed .

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

This information was not recorded.

Number of flowers or bolls per plant

This information was not recorded.

Monitoring for Plant Growth Characteristics

On June 1 the plants were observed and noted that they were much delayed in growth due to the late planting which made this comparison difficult to make.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 1 and October 1, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. The field had very light insect pressure.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 1 and October 1, 1993. No differences in the susceptibility of the plants to diseases were observed. The field had a very light incidence of disease.

Tifton, GA site

Planted - May 21, 1993
Harvested -October 28, 1993

Field Monitoring for Weediness Characteristics

There was better emergence and seedling vigor in transgenic plans than in the non-transgenic plants, but differences were not significant (June 1)

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 1, June 8, June 15, June 22, June 29, July 6, July 13, July 20, July 27, August 3, August 10, August 17, August 24, August 31, September 7, September 14, September 21, September 28 and October 5. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 1, June 8, June 15, June 22, June 29, July 6, July 13, July 20, July 27, August 3, August 10, August 17, August 24, August 31, September 7, September 14, September 21, September 28 and October 5. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 1, June 8, June 15, June 22, June 29, July 6, July 13, July 20, July 27, August 3, August 10, August 17, August 24, August 31, September 7, September 14, September 21, September 28 and October 5. No differences in the susceptibility of the plants to diseases were observed.

Bossier City, LA site

Planted - May 18, 1993
Harvested -October 15, 1993

Field Monitoring for Weediness Characteristics

No differences were observed in emergence and seedling vigor between the transgenic and non-transgenic plants (May 27).

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 22, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on September 3, 1993.

Monitoring for Plant Growth Characteristics

The following observations were recorded:

June 18 - No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

July 14 - Saw 4 - 5 plants with silvered leaves, asymmetrical and usually one or more lobes on leaves appeared malformed. All plants small but within normal size range. This observation is limited to line 1076.

August 11 and September 14 - Same as on July 14.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 18, July 14, August 11 and September 14. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 18, July 14, August 11 and September 14. No differences in the susceptibility of the plants to diseases were observed.

St. Joseph, LA site

Planted - May 17, 1993
Harvested -August 27, September 3 and September 27

Field Monitoring for Weediness Characteristics
Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on June 18, 1993

Number of days from planting to flowering (75% of plants have initiated)
No differences were observed between the transformed and non-transformed. Observation made on all plants on June 18, 1993.

Number of flowers or bolls per plant
No differences were observed between the transformed and non-transformed.

Monitoring for Plant Growth Characteristics
All plants within the trial were observed on June 18, July 13, August 2 and August 27. No differences in plant vigor, leaf morphology plant height and other characteristics, other than expected varietal differences were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 18, July 13 and August 2. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 18, July 13, August 2 and August 27. No differences in the susceptibility of the plants to diseases were observed.

Catham, MS site

Planted - June 7, 1993
Harvested -October 27 and 28, 1993

Field Monitoring for Weediness Characteristics

Two plots with non-transgenic plants have a poor stand. Appears to be the result of non-uniform irrigation in this area. Too much water (June 30, 1993).

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

Coker 312 plants were observed to have blooms on July 23, ,line 1076 had no blooms. Line 1076 had a later fruit setting and the cause was not determined but did not appear to be early insect damage (July 23, 1993).

Number of flowers or bolls per plant

Coker 312 appears to have more and larger bolls than line 1076. Does not appear to be insect related (August 20, 1993).

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 30, July 23 and August 20. The following observations were recorded:
June 30 - No differences in plant vigor, leaf morphology plant height and other characteristics were observed.
July 23 - non-transgenic had blooms while line 1076 hod no blooms.
August 20 - line 1076 had fewer and smaller bolls than the non-transgenic Coker 312.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 30, July 23 and August 20. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 30, July 23 and August 20. No differences in the susceptibility of the plants to diseases were observed.

Morgan City, MS site

Planted - June 2, 1993
Harvested -November 11, 1993

Field Monitoring for Weediness Characteristics

One Hundred of each line were compared and no differences were observed between the transgenic and non-transgenic (June 15, 1993).

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

One Hundred of each line were compared and no differences were observed between the transgenic and non-transgenic (August 10, 1993).

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics between Coker 312 and line 757 were observed throughout the growing season.

Field Monitoring for Insect Susceptibility

There was a tendency to have a higher population of *Lygus* spp. in the line 757 plot versus the Coker 312 plot.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to diseases to the Coker 312 and line 757 were observed throughout the growing season.

Scott, MS site

Planted - May 21 and May 27, 1993
Harvested -

Field Monitoring for Weediness Characteristics

This information was not recorded.

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

This information was not recorded.

Number of flowers or bolls per plant

This information was not recorded.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics were observed between the transgenic and non-transgenic lines.

Field Monitoring for Insect Susceptibility

No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to any disease were observed between the transgenic and non-transgenic lines.

Volunteers

No volunteer plants were observed on January 17, 1994.

Scott, MS site

Planted - May 13 and May 19, 1993
Harvested -September 20, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic lines.

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

No differences were observed between the transgenic and non-transgenic lines.

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics were observed between the transgenic and non-transgenic lines.

Field Monitoring for Insect Susceptibility

No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to any disease were observed between the transgenic and non-transgenic lines.

Florence, SC site

Planted - May 25, 1993
Harvested - October 21, 1993

Field Monitoring for Weediness Characteristics

Stand counts on June 9 indicated that the germination percentage was slightly higher for the transgenic plants when compared to the non-transgenic plants

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

Transgenic plants bloomed later than the non-transgenic plants (July 16, 1993).

Number of flowers or bolls per plant

Transgenic plants had higher numbers of bolls than did the non-transgenic plants. On July 30, bolls per 100 plants were 613 for non-transgenic treated, 548 for non-transgenic not treated and 833, 730, 695, 825 and 710 for treatments 1,2,3,4 and 6 respectively.

Monitoring for Plant Growth Characteristics

On June 21, the transgenic plants were smaller than the non-transgenic plants. Plant height was 8.9 inches compare to 9.7 inches.

Field Monitoring for Insect Susceptibility

The entire plot was observed on July 6, July 22, August 3 and August 4. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to any disease were observed between the transgenic and non-transgenic lines on June 25 and August 3.

Grand Junction, TN site

Planted - May 21, 1993
Harvested - October 25 and November 11, 1993

Field Monitoring for Weediness Characteristics

More than 50 plants were observed on June 10, 1993 and no differences were observed between the transgenic and non-transgenic.

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

More than 50 plants were observed on July 16, 1993 and no differences were observed between the transgenic and non-transgenic.

Number of flowers or bolls per plant

More than 50 plants were observed on July 30, 1993 and no differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics between Coker 312 and line 1076 were observed throughout the growing season. Observations were taken on June 10, June 22, July 2, July 16, July 30, August 16 and August 26, 1993.

Field Monitoring for Insect Susceptibility

Observations were taken on June 10, June 22, July 2, July 16, July 30, August 16 and August 26, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 10, June 22, July 2, July 16, July 30, August 16 and August 26, 1993. No differences in the susceptibility to diseases to the Coker 312 and line 1076 were observed throughout the growing season.

Corpus Christi, TX site

Planted - May 18, 1993
Harvested - N/A

This site was lost to excessive rains following planting. All plants were reported as dead by August 1993.

Monitoring for Volunteers

Following termination of this trial, tbe field has been monitored for volunteers. Observations were taken on 9/15, 10/13, 11/17/1993, 1/7, and 3/8/1994. NO volunteer cotton plants were ever observed at the plot site.

Halfway TX site

Planted - May 19, 1993
Harvested - November 23, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic.

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

No differences were observed between the transgenic and non-transgenic.

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics between Coker 312 and line 531 were observed throughout the growing season. Observations were taken on June 14, June 25, July 15, August 10, August 26, September 13, October 11, October 28 and November 11, 1993.

Field Monitoring for Insect Susceptibility

Observations were taken on June 14, June 25, July 15, August 10, August 26, September 13, October 11, October 28 and November 11, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 14, June 25, July 15, August 10, August 26, September 13, October 11, October 28 and November 11, 1993. No differences in the susceptibility to diseases to the Coker 312 and line 531 were observed throughout the growing season.

Sinton TX site - Efficacy Trial

Planted - May 17, 1993
Harvested - September 17 and 20, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic.

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

Line 1076 was rated approximately 6 days slower to develop and mature than was Coker 312.

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

Observations were taken on June 8, July 2, July 29 and August 26, 1993. The following observations were recorded:

Line 1076 appeared slightly shorter and slower to develop than Coker 312 (July 2).

Line 1076 was later in flowering than Coker 312 (July 29).

Line 1076 has smaller bolls which opened more slowly than Coker 312 (August 26).

Field Monitoring for Insect Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences in the susceptibility to diseases to the Coker 312 and the transgenic lines tested were observed throughout the growing season.

Field Monitoring for Volunteers

The filed was monitored for volunteers on March 8, 1994. None were observed.

Sinton TX site - Gene Evaluation Trial

Planted - May 17, 1993
Harvested - September 17 and 20, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic (May 28).

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

Lines 931, 1076, 1172 and 1195 were slower to produce and develop flower buds versus the Coker 312. This was possibly a function of more fruit on the *B.t.k.* lines than on Coker 312 (July 15, 1993).

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines (August 26, 1993).

Monitoring for Plant Growth Characteristics

Observations were taken on June 8, July 2, July 29 and August 26, 1993. The following observations were recorded:

Line 931 appears shorter and with less mainstem nodes than Coker 312 (July 2).
Line 931 is still shorter in plant height. Lines 931, 1076, 1172 and 1195 all flowered later than Coker 312 (July 29).
Lines 931, 1076 and 1172 have slower boll opening than Coker 312 (August 26).

Field Monitoring for Insect Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences in the susceptibility to diseases to the Coker 312 and the transgenic lines tested were observed throughout the growing season.

Field Monitoring for Volunteers

The filed was monitored for volunteers on; October 13, November 17, 1993 and January 7, February 8 and March 8, 1994. None were observed. In December 1993, some volunteers emerged and were destroyed by disking.

Appendix VI

Expert Opinion Letters

United States Department of Agriculture
Agricultural Research Service
Mid South Area
Crop Science Research Laboratory
P. O. Box 5367
Mississippi State
Mississippi 39762

May 5, 1994

SUBJECT: Boll Size, Lint Percent, and Yield of M 531 Comparison Lines

TO: Bob Buehler
Monsanto Company
700 Chesterfield Parkway North - GG6A
St. Louis, Missouri 63198

FROM: Johnie N. Jenkins
Director
Crop Science Research Laboratory

Johnie N Jenkins

The 1993 data on boll size, lint percentage, yield, and fiber properties, of each of the lines in the 1993 P1 test are shown on the enclosed sheet.

M 531 had significantly smaller bolls than Coker 312; however, the lint percentage was not different from Coker 312. The yield of M 531 was significantly higher than Coker 312 when all insects were controlled. Thus, the smaller bolls did not result in a reduction in yield.

Enclosure

BZLP93M 1993 DATA FROM JOHNIE N. JENKINS, ARS, MISSISSIPPI STATE, MS

TO: BOB BEUHLER, MONSANTO

FROM: JOHNIE N. JENKINS

	MICRONAIRE		50% SL		2.5% SL		E1		STRENGTH		BOLL SIZE		LINT PERCENT		LINT YIELD KG PER HA	
ENTRY	SKIP	SOLID	SKIP	SOLID	SKIP	SOLID	SKIP	SOLID	SKIP	SOLID	SKIP	SOLID	SKIP	SOLID	SKIP	SOLID
M 531	4.2	3.9	14.27	14.41	29.29	29.42	7.98	8.23	203.5	205.5	4.26	3.85	35.88	37.03	1316	808
M 757	4.1	3.9	14.28	14.23	28.97	29.13	8.14	8.29	204.3	207.1	4.17	3.76	35.89	36.08	1227	734
M 931	4.2	3.9	14.62	14.58	29.44	29.18	8.12	8.50	207.1	212.6	3.74	3.60	38.54	38.67	832	461
M 1076	4.0	3.8	14.07	14.00	29.10	29.26	7.58	7.97	205.0	209.6	4.09	3.73	32.57	32.83	999	616
M 1172	4.3	4.2	14.00	13.78	28.59	28.37	8.24	8.10	208.1	218.3	3.68	3.43	34.00	34.04	823	484
M 1195	4.2	4.1	13.94	13.79	27.79	27.79	8.23	8.29	196.7	199.2	3.70	3.38	38.83	39.52	788	466
COKER 312	3.9	3.7	14.43	14.52	29.80	30.02	7.79	7.96	202.7	204.1	4.64	4.24	35.89	36.31	1149	667
DES 119	4.4	4.3	14.48	14.55	28.64	28.92	9.00	9.20	208.0	211.2	4.33	4.22	38.08	37.71	1338	843
LSD 0.05	0.2	0.2	0.29	0.28	0.55	0.41	0.34	0.36	5.9	6.5	0.18	0.19	0.87	0.86	99	104

ENTRY	MIC MEAN	50% SL MEAN	2.5% SL MEAN	E 1 MEAN	STRENGTH MEAN	BOLL SIZE MEAN	LINT % MEAN	LINT YIELD MEAN KG/HA
M 531	4.1	14.34	29.35	8.11	204.5	4.06	36.46	1062
M 757	4.0	14.26	29.05	8.22	205.7	3.97	35.99	981
M 931	4.1	14.60	29.31	8.31	209.9	3.67	38.61	647
M 1076	3.9	14.04	29.18	7.78	207.3	3.91	32.70	808
M 1172	4.3	13.89	28.48	8.17	213.2	3.56	34.02	654
M 1195	4.2	13.87	27.79	8.26	198.0	3.54	39.18	627
COKER 312	3.8	14.48	29.91	7.88	203.4	4.44	36.10	908
DES 119	4.4	14.52	28.78	9.10	209.6	4.28	37.90	1091
LSD 0.05						0.15	0.66	103

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May 3, 1994

To Whom It May Concern:

Boll size is not a trait that is commonly measured by the plant breeders at Delta and Pine Land Company. There is no definite correlation between boll size and yield. Since the commercial cotton grown in the U.S. is all mechanically harvested, there is no real value to any particular boll size.

Early indications are that transgenic cotton's, containing Bt Construct 531, produce at least as much lint as the recurrent parents from which they were derived. Preliminary data shows no reduction and possibly an increase in lint production from these lines.

I have not measured the size of the bolls in this cotton. However, if the boll size turns out to be smaller than that of the recurrent parent, it will not matter. Total yield and not boll size will be used to evaluate these plants.

Sincerely,

Keith Jones, Ph.D.
Senior Cotton Breeder

Louisiana State University
Agricultural Center
Louisiana Agricultural Experiment Station

Red River Research Station
Hwy. 71 South
Post Office Box 8550
Bossier City, LA 71113-8550
(318) 741-7430
Fax: (318) 741-7433

May 6, 1994

Mr. Bob Buehler
Monsanto Company GG6A
700 Chesterfield Parkway North
St. Louis, Missouri 63196

Dear Mr. Buehler:

Enclosed is the information you requested on the comparison of Coker 312 and Strain 531. Please let me know if you need additional information .

Sincerely,

William D. Caldwell

William D. Caldwell
Professor

WDC:mm

1993 Monsanto Bt Cotton Trials
Louisiana Agricultural Experiment
Red River Research Station
Bossier City, Louisiana

The lint yield of Strain 531 was greater than Coker 312 regardless of spray schedule as shown on the following page. The boll size was smaller for Strain 531 than Coker 312 in both sprayed and non-sprayed plots. However, no significant differences occurred in boll size in the nonsprayed plots. The smaller boll size appears to have no influence on the yields observed in Strain 531 and Coker 312. Visual ratings were made on the picker efficiency following harvest with a mechanical harvester. Rating were made on a scale of 0-5, 0=excellent efficiency, 5=very poor efficiency. Picker efficiency ratings were the same for Coker 312 and Strain 531. Therefore, boll size had no influence on harvest efficiency. The lint percent and fiber quality were similar for both Coker 312 and Strain 531. However, fiber strength was improved in Strain 531. The differences between Strain 531 and Coker 312 are shown on the following page. Greater differences are found among strains developed from the same parental cross. Differences of similar magnitude also occur among the varieties that are recommended for planting in Louisiana.

Coker 312 - Strain 531 Comparison

Plots	Nonsprayed		Sprayed	
Entries	Coker 312	Strain 531	Coker 312	Strain 531
Lint yield (lbs/A)	711	858	797	802
Boll weight (grams)	4.8	4.7	4.9	4.4
Lint percent	37.8	38.1	37.7	38.1
Picker efficiency	1	1	1	1
Rating (0-5)				
0 = excellent efficiency				
5 = very poor efficiency				
Fiber Quality				
Micronaire	4.8	5.1	4.8	4.9
Length	1.18	1.15	1.16	1.15
Strength (grams/tex)	26.5	28.6	26.2	28.4

TEXAS AGRICULTURAL EXPERIMENT STATION
THE TEXAS A&M UNIVERSITY SYSTEM
Lubbock, Texas

TEXAS A&M UNIVERSITY AGRICULTURAL RESEARCH AND EXTENSION CENTER

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May 12, 1994

Dr. Frank Serdy
Monsanto GG6A
700 Chesterfield Parkway North
St. Louis, MO 63198

Dear Dr. Serdy:

I have performance tested cotton line 531 containing the Bt gene for several years. The performance of this line in our tests has been very good. Almost all traits of line 531 are equal, or superior, to the Coker cultivar which was used as the recurrent parent in the developmental process. The only attribute that some might consider detrimental is boll size. Line 531 produces a boll that is about 5% smaller than the recurrent parent. However, numerous cultivars currently available exhibit somewhat smaller bolls than the cultivars they are replacing. The reason for the smaller boll size is smaller seed and a thinner carpel wall. These are not disadvantageous traits. Cultivars with smaller seed are of equal vigor and tend to germinate as well as larger seeded cultivars.

I feel that line 531 is a very promising cotton genotype that will be well accepted by cotton producers.

Sincerely

John R. Gannaway

John R. Gannaway
Professor

JRG:ss

Appendix VII

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 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

Cotton leaf, seed and whole plant tissues to conduct safety assessment studies were collected from 6 sites throughout the cotton growing regions of the United States. The six field sites were as follows: Starkville, Mississippi; Bossier City, Louisiana; College Station, Texas; Tifton, Georgia; Maricopa, Arizona; and Loxley, Alabama. Expression levels of the *B.t.k.* HD-73 and NPTII proteins were estimated in each of these tissues and in leaf tissue sampled throughout the cotton growing season. Analysis for AAD was only performed for the young leaf and seed samples. Since none was detected in either of these tissues, no analysis for AAD was performed for leaves harvested throughout the season or in whole plants. Compositional analysis of the important cottonseed components (protein, oil, carbohydrate, ash, moisture and calories), as well at the composition of individual fatty acids and natural toxicants (gossypol, cyclopropenoid fatty acids and aflatoxin) present in Bollgard™ Cotton Line 531 were compared to the Coker 312 parental control to verify that the genetic engineering process did not alter these important seed components. Cottonseed from across four of the locations was pooled and processed to commercially representative fractions to compare the processing and processed fractions (particularly the toasted meal and refined oil) derived from cottonseed from the Bollgard™ Cotton Line 531 to those from the Coker 312 control. In addition, the levels of the *B.t.k.* HD-73 and NPTII proteins in the processed fractions were determined to facilitate exposure assessment of these proteins in human food and animal feed.

The following is a summary of the methods used to analyze these plant fractions.

Samples

Representative plant tissue samples were collected at various times during the growing season from Bollgard™ Cotton Line 531 and from the Coker 312 control. These samples included representative samples of the first true leaves, young leaves sampled approximately each month after the first true leaf samples were obtained, mature whole plants sampled just prior to harvest at one location (Mississippi), analytical seed samples and bulk seed samples (collected and pooled across replicates at each location). Nectar and pollen from these lines was collected from cotton plants grown in the greenhouse.

Protein Extraction from Cotton Leaf Tissue

For analyses, each leaf sample (containing four leaves) was mixed, sampled and extracted in a single vessel, according to SOP # BtC-PRO-019-02. Briefly, frozen leaves, as shipped from the field, were crushed to a course powder and mixed while in the sample container bag on dry ice. Frozen tissue was weighed and cold Tris-Borate (T-B) extraction buffer added to a final ratio of approximately 1 mg leaf tissue/40µL buffer (1:40). The T-B extraction buffer is 100 mM Tris-HCL, pH 7.5, 10mM sodium borate, 0.05% (v/v) Tween-20, 5mM $MgCl_2$, 0.2% (w/v) L-ascorbate. The tissue

was extracted with a Polytron PT3000 tissue homogenizer (Brinkman, Inc. Westbury, NY) equipped with a PTA 10TS generator for 1 minute at approximately 22,000 rpm and immediately placed on ice. Insoluble material was removed by centrifugation at approximately 10,000 x g for approximately 10 minutes at approximately 4°C. The supernatant was removed, aliquoted and used as the "cotton leaf extract" in further analyses. Aliquots of leaf extract were stored at approximately -80°C until analyzed.

Protein Extraction from Cotton Seed Tissue

Five cotton seeds were weighed from each sample of delinted seed (analytical seed samples) and extracted in a single vessel, according to SOP # BtC-PRO-019-02. The seeds were individually cracked, placed in a plastic tube, and cold T-B extraction buffer (described above) added to a final ratio of approximately 1 mg seed tissue/20µL buffer (1:20). The seeds were homogenized with a Polytron PT3000 tissue homogenizer (Brinkman, Inc., Westbury, NY) equipped with a PTA 10TS generator using four bursts of approximately 15 seconds, allowing cooling and settling of the tissue to occur between bursts; after extraction the homogenate was immediately placed on ice. The homogenate was clarified by centrifugation at approximately 10,000 x g for approximately 10 minutes at approximately 4°C. The supernatant was removed, aliquoted and used as the "cotton seed extract" in further analyses. Aliquots of leaf extract were stored at approximately -80°C until analyzed.

Protein Analysis

Crude protein content in the toasted meal fractions from processing was measured by Kjeldahl analysis (AOAC official method 976.06, 1990) according to SOP at the Delta Branch Experiment Station in Stoneville, Mississippi.

Total protein in tissue extracts was measured by the method of Bradford (1976) using the microtiter plate application of the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). the procedure (SOP # PRO-90-015-00) was validated, showing acceptable variability and appropriateness for evaluating total protein in cotton tissue extracts. Bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO) dissolved in T-B extraction buffer, was chosen as the appropriate standard by comparing protein assay results to amino acid composition of the same extracts (Rogan, *et al.*, 1992).

Quantitation of the levels of B.t.k. HD-73, NPTII and AAD proteins

The amount of *B.t.k.* HD-73, NPTII and AAD proteins in the extracts prepared from cotton leaf and seed samples were determined by validated Enzyme-Linked Immuno-Sorbent methods (ELISAs). Each ELISA was shown to be sensitive to the specific protein analyzed. The accuracy, precision and ruggedness of each of these assays was assessed. Spike-and-recovery and extraction efficiencies for each of the proteins measured in each of the matrices was evaluated for young leaf and seed tissue, for young leaves over the season and for whole plants. Stability of these proteins in the respective cotton tissue matrices was assessed and all assays were performed within the known limits of stability for each protein.

For *B.t.k.* HD-73, the full length protein expressed in the respective tissue was treated with trypsin to convert this protein to the trypsin-resistant core, which was then quantitated in the validated ELISA. Trypsinization was required to accurately estimate the amount of *B.t.k.* HD-73 protein present in these tissues.

Validated computer systems and software were used for data collection and reduction. Statistical analyses were performed as described in each of the attached reports.

Western Blot Analyses

Western blot analysis was completed according to SOP # BtC-PRO-002-02, a procedure similar to that described by Matsudaira (1987). Briefly, acrylamide gels from SDS-PAGE were equilibrated in the same buffer used for electrolution (transfer). Proteins were transferred out of the acrylamide gel onto nitrocellulose membrane. Additional protein binding sites on the membrane were blocked using 3% bovine serum albumin (BSA) in Tris-HCl (pH 8.0)/saline/Tween-20 buffer (TBST). The blots were incubated with a 1:1500 dilution (in TBST/1% BSA) of F204 antibody (bleed 9) specific for the HD-73 protein followed by incubation with goat anti-rabbit antibody-alkaline phosphatase conjugate (Promega Corp, Madison, WI). Protein bands bound by antibody were visualized using the NBT/BCIP colorimetric substrate system (Promega, Corp., Madison, WI). Levels of the *B.t.k.* HD-73 protein were quantitated by comparison to standards spiked into the same matrix and contained on the same blot.

Compositional analysis of cottonseed

The levels of protein, fat, ash, carbohydrates, calories and moisture (proximate analysis) were determined for cottonseed obtained from each site and each line (the seed were pooled across plots at each field test site). The analyses were conducted at Hazelton Laboratories, Madison, WI. The analytical methods utilized are as follows:

Protein (N x 6.25)

Official Methods of Analyses (1990), 15th Edition, Method 955.04C, 979.09, AOAC, Arlington, Virginia, (Modified).

The Kjeldahl method for Organic Nitrogen, R.B. Bradstreet, Academic Press, New York, New York (1965)

Quantitative Inorganic Analysis, Kelthoff and Aandell (1948), Revised Edition.

Fat

Official Methods of Analysis (1990), 15th Edition, Method 960.39, AOAC, Arlington, Virginia, (Modified).

Ash

Official Methods of Analysis (1990), 15th Edition, Method 923.03, AOAC, Arlington, Virginia, (Modified).

Carbohydrates

The total carbohydrate level is determined by difference after the percentages of protein, moisture, ash and fat are known. SOP #MP-CHO-MA.

Calories

The total calories in the proteins, carbohydrates and fats of various food and feed types have been determined by bomb calorimetry and feeding studies. The 4 cal/g (protein), 9 cal/g (fat) and 4 cal/g (carbohydrate) factors are averages of the values derived from these tests. SOP #MP-CALC-MA.

Moisture, 100 Degree Vacuum Oven.

Official Methods of Analysis (1990), 15th Edition, Method 926.08, 925.09, AOAC, Arlington, Virginia, (Modified).

Aflatoxin

Proceeding of the 3rd International Congress of Food Science and Technology, Pages 705-711 (Modified).

1 Determination by High Performance Liquid Chromatography: Journal of Assoc. Official Analytical Chemist, Volume 71, No.1, 26.052-26.060 (1988) (Modified).

2 Determination by One Dimensional Thin Layer Chromatography: Journal Assoc. Official Analytical Chemist, Volume 71, No.1, 26.031 (1988) (Modified).

3 Determination by Two Dimensional Thin Layer Chromatography: Journal Assoc. Official Analytical Chemist, Volume 71, No.1, 26.074 (1988) (Modified).

The levels of aflatoxins B_1, B_2, G_1 and G_2 were determined for each line from each of the six field test sites, and calculated according to OP-AC 103.

Cottonseed processing

Seed cotton from four of the six field sites (Mississippi, Louisiana, Texas and Georgia) were ginned and pooled (by line) across all four sites as a source of seed cotton for processing. Cottonseed was processed at the Food Protein Research & Development Center at Texas A&M University using a solvent extraction method, according to SOP# 8.27 R02, "Small- Scale Processing of Glanded Cotton to Bind Gossypol", SOP# 8.33 R01, "Small-Scale Toasting of Meal", and SOP# 8.1 R04, "Small Scale Processing of Cottonseed". The processing procedure used for this experiment was a scaled down version of the commercial procedure. The *B.t.k.* HD-73 content in the cottonseed meal before and after processing was estimated by measuring the bioactivity of these samples against tobacco budworm and by western blot analysis. NPTII protein levels were also

estimated in the cottonseed meal before and after processing using both an enzymatic assay specific for NPTII (similar to McDonnell, 1987) and by western blot analysis. The proximate composition of the toasted meal and the free and total gossypol levels in the raw and processed cottonseed meal was assessed. The amount or lack of total protein in the refined oil was also assessed.

Preparation of Seed Kernel Material for Gossypol and Fatty Acid Analyses

Cottonseed were dehulled with a Bauer Mill and the kernels separated from the hulls by hand. The kernels were ground using either of two techniques: 1) on dry ice using a stainless steel Wiley mill and passage through a 10 mesh screen, or 2) by hand with a mortar/pestle and passage through a 20 mesh screen. Duplicate samples of ground kernel, weighing approximately 3 grams each, were placed in glass vials, one set used for gossypol analysis, the second for fatty acid analysis.

Moisture Determination for Gossypol and Fatty Acid Analysis

Percent moisture in each samples of the kernel material was determined by weight difference before and after lyophilization. Samples were lyophilized in tared flasks to remove all water and obtain a true dry weight to the nearest 0.1 mg.

Measurement of Free and Total Gossypol Levels

Free and total gossypol levels were measured in the cottonseed kernel (prior to processing), toasted cottonseed meal (processed), and refined cottonseed oil at the USDA-ARS Southern Crop Research Laboratory, College Station, Texas. Evaluation of free gossypol levels was completed using high performance liquid chromatography (HPLC) according to the procedure described by Stipanovic, *et al.*, 1988 and A.O.C.S. Official Method Ba 7-58. Total gossypol levels (corrected for moisture) were measured spectrophotometrically using aniline as a complexing agent (Pons, *et al.*, 1958 and A.O.C.S. Official Method Ba 8-78).

Quantitation of Fatty Acid Levels

Lipids were extracted using a double Bligh and Dyer procedure (Bligh and Dwyer, 1959), as recently described by Wood (1991).

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, 1968a). The free fatty acids were converted quantitatively to phenacyl derivatives according to the procedure of Wood and Lee (1983).

Approximately 400 µg of the phenacyl derivatives were analyzed by high performance liquid chromatography (HPLC) according to the procedure used to examine the fatty acids of cottonseed (Wood, 1986a and 1986b). Peak elution order and peak shape were monitored by a strip recorder. 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. Peak area for each fatty acid is directly proportional to the percent of each fatty acid contained in total lipid.

Tobacco budworm bioassays.

Tobacco budworm diet incorporation assays (SOP #BUG-PRO-022-02) were used to assess the insecticidal activity of the *B.t.k.* HD-73 protein as well as to estimate the amount of *B.t.k.* protein expressed in cottonseed and processed cottonseed meal. Insecticidal activities were estimated in terms of EC_{50} values. EC_{50} is the concentration of *B.t.k.* HD-73 protein that is required to reduce the weight of the treated tobacco budworm larvae to 50% of the untreated larvae.

Insect feeding assay

The biological activity of purified and seed-expressed CryIA(c) protein was evaluated using a pinto bean-based (PB) insect diet incorporation assay (Reese et al. 1972, MacIntosh et al. 1990). *H. virescens* were obtained from the USDA-ARS, Stoneville, MS. Liquid agar-based pinto bean diet with 20% of the water omitted (24 mL) was added to 6 mL samples of test liquid (distilled water containing doses of the test, reference, or control substance). Treated diet was blended using a Vortex mixer, poured into 96-well insect assay trays, and allowed to cool and harden. One first instar *H. virescens* larva was added to each well. Apparently healthy, motile TBW larvae were impartially assigned to treatments. Wells were covered with Mylar® plastic and ventilated with a single insect pin hole. Assays were incubated at 28 ± 2°C and evaluated after 7 days.

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May 4, 1992

Dr. Paul Hedin
USDA-ARS Crop Science Research Laboratory
P.O. Box 5367
Starkville, MS 39762

Dear Dr. Hedin,

As per our telephone conversation, this letter confirms our agreement for you to analyze allelochemical levels in genetically modified and unmodified cotton squares and termini from the 1992 field trials. This will be the same type of analysis you completed last year for Dr. Roy Fuchs (levels of gossypol, anthocyanin, flavenoids, tannins).

This year, we need analysis of lines 531, 931 and C312. We will need final results by August 30, 1992.

I understand that no additional compensation will be made by Monsanto for these analyses -- compensation will be made through our agreement with Dr. Jenkins for the 1992 *B.t.* cotton field trials.

Please call me if you have any questions or feel you cannot make the stated deadlines. Thank-you for your cooperation.

Sincerely,

Sharon A. Berberich
Sr. Research Biologist
Regulatory Sciences
Phone: 314-537-6054
FAX: 314-537-6567

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Report

Analysis of Allelochemicals in Transgenic Cottons and Controls: 1992

Cotton Host Plant Resistance Unit, USDA-ARS
Mississippi State, MS 39762

The female moths of the tobacco budworm Heliothis virescens (F.), oviposit in the terminals of the cotton plant, Gossypium hirsutum L. There is no evidence that they are specifically attracted to the terminals by chemical cues, but rather that they are most proximate to the terminals during an overflight. Most of the eggs are placed singly on the small terminal leaves approximately 12 cm^2 in size (Ramalho, 1983). Three days after oviposition, the eggs hatch. Immediately after hatch, the young larvae will feed on the leaf tissue for a brief period before they migrate into the terminal area, which is comprised of meristematic tissue (immature squares and leaves). The young larvae spend about three days feeding on small squares with the potential of destroying a maximum of four squares each during this feeding period (Parrott, unpublished data).

During this period, gossypol is toxic to the larvae (Hedin et al., 1988), which also are observed to avoid consuming glands that contain gossypol. However, when the larvae molt into the second instar at about 72 hr, they can non-selectively consume the glands (Parrott et al., 1983). Earlier, Shaver and Parrott (1970) reported young larvae to be more sensitive to gossypol than older larvae. This finding was later supported by laboratory studies in which gossypol and two other allelochemicals were fed in diets to 1-, 3-, and 5-day old tobacco budworm (TBW) larvae. All three allelochemicals were toxic to 1- and 3-day old larvae, but they were not toxic to 5-day old insects. Evidence was obtained that the insects biosynthesized detoxifying enzymes, mixed function oxidases (MFO's), because piperonyl butoxide, a known inhibitor of MFO's, inhibited growth when added to the diets (Hedin et al., 1988).

Origin

Gathering and Processing of Plant Tissue. Cotton seed was provided by Monsanto. The plant samples were collected under the supervision of Drs. Jenkins and McCarty. Terminal leaves and squares were harvested, frozen, freeze-dried, and then ground through a 40-mesh screen. The powders were stored in vials at -20°C until they were evaluated. The plant material was provided by the USDA Cotton Research Unit at Mississippi State, MS.

Analysis of Allelochemicals. Analyses for gossypol and related terpenoid aldehydes were performed on cyclohexane-ethyl acetate-acetic acid (500:500:1; CHEA) extracts of plant tissues by the phloroglucinol reaction [2% in absolute EtOH-concentrated HCl (1:1)]; let stand 1 hr, with subsequent spectrometric analysis at 550 nm. The concentration was determined by comparison with data obtained from authentic gossypol and is expressed as gossypol equivalents. Condensed tannin analyses were performed on 70% aqueous methanol (MW) extracts of tissue. The

anthocyanidin chromophore was developed from the tannin by boiling 1 hr with 1-butanol-HCl (95:5) (Hedin et al., 1983b). The concentration was determined by comparison with the color obtained at 550 nm from a purified cotton condensed tannin sample, the structure of which was elucidated by Collum et al. (1981). The anthocyanin content was determined by measuring the absorbancy at 540 nm of a freeze-dried tissue extracted with methanol-water-HCl (79:19:3), using the molar extinction coefficient (E) of cyanidin 3-β-glucoside (Hedin et al., 1967). Flavonoids were determined after extraction of freeze-dehydrated tissue with 70% aqueous acetone. Diphenylboric acid-ethanolamine complex (Natural Product Reagent A, Aldrich Chemical Co., 1%) in methanol was added, and the chromophore absorptivity at 440 nm was determined and compared to that obtained from a purified sample of isoquercitrin, the most prevalent flavonoid in cotton. The results obtained were the average of three analyses. The data was collected from a Lotus 1-2-3 program interfaced with a Perkins Elmer Lambda 4B UV-Vis spectrophotometer. Spectrophotometer readouts were translated into percent of total using the following factors: % Gossypol = ABS X 0.563, % Tannin = ABS X 17.06, % Flavonoids = ABS X 0.97, and % Anthocyanin = ABS X 0.484. Analyses were performed by Mrs. M. Petty under the supervision of Drs. Hedin and McCarty.

Results and Discussion

Analyses of leaves and squares for the four allelochemicals were obtained from Coker 312 and two transgenic lines supplied by Monsanto, Mon 249 and Mon 531. The averages from analyses of six replicates of each were determined and standard errors were calculated. Very few changes, all of apparently minimum significance, were noted. Gossypol was marginally higher in squares of Coker 312, but intermediate to the two transgenic lines in leaves. Flavonoids were 6 and 9% higher, respectively, in leaves and squares of Mon 531 as compared with those of C 312. However, this is probably not nutritionally important. There were no statistical differences in anthocyanins. Tannins were lower in squares of C 312, but higher in leaves compared with Mon 531.

In general, the levels were representative for analyses of these allelochemicals in G. hirsutum lines. Results can vary by 2-fold or more depending on the seasonal time of sampling.

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Table 1. Analysis of Coker 312, Mon 249 and Mon 531 for gossypol, flavonoids, anthocyanins, and tannins in squares and leaves.

MONCHEM

1992 ROW NO.	REP	ENTRY	% GOSSY SQUARE	% GOSSY LEAF	% FLAV SQUARE	% FLAV LEAF	% ANTHO SQUARE	% ANTHO LEAF	% TANNIE SQUARE	% TANNIE LEAF
6397	1	249	0.199	0.156	0.384	0.897	0.080	0.246	9.820	13.728
6429	2	249	0.249	0.155	0.361	0.870	0.096	0.237	12.870	18.122
6473	3	249	0.198	0.150	0.334	0.846	0.082	0.212	8.336	9.986
6497	4	249	0.207	0.146	0.417	0.819	0.089	0.199	9.099	10.900
6525	5	249	0.201	0.138	0.512	0.848	0.088	0.201	8.840	9.302
6553	6	249	0.203	0.152	0.486	0.866	0.074	0.232	9.265	11.232
@AVG			0.210	0.150	0.416	0.858	0.085	0.221	9.705	12.212
@STD			0.018	0.006	0.064	0.024	0.007	0.018	1.484	2.981
SE=STD/SQRT N			0.007	0.002	0.026	0.010	0.003	0.007	0.606	1.217
6401	1	531	0.194	0.117	0.419	0.911	0.081	0.210	9.612	14.786
6433	2	531	0.204	0.132	0.421	0.764	0.091	0.226	9.161	13.961
6469	3	531	0.219	0.133	0.446	0.879	0.085	0.200	9.278	11.637
6513	4	531	0.185	0.113	0.476	0.820	0.080	0.182	9.525	12.653
6537	5	531	0.180	0.122	0.349	0.928	0.083	0.199	11.011	14.922
6565	6	531	0.231	0.122	0.450	0.947	0.075	0.202	9.944	12.209
@AVG			0.202	0.123	0.427	0.875	0.083	0.203	9.755	13.361
@STD			0.018	0.007	0.040	0.064	0.005	0.013	0.615	1.267
SE=STD/SQRT N			0.007	0.003	0.016	0.026	0.002	0.005	0.251	0.517
6393	1	312	0.225	0.154	0.353	0.771	0.080	0.242	10.159	17.097
6441	2	312	0.221	0.147	0.432	0.861	0.078	0.205	10.089	14.041
6477	3	312	0.207	0.136	0.371	0.816	0.079	0.209	9.127	11.119
6489	4	312	0.218	0.142	0.376	0.768	0.089	0.208	10.098	14.533
6533	5	312	0.235	0.136	0.398	0.855	0.081	0.232	8.281	13.842
6557	6	312	0.234	0.146	0.408	0.858	0.076	0.221	9.358	10.964
@AVG			0.223	0.144	0.390	0.822	0.081	0.220	9.519	13.599
@STD			0.010	0.006	0.026	0.040	0.004	0.014	0.681	2.100
SE=STD/SQRT N			0.004	0.003	0.011	0.016	0.002	0.006	0.278	0.857
AVG		MON 249	0.210	0.150	0.416	0.858	0.085	0.221	9.705	12.212
SE			0.007	0.002	0.026	0.010	0.003	0.007	0.606	1.217
AVG		MON 531	0.202	0.123	0.427	0.875	0.083	0.203	9.755	13.361
SE			0.007	0.003	0.016	0.026	0.002	0.005	0.251	0.517
AVG		C 312	0.223	0.144	0.390	0.822	0.081	0.220	9.519	13.599
SE			0.004	0.003	0.011	0.016	0.002	0.006	0.278	0.857

REMEMBER THAT MON 249 IS NOT REALLY MON 249. IT WAS COMPLETELY DAMAGED BY TBW.

Appendix IX

Boll Rot Count of Selected Monsanto Transgenic Cottons

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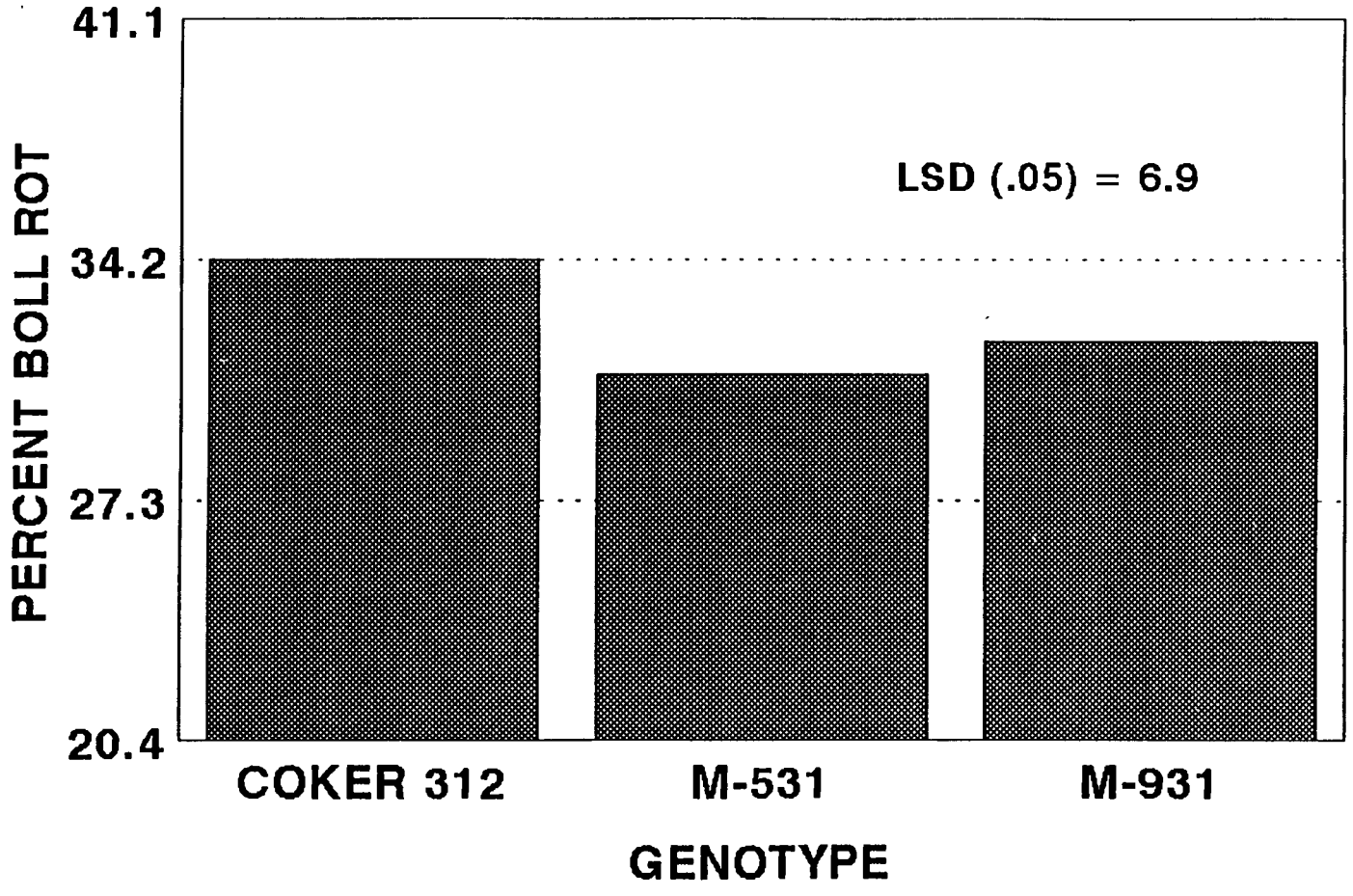
BOLL ROT EVALUATIONS
TRANSGENIC COTTONS



MONSANTO
BATSON/FUCHS

MAFES-Mississippi State University

BOLL ROT COUNT OF SELECTED MONSANTO TRANSGENIC COTTONS

Project Code:
Cooperator :FUCH

Location :PLANT SC. RES. CENTER
By:Dept. Plant Pathology & Weed Science

Trt No	Treatment Name	Form Amt	Fm Ds	Rate	Pathogen Code	BOLL ROT	BOLL ROT	BOLL ROT
					Crop Code	COTTON	COTTON	COTTON
					Rating Date	10-1-92	10-1-92	10-1-92
					Rating Data Type	TOT BOLL	ROT	ROT
					Rating Unit	NO.	NO.	%BOLLROT
1	COKER 312					208.0	71.2	34.2
2	MONSANTO 531					208.5	64.3	30.9
3	MONSANTO 931					201.5	64.2	31.8
LSD (.05)	=					8.1	15.2	6.9
Standard Dev.	=					6.29020	11.8542	5.33666
CV	=					3.05	17.81	16.52

MAFES-Mississippi State University

BOLL ROT COUNT OF SELECTED MONSANTO TRANSGENIC COTTONS

Project Code: Location :PLANT SC. RES. CENTER

Cooperator :FUCH By:Dept. Plant Pathology & Weed Science

ANALYSIS OF VARIANCE FOR BOLL ROT, COTTON, 10-1-92, TOT BOLL, NO.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	Prob(F)
Total	17	768.000000			
Block	5	189.333333	37.866667	0.957	0.4869
Treatment	2	183.000000	91.500000	2.313	0.1495
Error	10	395.666667	39.566667		

ANALYSIS OF VARIANCE FOR BOLL ROT, COTTON, 10-1-92, ROT, NO.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	Prob(F)
Total	17	2678.444444			
Block	5	1081.777778	216.355556	1.540	0.2622
Treatment	2	191.444444	95.722222	0.681	0.5280
Error	10	1405.222222	140.522222		

ANALYSIS OF VARIANCE FOR BOLL ROT, COTTON, 10-1-92, ROT, %BOLLROT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	Prob(F)
Total	17	618.829725			
Block	5	300.336228	60.067246	2.109	0.1476
Treatment	2	33.694444	16.847222	0.592	0.5717
Error	10	284.799052	28.479905		

DATA SUMMARY COMMENTS

A section of the fourth row of each plot sampled, sufficient to yeild at least 200 open bolls, was selected at random. All open bolls on these plants were examined and catagorized as healthy or exhibiting symptoms consistent with those described for boll rot. Six replications of plots of Coker 312, M-531, and M-931 that had received full-season insecticide appliccations were evaluated. Three colums of data are given: 1) total no. of bolls examined, 2) no. of bolls exhibiting symptoms of boll rot, and 3) percent boll rot. Analysis of the data at P=.05 indicated no significant difference in the number of bolls sampled for each treatment, number of rotten bolls, or incidence of boll rot among the cultivar and strains tested.
The amount of boll rot was the same in Coker 312, M-531, and M-931.

- End of Report -

MAFES-Mississippi State University

BOLL ROT COUNT OF SELECTED MONSANTO TRANSGENIC COTTONS

Project Code:
Location :PLANT SC. RES. CENTER
Cooperator :FUCH
By:Dept. Plant Pathology & Weed Science

Trt No	Treatment Name	Form Amt	Fm Ds	Rate	Plot No.	BOLL ROT COTTON 10-1-92 TOT BOLL NO.	BOLL ROT COTTON 10-1-92 ROT NO.	BOLL ROT COTTON 10-1-92 %BOLLROT
1	COKER 312				101	206.0	55.0	26.7
					203	200.0	68.0	34.0
					303	207.0	92.0	44.4
					401	204.0	57.0	27.9
					501	217.0	72.0	33.2
					601	214.0	83.0	38.8
					Mean =	208.0	71.2	34.2
2	MONSANTO 531				102	224.0	67.0	29.9
					201	200.0	82.0	41.0
					302	211.0	63.0	29.9
					403	208.0	66.0	31.7
					503	207.0	55.0	26.6
					603	201.0	53.0	26.4
					Mean =	208.5	64.3	30.9
3	MONSANTO 931				103	201.0	55.0	27.4
					202	201.0	72.0	35.8
					301	203.0	85.0	41.9
					402	200.0	69.0	34.5
					502	202.0	53.0	26.2
					602	202.0	51.0	25.2
					Mean =	201.5	64.2	31.8

Appendix X

Comparison of the *B.t.k.* HD-73 Protein Expressed by Insect Resistant Cotton with Commercially Available Microbial Pesticides Containing *B.t.* Proteins

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Pages 245-251

Appendix XI

Management of Insect Pests with Insect Resistant Plants: Recommended Approaches

MANAGEMENT OF INSECT PESTS WITH INSECT RESISTANT PLANTS: RECOMMENDED APPROACHES

Monsanto Agricultural Group
St. Louis, MO

Abstract

Insect resistant corn, cotton, and potatoes, which exhibit a high level of protection to damage and yield loss by lepidopteran pests (cotton and corn) and the Colorado potato beetle (potatoes) have been developed through the expression of *B.t.* genes in plants. Monsanto has developed recommended approaches to utilize these plants to maximize the utility and durability of these new insect control products. These approaches are being tested and will be optimized in the field prior to commercial introduction of insect resistant crops.

Introduction

Insect resistant crops represent an important new management tool to control crop damage and loss due to insect pests. These plants offer significant benefits to the grower, the consumer and the environment. Insect resistance has been developed through the expression of genes that produce insecticidal proteins from *Bacillus thuringiensis* (*B.t.*) in the cells of the plants. The particular genes being developed by Monsanto for cotton and corn are derived from the *B.t. kurstaki* strain, and for potatoes from *B.t. tenebrionis*. These proteins are the basis of several commercially available microbial insecticides, which have been demonstrated as highly selective for insects, with no activity against other types of living organisms such as mammals, fish, birds or non-insect invertebrates (earthworms, spiders, etc.) (EPA, 1991; EPA, 1988). In addition, these proteins show a remarkable insect specificity (MacIntosh *et al.*, 1990). The *B.t.* genes developed for cotton and corn produce proteins that are active only against certain lepidopteran larvae with no activity against other orders of insects. Importantly, this activity spectrum overlaps with several important pests of these crops which include the tobacco budworm, cotton bollworm or corn earworm, European corn borer, pink bollworm and several others such as cabbage looper, salt marsh caterpillar and cotton leaf perforator. Likewise, the *B.t.t.* gene developed for potatoes produces a protein active only against the Colorado potato beetle (CPB). Because these control agents are proteins, they have been found to break down rapidly in the environment and in mammalian digestive systems (Monsanto, 1993; Monsanto, 1994).

The use of insect resistant plants will provide important benefits to growers, society and the environment (McGaughey and Whalon, 1992; Gasser and Fraley, 1989; Gould, 1988). First and foremost, these plants offer an alternative to chemical insecticides currently used to control susceptible insect pests with efficacy equal to or better than that of current control methods. The use of insect resistant cotton, corn and potatoes will significantly reduce the application of chemical insecticides directed at these pests. The reduction of insecticide use will have direct benefits to the grower, such as less time and effort spent on insect control and reduced exposure to chemical insecticides.

Insect resistant crops are also likely to produce secondary benefits in pest control as an indirect result of the reduction in use of chemical insecticides. Chemical insecticides like pyrethroids are relatively non-specific and have the effect of killing beneficial predatory and parasitic insects (Roush and Tingey, 1993; Van den Bosch and Stern, 1962). Because the *B.t.* proteins produced by insect resistant plants are not active against these beneficial insects, populations have been shown to rise significantly in fields planted with insect resistant cotton and CPB resistant potatoes compared to nontransgenic cotton and potatoes treated with chemical insecticides (Feldman, *et al.*, 1993; Reed *et al.*, 1992; Stone *et al.*, 1992; Luttrell, pers. comm.). Preserving the beneficial insect population should enhance the biological control of both target pests and non-target pests such as mites, aphids, and leafhoppers, which increase as problems as their natural predators are removed. In addition, insect resistant cotton and corn and CPB resistant potatoes are equally capable of controlling target pest populations, which are beginning to lose their sensitivity to chemical insecticides (Everich, 1994; Stone and Sims, 1993), thus filling a need that is likely to grow in coming years.

The use of insect resistant plants will provide important benefits to growers, society and the environment. To achieve these benefits, it is important that insect resistant plant strategies be implemented and managed properly. In this respect, these plants are no different than any other pesticide. There are two aspects of this management. First, is the development of pest management techniques that allow the farmer to maximize the ability of these plants to control target pests. In essence, this is the development of a total insect management package that will be centered around a new tool, insect resistant cotton, corn or potatoes. Second, is the development of appropriate strategies to maximize the product durability and the utility of insect resistant crops. Part of this management program is the development and implementation of strategies targeted to prevent the development of insect resistance to the *B.t.* proteins produced by these plants. Because both management aspects can affect the way in which insect resistant plants are used by the grower, these two types of management, total pest management and insect resistance management, are interconnected.

Resistance management is not an issue particular to insect resistant plants, given the development of insect resistance to chemical insecticides. Monsanto scientists have addressed insect resistance for several years in laboratory and field studies and with outside collaborators we have examined nearly every suggestion that has been made for resistance management in insect resistant plants (Everich, 1994; Roush, 1994; Sachs, 1993; Stone and Sims, 1993). As the following discussion will demonstrate, promising strategies for resistance management for insect resistant plants are available and can be recommended. These strategies have been developed in consultation with an expert advisory panel established for each crop taking into account existing research and an understanding of crop production and agronomic practices. Consequently, these strategies may be specific for each crop and target pest. It is evident, however, that insect resistant plants offer some unique options in pest and resistance management that are not available with traditional pesticides.

Integrated Pest and Resistance Management with Insect Resistant Plants

As part of a package to provide economic control of insect loss and damage in cotton, corn, and potatoes, these insect resistant crops will provide a central focus around which other insect management practices will be applied. In many areas lepidopteran pests are the primary damaging insects of cotton and corn, so the use of these insect resistant plants to control these pests will be a major portion of total insect control. The primary pest in potato production is the CPB. Its control impacts the populations of other pests such as aphids and leafhoppers. By substituting genetically modified cotton, corn or potatoes for chemical pesticides directed at their target pests, a positive impact on overall insect management will result. Many of the details of pest management with insect resistant plants can only be determined by multi-year large scale field tests designed to incorporate these genetically modified crops into current production practices. Such field trials are in progress and are providing the data needed for developing a pest and resistance management program for these crops. These trials involve collaborations between Monsanto, HybriTech Seed International (a wholly owned subsidiary of Monsanto), seed company partners, and academic and extension entomologists. They are examining the impact of insect resistant plants on populations of beneficial and pest insects endemic to the crops and the impact on the use of conventional insecticides for controlling non-target pests (Feldman, *et al.*, 1993; Reed *et al.*, 1992; Stone *et al.*, 1992; Luttrell, pers. comm.), the establishment of the baseline susceptibility of our insect targets to *B.t.* protein (Stone and Sims, 1993; Everich, 1994; Luttrell, pers. comm.) and the impact of mixtures of resistant and non-resistant plants on yield loss (Roush, 1994).

Insect resistant cotton, corn and CPB resistant potatoes will be important additions to the available methods of controlling insect pests. The implementation of these plants is fully consistent with the goals of integrated pest management because:

a) the *B.t.* protein produced by the plants is insect specific, affecting only a few targeted pest species

b) the *B.t.* protein is active only against insects feeding on the plant and thus doing damage

c) use of the plants will reduce the application of chemical insecticides

d) use of the plants will preserve beneficial insects, which will enhance the biological control of non-target pests

Because pest and resistance management are interconnected, it is important to develop both of these approaches in tandem for each insect resistant crop.

Combination of Insect Resistant Plants with Chemical Insecticides

One aspect of the use of insect resistant plants for integrated pest management in corn, cotton, and potatoes is the continued use of chemical insecticides. Some insecticides will continue to be used in these crops for non-target pests. If possible, these insecticides need to be chosen so as to not negatively impact beneficial arthropods, which are integral

in the biological control of non-susceptible species. The combination of insect resistant crops with chemical insecticides, while part of a total insect control package, is not a resistance management option for insect resistant plants per se. Chemical insecticides can reduce the population size of insects selected for resistance to *B.t.* but cannot alter the gene frequencies within this population (Roush, 1989). Alternatively, insect resistant plants should positively impact current chemical insecticides by helping slow resistance development and prolonging the life of these important agricultural chemicals.

Resistance Management for Insect Resistant Plants

As described above, part of managing the implementation of insect resistant plants is the design and implementation of appropriate strategies to delay or prevent the development of insect resistance to *B.t.* protein in cotton, corn or potatoes. Described below are approaches that will help manage resistance development in these crops. It is important to note that: 1) as insect resistance development is a biological phenomenon, the rate of development is difficult if not impossible to predict and consequently, the efficacy of a strategy to delay or prevent its development may be impossible to demonstrate; 2) because of the available technology, biology of the pest, and the production practices of the crop, implementation of these strategies will be dependent on the crop and the target pest; and 3) field research must be conducted to determine the practical implementation of these strategies within current crop production practices. These strategies have been recommended by several researchers (Gould, 1988; Stone *et al.*, 1991; McGaughey and Whalon, 1992) and are summarized briefly below and then expanded in greater detail in the next section.

Summary of Considered Resistance Management Strategies for Insect Resistant Cotton, Corn and Potatoes

- High dose expression of *B.t.* protein in plants to control insects heterozygous for resistance alleles.
- Refugia as hosts for sensitive insects provided through non-insect resistant plants or other non-modified hosts.
- Monitoring of insect populations for susceptibility to *B.t.* protein.
- Agronomic practices that minimize insect exposure to *B.t.* plants.
- Integrated pest management (as described above).
- Combination of multiple genes within the same cotton plant, both of which are active against targeted insects but with different sites/modes of action.
- Incorporation of host plant resistance traits into insect resistant cotton and corn as they are proven effective.
- Incorporation of novel proteins that provide effective control of targeted pests.

Details of Resistance Management Strategies

High Dose Expression

High dose expression for resistance management is based on three assumptions:

1) Resistance will most likely be controlled by one major locus with recessive esistance alleles McGaughey and Beeman, 1988; MacIntosh *et al.*, 1991; Sims and Stone, 1991).

2) Insects developing resistance to the *B.t.* protein will be rare initially and will almost always mate with susceptible insects giving rise to heterozygous progeny (Gould, 1986).

3) More than 95% of the heterozygous progeny will be disabled or killed by insect resistant plants with the same dose as the homozygous susceptible larvae.

The high dose expression strategy uses plant expression of *B.t.* protein in quantities sufficient to kill those insects heterozygous for resistance to *B.t.* (McGaughey and Whalon, 1992; Roush, 1989). This resistance strategy fits nicely with the fact that high dose expression is essential for commercial efficacy of CPB resistant potatoes and insect resistant cotton and corn because of the range of sensitivity to the *B.t.* protein in corn and cotton insect targets (e.g., at least a 10-fold difference between tobacco budworm and European corn borer and cotton bollworm). High dose expression is also necessary to maintain consistent control across environments and genotypes. We plan to evaluate and develop the high dose expression strategy.

Refugia for Sensitive Insects

Refugia means providing a refuge for sensitive insects within a population so they will not be exposed to *B.t.* protein and not be selected for resistance. As a resistance management technique, refugia is based on the concept that control failure due to resistance is a population genetics phenomenon. Control failures are observed when the frequency of resistant insects in the population reaches a critical level. Refugia supply susceptible non-selected individuals to the general population. With adequate refugia, the frequency of resistance genes will be very low and spread only very slowly through the population. Refugia is an important component of our insect resistant crop resistance management strategies.

Refugia can be provided either within the crop or outside it. The refuge can also be planted specifically as such or exist naturally. In all of these approaches, the effectiveness of the refuge is based on those insects that survive on the refuge crop rather than its total acreage. This is an important point because, if the refuge is chemically treated, the refuge population is reduced and the amount of acreage required is increased. Examples of refugia that can be utilized are:

1) Refuge outside of the crop: Non-insect resistant cotton, corn or potatoes.

This type of refuge will exist in all the acres not covered by these insect resistant plants. This area will be substantial in the early years after introduction and could supply a sufficient refuge for several years. As insect resistant seed becomes more available and widely grown, this refuge will be reduced. Consequently, over time, reliance on non-insect resistant cotton, corn or potato fields for refugia may not be adequate.

2) Refuge outside of the crop: Non-modified crop hosts.

The European corn borer and the cotton bollworm or corn earworm have many non-corn or cotton hosts including other crops in all locations, which may provide an adequate refuge. The tobacco budworm and Colorado potato beetle have fewer alternatives and the pink bollworm has none. In some locations corn, cotton and potatoes may be the only host for at least one insect generation per season. The use of *B.t.* microbials or transgenic *B.t.* plants on other crops will also impact their utility as a refuge for insect resistant plants. This option must be evaluated carefully based on the crop, pest biology, and growing regions.

3) Refuge within the crop: Non-insect resistant plants.

In certain cases a likely solution is to provide an "in crop" refuge of non-insect resistant plants. For this in crop refuge, the choices are: a) random mixture of seed of insect resistant and non-resistant plants or b) non-insect resistant plants planted within the same field. The optimum refuge area required must be determined for each crop.

Mixed seed lines (*B.t.* and non-insect resistant seed within the same bag) have a certain appeal due to the "automatic" implementation. A possible problem with mixed seed arises from larvae that survive on a non-insect resistant plant and migrate to a modified plant where they may be less sensitive to *B.t.* protein because of their size. This could compromise insect control and increase selection pressure for resistance. The likelihood of this occurring is being investigated experimentally before this strategy is implemented.

There may also be economic and logistical problems if a mixed seed strategy is implemented. However, Monsanto, HybriTech and seed company partners are interested in determining the viability of the mixed seed approach. It is clear that field research is required to determine the percentage of non-insect resistant plants needed as a refuge, and what the impact of this percentage on over all yield, quality and seed company economics.

Another in-crop refuge could be non-insect resistant plants planted specifically by the farmer. Besides providing a refuge, such planting of separate indicator rows of non-insect resistant plants could potentially make scouting easier. Field research is needed to determine the optimum type of planting regime.

Agronomic Practices

Certain agronomic practices may need to be recommended for insect resistant plants. In particular, plow down dates to eliminate unnecessary insect exposure to *B.t.* protein from cotton regrowth or rotating CPB resistant potatoes with non-resistant potatoes may need to be recommended. The recommendation of these strategies will be determined on a regional basis, if necessary.

Monitoring Insect Resistance

Insect resistance monitoring is an important component of any insect resistance management strategy. A baseline frequency is in development. Resistance of major target pests to *B.t.* protein has not been detected in the field (Everich, 1994; Stone and Sims, 1993; Luttrell, pers. comm.). Baseline information should be collected on all *B.t.* products (engineered plants and *B.t.* microbials) to know when the frequency of resistant genotypes have increased within the population. This information must be developed on regional bases over several years so that susceptibility changes in populations can be identified and validated.

Pyramiding Traits

A set of strategies for the medium and long term focus on combining multiple insecticidal agents. The rationale is essentially the same for all of these: Expose the insects to two or more active agents with distinct modes of action at the same time, and the probability of any one insect being selected for resistance to both agents simultaneously is extremely low.

1) Combination with a Second Insect Resistance Gene

   A second gene within the same plant possessing a different mode of action will significantly reduce the frequency of resistant individuals (Peferoen, 1992; Stone *et al.*, 1991; Van Rie, 1991). Population models indicate that other alternative uses of a second gene such as seed mixture or using single genes in rotation, may be as effective as two genes within the same plant (Gould, 1988; Gould 1986). Assuming initial gene frequencies for *B.t.* protein resistance are low, initial introduction of a product with a single *B.t.* gene should not negatively compromise a second gene because the single gene product will be planted on limited acres in the first few years. In the medium term the best choice of second gene is an unrelated *B.t.* gene. In the long term, the use of novel, non-*B.t.* insecticidal genes holds great promise. This area is under active research.

2) Combination with Host Plant Resistance Traits

   This is a long term strategy to be implemented by seed companies or public breeders. Host plant resistance traits (HPR) used in combination with insect resistant cotton or corn need to be insecticidally effective and not negatively impact quality or yield. For example, Monsanto currently has funded research on HPR to help set direction on HPR traits that alone or in combination are useful in protecting the plant from lepidopteran insects in cotton (Sachs, 1993). Cotton seed companies are interested

in incorporating these traits if they are effective and have no negative effects on yield or quality. Similar work is planned with insect resistant corn. This strategy may have limited application to potatoes, however, as there are few varieties available that provide adequate CPB control and have desirable yield and quality characteristics.

Summary

Insect resistant cotton, corn and potatoes will offer great benefits in overall insect control in these crops. These plants will be developed to fit within existing pest management practices. Research programs for each crop have been in place for several years and will continue. With proper management and implementation, the development of insect resistance to *B.t.* will not be a technical or commercial problem that will limit the value or efficacy of these products. Monsanto has developed a package of strategies that will help effectively manage the potential development of insect resistance. The details of this program and its incorporation into existing pest management programs will be further developed and optimized in the field in the coming years.

Many aspects of the use of insect resistant plants in pest management and the implementation of resistance management strategies are unique to these products as compared to traditional chemical or microbial insecticides. For example, the use of refugia and the incorporation of multiple resistance traits through molecular biology or plant breeding are aspects that are ideally suited to insect resistant plants. This ability to utilize new methods in pest and resistance management make genetically modified insect resistant plants a critical component for successfully managing insect pests in the future.

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94-308-01p

Monsanto

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

January 26, 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: Bollgard™ Cotton Lines 757 and 1076
Monsanto #94-256

Dear Mr. Lidsky:

Monsanto has filed two separate Petitions for the Determination of Non-Regulated status for Bollgard™ Cotton Lines 531, 757 and 1076, all of which express a form of the insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*). The first petition submitted on November 3, 1994 requests Bollgard™ Cotton Line 531 be determined not to be a regulated article under 7 CFR §340.1. This petition has been designated number 94-308-01p and accepted by BBEP in a letter dated December 19, 1994. The second petition dated December 30, 1994 and submitted January 6, 1995 requests that Bollgard™ Cotton Lines 757 and 1076 also be determined not to be regulated articles. Due to the similarity of all three of these cotton lines, we hereby request that USDA/APHIS/BBEP consider the petition for Bollgard™ Cotton Lines 757 and 1076 to be an amendment to petition number 94-308-01p.

We believe that this request is justified for the following reasons. All three of the cotton lines contain the identical *cry1A(c)* gene and express the identical Cry1A(c) insect control protein. This protein is commonly referred to as the *B.t.k.* HD-73 protein. Line 757 was transformed using the same plasmid vector (PV-GHBK04) as was line 531. Line 1076 was transformed with plasmid vector PV-GHBK03. The only difference between these two vectors is the use of a different viral promoter for the *cry1A(c)* gene.

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Field tests of these lines has not demonstrated any difference in the growth, morphology, physiology or survivability of these cotton lines as compared to the parental non-transformed cotton. The only difference being the expression of low levels of the Cry1A(c) and NPTII proteins. Based upon all of these similarities, we believe our request to amend petition 94-308-01p with the new information submitted for lines 757 and 1076 is fully justified.

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

Frank Serdy Ph.D.
Regulatory Affairs Director

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

94-308-01? Supplement

Petition for Determination of Non-regulated Status:

Bollgard™ Cotton Lines 757 and 1076 (*Gossypium hirsutum* L.) with the gene from *Bacillus thuringiensis* subsp. *kurstaki*.

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

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December 30, 1994
#94-256

Prepared by:
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Steven R. Sims

Confidential Business Information Deleted

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 Bollgard™ Cotton Lines 757 and 1076 which express a form of the insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*). This petition requests a determination from APHIS that Bollgard™ Cotton Lines 757 and 1076 and any progeny derived from crosses between Bollgard™ Cotton Lines 757 and 1076 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 with approximately 13 million acres grown primarily in the tier of 15 southern states stretching from North Carolina to California. Lepidopteran insects (primary cotton bollworm, tobacco budworm and pink bollworm) are the main insect pest problem on these acres with approximately 80% of the planted acres infested, and approximately $180 M is spent annually on chemical insecticides for their control.

Bollgard™ Cotton Lines 757 and 1076 developed by Monsanto produce the insect control protein *B.t.k*. This protein is effective in controlling the cotton bollworm, tobacco budworm and pink bollworm. Microbial formulations containing these insecticidal proteins have been registered by EPA and commercially available for lepidopteran caterpillar control for nearly 30 years. Growers planting Bollgard™ Cotton Lines 757 and 1076 are not likely to require insecticide applications to control these destructive caterpillars. This substantial reduction in insecticide use will enhance the effectiveness of biological control and implementation of pest management strategies for other cotton insect pests.

The protein produced by Bollgard™ Cotton Lines 757 and 1076 is nearly identical in structure and activity to that found in nature and in commercial *B.t.k.* formulations registered with the EPA. Field experiments were conducted in 1993 and 1994 in over 75 experiments (line 757, approximately 35 experiments and line 1076 approximately 40 experiments) at locations throughout the United States cotton growing region demonstrated that the Bollgard™ Cotton Lines 757 and 1076 are protected season long from feeding damage caused by these lepidopteran caterpillars. Beneficial insects are unaffected and may increase in number. In addition, these plants exhibit no plant pathogenic properties, are no more likely to become weeds than the non-modified parental cotton

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Bollgard™ is a registered trademark of Monsanto Company, St. Louis Missouri.

lines, are unlikely to increase the weediness potential for any other cultivated plants or native species and are equivalent compositionally to the parental cotton line.

The use of Bollgard™ Cotton Lines 757 and 1076 will have a more positive impact on the environment than the use of chemical insecticides to control lepidopteran caterpillars. The *B.t.k.* protein is ecologically benign, i.e., it breaks down rapidly in the soil, is safe to non-target organisms such as fish, birds and mammals and specifically controls many species of lepidopteran caterpillars on cotton. In addition, the risk of an uncontrolled introduction of this cotton into the environment through hybridization or out-crossing to a native species resulting in a new weed variety is virtually non-existent on the mainland of the United States, where all of the United States cotton production takes place.

The determination that Bollgard™ Cotton Lines 757 and 1076 and their progeny are no longer regulated articles and their subsequent commercialization will represent an efficacious and environmentally compatible addition to the existing options for cotton insect pest management. In addition, Bollgard™ Cotton Lines 757 and 1076 will provide significant benefits to growers, the general public and the environment, including:

1. A more reliable, economical and less labor intensive means to control lepidopteran insect pests.

2. Insect control without harming non-target species, including humans.

3. A means for growers to significantly reduce the amount of chemical insecticides now applied to the crop while maintaining comparable yields. Therefore, lepidopteran insect control can be achieved in a more environmentally compatible manner than is currently available.

4. A reduction in the manufacturing, shipment and storage of chemical insecticides used on cotton.

5. A reduction in the exposure to workers to the pesticide and pesticide spray solution.

6. A reduction in the number of empty pesticide containers and amount of pesticide spray solution that must be disposed of according to applicable environmental regulations.

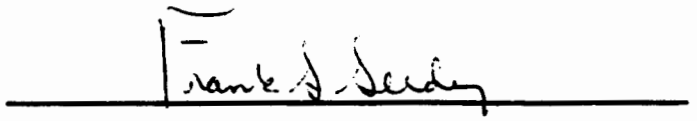
7. An ideal fit with Integrated Pest Management Programs (IPM) and sustainable agricultural systems.

In conclusion, the consistent lepidoptera control offered by Bollgard™ Cotton Lines 757 and 1076 will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop for control of cotton bollworm, tobacco budworm and pink bollworm. As a result, they will be able to utilize a host of IPM practices that cannot presently be implemented because of the lack of options other than use of chemical insecticides to control these pests. An increase in the biological and cultural control of non-target cotton pests and a more judicious use of chemical insecticides will result in a positive impact on the environment, which will ultimately be advantageous to the grower and the public as well.

Therefore, the Agricultural Group of Monsanto requests a determination from APHIS that Bollgard™ Cotton Lines 757 and 1076 and any progenies derived from crosses between Bollgard™ Cotton Lines 757 and 1076 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.



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NOTE TO THE REVIEWER

Justification for the Use of the CryIA(c) Designation for the protein expressed in Bollgard™ Cotton Lines 757 and 1076.

The insecticidal protein produced in Bollgard™ Cotton Lines 757 and 1076 is classified as CryIA(c). This designation is appropriate even though the *B.t.k.* gene producing the protein is the result of fusing a 5' portion of a *cryIA(b)* gene and the majority of the *cryIA(c)* gene. The appropriate classification of the expressed *B.t.k.* protein is dictated by the protein produced and not the gene or the method by which the gene was constructed. The portion of the *cryIA(b)* gene used encodes an N-terminal amino acid sequence that is highly homologous to the N-terminal amino acid sequence encoded by the *cryIA(c)* gene. The protein produced contains only 6 amino acid differences in this region, resulting in only 6 amino acid changes for the entire 1178 amino acid *B.t.k.* protein that result from using the sequence of the *cryIA(b)* gene portion. Furthermore, these 6 changes occur in the N-terminal, highly conserved portion of the *B.t.k.* protein. These changes are not located in the hypervariable region, which has been shown to be responsible for determining the insecticidal activity of the *B.t.* proteins (Geiser, *et al.*, 1986). These amino acid changes did not, as expected, affect the insecticidal specificity of the resulting *B.t.k.* protein.

The protein encoded by the *B.t.k.* gene introduced into Bollgard™ Cotton Lines 757 and 1076 is identical in length (1178 amino acids) and 99.4% identical in amino acid sequence to the protein encoded by the *cryIA(c)* gene (Adang, *et al.*, 1985). The Cry nomenclature developed by Hofte and Whiteley (1989) categorizes the vast array of *B.t.* proteins in classes, rather than single, distinct proteins, based on their structural relatedness and insect toxicity spectra. The CryIA(c) protein reported by Adang *et al.* (1985) was the only member of the CryIA(c) class at the time of Hofte and Whiteley's (1989) publication. Since that report, four other CryIA(c) proteins have been reported (Dardenne, *et al.*, 1990; Von Tersch, *et al.*, 1991; M73248; M73249). For example, the CryIA(c) protein that was characterized by Von Tersch *et al.* (1991) contains seven amino acid differences (6 amino acid substitutions and one amino acid deletion) and was isolated from a different subspecies (*B.t.* subsp. *kenyae*) than the protein characterized by Adang *et al.* (1985). All five of these CryIA(c) proteins plus the CryIA(c) protein used to produce Bollgard™ Cotton Lines 757 and 1076 are ≥99% identical at the amino acid level. In contrast, the CryIA(b) proteins, which are the next most homologous class of *B.t.* proteins, show <90% amino acid identity to any of the members of the CryIA(c) class of *B.t.* proteins. These homologies clearly establish that all six of the CryIA(c) proteins (including the *B.t.* protein expressed in the Bollgard™ Cotton Lines 757 and 1076) are closely related and are all appropriately classified as CryIA(c) proteins. The CryIA(c) class designation for the protein expressed in Bollgard™ Cotton Lines 757 and 1076 is, therefore, accurate and consistent with the established nomenclature of Hofte and Whiteley (1989).

Therefore, it is appropriate to refer to the protein expressed in Bollgard™ Cotton Lines 757 and 1076 as a CryIA(c) protein and the gene expressing this protein as *cryIA(c)* gene.

References

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Hofte, H. and Whiteley, H.R. 1989. Insecticidal Crystal Proteins of *Bacillus thuringiensis*. Microbiol. Reviews 53:242-255.

M73248. Not published but accessible through GenBank (Genpept), accession number M73248.

M73249. Not published but accessible through GenBank (Genpept), accession number M73249.

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Abbreviations Used in this Petition for the Determination of Non-Regulated Status of Bollgard™ Cotton Lines 757 and 1076

aad	Gene for 3"(9)-O-aminoglycoside adenylyltransferase
AAD	3"(9)-O-aminoglycoside adenylyltransferase
APHIS	Animal Plant Health Inspection Service
ATP	Adenosine triphosphate
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
C	Centigrade
C312	Coker cotton variety 312
CFR	Code of Federal Regulations
crylA(c)	Class I (Lepidoptera-specific) crystal protein gene
DNA	Deoxyribonucleic Acid
E35S	Promoter for *cryIA(c)* gene
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EUP	Experimental Use Permit
F	Fahrenheit
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
g	gram
GLP	Good Laboratory Practice
IPM	Integrated Pest 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
ppm	part per million
sp	species
T-DNA	Transfer-DNA
µg	microgram
USDA	United States Department of Agriculture
w/w	weight/weight

CONFIDENTIAL BUSINESS INFORMATION JUSTIFICATION

The information claimed as confidential within this application concerns the gene description. The gene description category includes (a) names and information about genes and promoters and (b) reference articles published in various scientific journals.

Legal Background

The Freedom of Information Act ("FOIA"), 5 U.S.C. §552, specifically exempts from release "trade secrets and commercial or financial information obtained from a person and privileged or confidential" ("Exemption 4"). 5 U.S.C. § 552(b)(4). Exemption 4 applies where the disclosure of information would be likely to cause substantial harm to the competitive position of the owner, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to share data with the agency voluntarily. National Parks & Conservation Association v. Morton,498 F.2d 765, 770 (D.C.Cir. 1974); Gulf & Western Industries, Inc. v. U.S., 615 F.2d 527 530 (D.C.Cir. 1979).

A party seeking to demonstrate "substantial competitive harm" need not show actual competitive harm, but must only demonstrate the presence of competition and the likelihood of substantial competitive injury. Id.at 530; National Parks & Conservation Association v. Kleppe, 547 F.2d 673, 679 (D.C.Cir. 1976); Miami Herald Pub. Co. v. U.S. Small Business Administration, 670 F.2d 610, 614 (5th Cir. Unit B 1982).

For the purposes of FOIA, courts have defined the term "trade secret" to mean a "secret, commercially valuable plan, formula, process, or device that is used for the making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort. Public Citizen Health Research Group v. FDA, 704 F.2d 1280, 1288 (D.C.Cir. 1983); Anderson v. Dept. of Health & Human Services, 907 F.2d 936, 943-44 (10th Cir. 1990).

Information on gene description falls squarely within this definition, and is the type of information accorded trade secret protection by the courts under Exemption 4 of the Freedom of Information Act. It is well established that information on the formulation and chemistry of a product should be treated as confidential for FOIA purposes. See, e.g., Anderson v. Dept. of Health & Human Services,907 F.2d 936 (10th Cir. 1990). This is exactly the type of information provided by each and every subcategory listed above in the gene description category. Where, as in the case of the Monsanto products subject to this FOIA request, the development time and costs of the product have been substantial and the information can only be obtained by competitors at considerable cost, disclosure is prohibited. Greenberg v.

Food and Drug Administration, 803 F.2d at [213, 1216-1218 (D.C. Cir. 1986); Worthington Compressors, Inc. v. Costie, 622 F.2d 45, 51-52 (D.C.Cir. 1981). The existence of confidentiality agreements binding employees not to reveal the information is another factor considered by the courts. Greenberg v. FDA,803 F.2d at 1216-1218.

Gene Description

The essence of the commercial value of the Monsanto biotechnology products is the particular genetic information that confers the desired properties on the plant product, as well as the technical know-how inherent in this information. Monsanto is at the leading edge in the development of biotechnology products in a rapidly growing and highly competitive industry. This expertise has been gained through many person years of effort, and the expenditure of tens of millions of dollars on biotechnology research.

Monsanto has been working on the development of the insect resistant cotton since 1980, and has expended over 10 million dollars in research and testing costs. Monsanto can document the development and testing costs by means of monthly summaries of the person hours devoted to these projects, budgetary documents, field test agreements, and project documents for the Chesterfield facility.

The uniqueness of this product lies in the particular combination of genetic components in the vectors transferred to these plants. Each genetic entity in these vectors has three pieces of information: a promotor region, the gene for the expression of the trait, and a stop signal. Although the information on each of these vector components may be in the public domain, the particular combination of the components put together by Monsanto is unique and represents years of effort and millions of dollars of expense.

To achieve the products which are the subject of this FOIA request, Monsanto has developed and tested many different plant strains using different combinations of genetic components. The plant products developed by Monsanto represent the best fit of the components, and the best mode of gene expression of the desired traits. The specific combination of genetic information on the vectors transferred to the Monsanto products has been kept strictly confidential. Monsanto employees and contractors under contract to Monsanto are contractually obligated to keep this information confidential.

There are many competitors of Monsanto, both national and international, who have the expertise not only to replicate Monsanto's products, but also to use Monsanto's technology to develop other products which would be competitive with Monsanto, thereby saving millions of dollars and years of development effort. These competitors include Calgene, Rhone-Poulenc, ICI, and Ciba-Geigy.

Monsanto's competitors cannot presently duplicate Monsanto's commercially valuable products from information in the public domain without going through the same painstaking trial and error development and testing of many different combinations of genetic information. It is important to emphasize that although there may be information about Monsanto products available in patent applications, this information is voluminous and general in nature, and does not identify the specific combinations of genetic information which Monsanto has found to be most effective. A competitor cannot determine from the patent applications which particular combination of genes and transgenic products will prove to be commercially valuable.

Access to gene description information for Monsanto's products would allow competitors to create essentially "copy-cat" products (avoiding any technical patent infringement) that would result in a market share loss for Monsanto of millions of dollars. By performing simple copy work, these competitors would avoid the millions of dollars and many years of research and development effort expended by Monsanto to develop its commercial products.

The release of gene description information would also provide competitors with commercially valuable knowledge about the particular products that Monsanto is planning to commercialize and the likely time frame for commercialization. This information would be extremely helpful to these companies in developing their own marketing strategies and development plans in a highly competitive market.

Names And Information About Genes, Promoters, And Expressed Traits.

The release of information about the genes and promoters in the vectors will directly provide competitors with the knowledge of the precise genetic sequence that Monsanto has found to be most desirable. If this information is disclosed the competitors will have access to the structure of the Monsanto products, with the consequences outlined above. Patents for the products at issue in this matter are pending, but have not been issued.

Information on the expressed trait of the genes is tantamount to providing the name of the genes, and will allow Monsanto's competitors to readily identify the particular genes that have been transferred to the Monsanto products. The release of any information relating to changes made to an original gene to facilitate fusion with another gene would explicitly reveal Monsanto's trade secret technology for developing gene combinations.

Identity And Characteristics Of Donor Organism

A donor organism is not claimed as CBI when the gene from such organism appears alone. CBI is only claimed for the name and/or identifying characteristics of a donor organism when the gene from this organism is used in a new and unique combination with another gene to give greatly enhanced expression of the desired trait.

The identity of the donor organisms for soybean plants genetically engineered to have tolerance to glyphosate herbicide and potato plants genetically engineered to be resistant to certain insects and viruses and to have a higher percentage of solids has been claimed as confidential by Monsanto because the disclosure of this information will essentially reveal to Monsanto's competitors the nature of the genes for the expressed traits. Likewise, information on the characteristics of the donor organisms and the source of the characterization of the donor will reveal directly or with little difficulty the identity of the donor organism. With this information in hand, even without information on the other components of the vector, Monsanto's competitors will be accorded a tremendous advantage in their search for competitive products, and will be able to unfairly take advantage of the expensive and time intensive effort by Monsanto to identify this donor as the most suitable organism for providing the genetic information necessary to best express the desired traits.

Reference Articles

Monsanto has not claimed as confidential references which do not directly reveal information pertaining to the specific combination of genes created by Monsanto. The disclosure of the specific references claimed as confidential would allow Monsanto's competitors to readily identify the precise biochemical structure of the genetic information transferred into Monsanto's products, and the technology for achieving the unique combinations developed by Monsanto.

Specifically, these references name and describe the precise piece of genetic information for each component of the Monsanto vector, provide the nucleotide sequence for these components, and describe the cloning of the genetic information. With all the references in hand, a competitor can determine the exact sequence of the genetic information in the Monsanto products, and the technology for creating the Monsanto combinations. He can then not only reproduce the Monsanto products, but also apply the knowledge of the Monsanto system to the development of competitive products. Without these reference cites, the literature in the field is so voluminous that competitors would be unable to determine how to best combine the many available components to produce a commercially viable product.

Monsanto also notes that these reference articles were submitted voluntary, as they were not required to support the Monsanto applications. Monsanto would not provide such reference information to the public, any more than it would publically disclose other information allowing the identification of the components of the vectors and the genetic sequence of the vectors. Accordingly, under the recently decided Critical Mass Energy Project v. NRC, these references are entitled to protection from disclosure under FOIA Exemption 4.

For each piece of information claimed as confidential, we are indicating in the attachment to this letter the category into which the information falls and referencing the above discussion. Additional justification information is provided for certain items, as necessary.

Page	Category of Information	Justification
III-3	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-3	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-3	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, expressed traits and referenced articles.
III-20	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-32	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-33	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.

III-34	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-35	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-36	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, and expressed traits.
III-42	Description of Gene and Donor Organism	See discussion on gene description - names and information about genes, donor organisms, expressed traits and referenced articles.

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Appendices

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Part I. Introduction

A. Rationale For Development of the Insect Resistant Cotton Plant

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. Lepidopteran insects are the main insect pest problem on these acres. During the growing season other insects (e.g., cotton boll weevil, lygus bugs, fleahoppers, spider mites, thrips, and aphids) are also present. The primary lepidopteran pests infesting cotton are cotton bollworm, tobacco budworm and pink bollworm. These insect pests infest approximately 80% of the planted acres and approximately $180 M is spent annually for chemical control.

Monsanto has developed genetically modified cotton plants that control many of the lepidopteran caterpillars which are serious pests in cotton production. These cotton plants, named Bollgard™ Cotton Lines 757 and 1076, produce an insect control protein derived from the common soil bacterium *Bacillus thuringiensis* var. *kurstaki* (*B.t.k*). Microbial formulations containing these insecticidal proteins have been registered by the Environmental Protection Agency (EPA) and commercially available for lepidopteran caterpillar control for nearly 30 years. The protein produced by Bollgard™ Cotton Lines 757 and 1076 is nearly identical in structure and activity to that found in nature and in commercial *B.t.k.* formulations registered with the EPA. This protein is highly selective in controlling many lepidopteran caterpillars and is expressed at an effective level in plant tissue throughout the growing season. Bollgard™ Cotton Lines 757 and 1076 were evaluated in the years 1993 and 1994 in over 75 experiments (line 757, approximately 35 experiments and line 1076, approximately 40 experiments) at locations throughout the United States cotton growing region under permits from the United States Department of Agriculture (USDA) (#93-011-05, 93-056-05, 94-025-01, 94-026-03, 94-027-03 and 94-054-02), and an Experimental Use Permit granted by EPA (#524-EUP-73). Results from these experiments have demonstrated that the Bollgard™ Cotton Lines 757 and 1076 are protected season long from feeding damage caused by many lepidopteran caterpillars. Beneficial insects are unaffected and may increase in number, providing predatory control of the lepidopteran pests. Growers planting Bollgard™ Cotton Lines 757 and 1076 are not likely to require insecticide applications to control these destructive caterpillars. This substantial reduction in insecticide use will enhance the effectiveness of biological control and implementation of pest management strategies for other cotton insect pests.

Safety studies summarized in this submission, as well as data generated by manufacturers of commercial *B.t.k.* products, have demonstrated that non-target animals such as fish, birds and mammals are unaffected by the *B.t.k.* protein. In addition, agronomic evaluations consisting of plant vigor, growth habit characteristics and general disease susceptibility, have shown Bollgard™ Cotton Lines 757 and 1076 to be equivalent to the parental Coker 312 cotton.

The commercialization of Bollgard™ Cotton Lines 757 and 1076 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 insect pest management. In addition, it will provide significant benefits to growers, the general public and the environment, including:

1. A more reliable, economical and less labor intensive means to control lepidopteran insect pests.

2. Insect control without harming non-target species, including humans.

3. A means for growers to significantly reduce the amount of chemical insecticides now applied to the crop while maintaining comparable yields. Therefore, lepidopteran insect control can be achieved in a more environmentally compatible manner than is currently available.

4. A reduction in the manufacturing, shipment and storage of chemical insecticides used on cotton.

5. A reduction in the exposure to workers to the pesticide and pesticide spray solution.

6. A reduction in the number of empty pesticide containers and amount of pesticide spray solution that must be disposed of according to applicable environmental regulations.

7. An ideal fit with Integrated Pest Management Programs (IPM) and sustainable agricultural systems.

B. Benefits of Insect Resistant Cotton

1. Summary

Lepidopteran insects are the main pest problem on most of the 13 million acres of cotton produced in the United States. During the growing season other insects (e.g., cotton boll weevil, lygus bugs, fleahoppers, spider mites, thrips, and aphids) are also present. The primary

lepidopteran pests infesting cotton are cotton bollworm, tobacco budworm and pink bollworm. These insect pests infest approximately 80% of the planted acres with approximately $180 M spent annually for chemical insecticides for their control. These insect resistant cotton plants are expected to replace a significant part of the chemical insecticides now applied to control lepidopteran insect pests.

There are additional reasons why these insect resistant cotton plants have advantages over cotton plants which must be sprayed with insecticides to control lepidopteran pests, including:

a. Chemical insecticides are costly and sometimes unreliable under intended use conditions. New chemical insecticides are expensive to develop and register and as a result must be sold at ever increasing prices so that the developer can recover these costs. The effectiveness of these chemicals can also be negatively influenced by environmental conditions. Rain following application, for example, reduces the length of control, and a dense canopy of foliage reduces penetration and effectiveness. Areas of the field that do not receive the spray will be damaged by insects. All of these conditions result in increased production costs and potentially lower yields for the grower.

b. Many chemical insecticides have the potential to cause environmental damage if not used as labelled.

c. Insect resistant cotton plants provide an ideal fit with existing IPM and sustainable agricultural programs. Essentially all cotton produced in the United States is grown under IPM programs. By reducing the use of non-selective insecticides, insect resistant cotton plants will enhance the effectiveness of these programs, due to the presence of increased numbers of beneficial insects and other predators. Natural pest defense systems are compatible with the goals of sustainable agriculture production systems.

d. Applicator and field worker exposure to chemical insecticides will be reduced.

e. Many insects have or are developing resistance to the available chemical insecticides. This resistance requires farmers to apply chemicals at higher rates and/or more frequently, with the prospect of eventually not being able to use them at all.

f. Bollgard™ Cotton Lines 757 and 1076 will likely have reduced levels of aflatoxin in the seed compared to non-modified cotton

The following are summaries of the Agronomic and Economic Benefits of Bollgard™ Cotton Lines 757 and 1076 as prepared by Luttrell *et al.* (1993) and Spurlock (1993) respectively. Copies of these full papers are found in Appendices I and II, respectively.

2. Agronomic Benefits

Cotton production in the United States is highly mechanized and dependent upon maximum utilization of new technology to remain competitive in a worldwide market. Pest problems, particularly insects, and environmental constraints, such as inadequate temperature and moisture, are major limiting factors to optimum cotton production. Most cotton production regions of the United States rely on extension specialists and crop consultants to design and implement effective IPM programs. Insect control decisions are largely based on routine field monitoring by agricultural consultants, extension personnel and growers. The intensity of monitoring varies among locations and is associated with production capabilities, potential insect damage and availability of consultants (Luttrell 1994). Numerous advances in IPM technology (Frisbie and Adkisson 1985, Frisbie *et al.* 1989) have encouraged a systems approach to insect management in United States cotton where insect control decisions are integrated into an overall crop production and management scheme. Perhaps the best example of this is the wide acceptance of early-maturing varieties and short-season cotton production systems first recommended in Texas. The Texas system of short-season cotton production (Walker *et al.* 1978) has been widely adopted across United States cotton and is recommended by agronomists and entomologists because it optimizes the production of valuable fiber and encourages the "avoidance" of damaging late-season populations of insects.

Although advances in IPM technologies have fostered improved cotton insect management systems, insect control is still largely based on the use of chemical insecticides (Herzog *et al.* 1993). Estimates of insect control costs and losses (Head 1991, 1992, 1993) averaged for the 1990's indicate that United States cotton growers apply an average of 4.86 applications of insecticide to 11.8 million acres of cotton and spend more than $400 million each year for control of cotton insects. This represents a large portion of total insecticide use in the United States. Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests (Luttrell 1994). The need for alternative insect control measures is becoming more critical to profitable cotton production in the United States. Environmental concerns limit the availability of existing insecticide chemistry and increase the developmental costs of new chemistry. Because of the high costs of developing and registering new insecticide chemistry, availability has declined over the past few years.

Bollgard™ Cotton Lines 757 and 1076 offer unique, innovative alternatives to traditional chemical control measures. Although alternative insect control tactics are often cited as major components of cotton IPM and research is continuously pursuing improved management methods (Frisbie *et al.* 1989), few alternative insect control methods are of sufficient efficacy to replace chemical control methods.

Other methods, such as biological control, host plant resistance and cultural control, provide suppression of pest populations without disrupting natural control, but generally lack the high efficacy and curative action of conventional insecticides. Bollgard™ Cotton Lines 757 and 1076 are the first major exception to this historical trend.

Bollgard™ Cotton Lines 757 and 1076 offer new mechanisms to produce and deliver a highly effective insecticide to target pests (i.e. production by cells of the crop plant rather than industrial facilities and application by spray equipment). The technology couples the environmental advantages of host plant resistance with the efficacy of an effective biological insecticide. Since the insecticidal activity is expressed throughout the plant for the entire season, improved control of some pest species over that provided by conventional insecticides is likely. Current technology which depends on foliar application of insecticides cannot dependably deposit insecticides to some regions of the plant canopy infested by pest species. This is especially true of pests that burrow and feed inside plant tissue (e.g. pink bollworms). Because Bollgard™ Cotton Lines 757 and 1076 express the *B.t.k.* protein that only has activity against certain Lepidoptera (moths and butterflies) and must be ingested to kill the pest, the technology offers selective activity against susceptible lepidopteran pest complexes without directly disrupting pest suppression by natural enemies, such as parasites and predators.

3. Economic Benefits

Pesticide regulation has become more restrictive in the United States resulting in the ban or severe restriction of the use of particular insecticides. Economic studies have been conducted to examine the likely impacts from such restrictive pesticide regulations. Taylor *et al.* (1991) developed a regional model and concluded that agricultural income in the South would be negatively impacted by more restrictive pesticide regulations. Richardson *et al.* (1991) 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. It is possible that genetically modified plants which are designed to control insects without the use of insecticide sprays will be able to offset some of the negative impacts from increased pesticide regulations.

Bollgard™ Cotton Lines 757 and 1076 are designed to sufficiently control infestations of lepidoptera, eliminating the need to control these pests with conventional insecticide applications. Revenue-related factors such as lint yields and quality characteristics are expected to be similar under both conventional cotton and Bollgard™ Cotton Lines 757 and 1076 production systems. However, per-acre production costs of Bollgard™ Cotton Lines 757 and 1076 are expected to be lowered due to

the reduction in insecticide use with the substitution of Bollgard™ Cotton Lines 757 and 1076 seed for conventional cotton seed. Growers who adopt Bollgard™ Cotton Lines 757 and 1076 will simply substitute this seed for conventional cotton seed and certain types of insecticides. Thus, the added cost of the Bollgard™ Cotton Lines 757 and 1076 seed must be compared with the savings obtained from replacing conventional seed and some insecticides.

Due to the diverse and complex interactions throughout the agricultural sector and related sectors of the economy, it is difficult (if not impossible) to predict future magnitudes of key variables with a high degree of accuracy. However, it is possible to speculate on the direction of change in these variables. For instance, pesticide regulations in the United States will likely become more restrictive over time. Reductions in insecticide use without Bollgard™ Cotton Lines 757 and 1076 will cause cotton yields to decline, farm profits to decline and acres devoted to cotton production to decline, especially in those regions where insecticide use is an integral production practice. A scenario which allows for the introduction of Bollgard™ Cotton Lines 757 and 1076 results in a very different forecast. Reductions in insecticide 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 adoption by large farms over that of 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 Bollgard™ Cotton Lines 757 and 1076 is not expected to be related to farm size; i.e., small and large farms will have the same per-acre costs and benefits from the adoption of this improved cotton and, thus, will likely have equal adoption rates.

In summary, the introduction of Bollgard™ Cotton Lines 757 and 1076 will have significant positive impacts on the profitability of some farmers and agribusinesses. It will allow cotton growers to eliminate some conventional insecticide applications and thus reduce pesticide expenses. Based on available cost and acreage data and assumptions concerning the portion of current cotton acres that would be converted to Bollgard™ Cotton Lines 757 and 1076, it is estimated that cotton producers could save over $77 million per year on insect control costs.

C. Regulatory Approvals

Note: Monsanto is pursing commercialization of 3 different lines or cultivars of Bollgard™ Cotton. These are named Lines 531, 757 and 1076. All three of these cotton lines are separate transformation events, with lines 531 and 757 being transformed with the same plasmid vector (PV-GHBK04) and line 1076 transformed with plasmid vector PV-GHBK03. All three encode the identical *B.t.k.* HD-73 (CryIA(c) insect control protein), the only difference in the vectors being the viral promoter which drives the *cryIA(c)* gene. Monsanto has applied for regulatory approval of Bollgard™ Cotton Line 531 by filing a petition for registration with EPA (PP4F4331, filed February 15, 1994, pending), Petition for Determination of non-regulated status with USDA/APHIS (filed November 3, 1994, #94-308-01p, pending) and continued the consultation with FDA by filing a summary of the food and feed safety data and information (November 21, 1994, pending) under the FDA May 29, 1992 policy statement concerning foods derived from new plant varieties. We expect this consultation to be completed in early 1995.

Prior to the commercialization of Bollgard™ Cotton Lines 757 and 1076, Monsanto will obtain the following regulatory approvals:

1. This determination from USDA/APHIS that Bollgard™ Cotton Lines 757 and 1076, and all progenies derived from crosses between Bollgard™ Cotton Lines 757 and 1076 and other cotton cultivars, are no longer a regulated articles according to 7CFR §340.6.

2. Regulatory approval from the EPA of the *B.t.k.* insecticidal protein as expressed in Bollgard™ Cotton Lines 757 and 1076 under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). This petition was submitted December 1994 (Dated December 20, 1994).

3. An exemption from the requirement of a tolerance for the *B.t.k.* insecticidal protein under sections 408 of the Federal Food Drug and Cosmetic Act (FFDCA) from the EPA and Food and Drug Administration (FDA). The petition for the exemption from the requirement of a tolerance for the *B.t.k.* protein was submitted to EPA on February 15, 1994 (PP4F4331).

The EPA has exempted the NPTII protein and the genetic material necessary for the production of the protein from the requirement of a tolerance in or on all agricultural commodities when used as a plant-pesticide inert ingredient (EPA 1994). FDA has approved the request from Calgene Inc. to amend the food additive regulations to provide for the safe use of NPTII as a processing aid in the development of new varieties of tomato, oilseed rape and cotton (Calgene, Inc., 1993, FDA 1994). No additional regulatory approvals are planned for the NPTII protein.

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 Coker and McNair cultivars account for most of the production in Georgia and the Carolinas.

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. Adaption 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 virtually nonexistent 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

Bollgard™ Cotton Lines 757 and 1076 contain the following 3 genes inserted via genetic engineering techniques:

- The *cryIA(c)* gene which encodes for an insecticidal protein, *B.t.k.* HD-73, derived from the common soil microbe *Bacillus thuringiensis* variety *kurstaki* (*B.t.k.*).

- The *nptII* gene which encodes the selectable marker enzyme neomycin phosphotransferase II (NPTII), was needed to identify transformed cells that contained the *B.t.k.* protein. It served no other purpose and has no pesticidal properties.

- The *aad* gene which encodes the bacterial selectable marker enzyme 3"(9)-O-aminoglycoside adenylyltransferase (AAD), allowed for the selection of bacteria containing the PV-GHBK04 or PV-GHBK03 plasmid on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter and the encoded protein is not detected in Bollgard™ Cotton Lines 757 and 1076.

Bollgard™ Cotton Lines 757 and 1076 were produced using the *Agrobacterium tumefaciens* transformation system. Line 757 was transformed using plasmid vector PV-GHBK04. Line 1076 was transformed with plasmid vector PV-GHBK03. The only difference between these two vectors is the use of a different viral promoter for the *cryIA(c)* gene. Both lines express the same proteins. The transformation system and related genes are described below.

A. Characteristics of the Non-transformed Cultivar

Bollgard™ Cotton Lines 757 and 1076 were developed by transforming the parental cotton cultivar Coker 312 (*Gossypium hirsutum* L.). This cotton was released by the Coker Pedigree Seed Company in 1974, and the variety is currently owned by the SeedCo Corporation of Lubbock, Texas. This is an older cotton variety, and little to none is being grown today. Therefore Monsanto does not intend to introduce Bollgard™ Cotton Lines 757 and 1076, but will allow our seed company partners to transfer the trait into 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. Therefore, Bollgard™ Cotton Lines generated with a Coker 312 background are acceptable from an agronomic perspective for testing purposes.

B. *Agrobacterium* Vectors and Transformation

Generally when using *Agrobacterium* vectors, only the T-DNA is transferred and integrated into the plant genome (Zambryski, 1992). It is generally 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.

The transformation vector contains well-characterized DNA segments required for selection and replication of the plasmid vector in bacteria and transfer of the T-DNA into plant cells. The plant expression vector was assembled and then transformed into *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 pMP9ORK (Koncz and Schell, 1986), in a chloramphenicol resistant derivative of the *Agrobacterium tumefaciens* strain A208. The disarmed pMP9ORK Ti plasmid does not carry the T-DNA phytohormone genes and is no longer considered a threat as a plant pest (Huttner, *et al.*, 1992). The pMP9ORK 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 ABl::plasmid vector conjugate, the T-DNA vector is transferred to the plant cells via the *vir* functions encoded by the disarmed pMP9ORK Ti plasmid (Klee, *et al.*, 1983 and Stachel and Nester, 1986). The Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium*.

The T-DNA, which includes the *cryIA(c)*, *nptII* and *aad* genes, was transferred into the genome of individual cotton cells thereby allowing selection on kanamycin. After a few days, the residual *Agrobacterium* cells were killed using different antibiotics. Procedures for *Agrobacterium* transformation of cotton hypocotyl sections were performed with modifications as described by Umbeck *et al.* (1987). Plants were regenerated with modifications of those as described by Trolinder and Goodin (1987). Subsequently, the cotton tissues were treated to stimulate regeneration of transgenic cells into shoots and ultimately plantlets were grown in soil and assayed for insect resistance.

C. Plant Expression Vectors

Bollgard™ Cotton Lines 757 and 1076 were transformed with plasmid vectors PV-GHBK04 and PV-GHBK03, respectively. The plasmid vectors, PV-GHBK04 and PV-GHBK03, are 11.4 Kb single border binary transformation vectors (Figure III-1 and III-2). They 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) integrated into the plant genomic DNA. The host for all DNA cloning and vector construction was *E. coli* MM-294, a derivative of the common laboratory *E. coli* K-12 strain. Both vectors are composed of several genetic components including non-functional DNA needed for cloning events. Table III-1 summarizes and references all the genetic components of PV-GHBK04 and PV-GHBK03.

The *cryIA(c)* and *nptII* genes were introduced into Coker 312 cotton plants using *Agrobacterium tumefaciens* binary single border transformation vectors (Bevan, 1984 and Wang, *et al.*, 1984). The plasmid vectors, PV-GHBK03 and PV-GHBK04, 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. It is composed of several genetic components. The 0.70 Kb *oriV* fragment from the RK2 plasmid (Stalker, *et al.*, 1981) provides the origin of replication for maintenance in *Agrobacterium tumefaciens* and is fused to the 3.0 Kb segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (*ori322*) and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* (Boliver, *et al.*, 1977 and Sutcliffe, 1978). This was fused to a 0.09 Kb DNA fragment from the pTiT37 plasmid which contains the 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 chimeric genes (genes with signals for plant expression), that encode the *B.t.k.* HD-73 and NPTII proteins and a bacterial selectable marker protein gene (*aad*) under the control of a bacterial promoter.

The chimeric gene in PV-GHBK04 responsible for the efficacious control of Lepidoptera (E35S/*cryIA(c)*/7S 3') consists of the enhanced 35S promoter (Kay *et al.*, 1987; Odell *et al.*, 1985), the *cryIA(c)* gene which encodes the *B.t.k.* HD-73 protein and the non-translated region of the soybean alpha subunit of the beta-conglycinin gene which provides the mRNA polyadenylation signals (Schuler, *et al.*, 1982) referred to as 7S 3' terminator sequence. The same chimeric gene in PV-GHBK03 ([ ]/*cryIA(c)*/E9 3') is under the control of the [ ]. The 3' end of the gene is from the E9 termination sequence and provides the mRNA polyadenylation signals (Coruzzi, *et al.*, 1984). These are fused to the 0.93 Kb fragment containing the *aad* gene, isolated from transposon Tn7, which encodes a protein that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985). Downstream of the *aad* gene is the chimeric gene for selection on kanamycin (35S/*nptII*/NOS 3') which

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consists of the cauliflower mosaic virus 35S promoter, the neomycin phosphotransferase type II (*nptII*) gene and the non-translated region of the 3' region of the nopaline synthase gene referred to as NOS 3' (Rogers *et al.*, 1985).

D. Inserted Genes

1. The *cryIA(c)* gene

The *cryIA(c)* gene contained within PV-GHBK04 and PV-GHBK03 was constructed by combining the first 1398 nucleotides of the *cryIA(b)* gene (corresponding to amino acids 1 to 466) (Fischhoff *et al.*, 1987) with nucleotides number 1399 to 3534 of the *cryIA(c)* gene (corresponding to amino acids 467 to 1178) (Adang *et al.*, 1985). With the exception of 6 amino acid differences, the *cryIA(b)* region is identical to the analogous region of the *B.t.k.* HD-73 protein encoded by the *cryIA(c)* gene as described by Adang *et al.* (1985). The *cryIA(c)* portion of the gene encodes a protein that is identical to the CryIA(c) protein present in nature (Adang *et al.*, 1985) with the exception of one amino acid at position 766. The protein found in nature contains a leucine at amino acid 766 and the *cryIA(c)* gene within PV-GHBK04 and PV-GHBK03 encode a serine at position 766. The discrepancy was unintentional and occurred during the genetic design of the gene for plant expression. Since the *B.t.k.* HD-73 protein produced in Bollgard™ Cotton Lines 757 and 1076 yields an insecticidally active trypsin-resistant core product of approximately 600 amino acids in size, the amino acid at position 766 will be lost in the insecticidally inactive fragment upon exposure to trypsin (or the proteases within the insect gut) and, therefore, will not affect the host range of the active N-terminal portion of the protein (Bietlot *et al*, 1989).

Both regions of the *B.t.k.* HD-73 gene were genetically improved for increased plant expression using a strategy comparable to that described by Perlak *et al.*, 1990 and 1991. Since the *B.t.k.* HD-73 protein present in Bollgard™ Cotton Lines 757 and 1076 contains the hypervariable region of the CryIA(c) protein, which has been shown to be responsible for insecticidal specificity (Geiser *et al.*, 1986), the gene in PV-GHBK04 and PV-GHBK03 is referred to as a *cryIA(c)* gene. The *cryIA(c)* gene contained within both plasmid vectors encodes a near-nature identical *B.t.k.* HD-73 protein as described by Adang *et al.* (1985) with the encoded protein produced in the Bollgard™ Cotton Lines being 99.4% identical to the naturally occurring *B.t.k.* HD-73 protein.

The *cryIA(c)* gene sequence, as introduced in Bollgard™ Cotton Lines 757 and 1076, is shown in Figure III-3. The corresponding amino acid sequence is shown in Figure III-4.

2. The *nptII* Marker Gene

The *nptII* gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). The NPTII enzyme uses ATP to phosphorylate neomycin and the related kanamycin, thereby inactivating these aminoglycoside antibiotics and preventing them from killing the cells producing NPTII. The coding sequence for the *nptII* gene is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982). The sole purpose of inserting the *nptII* gene into cotton cells with the *cryIA(c)* gene is to have an effective method of selecting cells that contain the insecticidal gene. In general, the frequency of cells that are transformed is often as low as 1 in 10,000 or 1 in 100,000 of the cells treated (Fraley *et. al.,* 1983). Therefore, to facilitate this process, a selectable marker gene, *nptII*, and selective agent, kanamycin, are used. Consequently, cells selected for plant generation that contain the *cryIA(c)* gene also contain the *nptII* gene.

The *nptII* gene sequence, as introduced into Coker 312 to produce Bollgard™ Cotton, is shown in Figure III-5. The corresponding amino acid sequence is shown in Figure III-6.

3. The *aad* Bacterial Marker Gene

The *aad* gene was isolated from transposon Tn7 (Fling, *et al.*, 1985) and is under the control of its own bacterial promoter which provided a selectable marker for genetic manipulations in the bacterial hosts. The *aad* gene encodes the enzyme 3"(9)-O-aminoglycoside adenylyltransferase (AAD) which allows for the selection of bacteria containing the PV-GHBK04 or PV-GHBK03 plasmid on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter and its lack of detectable expression was confirmed by an ELISA developed for the AAD protein (Berberich *et al.,* 1994). The *aad* gene sequence, as introduced into Coker 312 to produce Bollgard™ Cotton Lines 757 and 1076 is shown in Figure III-7. The corresponding amino acid sequence is shown in Figure III-8.

E. Genetic Analysis

1. Insert number, copy number and insert integrity

As described in Part III-B, Bollgard™ Cotton Lines 757 and 1076, were generated by *Agrobacterium tumefaciens* mediated transformation with the plasmid PV-GHBK04 and PV-GHBK03, respectively. DNA analyses were performed to characterize the inserted T-DNA in terms of insert number (number of integration events), copy number (number of T-DNA copies at a particular genetic locus) and insert integrity (gene size, composition and linkage). The characterization was performed by Southern blot analysis (Southern, 1975) on genomic DNA isolated from leaf tissue of the control (Coker 312) and Bollgard™ Cotton Line 757 or Line 1076 Cotton plants.

a. Bollgard™ Cotton Line 757

Genomic (chromosomal) DNA was isolated from control Coker 312 and Bollgard™ Cotton Line 757 plants. The molecular characterization conclusions were drawn from digestions of the DNA from Bollgard™ Cotton Line 757 with the enzymes *Ssp*I, *Rca*I, *Hin*dIII, or *Xba*I in combination with *Bsi*WI, *Bst*1107I, *Nhe*I or *Spe*I.

Ssp*I *digestion. The plasmid map of PV-GHBK04 in Figure III-1 shows two *Ssp*I restriction sites. If a single complete copy of the plasmid integrated into the plant genome, then upon restriction digestion with *Ssp*I, an approximately 7.4 Kb expected size fragment (Figure III-1) would be released along with two border fragments. If the entire plasmid integrated into the genome, then one border fragment greater than 4 Kb was expected and the other border fragment (released near the right border and containing approximately 0.1 Kb of T-DNA) was expected to be too small for detection.

Figure III-9, lane 3 shows the Southern analysis results from Bollgard™ Cotton Line 757 when cleaved with *Ssp*I and probed with the entire plasmid, PV-GHBK04. The expected size fragment of 7.4 Kb was observed, along with two additional fragments of 4.3 Kb and 3.8 Kb in size. These two bands appear as a single band in Figure III-9A, but are distinguishable as two bands on the autoradiograph of the Southern blot. The 7.4 Kb fragment hybridized to both the *cryIA(c)* and *nptII* probes (Figures III-10A and III-11A, lane 3, respectively) thereby confirming that a copy of T-DNA containing the two *Ssp*I restriction sites integrated into the plant genome. The 4.3 Kb fragment did not hybridize to the *cryIA(c)* or *nptII* probes but did hybridize to the EP (end of plasmid, Figure III-1) probe (Figures III-10A, through -12A, lane 3) thereby establishing that the 4.3 Kb fragment was a detectable border fragment. The 3.8 Kb fragment hybridized to the *cryIA(c)* probe but not the *nptII* or EP probes (Figures III-9A through -12A, lane 3, respectively) thereby establishing that a second partial T-DNA integrated into the cotton genome. The *Ssp*I digestion results do not establish whether the two copies inserted in tandem at a common genetic locus or at separate genetic loci.

Initiation of the T-DNA transfer at the right border by both copies was verified by the hybridization of a 7S probe (0.46 Kb in size) with Bollgard™ Cotton Line 757 DNA cleaved with *Xba*I in combination with a variety of four other enzymes (*Bsi*WI, *Bst*1107I, *Nhe*I or *Spe*I), Figure III-13A, lanes 3, 7, 11 and 15, respectively. Since the first of two *Xba*I sites within PV-GHBK04 is located at the 5' end of the 7S region (Figure III-1), each copy was expected to release a border fragment containing the 7S region. Since each of the four digestions showed two bands hybridizing to the 7S probe, this verified that each copy initiated the T-DNA transfer at the right border, as expected, and that the two copies did not insert in a head-to-head (right border to right border)

arrangement. If the integration had occurred in a head-to-head manner, then a single 7S fragment of minimally 0.92 Kb (0.46 Kb times 2) would have been predicted.

Since the second copy is approximately 3.8 Kb in size and initiation of the second T-DNA was shown to be from the right border, then maximally 3.3 Kb (3.8 Kb minus the 7S 3' region) of the 3' region of the *cryIA(c)* gene integrated into the cotton genome. The full length *cryIA(c)* gene is 3534 bp in size and encodes 1178 amino acids. Previous work has demonstrated that amino acids 29-607 are necessary for bioactivity (Hofte and Whiteley, 1989). Since the maximum amount of the *cryIA(c)* gene that could have inserted could only encode an 1100 amino acid protein, the first 78 amino acids could not be present and therefore an active *B.t.k.* protein is not possible from this second T-DNA insert.

Rca*I *digestions. The plasmid map of PV-GHBK04 shows two *Rca*I restriction sites at approximately 4.8 and 10.5 Kb downstream from the T-DNA right border. If a complete copy of the T-DNA integrated into the plant genome, including both *Rca*I restriction sites then three fragments were predicted (Figure III-1): a border fragment (of minimum size 4.8 Kb) containing the *cryIA(c)* gene, a 5.7 Kb internal fragment containing the *nptII* gene, and a second border fragment upstream of the *ori 322* region.

Figure III-9A, lane 5 shows the Southern analysis results using the entire PV-GHBK04 plasmid as a probe. Three fragments were observed: a 5.7 Kb fragment that did not hybridize to the *cryIA(c)* probe but did hybridize to the *nptII* probe (Figures III-10A and III-11A, lane 5), a 10.1 Kb fragment that hybridized to the *cryIA(c)* probe but not the *nptII* probe (the border fragment), and a 1.6 Kb fragment that hybridized to the EP probe (Figure III-12A, lane 5) thereby identifying it as the second border fragment from the complete T-DNA copy. These results indicated that a complete or nearly complete copy of T-DNA integrated into the plant genome past the *Rca*I site at position 10471 bp (Figure III-1).

Although the *Ssp*I digestion results described above and the *Hin*dIII and *Xba*I digestion results described below established the presence of a second T-DNA insert in Line 757, a second fragment that hybridized to the *cryIA(c)* probe was not detected in the *Rca*I digestion. This indicates that either the size of the *Rca*I fragment from the incomplete copy was below our limits of detection with Southern blot analysis or the additional fragment from the incomplete copy is co-migrating with the 10.1 Kb fragment (that hybridized with the *cryIA(c)* probe) from the complete copy of T-DNA.

***Hind*III digestions.** The genomic DNA of Bollgard™ Cotton Line 757 was cut with *Hind*III to determine the number of T-DNA insertion events that integrated into the plant genome. The plasmid map in Figure III-1 shows a single *Hind*III restriction site within PV-GHBK04. If a single complete copy of the plasmid integrated into the plant genome, then upon restriction digestion with *Hind*III, two border fragments greater than 4.6 Kb in size were predicted. One would contain the *cryIA(c)* gene and the second would contain the *nptII* gene.

In Figure III-9A, lane 6, three DNA fragments (21.1, 10.9 and 7.3 Kb) were shown to hybridize to the entire plasmid probe upon digestion with *Hind*III. The 21.1 and 10.9 Kb fragments hybridized to the *cryIA(c)* probe (Figure III-10A, lane 6) but not the *nptII* or EP probes (Figures III-11A and III-12A, lane 6). These data showed that these two fragments represented one of the border fragments from the complete T-DNA copy and a second incomplete T-DNA copy. This digestion result could not distinguish which was the border fragment for the complete copy and which was the fragment from the second T-DNA insert. The 7.3 Kb fragment did not hybridize to the *cryIA(c)* probe but did hybridize to the *nptII* and EP probes (Figures III-10A, III-11A and III-12A, lane 6) thereby identifying it as the second border fragment from the complete T-DNA copy.

The *Hind*III digestions showed that the two T-DNA copies integrated at two separate loci in the genome of Bollgard™ Cotton Line 757. If the two copies (complete and incomplete) had integrated at a single locus, then the *Hind*III digestion would have released two border fragments. Although the two copies integrated at separate loci, it is assumed that the two copies are linked since both copies are present in the R_2 and R_4 generations, as described below (i.e., they did not segregate out of the backcrossed lines).

The Southern analyses of Bollgard™ Cotton Line 757 indicates that a complete copy of the T-DNA as well as an incomplete copy of the T-DNA inserted at separate sites within the genome. The complete copy consist of the entire plasmid or almost the entire plasmid as indicated by the strong hybridization with the EP probe. The incomplete copy consists of the 7S 3' termination sequence and maximally a 3.3 Kb of the *cryIA(c)* gene. Based on the size of the incomplete copy observed from the *Ssp*I digestion, it cannot contain an active *cryIA(c)* gene. Segregation data from Bollgard™ Cotton Line 757 supports the presence of a single active gene.

b. Bollgard™ Cotton Line 1076

Genomic DNA was isolated from R_2 and R_4 Bollgard™ Cotton Line 1076 plants. The characterization results are derived from DNA digestions with the enzymes *Ssp*I, *Eco*RI, *Hind*III and *Rca*I.

SspI digestions. The plasmid map of PV-GHBK03 in Figure III-2 shows three *Ssp*I restriction sites. If a single complete copy of the plasmid integrated into the plant genome, then upon digestion with *Ssp*I, an approximately 7.0 Kb fragment (Figure III-2) would be released along with two border fragments. If the entire plasmid integrated into the genome, then one border fragment was expected to be greater than 4 Kb and the other (released near the right border and containing approximately 0.1 Kb of T-DNA) was expected to be too small for detection. A small fragment of about 350 bp (Figure III-2) would also be released but not normally detectable by Southern analysis.

Figure III-14A, lane 3 shows the Southern analysis results from Bollgard™ Cotton Line 1076 when cleaved with the restriction enzyme *Ssp*I and probed with the entire plasmid, PV-GHBK03. The expected size fragment of 7.0 Kb was observed, along with two additional fragments of 4.0 and 1.9 Kb in size. The 7.0 Kb fragment hybridized to the *cryIA(c)* and *nptII* probes but not the EP probe (Figures III-15A, III-16A and III-17A, lane 3) thereby confirming that a copy of T-DNA containing two internal *Ssp*I restriction sites integrated into the plant genome. The 4.0 Kb fragment did not hybridize to the *cryIA(c)* or *nptII* probes but did hybridize to the EP probe (Figures III-15A through 17A) thereby confirming this fragment as the border fragment from the complete copy of T-DNA. The presence of an additional 1.9 Kb fragment that hybridized to the *cryIA(c)* probe but not to the *nptII* or EP probes (Figures III-15A through -17A, lane 3) indicates a second incomplete copy of T-DNA inserted into the genome of Line 1076. However, the *Ssp*I digestion results did not establish if the two copies integrated in tandem at a common genetic locus or at separate genetic loci.

EcoRI digestions. Three *EcoR*I restriction sites are present in the plasmid PV-GHBK03 (Figure III-2). If a complete copy of the T-DNA integrated into the plant genome, then four fragments would have been released which would have included two border fragments (one greater than 6.9 Kb and the other greater than 0.6 Kb), and two internal fragments of approximate sizes 3.6 and 0.4 Kb.

Figure III-14A, lane 5 shows the results of cleaving DNA from Bollgard™ Cotton Line 1076 with *EcoR*I and probing with the entire plasmid, PV-GHBK03. Three fragments were observed which included a 7.36, 3.6 and 1.6 Kb fragments. The 3.6 Kb fragment hybridized to the *cryIA(c)* probe but not to the *nptII* or EP probes (Figures III-15A through -17A, lane 5), respectively, thereby identifying it as one of the internal fragments. The second internal fragment was not observed due to the sensitivity of this Southern blot. The 7.36 Kb fragment hybridized to the *nptII* and EP probes but not to the *cryIA(c)* probe (Figures III-15A through -17A, lane 5) thereby identifying it as one of the border fragments. The second, smaller border fragment was not detected due to the sensitivity of this Southern blot. The 1.6 Kb fragment hybridized to the *cryIA(c)* probe but not the *nptII* or EP probes (Figures III-15A

through 17A, lane 5) thereby identifying it as a second smaller T-DNA copy which inserted into the cotton genome. The 1.6 Kb *cryIA(c)* fragment cannot produce an active *B.t.k.* HD-73 protein. Previous work has demonstrated that amino acids 29-607 are necessary for bioactivity (Hofte and Whiteley, 1989). Since the maximum size of an encoded protein (from the 1.6 Kb *cryIA(c)* gene 3' end fragment) is 530 amino acids, an active *B.t.k.* protein is not possible from this second *cryIA(c)* gene.

The *EcoR*I digestion results established that one complete copy of the T-DNA integrated into the plant genome, along with an adjacent partial copy of T-DNA (the 1.6 Kb fragment) into the cotton genome to produce Bollgard™ Cotton Line 1076.

***Hind*III digestions.** Results for the *Hind*III cleavage of DNA from the R_4 generation of Bollgard™ Cotton Line 1076 are not available from this particular blot due to loss of the sample during the pre-loading procedure (Figures III-14A through -17A, lane 4). However, other blots done have demonstrated identical *Hind*III results for DNA isolated from the second (R_2) and fourth (R_4) generations of Bollgard™ Cotton Line 1076 DNA when probed with PV-GHBK03 and the *cryIA(c)* gene. Therefore, for simplicity, the results of the DNA digestions from the R_2 generation will be discussed (lane 9 in Figures III-14A through -17A).

A single *Hind*III restriction site is present within the plasmid PV-GHBK03 (Figure III-2). The genomic DNA of Bollgard™ Cotton Line 1076 was cleaved with *Hind*III to obtain information on the number of inserted T-DNA events. If a complete copy of the T-DNA integrated into the plant genome, then two border fragments greater than 4.7 Kb would have been released.

Figure III-14A, lane 9 shows that when DNA from Bollgard™ Cotton Line 1076 was cut with *Hind*III and probed with PV-GHBK03, two fragments of 5.7 and 19.7 Kb were generated. Both the 5.7 and 19.7 Kb fragments hybridize to the *cryIA(c)* probe while only the 19.7 Kb fragment hybridized to the *nptII* and EP probes (Figures III-15A through -17A, lane 9). These results indicate that the 5.7 Kb fragment is one of the border fragments while the 19.7 Kb fragment is the other border fragment connected to the second incomplete T-DNA copy.

Since the *Hind*III digestion released only two DNA fragments, this established that the two T-DNA copies integrated at the same genetic locus within the cotton genome to produce Bollgard™ Cotton Line 1076. If the two copies (incomplete and complete) had integrated at two separate loci, then the *Hind*III digestion would have released three border fragments.

RcaI digestions. The plasmid map of PV-GHBK03 shows two *Rca*I sites at 4880 bp and 10465 bp. If a complete copy of the T-DNA integrated into the plant genome, including both *Rca*I restriction sites, an approximately 5.6 Kb fragment (Figure III-2) was expected, along with two border fragments (one of at least 4.8 Kb and a second greater than 1 Kb, if the entire plasmid T-DNA integrated into the cotton genome).

Figure III-14A, lane 6 shows the results of cleaving DNA from Bollgard™ Cotton Line 1076 with *Rca*I and probing with the entire plasmid, PV-GHBK03. The expected size fragment of 5.6 Kb was observed, along with two additional fragments of 6.7 Kb and 11.6 Kb in size. The 5.6 Kb fragment hybridized to the *nptII* probe but not the *cryIA(c)* or EP probes thereby identifying it as the fragment released by the two internal *Rca*I sites (Figures III-15A through -17A, lane 6). Both the 6.7 Kb and the 11.6 Kb fragments hybridized to the *cryIA(c)* probe (Figure III-15A, lane 6) but not the *nptII* probe (Figure III-16A, lane 6). The 6.7 Kb fragment hybridized to the EP probe (Figure III-17A, lane 6). These results identified the 11.6 Kb fragment as a border fragment released from the complete or near complete T-DNA copy and the 6.7 Kb fragment as the second border fragment connected to the second T-DNA copy. The *Rca*I digestions support the *Hind*III digestion results which demonstrated a complete T-DNA copy and an incomplete T-DNA copy integrated in tandem into the cotton genome to produce Bollgard™ Cotton Line 1076.

2. Stability of Gene Transfer

The stability of the T-DNA after insertion into the cotton plants was demonstrated by comparing the DNA isolated from two different generations of Bollgard™ Cotton Lines 757 and 1076.

In order to assess the stability of the T-DNA insertion events over generations, genomic DNA from the R_2 generation was cleaved separately with *Ssp*I and *Hind*III and compared to the DNA isolated from the R_4 generation of Bollgard™ Cotton Lines 757 and 531 (Figures III-9A, III-10A, III-11A and III-12A: lane 3 compared to lane 8 and lane 6 compared to lane 9 for Bollgard™ Cotton Line 757 and lane 10 compared to lane 12 or lane 11 compared to lane 13 for Bollgard™ Cotton Line 531). No differences were observed in the DNA fragment sizes and hybridization patterns between the two generations, thereby confirming the genetic stability of the T-DNA insertion events.

Although the DNA from the R_4 generation of Bollgard™ Cotton Line 1076 cleaved with *Hind*III was not clearly detectable in the blot shown (Figures III-13A, III-14A, III-15A and III-16A, lane 4), other blots under this study demonstrated no differences between the banding patterns of DNA isolated from the R_2 and R_4 generations of Bollgard™ Cotton Line 1076. Since no differences were observed in the DNA fragment sizes and hybridization patterns between the two generations, the genetic stability of the T-DNA insertion events for Bollgard™ Cotton Line 1076 was confirmed.

The stability of the *cryIA(c)* gene has been demonstrated over four generations of backcrossed derivatives of Bollgard™ Cotton Line 757, Line 1076 and in several elite cultivar lines (Tables III-2 and III-3). The Chi square test for the BC3F1, BC3F2 and BC3F3 segregates were not different than expected. The Chi square test for the BC3F2 progeny test (expected segregation of 1 homozygote:2 heterozygotes) was significant at P=0.05 but not at P=0.01. By definition, we would expect a deviation of this magnitude from the expected ratio in approximately 5% of the cases. Thus, this result is most likely due to random sampling. Segregation data for R1 plants from Bollgard™ Cotton Line 757 and Line 1076 (progeny of the initial transformant, which is referred to as R0) and the progeny of the R1 plants are presented in Tables III-4 and III-5. These results are consistent with a tightly linked two-insertion event containing one active copy of the cryIA(c) gene.

Conclusions

In summary, southern blot analyses demonstrated a complete copy of T-DNA at least 10.6 Kb in length was inserted into the genome of cotton to produce Bollgard™ Cotton Line 757. A second incomplete copy of the T-DNA of no more than 3.8 Kb of T-DNA was inserted at a separate site in the genome of Bollgard™ Cotton Line 757. Southern analysis has also shown that one complete copy of T-DNA, at least 10.5 Kb in length, was inserted into the genome of cotton to produce Bollgard™ Cotton Line 1076 with a second incomplete copy of no more than 1.6 Kb of T-DNA (not including the 3' termination sequence) at the same site. The second incomplete copy of the T-DNA in both Bollgard™ Cotton Line 757 and Line 1076 contains a 3' non-translated region (termination sequence) and a portion of the *cryIA(c)* gene, which cannot encode a protein that is insecticidally active. No detectable rearrangements or insertions were observed in the genes contained on the complete T-DNA copy of Bollgard™ Cotton Line 757 or Line 1076 indicating that the T-DNA maintained its integrity during the transfer event. Additionally, DNA from the R_2 and R_4 generations of Bollgard™ Cotton Lines 531, 757 and 1076 showed the same T-DNA genetic composition thus confirming the genetic stability of the insertion events. The gene for *cryIA(c)* segregated in a manner consistent with two tightly linked insertion events and was stably transferred with crossing. The selfed data from the crosses further demonstrated the stability of transfer from generation to generation.

F. Description of the Expressed Proteins

1. *Bacillus thuringiensis* Crystal Proteins

a. Biochemistry

Bacillus thuringiensis is a crystalliferous spore-forming gram-positive bacterium that has been used commercially over the last 30 years to control insect pests. These microbes are found naturally in soil worldwide. Numerous different strains have been identified, characterized and used commercially. Several strains have been extensively studied and have been shown to be insecticidally active against selected insect pests as summarized below:

B. thuringiensis subsp. *israelensis* strains are active against Dipteran insects (mosquitoes and black flies);

B. thuringiensis subsp. *san diego* and *tenebrionis* strains are active against Coleoptera (potato beetle, elm leaf beetle);

B. thuringiensis subsp. *kurstaki*, *sotto* and *aizawai* strains are all active against Lepidoptera (tomato hornworm, gypsy moth, cabbage looper, tobacco budworm, cotton bollworm, etc.).

The protein produced in Bollgard™ Cotton Lines 757 and 1076, (*cryIA(c)*), is >99.4% identical to the protein produced by the *B.t.k.* HD-73 bacterial strain. This strain controls insect pests by the production of crystalline insecticidal proteins known as delta-endotoxins. These proteins are produced as the bacterium enters the sporulation phase and can account for approximately one-third of the weight of the bacterial cell. To be active against the target insect, the protein must be ingested. In the insect gut, the protein binds to specific receptors on the insect mid-gut, inserts into the membrane and forms ion-specific pores. These events disrupt the digestive processes and cause the death of the insect. Strains of *B. thuringiensis* have been used commercially to control selected insect pests. Commercial quantities of these microbes are prepared in large-scale cultures in which the bacteria are allowed to sporulate. The spores and proteins are then formulated for application to plants.

Two classes of insecticidal proteins (delta-endotoxins) are produced upon sporulation by *B.t.k.* strains. These are termed P1 and P2 proteins based on relative molecular weights. The *B.t.k.* HD-73 (CryIA(c)) protein falls in the P1 class. The P1 proteins range in molecular weight from 130,000 to 140,000 daltons and are comprised of 1100 to 1200 amino acids The P2 proteins are typically significantly smaller in size than the P1 proteins. The most well studied P2 proteins are 71 Kda in size and are comprised of 633 amino acids

(Widner and Whitely, 1989). The P1 proteins can be divided into an amino terminal and a carboxy terminal domain. The amino acid sequences of the carboxy terminal domain have been conserved (Thorne *et al.*, 1986; Jaquet *et al.*, 1987) across bacterial strains and contain a number of cysteine residues which form intramolecular bonds that are important in the formation of the protein crystal structure. The carboxy terminal domain is not essential for insect toxicity; it can be cleaved from the protein molecule without affecting the activity of the remaining protein towards insects (Adang *et al.*, 1987; Thorne *et al.*, 1986).

The amino terminal end of the P1 protein retains the insecticidal activity (Fischhoff *et al.*, 1987). Comparison of the amino acid sequence for various P1 proteins from several *B. thuringiensis* strains reveals considerable differences (22% homology in amino acid content for the *B.t. kurstaki* and *tenebrionis* subspecies) which account for the selectivity in activity against various insect orders.

b. Mode-of-Action

As stated previously, *B.t.k.* proteins must be ingested by the insect to exert insecticidal activity. The protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH (Bulla *et al.*, 1977); however, the pH of the larval insect gut is alkaline which favors solubilization of the protein crystal. The solubilized protein is subsequently activated by proteases in the insect gut. These proteases cleave the carboxy terminal domain from the rest of the protein (Chroma and Kaplan, 1990) as well as approximately 28 amino acids from the amino terminal end of the protein (Bietlot *et al.*, 1989). The activated protein, which consists of approximately 600 amino acids, diffuses through the peritrophic membrane of the insect to the midgut epithelium. There, it binds to specific high affinity receptors on the surface of the midgut epithelium of target insects (Wolfersberger *et al.*, 1986; Hofmann *et al.*, 1988; Hofmann *et al.*, 1988a; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990). Non-target insects, mammals, birds and fish do not possess such receptors. Pores are formed in the membrane leading to leakage of intracellular contents (e.g. K+) into the gut lumen and water into the epithelial gut cells (Sacchi, *et al.*, 1986; Knowles *et al.*, 1989). The larval gut epithelial cells swell due to osmotic pressure and lyse. The gut becomes paralyzed as a consequence of changes in electrolytes and pH in the gut causing the larval insect to quit eating and die.

c. Evidence for "Species-Selectivity"

The protein delta-endotoxins produced by the various subspecies of *B. thuringiensis*, although related, exhibit differences in the amino acid sequence for the amino terminal domain of the proteins. These differences account, in part, for their selective action against certain insect pests. More importantly, non-target insects lack receptors for the proteins on the surface of their gut cells. This has practical application in assessing the safety of *B. thuringiensis* protein delta endotoxins towards other non-target organisms such as fish, birds and mammals. No receptors for these proteins have been identified on intestinal cells of mammals such as rats and rabbits (Sacchi *et al.* 1986; Hoffman *et al.* 1988; Van Mellaert *et al.* 1988). This explains the absence of toxicity for the protein delta-endotoxins of *B. thuringiensis* subspecies such as ***kurstaki*** to non-target organisms. The *B.t.k.* HD-73 protein expressed in Bollgard™ Cotton Lines 757 and 1076 shows a strict host-range specificity for lepidopteran insects and has no deleterious effects on non-target organisms (see Part V(K)).

d. Human Food Safety Considerations

There are no receptors for the protein delta-endotoxins of *B. thuringiensis* subspecies on the surface of mammalian intestinal cells; therefore, humans are not susceptible to these proteins. This has been confirmed in numerous safety studies carried out in laboratory animals which are traditionally experimental surrogates for humans. The results of some of these studies have been published in scientific reviews (Ignoffo,1973; Shadduck *et al.*, 1983; Siegel and Shadduck, 1989). Results of unpublished safety studies generated by registrants of *B. thuringiensis* commercial preparations have also been summarized in a recently issued EPA Registration Standard for *Bt* Formulations (EPA, 1988). In published reviews and the EPA document, studies are referenced where large doses (5000 mg/kg) of *B. thuringiensis* formulations were administered as single or multiple oral doses (up to 2 years) to different laboratory animals, with no adverse effects. Avian and aquatic organisms have also been fed *B. thuringiensis* formulations, with no adverse effects. A typical formulation is composed of *Bt* spores and *Bt* protein endotoxin, the latter compromising up to one-third of the weight of the spores. While target insects are susceptible to oral doses of *B.t.k.* proteins (μg per gram of body weight), there was no evidence of any toxic effects observed in non-target laboratory mammals, fish or birds given the equivalent of up to 10^6 μg of protein per gram of body weight. No deleterious effects were observed on non-target insects at doses over 100 fold higher than needed to control target insects (EPA 1988).

In addition to the lack of receptors for the *B.t.k.* proteins, the absence of adverse effects in non-target animals is further supported by the poor solubility and stability of the *B.t.k.* proteins in the acid milieu of the stomach. The acid conditions in the stomach and the presence of bile acids denature the *B.t.k.* proteins facilitating their rapid degradation by pepsin. *In vitro* enzymatically activated delta-endotoxins are also non-toxic when administered orally to laboratory animals (Nishitsutsuji-Uwo *et al.* 1980). Even if activated *B.t.k.* protein toxins could enter the mammalian gastrointestinal tract, there are no receptors on the surface of gastrointestinal tissues to permit binding of the protein toxin to the cell surface.

These scientific considerations support the history of safe use of *B. thuringiensis* preparations. Based on the available scientific data, EPA and other regulatory scientists worldwide have determined that use of registered *B. thuringiensis* products pose no significant risks to human health or non-target organisms.

e. Lack of Exposure to Fish and Wildlife

As reported in the EPA Registration Standard for *Bacillus thuringiensis*, the naturally occurring *B.t.* proteins have been demonstrated to be virtually non-toxic to fish, avian species, mammals and other non-targets. Furthermore, 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 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 species should not feed on the large lint covered seed. In addition, the seed is not expected to enter aquatic habitats; therefore, fish should not be exposed.

Since the naturally occurring *B.t.k.* proteins have been demonstrated to be virtually non-toxic to fish, avian species, non-target insects, mammals and other non-target species and exposure to these species is not expected due their feeding preferences, no adverse effects are expected to wildlife from the commercialization of these plants.

Finally, no endangered or threatened lepidopteran insects, as listed in 50CFR 17.11 and 17.12, feed on cotton plants.

2. Biochemistry of the Neomycin Phosphotransferase II

The Neomycin Phosphotransferase II protein (NPTII), which has no insecticidal effect, is ubiquitous in the environment and found in microbes present on food and within the human digestive system (Flavell *et al.* 1992; Calgene, Inc., 1993). This protein has also been used as a selectable marker for animal and human cell transformation and for human gene therapy experiments (Culver *et al.*, 1991; Brenner *et al.*,

1993). The safety of NPTII and other selectable markers are addressed in recent reviews by Flavell *et al.* (1992) and Nap *et al.* (1992), and in two separate papers by Monsanto Scientists; Fuchs *et al.* (1993a) and Fuchs *et al.* (1993b). FDA has recently approved the request from Calgene Inc. to amend the food additive regulations to provide for the safe use of NPTII as a processing aid in the development of new varieties of tomato, oilseed rape and cotton (Calgene, Inc., 1993, FDA 1994). In addition, the EPA has exempted the NPTII protein and the genetic material necessary for the production of the protein from the requirement of a tolerance in or on all agricultural commodities when used as a plant-pesticide inert ingredient (EPA 1994).

These reviews and the approvals by the FDA and EPA support the safety of NPTII protein for use as a selectable marker in crops grown for human and animal consumption.

Conclusions

- The *Agrobacterium tumefaciens* transformation system utilized in the modification of this insect resistant cotton is well understood and has been utilized for many years in the modification of many dicotyledonous plants. The system is dis-armed and cannot transmit the crown gall disease.

- This transformation system stably inserts the genes into the chromosome of the plant cell.

- All of the elements of the plasmid vectors PV-GHBK04 and PV-GHBK03, which was utilized in the modification of Bollgard™ Cotton Lines 757 and 1076, respectively, are well characterized and understood. The function of each element is known and the genes have been cloned so they have no potential to transfer any plant pest characteristics to the host organism.

- The *cryIA(c)*, *nptII* and *aad* genes present in the PV-GHBK04 and PV-GHBK03 plasmid vectors have been completely sequenced.

- In separate transformation events, two T-DNA inserts, integrated in close proximity, have been inserted into the cotton genome to produce Bollgard™ Cotton Lines 757 and 1076. The *cryIA(c)* gene segregated in a manner consistent with a single active copy of the gene and was stably transferred with crossing.

- The amino acid sequences for the *B.t.k.* and NPTII proteins as present in Bollgard™ Cotton Lines 757 and 1076 have been elucidated based on nucleotide sequence.

- The *B.t.k.* protein produced in Bollgard™ Cotton Lines 757 and 1076, (CryIA(c)), is >99.4% identical to the protein produced by the *B.t.k.* HD-73 bacterial strain. To be active against the target insect, the protein must be ingested. In the insect gut, the protein binds to specific receptors on the insect mid-gut, inserts into the membrane and forms ion-specific pores. These events disrupt the digestive processes and cause the death of the insect.

- Strains of *B. thuringiensis* have been used commercially, for nearly 30 years, to control selected insect pests.

- The CryIA(c) protein produced in Bollgard™ Cotton Lines 757 and 1076 is considered non-toxic to non-target insects, birds, fish and mammals. These species lack receptors for the proteins on the surface of their gut cells.

- The NPTII enzyme expressed in Bollgard™ Cotton Lines 757 and 1076 functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation. It has no pesticidal activity and is not known to be toxic to any species.

- The *aad* gene, present in Bollgard™ Cotton Lines 757 and 1076, was used as a selectable marker for genetic manipulations in the bacterial hosts prior to plant transformation. The gene is under the control of its own bacterial promoter and the AAD protein was not detected in tissues from Bollgard™ Cotton Lines 757 and 1076.

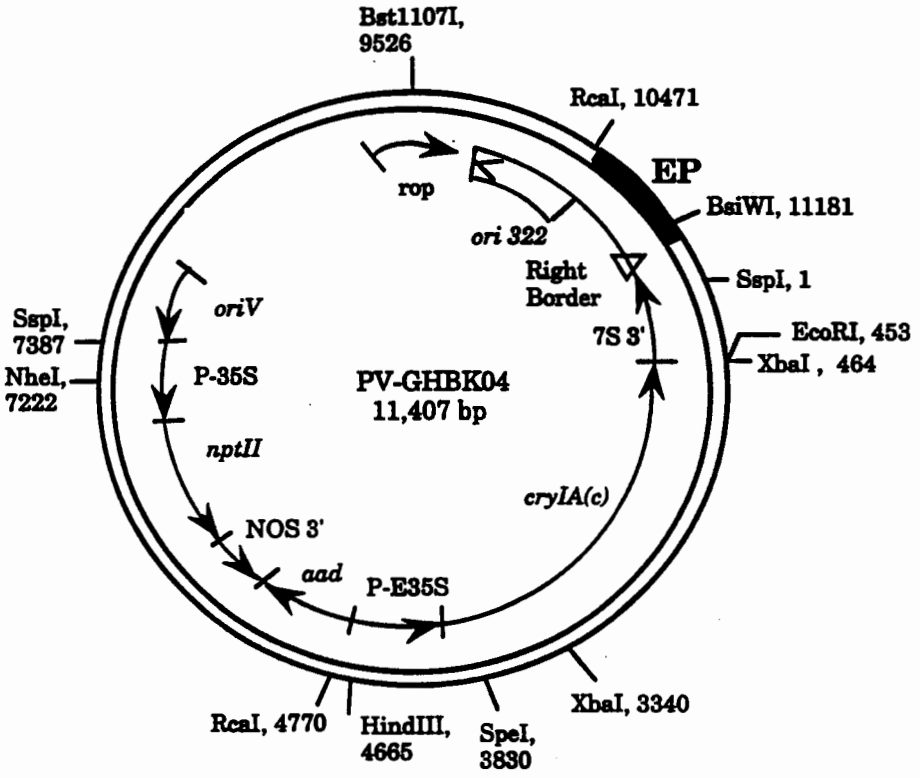

Figure III-1. Plasmid map of the 11.4 Kb binary vector PV-GHBK04.

Restriction sites and their locations in bp utilized during Southern hybridization analyses are shown. The right border is denoted by an open triangle. All probes were produced using the entire genetic region. The region used to produce the EP probe is denoted by the darkened area on the map.

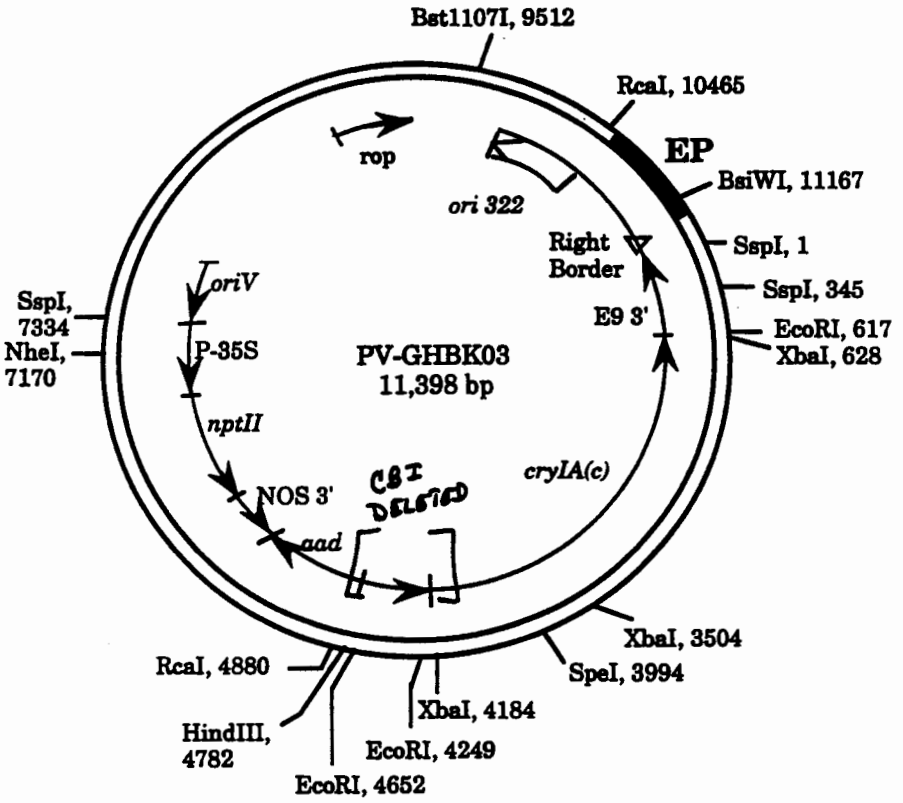

Figure III-2. Plasmid map of the 11.4 Kb binary vector PV-GHBK03.

Restriction sites and their locations in bp utilized during Southern hybridization analyses are shown. The right border is denoted by an open triangle. All probes were produced using the entire genetic region. The region used to produce the EP probe is denoted by the darkened area on the circle.

Figure III-3. Nucleotide sequence of the *B.t.k.* HD-73 protein encoded by *cryIA(c)* in Bollgard™ Cotton Line 757 and 1076 plants containing the PV-GHBK04 and PV-GHBK03 vectors, respectively. It is composed of the first 1-1398 nucleotides (1-466 amino acids) of *cryIA(b)* and 1399-3534 nucleotides (467-1178 amino acids) of *cryIA(c)*.

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Figure III-4. Amino acid sequence for the *B.t.k.* HD-73 full length protein which is present in Bollgard™ Cotton Lines 757 and 1076.

```
   1  MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51  VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101  EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151  QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201  GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251  DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301  NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351  VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401  YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451  PMFSWIHRSA EFNNIIASDS ITQIPAVKGN FLFNGSVISG PGFTGGDLVR
 501  LNSSGNNIQN RGYIEVPIHF PSTSTRYRVR VRYASVTPIH LNVNWGNSSI
 551  FSNTVPATAT SLDNLQSSDF GYFESANAFT SSLGNIVGVR NFSGTAGVII
 601  DRFEFIPVTA TLEAEYNLER AQKAVNALFT STNQLGLKTN VTDYHIDQVS
 651  NLVTYLSDEF CLDEKRELSE KVKHAKRLSD ERNLLQDSNF KDINRQPERG
 701  WGGSTGITIQ GGDDVFKENY VTLSGTFDEC YPTYLYQKID ESKLKAFTRY
 751  QLRGYIEDSQ DLEIYSIRYN AKHETVNVPG TGSLWPLSAQ SPIGKCGEPN
 801  RCAPHLEWNP DLDCSCRDGE KCAHHSHHFS LDIDVGCTDL NEDLGVWVIF
 851  KIKTQDGHAR LGNLEFLEEK PLVGEALARV KRAEKKWRDK  REKLEWETNI
 901  VYKEAKESVD ALFVNSQYDQ LQADTNIAMI HAADKRVHSI REAYLPELSV
 951  IPGVNAAIFE ELEGRIFTAF SLYDARNVIK NGDFNNGLSC WNVKGHVDVE
1001  EQNNQRSVLV VPEWEAEVSQ EVRVCPGRGY ILRVTAYKEG YGEGCVTIHE
1051  IENNTDELKF SNCVEEEIYP NNTVTCNDYT VNQEEYGGAY TSRNRGYNEA
1101  PSVPADYASV YEEKSYTDGR RENPCEFNRG YRDYTPLPVG YVTKELEYFP
1151  ETDKVWIEIG ETEGTFIVDS VELLLMEE
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Figure III-5. Nucleotide sequence for the neomycin phosphotransferase II (*nptII*) gene present in Bollgard™ Cotton Lines 757 and Line 1076.

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Figure III-6. Amino acid sequence for neomycin phosphotransferase II (NPTII) protein present in Bollgard™ Cotton Lines 757 and 1076.

1	MIEQDGLHAG	SPAAWVERLF	GYDWAQQTIG	CSDAAVFRLS	AQGRPVLFVK
51	TDLSGALNEL	QDEAARLSWL	ATTGVPCAAV	LDVVTEAGRD	WLLLGEVPGQ
101	DLLSSHLAPA	EKVSIMADAM	RRLHTLDPAT	CPFDHQAKHR	IERARTRMEA
151	GLVDQDDLDE	EHQGLAPAEL	FARLKARMPD	GEDLVVTHGD	ACLPNIMVEN
201	GRFSGFIDCG	RLGVADRYQD	IALATRDIAE	ELGGEWADRF	LVLYGIAAPD
251	SQRIAFYRLL	DEFF			

Figure III-7. Nucleotide sequence for the aminoglycoside adenylyltransferase (*aad*) gene present in Bollgard™ Cotton Lines 757 and 1076.

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Figure III-8. Amino acid sequence for the aminoglycoside adenylyltransferase (*aad*) gene present in Bollgard™ Cotton Lines 757 and 1076.

1	MREAVIAEVS	TQLSEVVGVI	ERHLEPTLLA	VHLYGSAVDG	GLKPHSDIDL
51	LVTVTVRLDE	TTRRALINDL	LETSASPGES	EILRAVEVTI	VVHDDIIPWR
101	YPAKRELQFG	EWQRNDILAG	IFEPATIDID	LAILLTKARE	HSVALVGPAA
151	EELFDPVPEQ	DLFEALNETL	TLWNSPPDWA	GDERNVVLTL	SRIWYSAVTG
201	KIAPKDVAAD	WAMERLPAQY	QPVILEARQA	YLGQEDRLAS	RADQLEEFVH
251	YVKGEITKVV	GK			

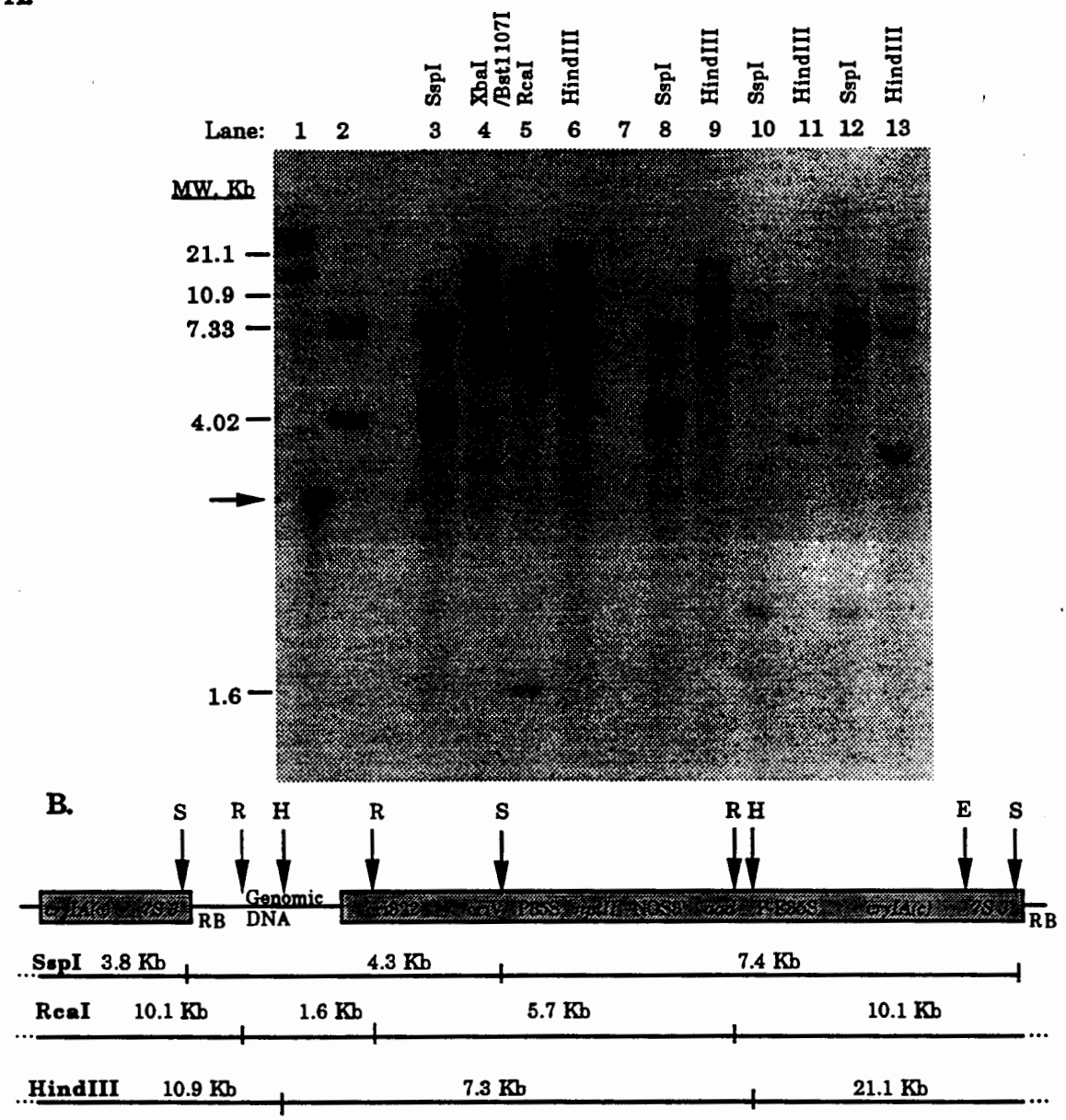


Figure III-9 A. Southern blot analysis of Bollgard™ Cotton lines 757 and 531 probed with PV-GHBK04. Lane 1 contains 13 µg of DNA from C312 digested with HindIII plus 50 pg of PV-GHBK04 cleaved with SspI. The enzyme digestion of the plasmid DNA was incomplete and therefore not used as a MW standard. Lane 2 contains 13 µg of C312 digested with HindIII plus 50 pg of PV-GHBK04 cleaved with EcoRI and HindIII simultaneously. Lanes 3-6 contain 13 µg of DNA from line 757 (R_4 generation) cleaved with the restriction enzymes SspI (lane 3), XbaI and Bst 1107I (lane 4), RcaI (lane 5), and HindIII (lane 6). Lane 7 contains 13 µg of DNA from C312 digested with HindIII. Lanes 8 and 9 contain 13 µg of DNA from line 757 (R_2 generation) cleaved with SspI or HindIII, respectively. Lanes 10 and 11 contain 13 µg of DNA from line 531 (R_4 generation) cleaved with SspI or HindIII, respectively. Lanes 12 and 13 contain 13 µg of DNA from line 531 (R_2 generation) cleaved with SspI or HindIII, respectively. The arrow indicates nonspecific hybridization. **B. Schematic illustration of the T-DNA region from line 757.** The right border is denoted by RB and is shown for orientation purposes (*i.e.*, an intact border sequence is not implied). Shaded areas indicate the portion of the plasmid radiolabelled to produce the probe used in Figure III-9A. The dotted lines indicate that the border fragments continue into the genomic DNA. The relative locations of RcaI and HindIII in the genomic DNA are unknown. SspI may also be present in the genomic DNA between the two copies. Abbreviations are as follows: S=SspI, R=RcaI, H=HindIII, E=EcoRI.

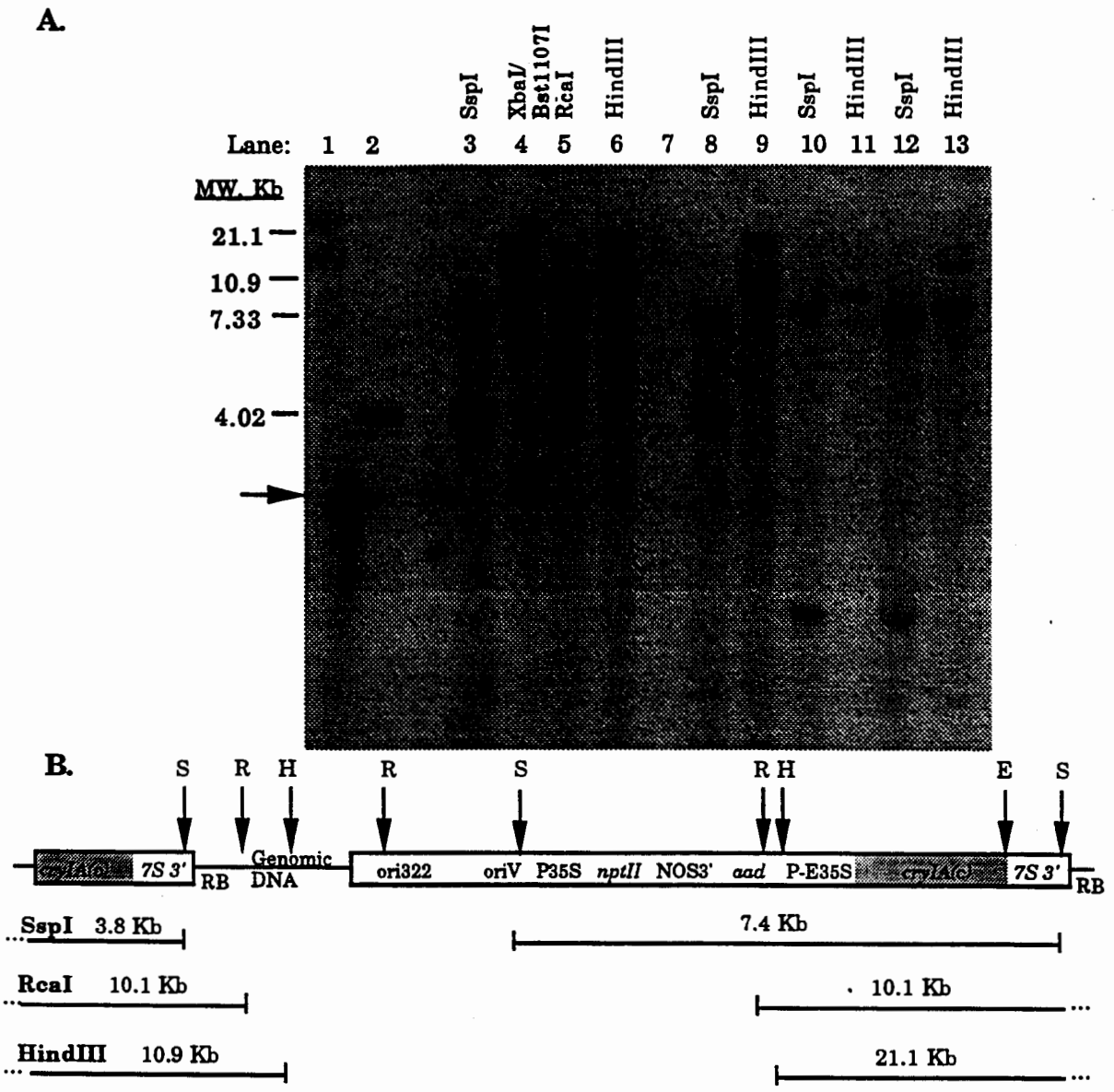


Figure III-10. A. Southern blot analysis of Bollgard™ Cotton lines 757 and 531 probed with the *cryIA(c)* gene. Southern blot from Figure III-9A, the plasmid probe removed, and reprobed with the *cryIA(c)* gene. Lane designations are the same as in Figure III-9A. **B. Schematic illustration of the T-DNA region of line 757.** All other designations are as in Figure III-9B.

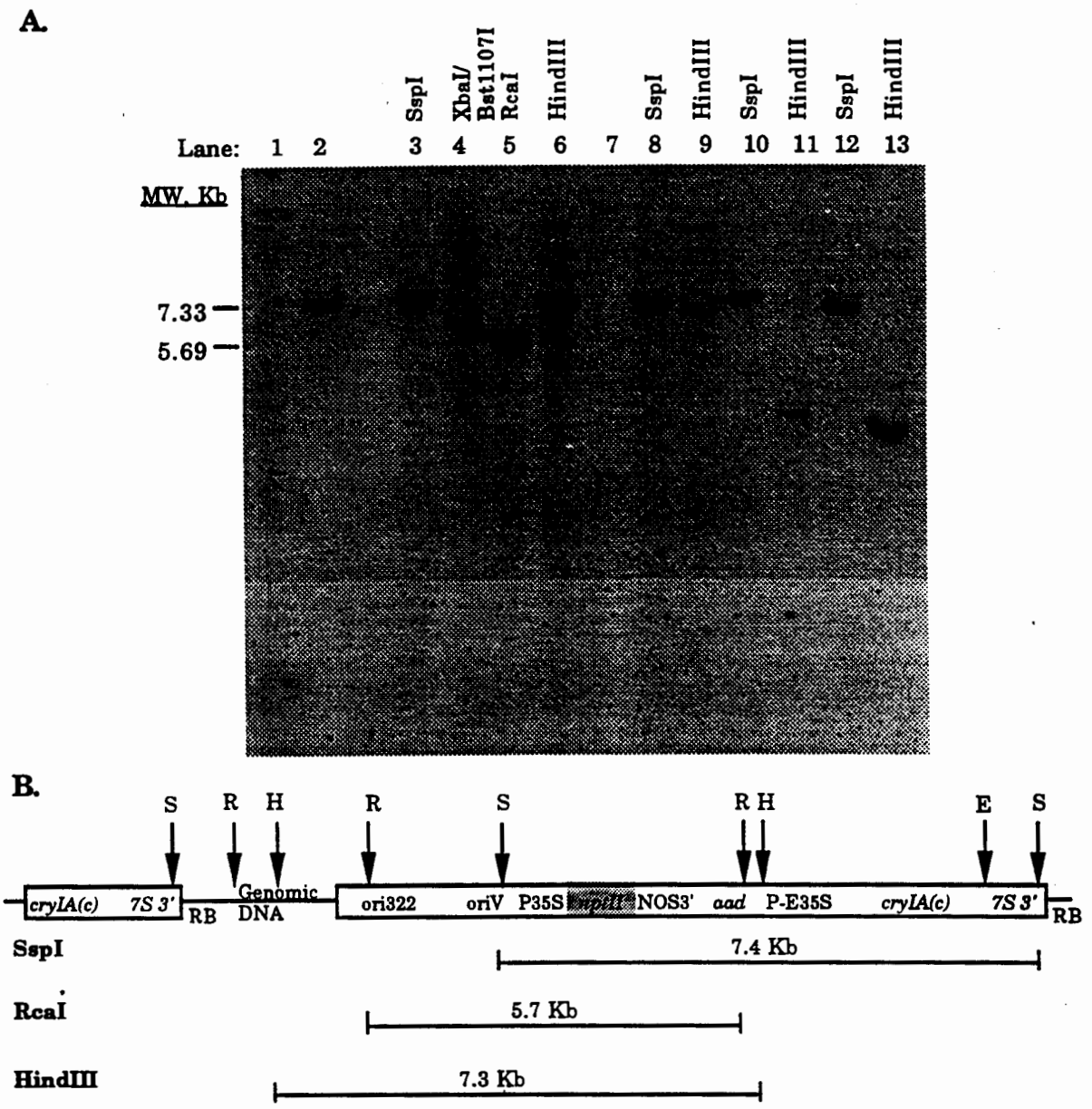
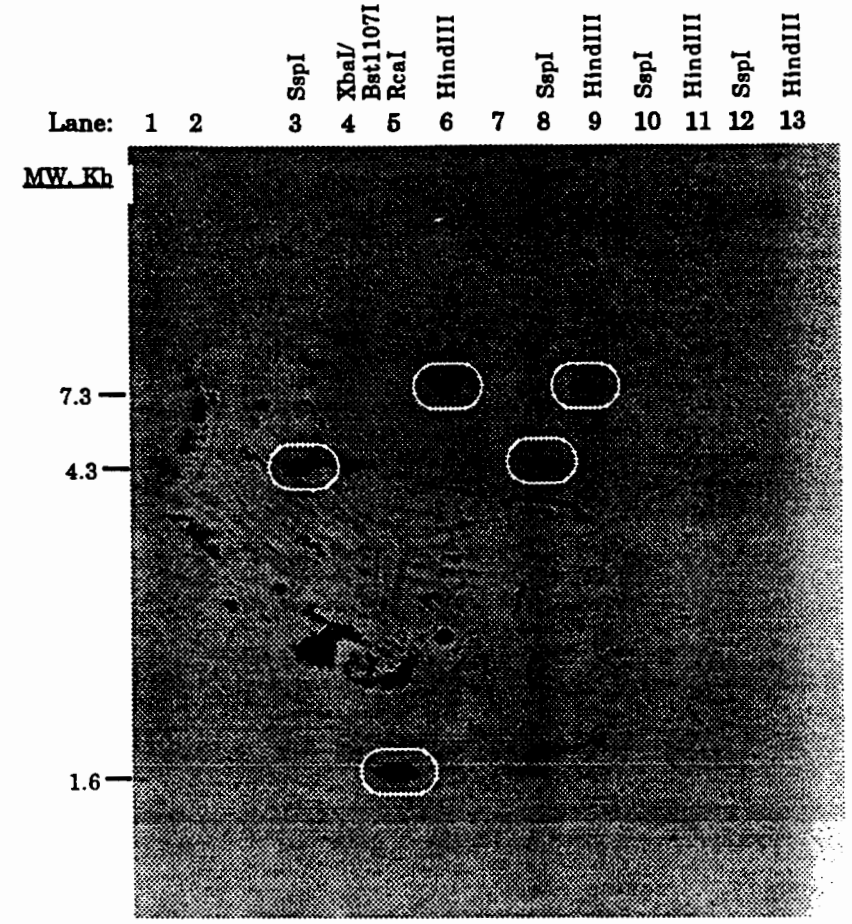


Figure III-11. A. Southern blot analysis of Bollgard™ Cotton lines 757 and 531 probed with the *nptII* gene. Southern blot from Figure III-9A, the plasmid probe removed, and reprobed with the *nptII* gene. Lane designations are the same as in Figure III-9A. **B. Schematic illustration of the T-DNA region of line 757.** All other designations are as in Figure III-9B.

A.



B.

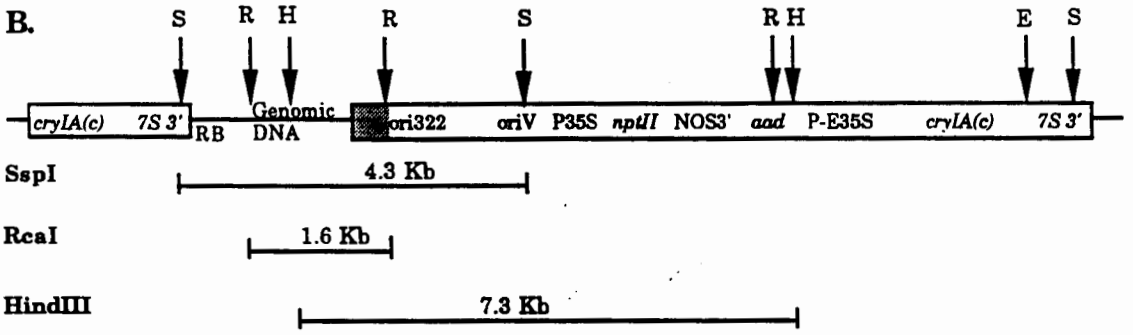
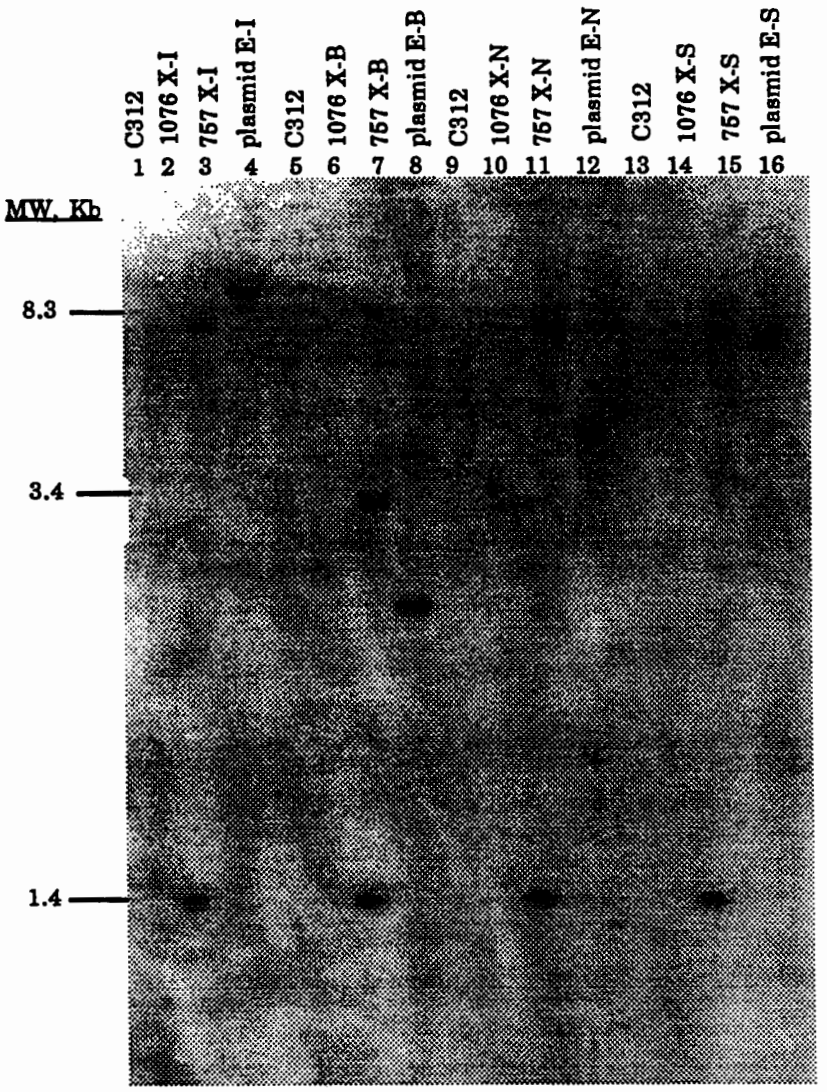


Figure III-12. A. Southern blot analysis of Bollgard™ Cotton lines 757 and 531 probed with the EP region. Southern blot from Figure III-9A, the plasmid probe removed, and reprobed with the EP region. Lane designations are the same as in Figure III-9A. **B. Schematic illustration of the T-DNA region of line 757.** All other designations are as in Figure III-9B.

A.



B.

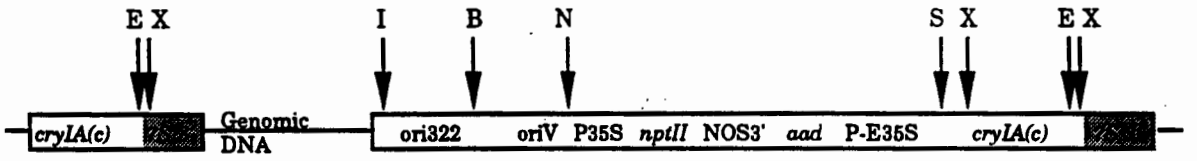
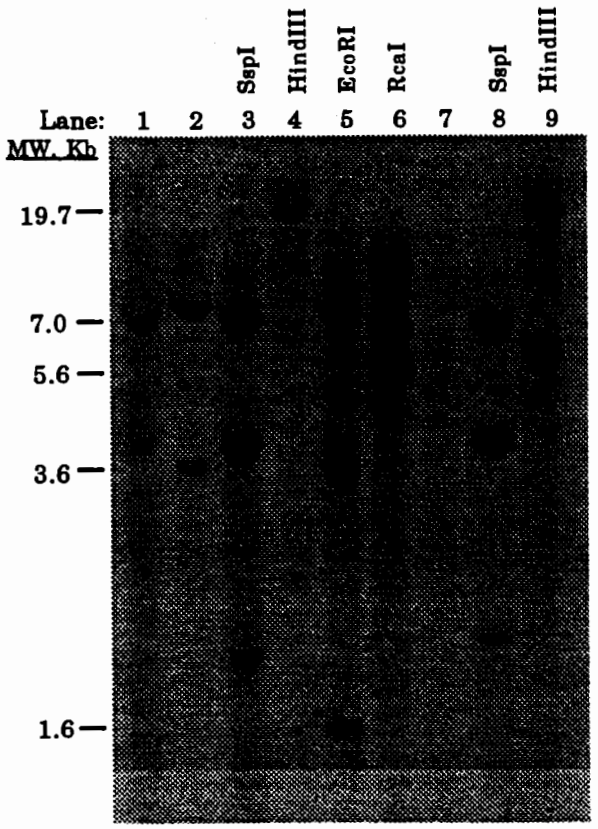


Figure III-13 A. Southern blot analysis of Bollgard™ Cotton lines 757 and 1076 probed with the 7S 3' region. Lanes 1-16 contain 5 µg of DNA each as labelled above the lane except for lanes 4, 8, 12, and 16. Lanes 4, 8, 12, and 16 contain 50 pg of plasmid PV-GHBK04 digested as labelled plus 5 µg of C312 control DNA cleaved with HindIII. **B. Schematic illustration of the T-DNA area of line 757.** Shaded areas indicate the portion of the plasmid radiolabelled to produce the probe used in Figure III-13A. Abbreviations are as follows: X=XbaI, E=EcoRI, S=SpeI, N=NheI, B=Bst1107I, I=BsiWI.

A.



B.

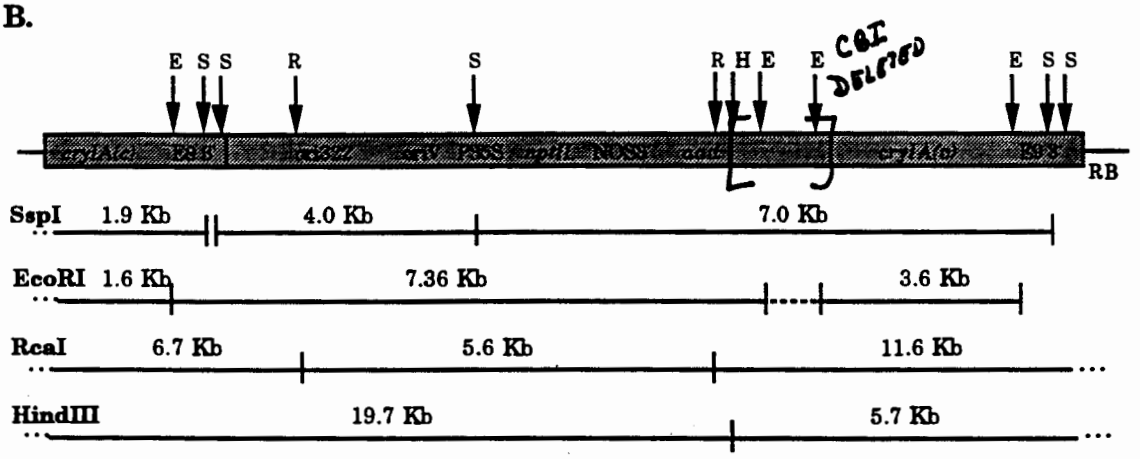


Figure III-14. A. Southern blot analysis of Bollgard™ Cotton line 1076 probed with PV-GHBK03. Lane 1 contains 13 µg of DNA from C312 digested with HindIII plus 50 pg of PV-GHBK03 cleaved with SspI. Lane 2 contains 13 µg of DNA from C312 digested with HindIII plus 50 pg of PV-GHBK03 cleaved with EcoRI. Lanes 3-6 contain 13 µg of DNA from line 1076 (R4 generation) cleaved with SspI, HindIII, EcoRI, and RcaI, respectively. Lane 7 contains 13 µg of DNA from C312 digested with HindIII. Lanes 8 and 9 contain 13 µg of DNA from line 1076 (R2 generation) cleaved with SspI and HindIII, respectively. **B. Schematic illustration indicating the locations of the restriction sites in the T-DNA of line 1076.** The right border is denoted by RB and is shown for orientation purposes (*i.e.*, an intact border sequence is not implied). Shaded areas indicate the portion of the plasmid radiolabelled to produce the probe used in Figure III-14A. The dotted lines at the edge of the schematic indicate that the border fragments continue into the genomic DNA. Abbreviations are as follows: S=SspI, E=EcoRI, R=RcaI, H=HindIII.

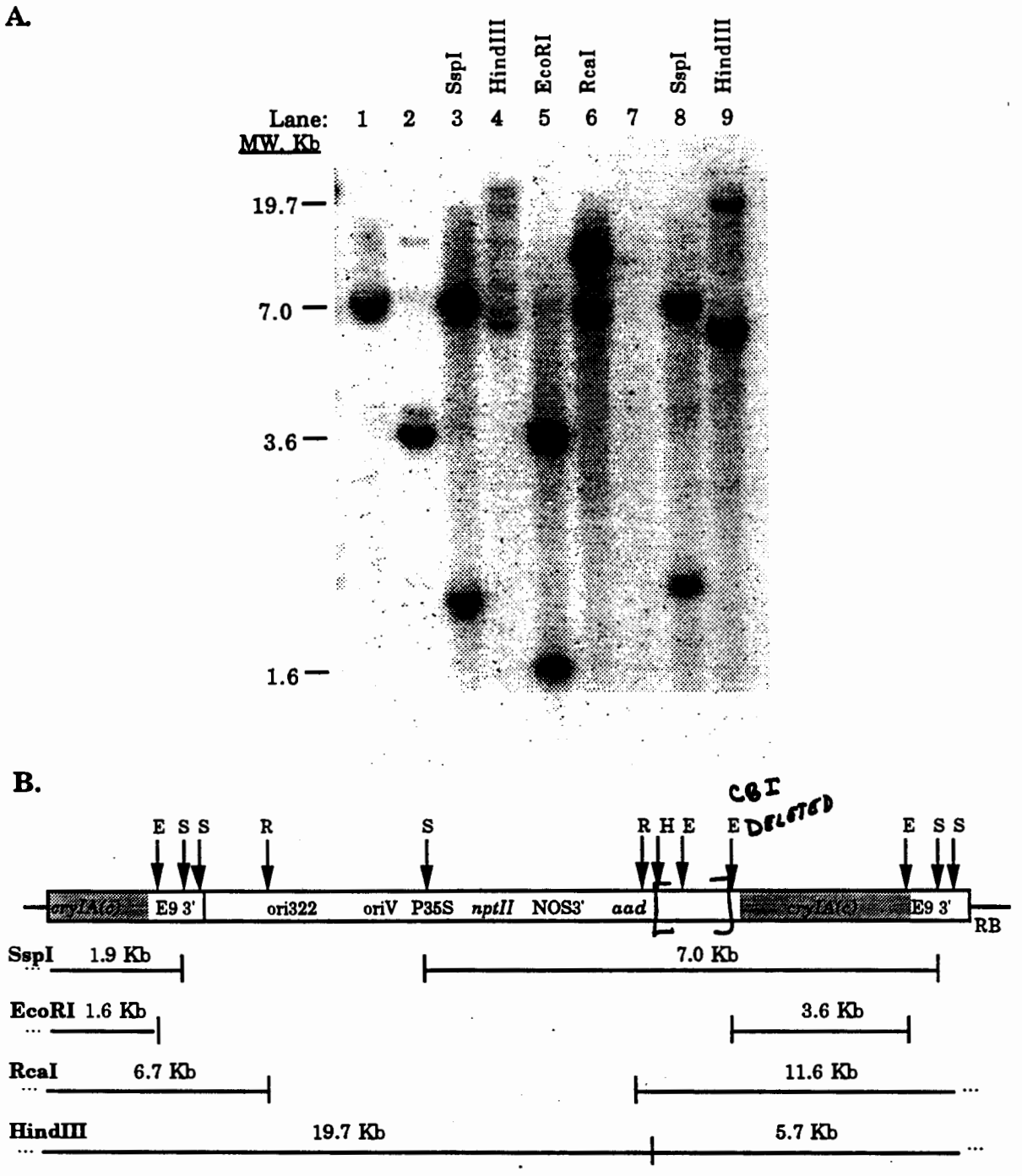
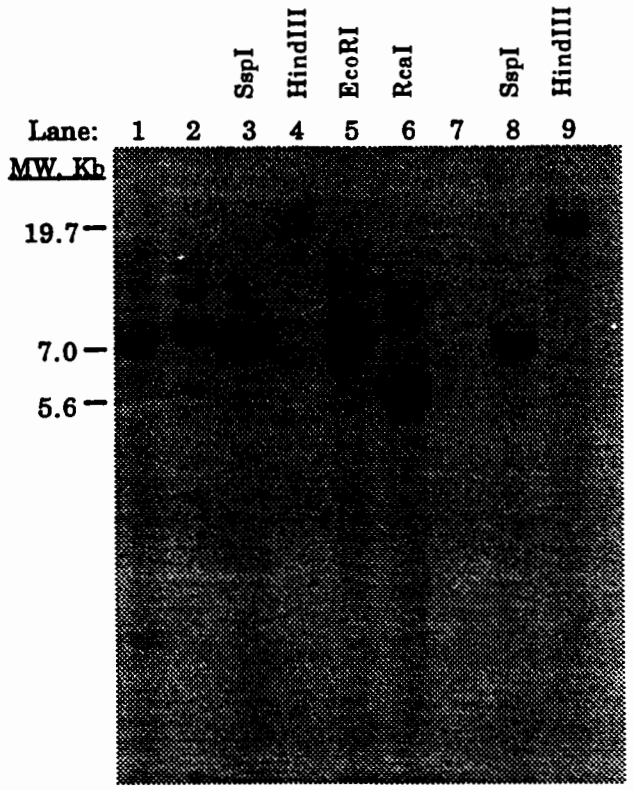


Figure III-15. A. Southern blot analysis of Bollgard™ Cotton line 1076 probed with the *cryIA(c)* gene. Southern blot from Figure III-14A, the plasmid probe removed, and reprobed with the *cryIA(c)* gene. Lane designations are the same as in Figure III-14A. **B. Schematic illustration of the T-DNA region of line 1076.** All other designations are as in Figure III-14B.

A.



B.

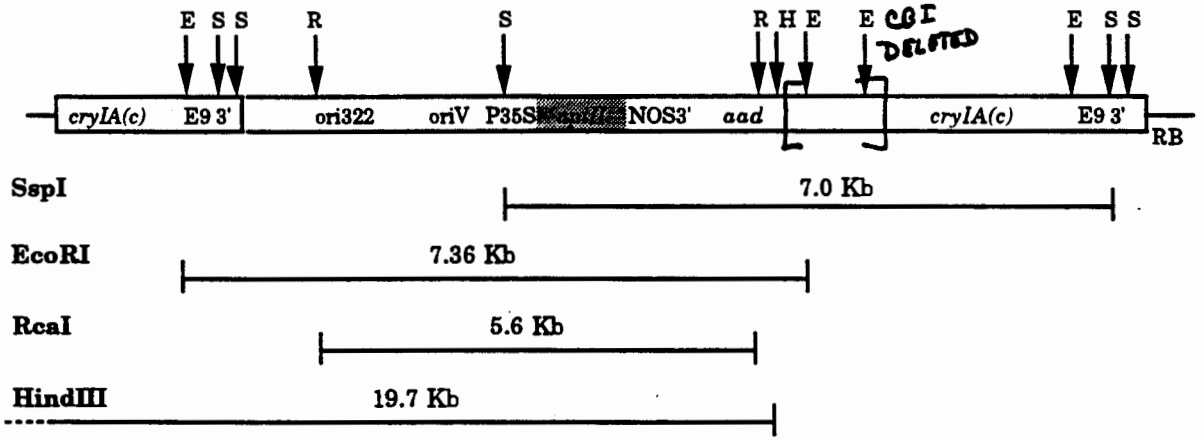
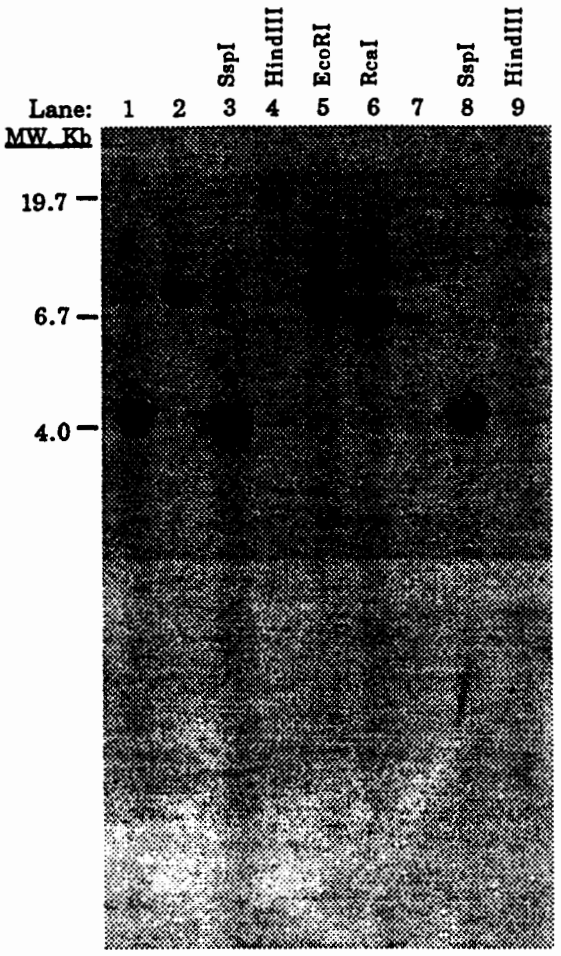


Figure III-16. A. Southern blot analysis of Bollgard™ Cotton line 1076 probed with the *nptII* gene. Southern blot from Figure III-14A, the plasmid probe removed, and reprobed with the *nptII* gene. Lane designations are the same as in Figure III-14A. **B. Schematic illustration of the T-DNA region of line 1076.** All other designations are as in Figure III-14B.

A.



B.

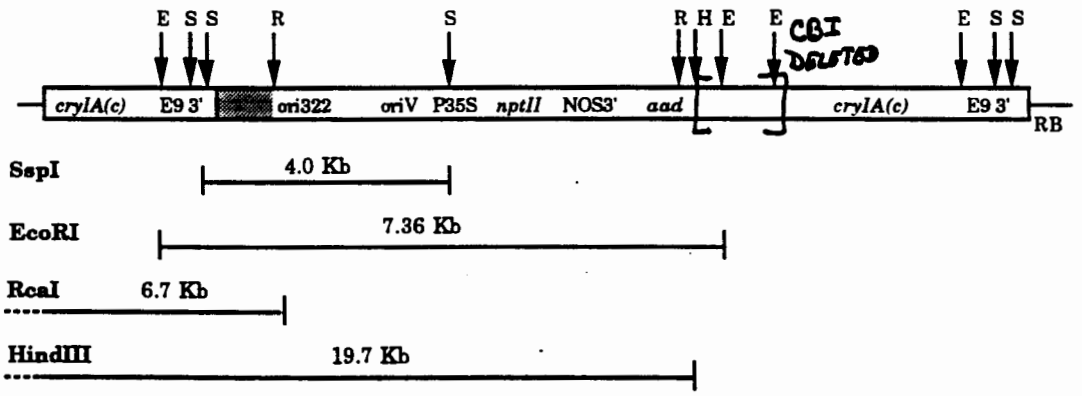


Figure III-17. A. Southern blot analysis of Bollgard™ Cotton line 1076 probed with the EP region. Southern blot from Figure III-14A, the plasmid probe removed, and reprobed with the EP region. Lane designations are the same as in Figure III-14A. **B. Schematic illustration of the T-DNA region of line 1076.** All other designations are as in Figure III-14B.

Table III-1. Summary of DNA Components in PV-GHBK04 and PV-GHBK03.

Genetic Element	Size, Kb*	Function
right border (RB)	0.09	A DNA fragment from the pTiT37 plasmid containing the 24 bp border nopaline-type T-DNA right border used to initiate the T-DNA transfer (RB) from *Agrobacterium tumefaciens* to the plant genome (Depicker *et al.*, 1982, and Bevan *et al.*, 1983).
P-E35S	0.62	(PV-GHBK04) The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1987).
[		] CBT DELETED
cryIA(c)	3.5	The gene which confers insect resistance. The modified gene encodes an amino acid sequence that is 99.4% identical to the *cryIA(c)* gene as described by Adang *et. al* (1985)
7S 3'	0.43	(PV-GHBK04) A 3' non-translated region of the soybean alpha subunit of the beta-conglycinin gene that provides the mRNA polyadenylation signals (Schuler *et al.*, 1982).
E9 3'	0.63	(PV-GHBK03) The termination sequence from pea ribulose-1,5,-bisphosphate carboxylase, small subunit (*rbcS*) (Coruzzi, *et al.*, 1984).
aad	0.79	The gene for the enzyme 3"(9)-O-aminoglycoside adenylyltransferase that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985).
P-35S	0.32	The 35S promoter region of the cauliflower mosaic virus (CaMV) (Gardner *et al.*, 1981; Sanders *et al.*, 1987).
nptII	0.79	The gene isolated from Tn5 (Beck *et al.*, 1982) which encodes for neomycin phosphotransferase type II. Expression of this gene in plant cells confers resistance to kanamycin and serves as a selectable marker for transformation (Fraley *et al.*, 1983).
NOS 3'	0.26	A 3' non-translated region of the nopaline synthase gene which functions to terminate transcription and direct polyadenylation of the *nptII* mRNA (Depicker *et al.*, 1982; Bevan *et al.*, 1983).
oriV	0.62	Origin of replication for ABI *Agrobacterium* derived from the broad-host range plasmid RK2 (Stalker *et al.*, 1981).
ori322/rop	1.8	A segment of pBR322 which provides the origin of replication for maintenance of the PV-GHBK04 plasmid in *E. coli*, the replication of primer (*rop*) region and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells (Bolivar *et al.*, 1977; Sutcliffe, 1978).

* Sizes given are the actual size of the genetic elements and do not include DNA border sequences, necessary for cloning purposes, unless otherwise indicated.

Table III-2. Segregation data for backcross (BC) derivatives of Bollgard ™ Cotton Line 757 with elite cultivar (EC) varieties. Values are in ratios of plants that are positive or negative for the *B.t.k.* HD-73 protein as determined by ELISA.

	actual	expected	Chi square value
BC3 F1 segregation (expect 1:1) BC3 F1 EC's	287:237	262:262	4.77**
BC3 F2 progeny test (expect 1 homozygote :2 heterozygote) BC3 F2 EC1,2	6:13	6.3:12.7	0.02*
BC3 F2 progeny test (segregation of heterozygotes, expect 3:1) BC3 F2 EC1,2	140:40	135:45	0.75

* not significant at P = 0.05
** significant at P=0.05, but not at P=0.01

Table III-3. Segregation data for backcross (BC) derivatives of Bollgard ™ Cotton Line 1076 with elite cultivar (EC) varieties. Values are in ratios of plants that are positive or negative for the *B.t.k.* HD-73 protein as determined by ELISA.

	actual	expected	Chi square value
BC3 F1 segregation (expect 1:1) BC3 F1 EC1,2,3	52:65	58.5:58.5	1.44*
BC3 F2 segregation (expect 3:1) BC3 F2 EC1,2	132:37	127:42	0.79*
BC3 F2 progeny test (expect 1 homozygote :2 heterozygote) BC3 F2 EC1,2	44:85	43:86	0.03*
BC3 F2 progeny test (segregation of heterozygotes, expect 3:1) BC3 F2 EC1,2	786:262	786:262	0.0*

* not significant at P = 0.05

Table III-4. Segregation data and analysis of progeny of Bollgard ™ Cotton Line 757.

		Single insert		Double insert	
	actual	expected (3:1)	Chi square value	expected (15:1)	Chi square value
R1 plants	34:6	30:10	2.13*	37.5:2.5	5.23+
R1 progeny¶	2:5	2.3:4.7	0.06*	- -	- -

* not significant at P = 0.05 (Chi Square value= 3.84).
+ significant at P = 0.05 (Chi square value= 3.84).
¶ data expressed as R1 homozygous pos: R1 heterozygotes

Table III-5. Segregation data and analysis of progeny of Bollgard ™ Cotton Line 1076.

		Single insert		Double insert	
	actual	expected (3:1)	Chi square value	expected (15:1)	Chi square value
R1 plants	31:5	27:9	2.37*	34:2	4.76+
R1 progeny¶	24:56:29	27:55:27	0.49*	--	--

* not significant at P = 0.05 (Chi Square value= 3.84).
+ significant at P = 0.05 (Chi square value= 3.84).
¶ data expressed as R1 homozygous pos: R1 heterozygotes: R1 homozygous neg.

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Part IV. Results of Field Trials

A. Field Test Permits and Locations

Bollgard™ Cotton Lines 757 and 1076 have been field tested in 1993 and 1994 at approximately 80 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: 93-011-05, 93-056-05, 94-025-01, 94-026-03, 94-027-03 and 94-054-02. The final reports for USDA permitted studies numbers 93-011-05 and 93-056-05 are included in Appendix V of this Determination. The final reports for the 1994 field trials will be completed early 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 Bollgard™ Cotton Lines 757 and 1076 to attack by non-target insects.
- Susceptibility of Bollgard™ Cotton Lines 757 and 1076 to disease infection.
- Monitoring for volunteers.

B. Plant growth and general observations

Bollgard™ Cotton Lines 757 and 1076 were compared to the non-transformed parental line Coker 312 at each location with the exception of the breeding sites at Wabbaseka AR, Scott MS, and Shafter CA. At selected locations, the yield and control of target insects were measured. 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 Bollgard™ Cotton Lines 757 and 1076 and the Coker 312 control.

No significant differences in weediness or survival characteristics were noted between Bollgard™ Cotton Lines 757 and 1076 and the Coker 312 control (Appendix V). All locations reported similar emergence of Bollgard™ Cotton Lines 757 and 1076 compared to Coker 312 except for Tifton GA, Florence SC, Wabbaseka AR, and Chatham MS in 1993. At

Tifton and Florence, there was slightly better emergence and seedling vigor for transgenic plants compared to the non-transgenic control. Differences were not significant according to the researchers.

At Wabbaseka in 1993, some dormancy was noted in transgenic lines harvested in the greenhouse immediately prior to planting. This is a frequent occurrence for cotton seed produced in the greenhouse and is not associated with the introduced gene(s). Differences were also reported in Chatham MS where two non-transgenic plots had a poor stand. These differences were attributed to non-uniform irrigation.

In addition to monitoring for weediness, morphological observations were also recorded at the field sites. Most locations reported no significant morphological, growth or developmental differences for Bollgard™ Cotton Lines 757 and 1076 in the field (Appendix V). These included; germination, morphology, time to flowering and fruiting, boll formation, boll development and yield (if insect damage was controlled in the Coker 312 control).

The researcher at Wabbaseka AR reported longer peduncles in the transgenic lines compared to the non-transgenics. This difference is not expected to effect weediness or survivability and was not reported at other locations. Bossier City LA reported 4 to 5 plants of Bollgard™ Cotton Line 1076 possessed asymmetrical, silvered leaves (out of several hundred plants). This phenotype did not appear to be associated with the insert since it was observed in only a small number of plants within this line.

One location reported premature defoliation in Bollgard™ Cotton plants. This was later attributed to a potassium deficiency. Not surprisingly, the potassium deficiency was more pronounced in the transgenic plants which had a higher boll load.

The most consistent difference reported was delayed flowering and/or delayed maturity of Bollgard™ Cotton Line 1076 compared to Coker 312 which was reported at Chatham MS, Florence SC, and West Sinton TX. Some locations also reported a smaller boll size with this line. The source of this variation is unknown. It could be due to the initial plant selection of Coker 312 for transformation with this particular line; considerable genetic diversity exists among plants within the Coker 312 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 whether these differences are present in all breeding material produced with Bollgard™ Cotton Line 1076 or whether lines without these differences can be recovered with back-crossing. This cannot be determined in the field tests which reported these differences since this material was selfed

progeny of the original transformant. The commercial acceptability of backcrossed derivatives of Bollgard™ Cotton Line 1076 will require lines without significant delay in maturity.

No differences in susceptibility to non-target insects were noted between Bollgard™ Cotton Lines 757 and 1076 and Coker 312 at any location, except for Morgan City MS (Appendix V). At Morgan City, there tended to be a higher population of *Lygus* spp. in the Bollgard™ Cotton Line 757 plots relative to Coker 312. This is not surprising since Bollgard™ Cotton Line 757 plot was not sprayed for control of lepidopteran pests while the Coker 312 was sprayed. *Lygus* spp. are sometimes controlled by applications of insecticides for lepidopteran pests. Significantly, the other locations did not report an increase in non-lepidopteran pests with the decrease in applications for lepidopteran control.

Specific notations were made for similar responses of Bollgard™ Cotton Lines 757 and 1076 and Coker 312 to the following pests: sweet potato whitefly, armyworm, leaf hoppers, leaf miners, Lygus bugs, aphids, and boll weevils.

Similarly, no differences in susceptibility to diseases were noted between Bollgard™ Cotton Lines 757 and 1076 and Coker 312 at any location (Appendix V). Additionally, the plants were monitored for symptoms of infection by *Agrobacterium*. No symptoms were noted at any location.

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 volunteers were observed in the fall at some locations where harvest was early. Three locations reported survival through the winter to the following spring: San Patricio County TX, Maricopa AZ, and Chatham MS. Survival in Texas and Arizona was more likely due to the relatively warm winters at these locations. Only three plants survived in Mississippi. None of the other locations reported survival to the following spring. All of these trials 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 season to ascertain whether differences exist between the over-wintering ability of the Bollgard™ Cotton lines and Coker 312.

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 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 only three 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 Bollgard™ Cotton Lines 757 and 1076 and Coker 312. The major difference observed in maturity between Bollgard™ Cotton Line 1076 and Coker 312 is common between cotton varieties and does not cause concern in the commercialization of the crop. Furthermore, this does not impart any special adaptive, competitive or survival characteristics to Bollgard™ Cotton Line 1076. Finally, no new variety expressing the *B.t.k.* protein will be commercialized unless it meets all morphological, yield and quality characteristics of cotton varieties produced in the United States.

C. Efficacy of Bollgard™ Cotton lines 757 and 1076

1. Introduction

Several hundred cotton lines have been transformed in Monsanto laboratories to contain a synthetic full length HD-73 gene derived from *Bacillus thuringiensis* var. *kurstaki*. These lines were initially assayed in the greenhouse for resistance to *Heliothis zea*. Those lines which provided the best insect control in the greenhouse, have a single insert, and exhibited no obvious agronomic limitations were tested in the field. The initial field evaluations with Bollgard™ Cotton lines were in line evaluation trials; the purpose of these trials was to determine the level of lepidopteran control provided by Bollgard™ Cotton lines in the field. Additional field evaluations such as economic threshold, population dynamics, refugia trials, etc. are also conducted with some of the lines initially tested in the line evaluation trials.

2. Materials and Methods

Line evaluation trials were conducted in 1993 and 1994 at six locations across the cotton belt: Tifton, GA, Loxley, AL, Starkville, MS, Bossier City, LA, College Station, TX, and Maricopa, AZ. Each location evaluated Bollgard™ Cotton Lines 757, 1076, several other Bollgard™ Cotton lines, and Coker 312.

All trials used a randomized complete block design with 6 replications. Treatments were assigned in a split plot fashion with the main plots being insecticide treatments (sprayed vs. unsprayed) and the sub-plots being the line treatments. Sprayed treatments were treated with weekly applications

of a lepidopteran insecticide (a labelled pyrethroid) while unsprayed treatments were not treated with lepidopteran insecticides. Spraying for lepidopteran control was initiated when an economic infestation was observed and was terminated after the infestation dropped below economic levels.

Data collection was initiated after the damage of the unsprayed, non-transgenic control(s) exceeded 5% and was continued on a weekly basis until the damage dropped below this level. For locations infested by *Heliothis spp.* (GA, AL, MS, LA, and TX), insect control was evaluated by counting the economically damaged squares and bolls on 20 randomly selected squares and bolls in the center two rows of each plot. Yield was determined by harvesting the middle two rows of each plot after approximately 75% or more of the bolls were open.

3. Results

Bollgard™ Cotton Lines 757 and 1076 provided excellent square and boll protection at all sites which had a significant infestation of *Heliothis spp.* in 1993 (Table IV-1). The season averages for square and boll damage was typically equivalent to or slightly better than Coker 312 sprayed weekly. Yield results comparing the Bollgard™ Cotton lines with Coker were variable. At Mississippi, the unsprayed plots were artificially and intensively infested with *H. zea* while the sprayed plots were not artificially infested. This could account for some of the reduction in yields between the Bollgard™ Cotton lines and Coker 312. In Texas, the significantly higher yields with Bollgard™ Cotton lines were probably due to improved insect control; the *H. virescens* in this area was resistant to synthetic pyrethroids.

Bollgard™ Cotton Lines 757 and 1076 also provided excellent square and boll protection in 1994 (Table IV- 2). Season long square protection with Bollgard™ Cotton Lines 757 and 1076 was better than weekly spraying of pyrethroids in Georgia and Texas. In Alabama, Mississippi, and Louisiana season long square protection was equivalent with Bollgard™ Cotton Lines 757 and 1076 and weekly spraying. Boll protection was essentially equivalent in Alabama and Mississippi. In Louisiana, weekly sprayings provided better boll protection, while Bollgard™ Cotton Lines 757 and 1076 performed better than sprayed Coker 312 in Texas. In summary, the Bollgard™ Cotton lines provided excellent square and boll protection across the cotton belt throughout the season.

Mean yield across locations for Bollgard™ Cotton Line 757 unsprayed was 102% of the sprayed Coker 312 control and ranged from 82% to 136% (Table IV-2). For Bollgard™ Cotton Line 1076, mean yield was 89% of the sprayed Coker across locations. Yield of Bollgard™ Cotton Line 1076 was similar to Coker 312 at Georgia, Alabama, Louisiana, and Texas. In Mississippi, Bollgard™ Cotton Line 1076 yielded significantly less than Coker 312. One possible explanation for this is the extremely high pressure from the artificial infestation at this site. Another possibility would be genetic differences between Bollgard™ Cotton Line 1076 and Coker 312, discussed below.

Yield comparisons between the Bollgard™ Cotton lines and Coker 312 may or may not be reliable indicators of performance. The Bollgard™ Cotton lines can differ genetically from Coker 312 due to the initial plant selected from tissue culture; Coker 312 possesses considerable genetic variability within the variety. Also, the tissue culture process itself can induce genetic changes within the Bollgard™ Cotton lines independent of the gene insertion. Finally, the insert itself can cause genetic changes. These potential sources of genetic variation and the changes in square and boll protection due to the Bollgard™ Cotton gene make yield comparisons problematical.

Please note that the square and boll damage which occurred in these trials was not cumulative i.e. the square or boll damage for a particular treatment could decrease with time. For example, the Bollgard™ Cotton lines could have 10% square damage in one week, but only 5% damage the following week. This is because those squares which are damaged tend to abort and, therefore, would not be counted in subsequent evaluations. Thus, cumulative damage would not be reflected in the weekly evaluations on insect damage, but instead would be reflected in yield.

In summary, these data indicate that Bollgard™ Cotton Lines 757 and 1076 provide excellent control from square and boll damage by lepidopteran pests. Typically, this control is equivalent to and sometimes superior to weekly applications of pyrethroid insecticides.

Table IV-1. Summary of 1993 season averages for square damage, boll damage, and yield for Bollgard™ Cotton Lines 757 and 1076 relative to Coker 312 sprayed weekly for control of lepidopteran pests.

	% damaged squares		
Line*	MS	LA	TX
757	6	1	0
1076	3	0	0
C312	7	5	5

	% damaged bolls		
Line*	MS	LA	TX
757	6	3	0
1076	3	1	0
C312	4	6	2

	Yield (% of C312 sprayed)		
Line*	MS	LA	TX
757	87%	92%	162%
1076	81%	86%	145%

* Bollgard™ Cotton lines were not treated with insecticides for control of lepidopteran pests. Coker 312 was treated weekly with insecticides during the primary lepidopteran infestation period.

Table IV-2. Summary of 1994 season averages for square damage, boll damage, and yield for Bollgard™ Cotton Lines 757 and 1076 relative to Coker 312 sprayed weekly for control of lepidopteran pests.

	% damaged squares				
Line*	**GA**	**AL**	**MS**	**LA**	**TX**
757	1	0	3	2	1
1076	1	1	2	1	1
C312	11	5	4	4	12

	% damaged bolls				
Line*	**GA**	**AL**	**MS**	**LA**	**TX**
757	-	0	1	6	3
1076	-	0	1	3	1
C312	-	2	4	1	8

	Yield (% of C312 sprayed)				
Line*	**GA**	**AL**	**MS**	**LA**	**TX**
757	109	100	83	82	136
1076	99	87	65	85	108

* Bollgard™ lines were not treated with insecticides for control of lepidopteran pests. Coker 312 was treated weekly with insecticides during the primary lepidopteran infestation period.

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Part V. Detailed Description of the Phenotype of Bollgard™ Cotton Lines 757 and 1076

INTRODUCTION

Data and information supplied in this Petition for Determination of Non-Regulated Status demonstrate that Bollgard™ Cotton Lines 757 and 1076 are substantially equivalent to the non-modified cotton line, Coker 312 (C312), except for the inserted genetic sequences, the expressed proteins [*B.t.k.* CryIA(c) protein and neomycin phosphotransferase II (NPTII) enzyme], and the ability of the plant to resist damage from Lepidopteran insects. The information supplied in this section and referenced from other sections of this petition will demonstrate that the modified Bollgard™ Cotton Lines 757 and 1076 are not likely to pose a greater plant pest risk than line C312 from which it was derived. This conclusion is based on evaluation of phenotypic characteristics, safety of the inserted proteins and cottonseed products, and the environmental characteristics.

A variety of studies were conducted to characterize the unique traits of the modified cotton lines and to establish that Bollgard™ Cotton Lines 757 and 1076 are substantially equivalent to the parental cotton line, C312. The inserted genetic material and insecticidal efficacy of Bollgard™ Cotton Lines 757 and 1076 were described in the previous sections (Part III and IV). The following characteristics of Bollgard™ Cotton Lines 757 and 1076 are described in this section:

- expression of the *B.t.k.* and NPTII proteins,
- the comparison of Bollgard™ Cotton Lines 757 and 1076 and line C312 on the basis of composition and quality of the cottonseed and processed cottonseed products,
- comparison of the natural toxicants of the seed,
- safety assessment of the *B.t.k.* HD-73 protein to non-target insects,
- demonstration of the wholesomeness of cottonseed food/feed products,
- the environmental fate of the *B.t.k.* HD-73 protein,
- the disease susceptibility of Bollgard™ Cotton Lines 757 and 1076 versus line C312, and
- the potential for out-crossing and weediness.

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 Bollgard™ Cotton Lines 757 and 1076

As described in Part III, Bollgard™ Cotton Lines 757 and 1076 have been modified to express a protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 [CryIA(c)] (abbreviated as *B.t.k.* HD-73) which has insecticidal activity against lepidopteran insect pests (Hofte and Whiteley, 1989; Perlak *et al.*, 1990; Perlak *et al.*, 1991; MacIntosh *et al.*, 1990). In addition to the *B.t.k.* HD-73 gene, a gene encoding the NPTII protein is present as a result of its use as a selectable marker during the development of the insect resistant cotton plants. A second selectable marker gene encoding aminoglycoside adenylyltransferase (AAD) is present in Bollgard™ Cotton Lines 757 and 1076 as a result of its use in selection for the microbial systems used for the genetic engineering process. The *aad* gene is controlled by a bacterial promoter; therefore, the protein was not expected to be expressed in the cotton leaf or seed tissue from Bollgard™ Cotton Lines 757 and 1076. The control line, C312 is the parental variety from which Bollgard™ Cotton Lines 757 and 1076 were generated and does not contain the genes encoding the *B.t.k.* HD-73, NPTII or AAD proteins.

Levels of the expressed proteins (*B.t.k.* HD-73, NPTII, and potentially AAD) were evaluated in young leaf (3-6 week plantlets) and seed tissues collected from six field locations during the 1993 growing season using Enzyme Linked Immuno-Sorbent Assay (ELISA) (Harlow and Lane, 1988) and western blot (Matsudaira, 1987) methods. The six field sites were as follows: Starkville, Mississippi; Bossier City, Louisiana; West Sinton, Texas; Tifton, Georgia; Maricopa, Arizona; and Loxley, Alabama. In addition, at one field site (West Sinton, Texas), young leaf tissue was collected at 3 time points throughout the season after the initial sampling and whole, mature cotton plants were collected just prior to defoliation and harvest to establish the consistency of expression throughout the season and to estimate the amount of *B.t.k.* HD-73 and NPTII protein that might enter the environment at the end of the growing season. The expression of the *B.t.k.* HD-73 protein was also evaluated in nectar and pollen collected from Bollgard™ Cotton Lines 757 and 1076 (plants grown in the greenhouse) to provide information on the degree of non-target insect exposure to the insecticidal protein via pollen and nectar produced by the modified cotton lines.

1. Young Leaf and Seed - These data show that the *B.t.k.* HD-73 and NPTII proteins were expressed at extremely low and relatively consistent levels in leaf and seed from Bollgard™ Cotton Lines 757 and 1076 across all six sites (Tables V-1 through V-6). Bollgard™ Cotton Line 757 contained approximately 12.6 and 9.9 µg *B.t.k.* HD-73 protein/gram fresh weight of tissue (fwt), and 6.9 and 3.3 µg NPTII protein/gram fwt in leaf and seed tissues, respectively. Bollgard™ Cotton Line 1076 contained approximately 12.2 and 12.7 µg *B.t.k.* HD-73 protein/gram fwt and 16.3 and 7.9 µg NPTII protein/gram fwt in leaf and seed tissues, respectively. These expression levels varied only two to three fold across the six field sites. *B.t.k.* HD-73

protein levels varied less than three fold in young leaf tissue collected over the growing season with the highest level observed at the second sampling date (7/6/93) at the one field site evaluated (Table V-7). Levels of *B.t.k.* HD-73 and NPTII proteins in whole plant tissue were much lower, on a fresh weight basis, than in leaf tissue (Table V-8). These results establish minimal variability in the expression of the *B.t.k.* HD-73 protein when the insect resistant cotton plants were grown at different geographical locations and different environmental conditions.

As predicted, no AAD was detected in leaf or seed tissue from Bollgard™ Cotton Lines 757 and 1076. The sensitivity of the ELISA for the AAD protein was approximately 0.008 µg AAD/gram fresh weight of leaf and 0.005 µg AAD/gram fresh weight of seed.

As expected, no *B.t.k.* HD-73 or NPTII protein was detected in leaf tissue from C312 control. The level of out-crossing of the insect resistance trait was evaluated in cottonseed from the control line, C312, grown in closely situated plots at all sites. Out crossing ranged from 0% (at Texas and Alabama) to 17% (at Mississippi). These levels of out-crossing are well within the previously established ranges of out-crossing for both commercial cotton varieties (Afzal and Khan, 1950; Green and Jones, 1953; Theis, 1953; Simpson and Duncan, 1956) and other genetically engineered cotton varieties (Umbeck *et al.*, 1992; Kareiva and Morris, 1992). Out-crossing was not detected in control cottonseed which was physically removed from the Bollgard™ Cotton lines by a minimum of 20 buffer rows of non-transgenic cotton.

These levels of out-crossing did not impact the evaluation of expression levels in seed from Bollgard™ Cotton Lines 757 and 1076. Expression levels of the *B.t.k.* protein in seed from isolated plots (separated by a minimum of 8 buffer rows of cotton) were not statistically different at the 5% level from expression measured in seed from plots of Bollgard™ Cotton Lines 757 and 1076 grown closely to control cotton and other varieties of Bollgard™ Cotton Lines. It was also concluded that the out-crossing did not significantly impact the quality and toxicant data or the quail feeding study since only seed from isolated plots was used for these studies (See paragraph K(2), Effects on Non-Target Organisms in this Part of this Petition of Determination of Non-Regulated Status).

2. Whole Plant - Levels of *B.t.k.* HD-73 and NPTII proteins in whole plant tissue were much lower, on a fresh weight basis, than in young leaf tissue. *B.t.k.* HD-73 is present in whole cotton plants from Bollgard™ Cotton lines 757 and 1076 at 1.1 and 1.7 µg/g fwt of the plant; NPTII protein levels were 3.7 and 14.6 µg/g fwt of the plant from Bollgard™ Cotton lines 757 and 1076, respectively (Table V-8). These measured concentrations were used to estimate the amount of *B.t.k.* HD-73 and NPTII protein that could enter the environment due to post-harvest incorporation of mature plants from Bollgard™ Cotton Lines 757 and 1076 (minus lint and seed): 12.2 and 23.4 g *B.t.k.* HD-73 protein/acre and 57.5 and 183 g NPTII protein/acre, for

Bollgard™ Cotton lines 757 and 1076, respectively (assuming 60,000 plants per acre). A soil degradation study was performed that confirmed the rapid degradation of the *B.t.k.* HD-73 protein expressed in the insect resistant cotton plants in the soil, (See paragraph M, Possible Impact on the Environment in this Part of this Petition of Determination of Non-Regulated Status).

3. Nectar and Pollen - The level of *B.t.k* HD-73 protein measured in pollen and nectar from Bollgard™ Cotton Lines 757 and 1076 was extremely low; the maximum levels of *B.t.k.* HD-73 protein expression level in pollen and nectar are 38 and 0.88 ng/g fwt respectively (Table V-9). The expression level of the *B.t.k.* HD-73 protein in the pollen and nectar from Bollgard™ Cotton Lines 757 and 1076 was obtained to serve as a basis of exposure of beneficial (non-target) insects to the *B.t.k.* HD-73 protein expressed in these plants (see paragraph K(1) and Lack of Effects on Non-Target Organisms (see paragraph K(2 & 3) in this Part of this Petition of Determination of Non-Regulated Status).

B. Composition, Quality, and Toxicant Analyses of the Cottonseed from BollgardTM Cotton Lines 757 and 1076

Field grown cottonseed from Bollgard™ Cotton Lines 757, 1076 and C312 were shown to be compositionally equivalent based upon analysis of the major cottonseed components (protein, lipid, moisture, ash, carbohydrate, calories), the fatty acid profile and the levels of important toxicants (gossypol, cylcopropenoid fatty acids and aflatoxin).

The levels of the major components (protein, oil, carbohydrate, moisture, ash and calories) and major fatty acids in the cottonseed from Bollgard™ Cotton Lines 757 and 1076 were comparable to C312 (Tables V-10, V-11). Gossypol levels in seed from Bollgard™ Cotton Lines 757 and 1076 and C312 fell well within the ranges previously reported for cotton varieties (Table V-12) (Pons *et al.*, 1958; Abou-Donia, 1976) and the variability across locations was consistent with previously reported data (Altman *et al.*, 1989; Berardi and Goldblatt, 1980). Levels of the toxicant, cyclopropenoid fatty acids (dihydrosterculic, sterculic and malvalic), for cottonseed from the six field sites showed no statistically significant differences between seed from Bollgard™ Cotton Lines 757 and 1076 and C312 (Table V-11). The four primary aflatoxins commonly found in cottonseed were undetectable at a sensitivity of 1 part per billion in seed from Bollgard™ Cotton Lines 757 and 1076 as well as the control cotton line at all six field sites.

There were small compositional differences upon comparison of the seed from Bollgard™ Cotton Lines 757 and 1076 versus seed collected from C312 (Tables V-10, V-11, V-12). Although these differences were statistically significant, the difference in the level of these components were biologically unimportant and well within the reported ranges for those components in commercial cottonseed (ranges referenced in Tables V-10a and V-12).

These minor differences in seed from Bollgard™ Cotton Lines 757 and 1076 and the control line represent inherent variability within cotton varieties and are not attributed to the insertion of the genes for insect resistance.

C. Cottonseed Processing

The quality of the processed cottonseed products from Bollgard™ Cotton Lines 757 and 1076 were shown to be equivalent to the control line. Seed cotton from four of the six field sites were ginned and pooled (by line) across sites as a source of seed for processing. The composite cottonseed sample was processed at the Food Protein Research & Development Center at Texas A&M University into defatted/ toasted meal and refined oil which are the primary cottonseed products used for animal feed (except cattle, which consume whole seed) and human food.

When compared for yield of processed fractions relative to starting material (linters, linter motes, delinted seed, hulls, kernels, toasted meal, crude oil and refined oil), the results were comparable for both Bollgard™ Cotton Lines when compared to C312 and similar to the means and ranges previously reported for processed cottonseed fractions from other cotton cultivars (Table V-13).

There were no meaningful differences in the levels of total and free gossypol in the raw cottonseed kernels, toasted meal and refined oil from both Bollgard™ Cotton Lines and C312 (Table V-14). Reduction of 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, 1990). Under the 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 the amount of free gossypol was reduced to trace levels in the toasted meal from both lines. Total gossypol levels were reduced by approximately 18% in the toasted meal for both lines.

The total protein content of the toasted meal and refined oil fractions from Bollgard™ Cotton Lines 757 and 1076 and C312, were shown to be consistent with commercial quality products. Cottonseed meal used as a feed additive (protein concentrate) for livestock feed is typically prepared at $\geq$ 41% total protein. The toasted meal fractions from Bollgard™ Cotton Lines 757 and 1076 and C312 contained approximately 40% total protein by weight. Refined cottonseed oil from Bollgard™ Cotton Lines 757 and 1076 and C312 were comparable in quality to commercially processed cottonseed oil (Table V-15)

Finally, processing cottonseed from Bollgard™ Cotton Lines 757 and 1076 dramatically decreased the amount of biologically active *B.t.k.* HD-73 and NPTII proteins in the toasted meal. The native, expressed *B.t.k.* HD-73 and NPTII proteins were reduced to undetectable levels ($\leq$ 4 parts per billion) in the processed meal fractions Bollgard™ Cotton Lines 757 and 1076. Denatured, inactive *B.t.k.* HD-73 and NPTII proteins were detected in the processed meal at much lower levels than raw meal (reduced by at least 80% in toasted meal). Therefore, processed cottonseed meal does not represent a source of significant exposure to either the *B.t.k.* HD-73 or NPTII protein.

These data establish that cottonseed from Bollgard™ Cotton Lines 757 and 1076 processes comparably to cottonseed from C312 and that the level of the important toxicant, gossypol, is comparable in cottonseed products produced from the Bollgard™ Cotton lines and the control line. Therefore, insertion of the genes to provide insect resistance did not alter the processing characteristics of the cottonseed or the quality of two major cottonseed products, toasted meal and refined oil.

D. Allelochemical Levels in Vegetative Tissues

Cotton contains allelochemicals, in addition to gossypol, that may be involved in pest control (Hedin, *et al.*, 1983; Hedin, *et al.*, 1988; Hedin, *et al.*, 1991). 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 analyzed from samples obtained from the 1993 field tests in Starkville, Mississippi. As expected, no meaningful differences in the levels of gossypol, flavonoids, tannins and anthocyanins were detected between Bollgard™ Cotton Lines 757 and 1076 and C312 and the levels of these allelochemicals were representative for *G. hirsutum* lines. The complete report for the 1993 analyses are found in Appendix VII. The results for Bollgard™ Cotton Lines 757, 1076, and C312 are summarized in Table V-16.

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 Bollgard™ Cotton Lines 757 and 1076 possess no disease or pest susceptibilities different than the parental non-transformed cotton.

F. Plant Pest Risk

In all field and green house trials, plants of Bollgard™ Cotton Lines 757 and 1076 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 the Bollgard™ Cotton Lines to express any plant disease (See part III). Bollgard™ Cotton Lines 757 and 1076 do not exhibit any different agronomic or morphological characteristics which may give them an advantage over other species within the ecosystem in which they are grown (see Part IV). The compositional and toxicant analyses comparing Bollgard™ Cotton Lines 757 and 1076 to C312 showed no differences (see section B, above). Therefore, it is concluded that Bollgard™ Cotton Lines 757 and 1076 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 Bollgard™ Cotton Lines 757 and 1076 or any *Gossypium* species crossing with these Bollgard™ Cotton 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 *B.t.k.* 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 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. Ultimately, if *B.t.k.* should be transferred to a wild population of a tetraploid, and if this was considered undesirable, the size of the plants, their perennial growth habit, their restricted habitat and their low natural fecundity would make control exceptionally easy (Stewart, 1992; Appendix III).

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 better able to withstand damage from foliar eating insects. This insect resistance would not be 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 *B.t.k.* and *nptII* genes. Expression of the gene products (*B.t.k.* HD-73 and NPTII proteins) in the modified cotton plant would not change any of the above listed attributes.

H. Germination and Vigor Results for BollgardTM Cotton Lines 757, 1076 and C312

Field germination studies comparing C312 and Bollgard™ Cotton Lines 757 and 1076 have not been conducted to date. For most studies, the seed for Bollgard™ Cotton Lines 757 and 1076 and Coker 312 were not produced in the same location, thus making it impossible to make such a comparison. For example, the seed for Bollgard™ Cotton Lines 757 and 1076 for most field studies was grown in a winter nursery while the C312 seed was purchased from SeedCo Co. in Lubbock, TX. Data is available from a laboratory study and two field locations which utilized seed from Bollgard™ Cotton Lines 757 and 1076 and the C312 produced at the same location. These results support the conclusion that no significant differences exist between the germination rates of Bollgard™ Cotton Lines 757 and 1076 and C312.

Laboratory Germination Study

Bollgard™ Cotton Lines 757 and 1076 and C312 were grown in multiple locations in 1993. Seed from Bollgard™ Cotton Lines 757 and 1076 and C312 grown in Alabama, Mississippi, and Texas was analyzed *in vitro* at Monsanto to determine the germination potential of these lines. Seeds were placed in moist germination paper and percent germination was recorded after seven days. Germination was determined in both warm (31° C day, 24° C night) and cool conditions (19° C constant).

As expected, germination varied considerably from location to location (Table V-17). For example, the Coker 312 had a mean germination of 90% from Mississippi and 35% from Texas. Seed quality is impacted by several factors including time of harvest, moisture at harvest, temperature, etc. The seed produced in Alabama came from a high rainfall area and was harvested relatively late; these factors reduced germination in this seed (55% warm germination) relative to the seed produced in Mississippi (90% warm germination).

Percent germination of Bollgard™ Cotton Lines 757 and 1076 was equivalent to Coker 312 at all three locations under both warm and cool conditions (Table V-17). The only statistically significant differences were between Lines 757 and 1076. The seed from Bollgard™ Cotton Line 1076 had a significantly higher germination percentage than Bollgard™ Cotton Line 757 in both warm and cool conditions for the seed produced in Alabama. Similarly, for the seed produced in Texas, Bollgard™ Cotton Line 1076 had a higher % germination than Line 757 under cool conditions. One possible explanation for this is the slightly different maturities of these two lines. Bollgard™ Cotton Line 1076 matures several days later than Bollgard™ Cotton Line 757. Thus, the bolls from Bollgard™ Cotton Line 1076 open later than Bollgard™ Cotton Line 757 leaving them less exposed to adverse conditions such as high moisture.

In summary, the percent germination in both warm and cool conditions for seed from Bollgard™ Cotton Lines 757 and 1076 was similar to C312 treated with commercial sprays. This was true for seed produced at all three locations. These results indicate that these Bollgard™ Cotton lines produced seed of similar quality as Coker 312.

Field Germination/Stand Counts

To assess germination under field conditions, stand counts were taken at two of the field sites: West Sinton, TX and Bossier City, LA. At West Sinton, stand counts were taken in each of the middle two rows of all 12 replications, for a total of 24 replications. The stand counts for C312 and Bollgard™ Cotton Lines 757 and 1076 did not differ significantly (Table V-18). Similarly, at Bossier City, stand counts were taken in four of the replications (Table V-19). Again, there was no meaningful difference in the stand counts between Bollgard™ Cotton Lines 757 and 1076 and C312. The field results support the conclusion that no meaningful differences exist in the germination or survival rates of Bollgard™ Cotton Lines 757 and 1076 and C312. Additionally, no cooperator has reported a difference in the overall growth and development of Bollgard™ Cotton Lines 757 or 1076 compared to C312.

I. Out-Crossing Potential

The potential for pollen transfer from cotton to other species and for Bollgard™ Cotton Lines 757 and 1076 to become a weed or pest is addressed in Part II and Appendices III & 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. Bollgard™ Cotton Lines 757 and 1076 (*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 Bollgard™ Cotton Lines 757 and 1076 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 Bollgard™ Cotton Lines 757 and 1076 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 *B.t.k.* 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 the *B.t.k.* cotton in 1990. One part of these studies was to study the out-crossing potential of these cotton plants. Sites where these tests were located were:

Casa Grande, Arizona
Maricopa, Arizona
Bossier City, Louisiana
Starkville, Mississippi
Brawley, California
College Station, Texas
Lubbock, Texas

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 Table V-20.

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 Texas sites.

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. Non-target Insects

There is extensive information about microbial preparations of *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) containing the *B.t.k.* proteins, including the CryIA(c) protein (*B.t.k.* HD-73). The literature has established that the *B.t.k.* proteins are:

- extremely selective for the lepidopteran insects (MacIntosh *et al.*, 1990; Klausner, 1984; Aronson *et al.*, 1986; Dulmage, 1981; Whitely and Schnepf, 1986),
- bind specifically to receptors on the mid-gut of lepidopteran insects (Wolfersberger *et al.* 1986; Hofmann *et al.* 1988a; Hofmann *et al.* 1988b; Van Rie, *et al.* 1989; Van Rie, *et al.* 1990), and
- have no deleterious effect on beneficial/non-target insects, including predators and parasitoids of lepidopteran insect pests or honeybee (*Apis mellifera*) (Flexner *et al.*, 1986; Krieg and Langenbruch, 1981; Cantwell et al., 1972; EPA, 1988; Vinson, 1990; Melin and Cozzi, 1989).

The chapters by Vinson (1990) and Melin and Cozzi (1989) provide comprehensive reviews of the extensive literature that has established the safety of the *B.t.k.* microbes and encoded proteins to an array of beneficial insects. To compliment these chapters, Monsanto conducted a study to compare the *B.t.k.* protein expressed in Bollgard™ Cotton Lines 757 and 1076 with commercially available microbial pesticides containing *B.t.* protein. The conclusion reached from the results of this study were that the protein expressed by Bollgard™ Cotton Lines 757 and 1076 was similar in molecular weight and immunological reactivity to one or more proteins contained in the commercial *B.t.* products Dipel® and Thuricide®. Thus the literature demonstrating the safety of these insecticides to non-target organisms is useful in predicting the safety of the *B.t.k.* HD-73 protein expressed in Bollgard™ Cotton Lines 757 and 1076. The complete report of this study is found in Appendix VIII.

To confirm the specificity of the *B.t.k.* HD-73 protein expressed in Bollgard™ Cotton Lines 757 and 1076, a study was completed to evaluate the insecticidal activity of the full-length *B.t.k.* HD-73 protein and the activated, trypsin-resistant core of the protein versus ten species from five different orders of insects, including Lepidoptera. Of the ten species tested, only the four species of Lepidoptera were sensitive to both forms of the *B.t.k.* HD-73 protein. These data confirm the insecticidal specificity of the protein expressed by Bollgard™ Cotton Lines 757 and 1076 for insect species in the Order Lepidoptera.

In addition, separate studies were undertaken to assess the potential toxicity of *B.t.k.* HD-73 protein to other non-target insects:

- parasitic Hymenoptera (*Nasonia vitripennis*), a beneficial parasite of the housefly (*Musca domestica)*,
- the larva and adult honey bee (*Apis mellifera* L.), a beneficial insect pollinator,
- ladybird beetles (*Hippodamia convergens*), a beneficial predaceous insect which feeds on aphids and other plant bugs commonly found on stems and foliage of weeds and cultivated plants, and
- green lacewing larvae (*Chrysopa carnea*), a beneficial predaceous insect commonly found on cotton and other cultivated crops.

In each study, the maximum nominal *B.t.k.* HD-73 protein (full-length) concentration tested (20 ppm) was greater than 500 times the maximum *B.t.k.* HD-73 protein expression level in pollen (< 38 ppb) and nectar (< 1 ppb) from either Bollgard™ Cotton Line 757 or 1076. These studies established that the LC50 for the *B.t.k.* HD-73 protein is greater than 20 ppm versus all the species tested. Therefore, the "no observed effect level" was 20 ppm.

2. Non-Target Birds and Fish

A study was conducted to assess the wholesomeness of insect resistant 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/day 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 historical data demonstrating safety of *B.t.* proteins to fish 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 *B.t.k.* proteins have been demonstrated to be virtually non-toxic to fish, avian species, non-target insects, mammals and other non-target 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

Based upon the results of these studies, the host range of toxicity of the *B.t.k.* HD-73 protein as produced in Bollgard™ Cotton Lines 757 and 1076 is comparable to the proteins produced in nature by the *Bacillus thuringiensis* variety *kurstaki* soil microorganism. This protein is accepted by EPA as being non-toxic to all non-target organisms (EPA, 1988).

L. Impact on Endangered Species

No endangered or threatened lepidopteran insects, as listed in 50CFR 17.11 and 17.12, feed on cotton plants.

M. Possible Impact on the Environment

Persistence in the environment following harvest - The *B.t.k.* HD-73 protein in Bollgard™ Cotton Lines 757 and 1076 is present in the plant tissue remaining in the field after harvest of the lint and seed. This cotton plant residue is typically tilled into the soil. The environmental fate of *B.t.k.* HD-73 protein in soil was determined by measuring the rate at which the bioactivity of the *B.t.k.* HD-73 protein dissipates when added to soil as the purified protein and as a component of insect resistant cotton tissue.

Two test substances were used in this study: 1) *B.t.k.* HD-73 that was purified from *E. coli*, characterized and shown to be equivalent to the *B.t.k.* HD-73 protein expressed in insect resistant cotton plants, and 2) lyophilized cotton tissue powder prepared from field-grown Bollgard™ Cotton Line 931 plants. Bollgard™ Cotton Line 931 expresses the same *B.t.k.* HD-73 protein as Bollgard™ Cotton Lines 757 and 1076. Bollgard™ Cotton Line 931 was used in this study due to its higher expression of the *B.t.k.* HD-73 protein. *B.t.k.* HD-73 purified protein was added to soil at the rates of 0.3, 0.8 and 1.5 µg/ g dry wt soil; Bollgard™ Cotton Line 931 tissue powder was added at 0.01, 0.03 and 0.05 g/ g dry wt soil. These samples were incubated in soil (Dupo silt loam) at approximately 24°C for up to 54 days at a relatively constant soil moisture level. Aqueous soil suspensions was prepared from incubated soil samples, incorporated into artificial insect diet and presented to tobacco budworm *Heliothis virescens* (TBW) larvae. Half-lives were calculated using the equation for first-order rate of dissipation. Recovery of *B.t.k.* HD-73 protein TBW activity was assessed for both test substances at all rates evaluated.

Purified *E. coli B.t.k.* HD-73 protein TBW bioactivity dissipates with an estimated half-life of 9.3 to 20.2 days, depending on the dose. *B.t.k.* HD-73 protein TBW bioactivity, added to soil at 0.01g tissue powder per g dry wt soil as a component of Bollgard™ Cotton Line 931 tissue, dissipates with an estimated half-life of 41 days. Recovery of *B.t.k.* HD-73 protein TBW bioactivity was high when added to soil as the purified protein and as a component of lyophilized cotton tissue powder.

The results of this study suggest that the *B.t.k.* HD-73 protein will degrade readily (estimated half-life of 41 days), when added to soil as a component of post-harvest insect resistant cotton plants. The measured half-life of the purified *B.t.k.* protein in soil is comparable to that measured for the microbial *B.t.k.* preparations (West, 1984; Pruett *et al.*, 1980).

Other potential effects that could conceivably be associated with the commercialization of Bollgard™ Cotton Lines were evaluated. A review of all available information including extensive field test results, safety studies and independent scientific research indicates that the commercial use of Bollgard™ Cotton Lines 757 and 1076 will not result in any adverse effects to the environment. In fact, it is likely that commercialization will have a positive impact on the environment by promoting integrated pest management practices and reduced reliance on traditional chemical insecticides.

N. Summary

1. Expression of the Inserted Genes

Bollgard™ Cotton Lines 757 and 1076 have been modified by the insertion of the PV-GHBK04 and PV-GHBK04 plasmids, respectively, which contain the *B.t.k. cryIAc* gene imparting the insect resistance trait. In addition to the insecticidally active *B.t.k.* HD-73 protein, Bollgard™ Cotton Lines 757 an 1076 express the selectable marker protein, NPTII.

The *B.t.k.* HD-73 and NPTII proteins were expressed at low and relatively consistent levels in Bollgard™ Cotton Lines 757 and 1076 across all six field sites. Bollgard™ Cotton Line 757 contained approximately 12.6 and 9.9 µg *B.t.k.* HD-73 protein/gram fresh weight of tissue (fwt), and 6.9 and 3.3 µg NPTII protein/gram fwt in leaf and seed tissue, respectively. Bollgard™ Cotton Line 1076 contained approximately 12.2 and 12.7 µg *B.t.k.* HD-73 protein/gram fwt and 16.3 and 7.9 µg NPTII protein/gram fwt in leaf and seed tissue, respectively. *B.t.k.* HD-73 protein levels varied less than five fold in young leaf tissue collected over the growing season with the highest levels observed early in the season at the one field site evaluated.

Levels of *B.t.k.* HD-73 and NPTII proteins in whole plant tissue were much lower, on a fresh weight basis, than in leaf or seed tissues. *B.t.k.* HD-73 is present in whole cotton plants from Bollgard™ Cotton Lines 757 and 1076 at 1.1 and 1.7 µg/g fwt of the whole plant, respectively; NPTII protein levels are 3.7 and 14.6 µg/g fwt for plants from Bollgard™ Cotton Lines 757 and 1076, respectively. These measured concentrations were used to estimate the amount of *B.t.k.* HD-73 and NPTII protein that could enter the environment due to post-harvest incorporation of mature plants from the Bollgard™ Cotton Lines (minus lint and seed): 12.2 and 23.4 g *B.t.k.* HD-73 protein/acre and 57.5 and 183 g NPTII protein/acre, for Bollgard™ Cotton Lines 757 and 1076, respectively (assuming 60,000 plants per acre).

Nectar and pollen collected from Bollgard™ Cotton Lines 757 and 1076 contain very low levels of *B.t.k.* HD-73 protein. The expression of *B.t.k.* HD-73 protein in pollen collected from Bollgard™ Cotton Line 757 and 1076 greenhouse-grown plants were 23.0 and 37.8 ng/g fresh wt, respectively. The *B.t.k.* HD-73 protein levels in nectar were 0.72 and 0.88 ng/g fresh wt collected from Bollgard™ Cotton Line 757 and 1076 plants, respectively. Thus, pollen and nectar produced by plants from Bollgard™ Cotton Lines 757 and 1076 present a low source of potential *B.t.k.* HD-73 protein exposure to non-target organisms.

A second selectable marker gene encoding aminoglycoside adenylyltransferase (AAD) is present in the Bollgard™ Cotton Lines 757 and 1076; 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 Bollgard™ Cotton Lines 757 and 1076.

2. Composition, Quality, and Processing of the Seed

The cottonseed and processed cottonseed products from Bollgard™ Cotton Lines 757 and 1076 are equivalent to the cottonseed and processed products from the C312 parental control on the basis of composition and quality.

The cottonseed from both Bollgard™ Cotton Lines were compared to the seed from the control cotton line on the basis of major seed components (protein, oil, 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 meaningful differences in the seed were observed between the Bollgard™ Cotton Lines and the C312 control line.

Cottonseed from Bollgard™ Cotton Lines 757 and 1076 processed comparably to the C312 control, with comparable reductions in the levels of gossypol in the processed meal prepared from all three lines. No gossypol was observed in refined cottonseed oil. Both *B.t.k.* HD-73 and NPTII proteins were reduced to non-detectable levels in processed cottonseed meal from both Bollgard™ Cotton Lines.

3. **Plant Pest Risk**

Bollgard™ Cotton Lines 757 and 1076 do not pose any different plant pest risk to other plants and the environment than non-transformed cotton varieties.

In all field and green house trials, plants of Bollgard™ Cotton Lines 757 and 1076 were repeatedly inspected for any signs of *Agrobacterium* infection and other disease symptoms, and none were found. Bollgard™ Cotton Lines 757 and 1076 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 insect resistance 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**

Bollgard™ Cotton Lines 757 and 1076 and the expressed proteins have no adverse effect on non-target organisms or the environment.

A series of safety studies were conducted with the purified, active ingredient in Bollgard™ Cotton Lines 757 and 1076 (*B.t.k.* HD-73 protein) on several non-target beneficial insects. No toxicity was observed at a level representing approximately 500 times the maximum *B.t.k.* HD-73 protein expression level in pollen and nectar in the Bollgard™ Cotton lines.

An additional 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 historical data demonstrating safety of *B.t.* proteins to fish and the unlikely event of exposure, a toxicity study with fish was not considered necessary.

No endangered or threatened lepidopteran insects, as listed in 50 CFR 17.11 and 17.12, feed on cotton plants.

The *B.t.k.* HD-73 protein was shown to degrade readily when added to soil as purified protein or as tissue from insect resistant cotton plants. The rate of degradation was similar to the degradation rates reported for commercial microbial pesticides containing *B.t.k.* protein.

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. In fact, use of Bollgard™ Cotton Lines 757 and 1076 will have a more positive impact on the environment than the use of chemical insecticides to control Lepidopteran caterpillars. The *B.t.k.* protein is ecologically benign, i.e. it breaks down rapidly in the soil, is safe to nontarget organisms such as fish, birds and mammals and specifically controls many species of Lepidopteran caterpillars on cotton. In addition, the risk of an uncontrolled introduction of this cotton into the environment through hybridization or out-crossing to a native species resulting in a new weed variety is non-existent on the mainland of the United States.

The consistent Lepidoptera insect control offered by Bollgard™ Cotton Lines 757 and 1076 will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop for control of cotton bollworm, tobacco budworm and pink bollworm. As a result, they will be able to utilize many IPM practices that cannot presently be implemented because of the lack of options other than use of chemical insecticides to control these pests. An increase in the biological and cultural control of non-target cotton pests and a more judicious use of chemical insecticides will result in a positive impact on the environment, which will ultimately be advantageous to the grower and the public as well.

Therefore, it is concluded that the Bollgard™ Cotton Lines 757 and 1076 do not pose any different plant pest risk to other plants and the environment than is now caused by non-transformed cotton varieties.

Table V-1. Mean Expression of the *B.t.k.* HD-73 and NPTII Proteins Across Sites in Bollgard™ Cotton Line 757, 1993 Field Trials.

Tissue	*B.t.k.* HD-73 µg/g fwt*	*B.t.k.* HD-73 Range**	NPTII µg/g fwt*	NPTII Range
Leaf	12.65 (2.899)†	6.85 - 18.45	6.86 (1.000)	4.86 - 8.86
Seed	9.87 (1.307)	7.25 - 12.48	3.31 (0.232)	2.84 - 3.77

* Mean expression level across all field test locations. N=36, 3-6 samples per each of six sites.
** The 95% confidence interval for the mean expression levels across field locations expressed as µg/g fresh/frozen weight of tissue (fwt).
† Numbers in parenthesis are the standard error for the mean expression level across all field locations.

Table V-2. Expression of *B.t.k* HD-73 and NPTII Proteins in Leaf Tissue from Bollgard™ Cotton Line 757 at each Site in the 1993 Cotton Field Tests

Site	*B.t.k.* HD-73 µg/g fwt*	*B.t.k.* HD-73 % CV†	NPTII µg/g fwt*	NPTII %CV
Texas	24.19	3.37	6.27	16.69
Mississippi	15.24	61.86	5.80	9.74
Georgia	3.76	20.80	5.28	10.64
Louisiana	14.08	22.19	9.50	17.85
Arizona	11.45	28.84	10.22	20.25
Alabama	7.19	52.34	4.08	67.05

* Mean value of samples taken at each site (N = 3-6 samples per site).
† Variability among plots in the same location, expressed as % coefficient of variation (%CV).

Table V-3. Expression of *B.t.k.* HD-73 and NPTII Proteins in Cottonseed from Bollgard™ Cotton Line 757 at each Site in the 1993 Cotton Field Tests

Site	*B.t.k.* HD-73 µg/g fwt*	% CV†	NPTII µg/g fwt*	%CV
Texas	7.13	27.37	3.43	12.24
Mississippi	5.44	14.32	2.75	11.72
Georgia	11.60	4.06	3.46	27.80
Louisiana	12.60	22.19	2.96	16.49
Arizona	9.17	28.19	2.78	15.27
Alabama	12.99	26.56	4.14	32.99

* Mean value of samples taken at each site (N = 3-6 samples per site).
† Variability among plots in the same location, expressed as % coefficient of variation (%CV).

Table V-4. Mean Expression of the *B.t.k.* HD-73 and NPTII Proteins Across Sites in Bollgard™ Cotton Line 1076, 1993 Field Trials.

Tissue	*B.t.k.* HD-73 µg/g fwt*	Range**	NPTII µg/g fwt*	Range
Leaf	12.23 (2.531)†	7.16 - 17.29	16.32 (1.539)	13.25 - 19.40
Seed	12.73 (0.642)	11.45 - 14.02	7.93 (0.330)	7.27 - 8.59

* Mean expression level across all field test locations. N=36, 3-6 samples per each of six sites.
** The 95% confidence interval for the mean expression levels across field locations expressed as µg/g fresh/frozen weight of tissue (fwt).
† Numbers in parenthesis are the standard error for the mean expression level across all field locations.

Table V-5. Expression of *B.t.k* HD-73 and NPTII Proteins in Leaf Tissue from Bollgard™ Cotton Line 1076 at each Site in the 1993 Cotton Field Tests

Site	*B.t.k.* HD-73 µg/g fwt*	*B.t.k.* HD-73 % CV†	NPTII µg/g fwt*	NPTII %CV
Texas	21.19	7.64	17.23	9.97
Mississippi	17.71	25.21	11.96	27.44
Georgia	4.11	17.90	18.21	56.97
Louisiana	11.26	15.00	20.33	34.80
Arizona	9.03	2.36	18.89	6.74
Alabama	10.05	12.02	11.32	16.38

* Mean value of samples taken at each site (N= 3-6 samples per site).
† Variability among plots in the same location, expressed as % coefficient of variation (%CV).

Table V-6. Expression of *B.t.k* HD-73 and NPTII Proteins in Cottonseed from Bollgard™ Cotton Line 1076 at each Site in the 1993 Cotton Field Tests

Site	*B.t.k.* HD-73 µg/g fwt*	*B.t.k.* HD-73 % CV†	NPTII µg/g fwt*	NPTII %CV
Texas	16.22	6.86	6.82	15.09
Mississippi	12.64	13.84	7.31	15.27
Georgia	11.59	8.35	8.37	47.55
Louisiana	13.41	8.45	7.76	25.81
Arizona	11.67	12.71	6.98	17.40
Alabama	12.59	25.11	8.96	27.72

* Mean value of samples taken at each site (n = 3 - 6 samples per site).
† Variability among plots in the same location, expressed as % coefficient of variation (%CV).

Table V-7. Expression of the *B.t.k.* HD-73 and NPTII Proteins in Young Leaf Tissue of Bollgard™ Cotton Lines 757 and 1076 throughout the Growing Season (1993, West Sinton, TX).

Line #	Sampling Date	Mean § µg/g *B.t.k.* HD-73	Mean % Extraction Efficiency	Mean % Recovery of Spike††	Corrected for Bias µg/g*
757	6/8/93	24.19 (0.81)†	95.27 (0)¥	78.73 (25)**	32.25
	7/6/93	14.48 (2.92)	54.35 (5)	64.18 (8)	41.49
	8/3/93	3.57 (1.46)	47.87 (10)	73.59 (25)	10.11
	9/1/93	1.49 (0.17)	32.72 (2)	56.32 (6)	8.09
1076	6/8/93	21.19 (1.62)	94.62 (0)	78.73 (25)	28.44
	7/6/93	23.02 (3.33)	58.82 (4)	64.18 (8)	61.06
	8/3/93	7.05 (3.31)	45.90 (13)	73.59 (25)	20.85
	9/1/93	5.54 (0.77)	42.74 (3)	56.32 (6)	23.08

* corrected for extraction efficiency and recovery of spike

§ Mean expression level from analysis of three leaf samples at each time point.

† numbers in parentheses are the standard deviation of the mean within each line and timepoint (N = 3)

†† Range of recovery from buffer (no matrix) was 56 to 79% for spike levels ranging from 50 to 400 ng/ml. Higher recovery is seen for lower level of spike.

** Numbers in parenthesis represent the standard deviation for mean percent recovery over 3 spikes, n=6 (duplicates at each level).

¥ Mean extraction efficiency for three samples, with standard deviation of the mean in parenthesis.

Table V-8: Expression of *B.t.k.* HD-73 and NPTII Proteins in Mature Bollgard™ Cotton Plants from West Sinton, TX, 1993.

Line	Mean g fwt Tissue/Plant	*B.t.k.* HD-73 µg/g fwt	*B.t.k.* HD-73 µg/plant	NPTII µg/g fwt	NPTII µg/plant
757	185 (78)†	1.071 (0.326)	202.9 (130.5)	3.7	958
1076	217 (75)	1.743 (0.223)	389.9 (180.3)	14.6	3056

† Numbers in parenthesis represent the standard deviation from three replicates.

Data represents the mean value of three replicates for *B.t.k.* HD-73 protein and a single sample analysis for NPTII protein.

Table V-9. Assessment of the *B.t.k.* HD-73 protein levels in Bollgard™ Cotton nectar and pollen.

Sample Type	Cotton Line	*B.t.k.* HD-73 (ng/g fwt)		Total Protein (mg/g fwt)	
Pollen*	C312	0.0	(8.6)	85.6	(2.9)†
	Line 757	23.0	(5.5)	80.0	(21.4)
	Line 1076	37.8	(50.2)	86.3	(1.8)
Nectar**	C312	0.00	(0.00)	0.04	(0.03)
	Line 757	0.72	(0.11)	0.06	(0.00)
	Line 1076	0.88	(0.17)	0.03	(0.01)

* Values represent the mean and standard deviation (in parentheses) of two replicate pollen samples for each line. Each replicate represents the pooled pollen collected from multiple plants on separate collection days. A small background response on the *B.t.k.* HD-73 ELISA for the Coker 312 control nectar (6.1 ng/g fresh wt) was subtracted from each line.

† Protein levels for pollen and nectar represent the mean and standard deviation () from three replicate samples

** Data presented represent the mean and standard deviation (in parentheses) from two replicate nectar samples per line. Each replicate represents nectar pooled from unique multiple collection days. *B.t.k.* HD-73 protein was not detected in the control (Coker 312) line; the detection limit of the *B.t.k.* HD-73 ELISA used in this study is 0.80 ng/ml, which corresponds to 0.80 ng/g fresh wt under the conditions used.

Table V-10. Summary of Proximate Analysis of Cottonseed from Bollgard™ Cotton Lines 1076 and 757 Collected from the 1993 Field Trials

Characteristic†	C312 Mean[1] (Range)¥	757 Mean[2] (Range)¥	1076 Mean[2] (Range)¥
Protein %	27.00 (23.3-28.4)	27.60 (23.4-30.5)	26.57 (23.5-28.9)
Fat %	22.96 (19.6-25.1)	22.95 (21.9-25.6)	20.80* (16.6-22.8)
Ash %	4.63 (4.3-5.0)	4.45 (3.8-4.8)	4.45 (4.1-4.7)
Carbohydrate %	45.40 (42.8-47.6)	44.99 (41.9-46.5)	48.17* (46.8-51.0)
Calories/100g	496.32 (479-508)	496.91 (495-510)	486.03* (464-496)
Moisture%	12.36 (9.6-15.9)	13.18 (8.0-16.4)	10.60 (9.4-12.6)

† Protein, fat, ash, carbohydrate, and calories reported as percent dry weight of sample.
¥ Range denotes the lowest and highest individual values across sites for each line.
* Statistically significant from control line, C312, at the 5% level (paired t-test).
1 Value reported is least squares mean of five samples, one from each field site where bulk seed from line C312 was collected (Study 93-01-36-01).
2 Value reported is least squares mean of four samples, one from each field site where bulk seed from IRC lines was collected (Study 93-01-36-01).

Table 10a. Literature References

Component	Literature range/mean value	Literature reference
Protein %	18.8-22.9	Turner, *et al.*, 1976.
	23.5-29.5	Cherry, *et al.*, 1978a.
	12-32	Kohel, *et al.*, 1985.
Fat (oil) %	23.2-25.7	Cherry, *et al.*, 1978b.
	23.6-25.0	Cherry, *et al.*, 1978a.
Ash %	4.1-4.9	Cherry, *et al.*, 1978b.
	3.8	Belyea, *et al.*, 1989.
Moisture	5.4-10.1	Cherry, *et al.*, 1978a.

Table V-11. Lipid and Fatty Acid Composition of Cottonseed from Bollgard™ Cotton Lines 757, 1076 and Control Cotton Line, C312

Component	C312[a,b]		757[a,b]		1076[a,b]	
	Mean	Range†	Mean	Range	Mean	Range
Lipid	33.5	30.9-35.5	33.6	32.1-36.9	33.7	31.4-36.3
Myristic (14:0)	0.94	0.67-1.07	0.97	0.79-1.10	1.02*	0.86-1.18
Pentadecanoic (15:0)	0.40	0.32-0.60	0.75	0.24-1.22	0.43	0.29-0.77
Palmitic (16:0)	26.5	24.8-27.8	26.8	23.8-28.1	26.8	24.4-28.1
Palmitoleic (16:1)	0.64	0.48-0.71	0.63	0.58-0.66	0.73*	0.66-0.78
Margaric (17:0)	0.16	0.13-0.20	0.17	0.13-0.22	0.19	0.17-0.22
Stearic (18:0)	2.63	2.32-3.26	2.90	2.74-3.19	2.54	2.46-2.63
Oleic (18:1)	15.3	14.8-16.0	15.7	13.4-17.2	15.2	13.5-16.7
Linoleic (18:2)	47.8	46.4-49.9	46.0	43.3-49.2	47.7	45.1-50.5
Linolenic (18:3)	0.20	0.13-0.29	0.17	0.13-0.24	0.17	0.11-0.29
Arachidic (20:0)	0.29	0.26-0.31	0.26	0.21-0.31	0.29	0.25-0.33
Behenic (22:0)	0.15	0.12-0.17	0.14	0.11-0.16	0.14	0.11-0.15
Malvalic (C-17)	0.37	0.22-0.45	0.42	0.23-0.62	0.28	0.26-0.37
Sterculic (C-18)	0.59	0.48-0.70	0.68	0.47-0.86	0.63	0.48-0.78
Dihydrosterculic (C-19)	0.36	0.29-0.50	0.76	0.28-1.41	0.31	0.15-0.75

[a] Value of lipid is % of dry sample weight. Value of fatty acid is % of total lipid.
[b] Values presented are least squares mean and ranges [five samples for C312 and four samples for IRC lines; one seed sample of each line from each site where bulk seed samples were collected (Study 93-01-36-01)].
† Range denotes the lowest and highest individual value across sites for each line.
* Significantly different from control, line C312, at the 5% level (paired t-test).

Table V-12. Gossypol Levels Determined in Cottonseed from Bollgard™ Cotton Lines 757 and 1076 and the Control Line, C312

Line	% Total Gossypol† Mean	Range
C312	1.16[a]	(0.97-1.43)[a]
757	1.08	(0.85-1.31)
1076	1.04*	(0.85-1.22)

† Gossypol expressed as percent dry weight of seed; literature range is 0.39 - 1.7% (Berardi and Goldblatt, 1980).
* Values are statistically significant compared to the Coker 312.
a Values reported for seed samples are the least squares mean (from statistical analyses); ranges represent the lowest and highest values among samples per line: one sample per site where bulk cottonseed was harvested, (N = 5 for C312, N = 4 for 757 and 1076).

Table V-13. Yield of Fractions from Processing Bollgard™ Cottonseed

Fraction	Percent Yield Line C312	Line 757	Line 1076	Reported Percent Yields
Delinted Cottonseed†	76	78	80	88 - 90[1]
**Hulls¥	8.2	7.6	9.1	30-35[1], 25.5[2]
Linters (all cuts)†	18	16	15	10-12[1], 9.9-12.4[3],8.4[2]
Kernels¥	48	41	44	65-70[1], 43.5-53.4[3],46[2]
Crude Oil††	26	24	20	25-31[1], 16.3[2]
Refined Oil	11	8.5	7.5	not available
Toasted Meal††	58	44	56	69 - 75[1]

** Kernel material from the small scale process contains hull material at 5 - 8%; therefore % yield for the hull material appear much lower when compared to reported yields.
† Percent weight of fuzzy cottonseed
¥ Percent weight of delinted seed
†† Percent weight of kernel
1 Yield ranges obtained from Texas A&M University GLP Processing Program.
2 Cottonseed and Its Products, 1989.
3 Cherry and Leffler, 1984.

Table V-14. Gossypol Levels Determined in Raw Cottonseed Meal, Toasted Meal, and Refined Oil from Bollgard™ Cotton Lines 757 and 1076 and the Control Line, C312*

Line	% Total Gossypol	% Free Gossypol
Raw Meal		
C312	1.06	0.667
757	1.09	0.661
1076	0.83	0.513
Toasted meal		
C312	1.11	0.011
757	0.81	ND[a]
1076	0.72	ND
Refined Oil[b]		
C312	0.09	ND[c]
757	ND	ND
1076	ND	ND

* Values were obtained from analysis of one composite sample comprised of seed from all field sites where bulk cottonseed were collected in the 1993 field test.

[a] ND= not detected (limit of detection for measurement of free gossypol in toasted meal = 0.007%)

[b] Literature reported as ≤ 0.01% (Cherry and Leffler, 1984).

[c] ND = not dectected (limit of detection was 0.04% and 0.002% for measurement of total and free gossypol in oil, respectively).

Table V-15. Summary of Oil Quality from Bollgard™ Cotton Lines 757, 1076, and Control Cotton Line, C312

Component	Lit Range	Refined Oil from Line: C312	757	1076
Fatty Acids†:				
Myristic (14:0)	(0.5-2.5) [1] (0.68-1.16)[2]	0.98	0.86	0.88
Palmitic (16:0)	(17-29)[1] (21.63-26.18)[2]	25.42	24.96	25.94
Palmitoleic (16:1)	(0.5-1.5) [1] (0.56-0.82)[2]	0.64	0.60	0.63
Margaric (17:0)	not available	0.19	0.11	0.11
Stearic (18:0)	(1.0-4.0)[1] (2.27-2.88)[2]	2.53	2.62	2.38
Oleic (18:1)	(13-44)[1] (15.17-19.94)[2]	14.92	15.49	13.64
Linoleic (18:2)	(33-58)[1] (49.07-57.64)[1]	50.27	50.09	50.80
Linolenic (18:3)	(0.1-2.1)[1] (0.23)[3]	0.16	0.15	0.15
Arachidic (20:0)	(<0.5)[1], (0.41)[3]	0.21	0.26	0.27
Behenic (22:0)	(<0.5)[1]	0.12	0.12	0.13
Sterculic	(0.08-0.56)[4]	0.48	0.58	0.60
Malvalic	(0.22-1.44)[4]	0.36	0.42	0.41
Dihydrosterculic acid (C-19)	not available	0.22	0.35	0.26
Vitamin E:				
alpha-Tocopherol	not available	638[5]	597	689

† Reported as % of total lipids. One sample of refined oil per line produced from a composite of seed across sites.

1 Ranges adopted by the FAO/WHO Codex Alimentarius committee on fats and oils {533}.

2 Cherry and Leffler, 1984.

3 Cherry, J.P., 1983.

4 Phelps, et.al., 1965. Values reported for crude cottonseed oil.

5 Reported as mg/kg

Table V-16. Allelochemical Levels in Vegetative Tissues from Bollgard™ Cotton Lines 757, 1076 and Control Line, C312.*

Line	Tissue	1993 Field Season† Gossypol	Anthocyanin	Flavonoid	Tannin
757	Square	0.280	0.13	0.36	11.59
1076	Square	0.258	0.11	0.38	12.53
C312	Square	0.294	0.11	0.39	14.89
757	Leaf	0.111	0.26	0.70	11.70
1076	Leaf	0.119	0.28	0.75	11.05
C312	Leaf	0.143	0.34	0.80	17.11

* Reported as percent of dry weight of tissue.
† Mean value reported from six samples taken from replicated plots for each line.

Table V-17. Germination results for seed from Bollgard™ Cotton Line 757, 1076, and Coker 312 grown in Alabama, Mississippi, and Texas.

	% germ (warm)			% germ (cool)		
Line	AL	MS	TX	AL	MS	TX
757	28 bc	95 ab	33 bc	28 bcd	85 ab	33 b
1076	68 a	85 ab	55 ab	53 a	88 a	70 a
C312	55 ab	90 ab	35 bc	48 ab	88 a	40 ab

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

Table V-18. Stand Counts* at West Sinton, Texas

Line	Avg. # plants/30 ft of Row
Coker 312	137.5 (18)†
Line 757	138.0 (2.4)
Line 1076	143.3 (3.1)

* Planted on 5/17/93 at a seeding rate of 5 seeds/foot .
† Standard deviation of the mean in parenthesis (n = 4 plots)

Table V-19. Stand Counts* at Bossier City, Louisiana

Line	Avg. # plants/30 ft of Row
Coker 312	107 (6.7)†
Line 757	107 (6.9)
Line 1076	103 (10)

* Planted on 5/12/93 at a seeding rate of 4.5 seeds/foot, counts taken on 6/30.
† Standard deviation of the mean in parenthesis (n = 12 plots)

Table V-20. Percent Outcrossing at varying distances from the Bollgard™ Cotton observed at six sites in 1990.

Approximate distance from test (ft)	College Station	Halfway		Brawley		Maricopa	Bossier City		Starkville	
	Location									
	%*	%	S.D.†	%	S.D.	%	%	S.D.	%	S.D.
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. Current Cotton Agronomic Practices and the Impact of Insect Resistant Cotton on Cotton Pest Management

Luttrell *et al.* 1993, reviews the current agronomic practices for cotton production and the potential impact of insect resistant cotton on cotton pest management. The following is a summary of this review, which can be found in Appendix I.

Cotton production in the United States is highly mechanized and dependent upon maximum utilization of new technology to remain competitive in a worldwide market. Pest problems, particularly insects, and environmental constraints, such as inadequate temperature and moisture, are major limiting factors to optimum cotton production. Most cotton production regions of the United States rely on extension specialists and crop consultants to design and implement effective IPM programs. Insect control decisions are largely based on routine field monitoring by agricultural consultants, extension personnel and growers. The intensity of monitoring varies among locations and is associated with production capabilities, potential insect damage and availability of consultants (Luttrell 1994). Numerous advances in IPM technology (Frisbie and Adkisson 1985, Frisbie *et al.* 1989) have encouraged a systems approach to insect management in United States cotton where insect control decisions are integrated into an overall crop production and management scheme. Perhaps the best example of this is the wide acceptance of early-maturing varieties and short-season cotton production systems first recommended in Texas. The Texas system of short-season cotton production (Walker *et al.* 1978) has been widely adopted across United States cotton and is recommended by agronomists and entomologists because it optimizes the production of valuable fiber and encourages the "avoidance" of damaging late-season populations of insects.

Although advances in IPM technologies have fostered improved cotton insect management systems, insect control is still largely based on the use of chemical insecticides, which include all classes of chemical insecticides such as pyrethroids, organophosphates, carbamates, etc. (Herzog *et al.* 1993). Estimates of insect control costs and losses (Head 1991, 1992, 1993) averaged for the 1990's indicate that United States cotton growers apply an average of 4.86 applications of insecticide to 11.8 million acres of cotton and spend more than $400 million each year for control of cotton insects. This represents a large portion of total insecticide use in the United States. Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests (Luttrell 1994). The need for alternative insect control measures is becoming more critical to profitable cotton production in the United States.

Environmental concerns limit the availability of existing insecticide chemistry and increase the developmental costs of new chemistry. Because of the high costs of developing and registering new insecticide chemistry, availability has declined over the past few years.

Bollgard™ Cotton Lines 757 and 1076 offer unique, innovative alternatives to traditional chemical control measures. Although alternative insect control tactics are often cited as major components of cotton IPM and research is continuously pursuing improved management methods (Frisbie *et al.* 1989), few alternative insect control methods are of sufficient efficacy to replace chemical control methods. Other methods, such as biological control, host plant resistance and cultural control, provide suppression of pest populations without disrupting natural control, but generally lack the high efficacy and curative action of conventional insecticides. Bollgard™ Cotton Lines 757 and 1076 ares the first major exception to this historical trend.

Bollgard™ Cotton Lines 757 and 1076 offer new mechanisms to produce and deliver a highly effective insecticide to target pests (i.e. production by cells of the crop plant rather than industrial facilities and application by spray equipment). The technology couples the environmental advantages of host plant resistance with the efficacy of an effective biological insecticide. Since the insecticidal activity is expressed throughout the plant for the entire season, improved control of some pest species over that provided by conventional insecticides is likely. Current technology which depends on foliar application of insecticides cannot dependably deposit insecticides to some regions of the plant canopy infested by pest species. This is especially true of pests that burrow and feed inside plant tissue (e.g. pink bollworms). Because Bollgard™ Cotton Lines 757 and 1076 express the *B.t.k.* protein that only has activity against certain lepidoptera insects and must be ingested to kill the pest, the technology offers selective activity against susceptible lepidopteran pest complexes without directly disrupting pest suppression by natural enemies, such as parasites and predators.

B. Development of Pest and Resistance Management Strategies for Insect Resistant Cotton

Some organisms are resistant to single or multiple pesticides in use today. It has not been established whether this resistance is because the organism has adapted metabolically to be able to tolerate the effects of the pesticide, or that a small segment of the population was naturally resistant and dominate as the numbers of the susceptible members have been reduced. Regardless of how resistance is obtained, it is a potentially serious problem with some pests.

Some insect resistance to the *B.t.k.* insect control protein has been reported in the past 5 years. Examples of insects for which resistance has been reported are the Indianmeal moth (*Plodia interpunctella*), almond moth

(*Caudra cautella*) and the diamondback moth (*Plutella xylostella*). There are also some examples of insecticides such as the organophosphates for which little resistance has been reported. In fact some of these chemical insecticides are able to control the same insects at the same dosages as when they were commercialized over 30 years ago.

It is currently not possible to accurately predict whether resistance will occur by an insect to an insecticide. Therefore, is important that every insecticide commercialized be used in a manner and as a part of an overall pest control program so as to maximize its usefulness. Monsanto is developing a pest control strategy aimed at reducing the probability of resistance becoming a problem. This strategy is included in Appendix IX of this Petition for Determination of non-Regulated Status. This will be offered to growers choosing this cottonseed. We believe by implementing these strategies, the development of resistance (if it occurs at all), can be managed to maximize the usefulness of this modified cotton.

To achieve the benefits described above, it is important that insect resistant cotton be implemented and managed properly. In this respect, these plants are no different than any other crop protection product that has been used over the last century. It is clear from the knowledge gained over that time, that to successfully maximize the long-term use of insect resistant cotton, two interconnected management components are required. First, is the development of integrated pest management techniques that allow the farmer to optimize the utility of these plants for cotton pest control. In essence, this is the development of a total insect management package that will be centered around insect resistant cotton. Second, to maximize the durability of this cotton, is the development and implementation of strategies targeted to prevent the development of insect resistance to the insect control protein produced by the plants.

For the last several years, extensive consultations have been held with the leading cotton pest and resistance management researchers to develop a program to maximize the use and durability of insect resistant cotton. Laboratory and field studies designed in collaboration with these experts from academia and extension are in progress and are providing the data needed for developing this management program. These studies are examining the impact of insect resistant cotton on populations of beneficial and pest insects endemic to the crop, the impact on the use of conventional insecticides for controlling non-target pests, the establishment of the baseline susceptibility of our insect targets to the *B.t.k.* insect control protein, and the impact of mixtures of resistant and non-resistant plants on yield loss.

Monsanto scientists have worked for several years on laboratory and field studies of insect resistance, and with outside collaborators nearly every suggestion made for resistance management in insect resistant cotton is being examined. These strategies, developed in consultation with an expert advisory panel, take into account existing research and an understanding of cotton production and agronomic practices. They include:

1) High dose expression of the *B.t.k.* insect control protein in cotton to control caterpillars heterozygous for resistance alleles.

2) Refugia as hosts for sensitive insects provided through non-insect resistant cotton.

3) Monitoring of insect populations for susceptibility to the *B.t.k.* insect control protein.

4) Agronomic practices that minimize insect exposure to the *B.t.k.* insect control protein.

5) Development of novel lepidopteran control proteins with a distinct mode of action from the *B.t.k.* insect control protein.

Those pest and resistance management strategies best suited for use in cotton production and with the potential for delaying or preventing the development of resistance will be recommended. In addition, an extensive effort has been initiated to educate cotton growers as to the most effective ways to integrate insect resistant cotton within their current production practices. This cooperative effort between growers, academia, extension, seed company partners and Monsanto will help ensure that the benefits of insect resistant cotton are fully realized and sustained.

C. 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 Bollgard™ Cotton Lines 757 and 1076 be grown in their 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 the *B.t.k.* insect control protein and the Bollgard™ Cotton plants.

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.

D. Potential for BollgardTM Cotton Lines 757 and 1076 to Become Weeds

Bollgard™ Cotton Lines 757 and 1076 are not expected to have any different weedy characteristics than other cotton grown in the United States. A detailed discussion of the potential for Bollgard™ Cotton Lines 757 and 1076 to become weeds is addressed in Part V paragraph G, of this Petition for Determination of Non-Regulated Status.

E. Increased Numbers of Beneficial Insects

Aside from the benefit of a decrease in the use of chemical insecticides an additional benefit has been identified, that being an increase in the numbers of beneficial insects present in the cotton fields.

The worst enemies of most insects are predatory insects. These predators feed on other insects thus providing a "natural" level of control. Most chemical insecticides used in cotton are fairly general in the range of insects controlled, and therefore, most insects including the beneficial predators are controlled. Over the period of a growing season their numbers can be depleted to the point that control of pests by the predators is essentially non-existent. Since the *B.t.k.* insect control protein is very specific in its range of control, an increase in the numbers of beneficial insects has been observed in the field and are expected to supplement the control of the cotton insect pests. This increased presence of beneficials will likely reduce the need for insecticide applications targeted to control of cotton pests not susceptible to the *B.t.k.* insect control protein.

Conclusion

None of the environmental consequences identified are of a nature as to justify that Bollgard™ Cotton lines 757 and 1076 should not be commercialized. Bollgard™ Cotton Lines 757 and 1076 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 *B.t.k.* protein and the Bollgard™ Cotton plants. The positive consequences of reduced pesticide use, increases in the numbers of beneficial insects, the substantial equivalence of Bollgard™ Cotton Lines 757 and 1076 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.

Finally, the potential for susceptible cotton insect pests to develop resistance to the *B.t.k.* protein has been considered and resistance management options developed. When one considers the benefits that this cotton will provide to the grower, the public and the environment, (the decreased use of chemical insecticides), it is justified to proceed in this careful manner versus the alternative of not allowing Bollgard™ Cotton Lines 757 and 1076 to be commercialized.

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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 Bollgard™ Cotton Lines 757 and 1076 developed using the plasmid vectors PV-GHBK04 and PV-GHBK03, respectively. Therefore, on the basis of the substantial potential benefits to the farmer, the environment, and the significantly reduced risk to public health, Monsanto requests that Bollgard™ Cotton Lines 757 and 1076 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 insect resistant cotton.

Appendix I

Agronomic Benefits of Insect Resistant Cotton

AGRONOMIC BENEFITS OF INSECT RESISTANT COTTON

Impact of Transgenic Cotton Expressing Endotoxin Proteins from Bacillus thuringiensis on Cotton Insect Management in the USA

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Background

Transgenic cotton expressing delta endotoxin proteins of *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*-cotton) represents one of the first implementable products of plant genetic engineering for production agriculture (Gasser and Fraley 1989, Meeusen and Warren 1989, Vaech *et al.* 1987). Development of *B.t.k.*-cotton has progressed from initial insertion of *B.t.k.* genes into cotton plants in 1987 (Umbeck *et al.* 1987) and 1988 (Deaton 1991, Perlak *et al.* 1990) to the current state of a commercial insect-control product with confirmed high levels of efficacy (Bartlett 1993, Benedict *et al.* 1991, 1992, 1993, Buehler 1993, Deaton 1991, Gannaway *et al.* 1991, Jenkins *et al.*. 1991, 1992, 1993, Micinski and Caldwell 1991, Williamson and Deaton 1991, Wilson and Flint 1991, Wilson *et al.* 1992, 1993). Early field tests in 1989 of initial B.t.k.-cottons developed by Agracetus (Middleton, Wis.) indicated low levels of protein expression in the plants and low levels of insect control (Benedict *et al.* 1992, Jenkins *et al.* 1990, Umbeck *et al.* 1990). Improved expression of the insect control protein genes as a result of coding sequence modifications by Monsanto (St. Louis, Missouri) scientists (Amstrong *et al.* 1990, Deaton 1991, Perlak *et al.* 1991) resulted in transgenic cottons with higher levels of insect control (Benedict *et al.* 1993, Jenkins *et al.* 1993, Wilson *et al.* 1992). Mortality rates of tobacco budworm, one of the most important insects pests of cotton, exposed to these improved *B.t.k.*-cottons (Benedict *et al.* 1992, 1993, DeSpain *et al.* 1993) were as high as those expected from efficacious chemical insecticides [i.e. greater than 85% mortality (Luttrell *et al.* 1987, Roush and Luttrell 1989)] and much higher that those obtained with conventional spray applications of *B. thuringiensis* var. *kurstaki* [i.e. less than 60% mortality (Luttrell *et al.* 1982)].

Cotton production in the USA is highly mechanized and dependent upon maximum utilization of new technology to remain competitive in a worldwide market. Pest problems, particularly insects, and environmental constraints, particularly adequate temperature and moisture, are major limiting factors to optimum cotton production.

Most cotton production regions of the USA rely on extension specialists and crop consultants to design and implement effective integrated pest management (IPM) programs. Insect control decisions are largely based on routine field monitoring by agricultural consultants, extension personnel, and growers. The intensity of monitoring varies among locations and is associated with production capabilities, potential insect damage, and availability of consultants (Luttrell 1994). Numerous advances in IPM technology (Frisbie and Adkisson 1985, Frisbie *et al.* 1989) have encouraged a systems approach to insect management in USA cotton where insect control decisions are integrated into an overall crop production and management scheme. Perhaps the best example of this is the wide acceptance of early-maturing varieties and short-season cotton production systems first recommended in Texas. The Texas system of short-season cotton production (Walker *et al.* 1978) has been widely adopted across USA cotton and is recommended by agronomists and entomologists because it optimizes the production of valuable fruit and encourages the "avoidance" of damaging late-season populations of insects.

Although advances in IPM technologies have fostered improved insect management systems in USA cotton, insect control is still largely based on the use of chemical insecticides (Herzog *et al.* 1993). Estimates of insect control costs and losses (Head 1991, 1992, 1993) averaged for the 1990's indicate that USA cotton growers apply an average of 4.86 applications of insecticide to 11.8 million acres of cotton and spend more than $400 million each year for control of cotton insects. This represents a large portion of total insecticide use in the USA. Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests (Luttrell 1994). The need for alternative insect control measures is becoming more critical to profitable cotton production in the USA. Environmental concerns are limiting the availability of existing insecticide chemistry and increasing the developmental costs of new chemistry. Because of the high costs of developing and registering new insecticide chemistry, availability of new insecticide chemistry has declined over the past few years.

Transgenic cotton plants expressing insecticidal proteins offer unique, innovative alternatives to traditional chemical control measures. Although alternative insect control tactics are often cited as major components of cotton IPM and research is continuously pursuing improved management methods (Frisbie *et al.* 1989), few alternative insect control methods are of sufficient efficacy to replace chemical control methods. Other methods, such as biological control, host plant resistance, and cultural control, provide suppression of pest populations without disrupting natural control,

but generally lack the high efficacy and curative action of conventional insecticides. *B.t.k.* cotton is perhaps the first major exception to this historical trend.

Transgenic cotton offers new mechanisms to produce and deliver an insecticide to target pests (i.e. production by cells of the crop plant rather than industrial facilities and application by spray equipment). The technology actually couples the environmental advantages of host plant resistance with the efficacy of an effective conventional insecticide. Since the insecticidal activity is expressed throughout the plant for the entire season, improved control of some pest species over that provided by conventional insecticides is likely. Current technology which depends on foliar application of insecticides cannot dependably deposit insecticides to some regions of the plant canopy infested by pest species. This is especially true of pests that burrow and feed inside plant tissue. Because *B.t.k.*-cotton expresses insecticidal proteins that only have activity against certain Lepidoptera (moths and caterpillar insects) and must be fed upon to kill the pest, the technology offers selective activity against susceptible lepidopteran pest complexes without directly disrupting pest suppression by natural enemies, such as parasites and predators.

The accomplishments of molecular biology and genetic engineering over the past 10 years have created an abundance of social and economic questions relative to transgenic plants. The unique characteristics of this new technology provide, perhaps, the best historical opportunity to reduce the inputs of conventional insecticides (most of which are nerve poisons) and still maintain optimum protection of cotton from economically damaging pest populations. Growers are anxious to obtain this new technology because of the demonstrated high levels of insect control afforded by *B.t.k.*-cotton. Other factors that contribute to the heightened interest in *B.t.k.*-cotton are recurring problems with insecticide resistant pests of cotton (Elzen *et al.* 1992), outbreaks of secondary and new pests, and increased societal demands for long-term, environmentally safe and biologically rational methods of pest control.

Questions about the environmental safety of transgenic plants have dominated much of the interest in the technology. There also has been a great deal of interest focused on the potential development of pest populations resistant to the *B. thuringiensis* endotoxins and recommended deployment strategies to manage resistance (McGaughey and Whalon 1992). These are important issues that must be considered in deployment strategies for *B.t.k.*-cotton. However, it is equally important to recognize that *B.t.k.*-cotton offers a truly efficacious, environmentally safe alternative to conventional insecticidal control.

This report examines the potential impacts of *B.t.k.*-cotton on current cotton IPM programs and speculates what future opportunities may develop as implementation and improvement of the technology advances. These projections are based on current knowledge of a new technology which is

less than 5 years of age and an appreciation of the importance of managing insect resistance to insecticides. Because cotton production and associated IPM programs vary across the different geographic regions of cotton production in the USA, regional perspectives of the possible role of *B.t.k.*-cotton in IPM programs are included. Estimates of the crop loss and control costs associated with cotton insect pests were developed from 1990, 1991, and 1992 data published by the Beltwide Cotton Conference (Head 1990, 1991, 1992). These estimates were used throughout the report as a standardized reference to the level of economic damage and control costs involved. Percent crop loss refers to amount of crop damage suffered in the presence of control measures. Total economic loss includes control costs and crop loss. All data in Tables 1, 2, and 3 were derived by averaging 1990-1992 annual estimates and arranged relative to the amount of crop loss and insecticide use due to: (a) tobacco budworm-bollworm complex (*Heliothis-Helicoverpa* complex) alone, (b) all Lepidoptera (includes tobacco budworm-bollworm complex), and (c) all insects (includes all Lepidoptera). Current research indicates that *B.t.k.*-cotton will provide a high level of control of the tobacco budworm-bollworm complex and pink bollworm. Less experimental data are available on the effects of *B.t.k.*-cotton on other Lepidoptera. Comparing estimates for tobacco budworm-bollworm complex and all Lepidoptera should provide a realistic range of possible estimates for the value of *B.t.k.*-cotton. Since *B.t.k.*-cotton only affects Lepidoptera, the impact of non-lepidopteran pests on cotton production (i.e. those not directly affected by *B.t.k.*-cotton) can be estimated by subtracting crop loss and control cost data for all Lepidoptera from that reported for all insects (Tables 1, 2, 3).

Potential Impact of *B.t.k.* Cotton on Cotton Pest Management

Pimental *et al.* (1989) suggested that genetic engineering would improve crop yields and improve the efficiency of crop production. In an early review of the potential effects of genetically engineered crops on insect control, Meeusen and Warren (1989) listed several potential advantages and disadvantages (or uncertainties) of the new technology from an agricultural industry perspective. The advantages envisioned were:

1. growers would be less dependant on favorable weather conditions for application of insecticides because insecticidal activity would be continuously expressed and not altered by inclement weather,

2. lower locations of plant canopies (or locations inside tissues) where insecticide sprays cannot be deposited dependably would be protected from insect damage because the insecticidal toxins could be expressed constitutively (i.e. in all tissues and cells) throughout the plant,

3. the need to scout crops would be reduced because of the continuous expression of insecticidal activity,

4. the costs of spraying crops would be eliminated or greatly reduced,

5. the cost of developing a commercial insect-resistant crop line (genetically engineered) would be less than that of developing a new chemical insecticide (currently at $60 to $100 million),

6. spray drift and groundwater contamination would be reduced because the active materials are produced directly in the crop tissue,

7. adverse effects on non-target organisms should be reduced because the only organisms able to receive a dose of the active material would be those feeding on the crop,

8. monitoring of crops for safety for human consumption should be easier since the insecticidal protein expression would be known in advance of harvest and the need for expensive toxicological and residue tests would be eliminated or reduced.

Disadvantages envisioned by Meeusen and Warren (1989) included the likely selection for pest populations resistant to the insecticidal toxins and uncertainties over regulatory and patent procedures and policies. Some of these issues have been resolved or are in the process of being resolved through private- and public-sector sponsored research.

Based on our current knowledge and perspectives as public supported entomologists, we believe that *B.t.k.*-cotton offers unique opportunities to improve existing IPM programs on cotton. Most of the opportunities are associated with the potential of *B.t.k.*-cotton to reduce the use of conventional insecticides. Reduced insecticide use will provide expanded opportunities for non-insecticidal control measures previously limited by the ecological disruptive nature of broad-spectrum, conventional insecticides.

Reduced Insecticide Use

The most obvious and direct effect of *B.t.k.*-cotton on existing cotton pest management is the likely reduction in use of chemical insecticides for control of susceptible lepidopterous pests, especially the tobacco budworm (*Heliothis virescens*) and the bollworm (*Helicoverpa zea*). Species of Lepidoptera vary in their inherent susceptibility to *B.t.k.* proteins (Hofte and Whiteley 1989, Krieg and Langenbruch 1981, MacIntosh *et al.* 1990). The tobacco budworm is more susceptible than the bollworm to the endotoxin proteins. However, initial field tests (Benedict *et al.* 1991, 1993, Jenkins *et al.* 1992) suggest that *B.t.k.*-cotton will provide a high level of field control of both pest species. Nationwide, the tobacco budworm-bollworm complex accounts for 39.6% of all acre applications (acre application = 1 application of an insecticide on 1 acre) of insecticide in cotton (Table 2). The percent of total insecticide applications directed at the tobacco budworm-bollworm complex is 58.2, 37.2, 30.3, and 3.6%, respectively, for the Southeast, Mid-

south, Southwest and West regions of cotton production in the USA. Considerable variation exists among (Table 1) and within (Table 3) different cotton production regions in the intensity of insecticide use for tobacco budworm-bollworm; however, eliminating insecticide use for this pest complex would be a major economic and ecological accomplishment for USA cotton production. During the past 3 years, USA cotton growers have annually spent an average of $180 million for control of this pest complex on cotton. Although *B.t.k.*-cotton may not eliminate all of the insecticide applied to control tobacco budworm-bollworm on all of the cotton acreage in the USA, current research indicates that the technology possesses efficacy necessary to have a major impact on insecticide use directed at tobacco budworm-bollworm. Actual use in the production system will be influenced by marketing policies and alternatives.

Potential effects of *B.t.k.*-cotton on species of Lepidoptera other than the tobacco budworm-bollworm complex are less defined. Armyworms (*Spodoptera* spp.) are more tolerant to endotoxin proteins than the tobacco budworm and the bollworm (Jenkins *et al.* 1992), and the level of control expected from *B.t.k.*-cotton is questionable at this time. However, large plot field studies have suggested that damage from the beet armyworm (*S. exigua*) will be reduced in *B.t.k.*-cotton (Luttrell personal observation., Wilson *et al.* 1992). Control of these less susceptible species may be higher than that suggested from results of laboratory assays because the continuous expression of insecticidal activity in the transgenic plants will insure continuous contact with the toxin. A cumulative toxic effect is likely. Several other pest species, particularly the pink bollworm (*Pectinophora gossypiella*), are very susceptible to endotoxin (Graves and Watson 1970), and *B.t.k.*-cotton offers an excellent opportunity to reduce insecticide use for these species. If one assumes that *B.t.k.*-cotton will effectively eliminate insecticide applications for all lepidopterous pests of cotton, insecticide use could be reduced by more than 45% (Table 2) and the total costs of controlling cotton insects could be reduced by approximately 50% (Table 2). However, the effects of *B.t.k.*-cotton on many species of Lepidoptera are not experimentally tested, and these savings do not include the price of *B.t.k.*-cotton seed. Projecting benefits of *B.t.k.*-cotton on the basis of eliminating the crop loss and insecticide costs of all Lepidoptera is likely an over-estimate of the actual benefits of *B.t.k.*-cotton. However, the vast majority of control costs and crop loss due to lepidopterous pest attacking cotton are associated with species that are very susceptible to *B.t.k.*-cotton (tobacco budworm, bollworm, pink bollworm). Most of the insecticide directed against lepidopterous pests in the Mid-south and Southwest is targeted at the tobacco budworm-bollworm complex. In the Southeast, more insecticide is used for control of other lepidopterous pests, especially the beet armyworm, European corn borer (*Ostrinia nubilalis*), and soybean looper (*Pseudoplusia includens*), but tobacco budworm-bollworm is the primary target of control measures. Most of the insecticide directed against lepidopterous pests of cotton in the West is targeted at pink bollworm. Because pink bollworm is extremely susceptible to endotoxin proteins (Graves and Watson 1970, Bartlett 1993), *B.t.k.*-cotton offers a

unique opportunity to reduce insecticide use in the West for pink bollworm and improve the marginal efficacy of current chemical control methods. Eliminating use of insecticide for lepidopterous pests of cotton in the entire USA would result in a savings of $206 million in insecticide costs to the cotton industry (Table 2).

Expanded Opportunities for Biological Control

Field surveys indicate that the number of arthropod species associated with the cotton may range from a few hundred to more than a thousand (Hearne and Fitt 1992). Most of these species are predators and parasites of the phytophagous species, and most of the crop damage can usually be explained by the presence of 5 to 10 pest species. Damaging populations of arthropod pests are often associated with insecticide use. Broad spectrum insecticides disrupt the ecological interrelationships among the numerous pest arthropods and their natural enemies, and often result in a rapid increase in pest densities when natural enemies are eliminated. Reductions in insecticide use due to planting *B.t.k.*-cotton would enhance natural control and provide a better opportunity for augmentative approaches to biological control which have historically been limited in cotton because of the disruptive nature of insecticides (King and Coleman 1989). The extent of expanded opportunities is difficult to estimate because *B.t.k.*-cotton will not eliminate the need for insecticides against non-lepidopterous pests of cotton. Some pest species have been historically controlled by applications directed at lepidopterous pests. If the applications directed at the lepidopterous pests are removed, additional applications may be required to suppress these previously unrecognized pest problems. Conversely, some pest species have reached pest status because insecticide applications directed at lepidopterous pests disrupted natural control agents. Reductions in applications for control of Lepidoptera would likely result in reduced need to control some insecticide induced pests. Although the extent of expanded opportunities for biological control is unknown, *B.t.k.*-cotton certainly represents one of the most realistic opportunities in the history of cotton IPM to enhance biological control of cotton insects.

Improved Control of Some Pest Species

Some lepidopterous pests of cotton, such as the pink bollworm and fall armyworm (*S. frugiperda*), possess behavioral characteristics which allow them to avoid contact with insecticide deposits on upper portions of the plant canopy. Insecticide sprays cannot dependably deliver insecticide deposits to lower portions of the plant canopy. Since *B.t.k.*-cotton expresses endotoxin proteins in all plant tissues, pest species which are commonly located in plant canopy levels shielded from insecticide deposits or within fruiting structures will not be able to escape contact with the insecticidal toxins.

Foliar applications of *B. thuringiensis* have historically resulted in variable levels of control of cotton insects (Phillips *et al.* 1979). This variable performance in efficacy has been associated with the need for the target insect pests to ingest spray deposits of *B. thuringiensis* on the upper plant canopy. These foliar sprays have limited residual activity, and fruit-feeding insects such as the tobacco budworm and bollworm typically feed in plant locations that receive reduced deposits of insecticide. Precise timing of treatments relative to larval development and location in the plant canopy is critical to obtain adequate control.

The continuous expression of insecticidal activity by *B.t.k.* plants should eliminate management decisions and risks associated with accurate timing of insecticide treatments for pests susceptible to *B.t.k.*-cotton. Routine crop and insect monitoring will continue to be an important component of cotton IPM programs because of the variation in susceptibility of different lepidopteran pests to endotoxin proteins (MacIntosh *et al.* 1990) and the presence of numerous non-lepidopterous pests in USA cotton. Changes in some pest management procedures will be necessary because lepidopteran insects must feed on the plant to receive a toxic dose of insecticide and current management techniques rely to some extent on detection of insect eggs to trigger control action. Ring *et al.* (1993a) describe changes that may be required in treatment threshold recommendations.

Environmentally Safe Mode of Action and Delivery System

The insecticidal proteins of *B.t.k.*-cotton are derived from one of the most studied and environmentally-safe biological insecticides, *B. thuringiensis* var. *kurstaki* (Burgess 1981, Burgess and Hussey 1971, Heimpel 1967). This bacterium is a common soil-borne pathogen of insects that produces a proteinaceous crystalline structure during sporulation. Use of *B. thuringiensis* as a microbial insecticide spans more than 100 years, and commercial products have been registered for use on a wide range of USA crops since the early 1960's. This environmentally-safe, microbial insecticide is used for control of lepidopterous pests in many environments ranging from home vegetable gardens to area-wide spraying of national forests.

Insecticidal activity of the bacteria is associated with the crystalline structure which must be consumed by an insect and activated in the insects midgut to become insecticidal. The insecticidal toxins or protein subunits of the intact crystal (endotoxins) are activated through the action of proteolytic enzymes on the crystalline structure in the insects midgut. These proteinaceous subunits bind to receptors on the midgut lining of the insect and create ruptures or pores in the midgut epithelial cells. As a result of this action, the contents of the insects gut and the insects hemolymph (blood) are no longer separated. The insect generally dies of gut paralysis, although septicemia may occur when an insect ingests an intact bacterial cell with spore and crystal. *B.t.k.*-cotton mimics the gut paralysis mode of action.

Considerable variation exists in the range of activity of different varieties and isolates of *B. thuringiensis* (Burgess 1981, Hofte and Whiteley 1989). The insecticidal activity of *B.t.k.*-cotton is derived from the insecticidal activity of *B. thuringiensis* var. *kurstaki* which is only toxic to lepidopteran insects. Other varieties or subspecies of *B. thuringiensis* exhibit activity against Coleoptera (beetles) and Diptera (flies).

Because insecticidal activity of *B.t.k.*-cotton is derived from the environmentally safe *B. thuringiensis* var. *kurstaki*, *B.t.k.*-cotton offers a unique mechanism to deliver an alternative insecticidal action (gut paralysis) to a limited range of insect pests (lepidopterans). Most conventional insecticides used in cotton are nerve poisons that potentially affect a wide range of target and non-target organisms. *B.t.k.*-cotton will only affect the lepidopteran insects that feed on the plant tissue, and it will only kill insects with appropriate binding sites in their midgut. Non-target exposure of insects belonging to other orders or other animals is eliminated or greatly reduced as compared to that associated with conventional insecticides.

The mode of action of *B.t.k.*-cotton also offers an efficacious alternative to the nerve poisons. Alternating and mixing insecticidal modes of action is an important component of some resistance management strategies. Conventional formulations of *B. thuringiensis* also offer an alternative mode of action, but their efficacy against fruit-feeding insects of cotton is limited (Phillips *et al.* 1979).

Since *B.t.k.*-cotton will deliver the toxic agent to the target insect by producing an insecticidal protein within the plant tissue that serves as a food source for the insect, non-target exposure to the toxic agent is greatly reduced. Application costs should also be reduced, and the need for manufacturing, shipping, storing, and handling costs of traditional chemical insecticides should be eliminated or reduced. This improved safety to farm workers and reduced exposure of non-target organisms should be viewed as a major advantage of transgenic technology.

Unique Opportunities for Population Regulation of Some Pests

Although the effects of *B.t.k.*-cotton on population growth of target pests are difficult to estimate and will ultimately be influenced by many biological and ecological factors (e.g. number of other plant species attacked by the pest, dispersal or migration range of species involved, extent of farmer adoption of *B.t.k.*-cotton, methods of *B.t.k.*-cotton deployment, host range of species involved, insertion of *B.t.k.* genes in other crops, etc.), the high levels of pest mortality observed in recent experiments suggests that *B.t.k.*-cotton could have a major impact on population growth of lepidopterous pest species susceptible to endotoxin, especially tobacco budworm and pink bollworm. Autocidal and some augmentative biological control methods of insect control are typically targeted at population suppression or eradication of pest species (King and Coleman 1989, Laster

et al. 1988). The success of these projects often depends on creating a high ratio of released insects to native insects. If *B.t.k.*-cotton were planted over a high portion of the cotton acreage in a given area, it is possible that populations of some lepidopterous pests, particularly the very susceptible tobacco budworm, could be dramatically reduced. While these populations were at extremely low levels, autocidal and biological control programs would have a unique opportunity to create high ratios of released insects to naturally-occurring insects. It is important to note that resistance management strategies require refugia (i.e. refuge locations where the pest's food plants do not contain *B.t.k.* genes and susceptible insects can survive) (Fischoff 1992, McGaughey and Whalon 1992). Therefore, it would not necessarily be advantageous to plant a high fraction of the total cotton acreage in an area to *B.t.k.*-cotton. Given that the high efficacy of *B.t.k.*-cotton may provide a unique opportunity for the release of autocidal or biological control agents, further examination and experimentation of these issues are warranted.

Relationships with Other Control Measures

B.t.k.-cotton is exceptionally compatible with many other ecologically sound methods of insect control such as biological control and host plant resistance. Research is actively investigating the pyramiding of traditional host plant resistance traits (plant secondary chemistries and morphologies) with the transgenic expression of endotoxin proteins (Benedict *et al.* 1993, Sachs *et al.* 1993). Certain secondary chemistries, such as increased concentrations of terpenes and tannins, have been utilized in some cottons to reduce injury from the tobacco budworm-bollworm complex. Traditional host plant resistance mechanisms to suppress plant damage from the tobacco budworm-bollworm complex, cabbage looper, and pink bollworm have low ability to suppress damage and kill pests (in the range of 20 to 60% larval mortality) compared to *B.t.k.*-cotton (90 to 100% larval mortality) (Benedict *et al.* 1976, 1985, Wilson *et al.* 1992, Zummo *et al.* 1983). Preliminary results show that pyramiding the traditional host plant resistance mechanisms with *B.t.k.*-cotton increases plant resistance to bollworm (Sachs *et al.* 1993). *B.t.k.*-cotton can be viewed as the first successful example of an antibiosis mechanism of host plant resistance in cotton. Because host plant resistance mechanisms are inherent to the plant's genome and they begin their pest defenses at plant emergence (Benedict *et al.* 1988), they are the foundation of all other IPM opportunities.

Preliminary field studies conducted by Monsanto and Mississippi State University suggest that *B.t.k.*-cotton does not exhibit a direct, negative impact on the major predators and parasites in cotton (Stone, unpublished data, Monsanto; Luttrell, unpublished data, Mississippi State University). Current preliminary research in large-plot experiments does suggest that densities of natural enemies, particularly some predators, are affected indirectly by the density of pest species present (Luttrell, unpublished data). Because densities of predators and parasites respond to the densities of the pest (i.e. prey or host species), a decrease in densities of parasites and

predators is expected as densities of pest species decline. Additional experimentation in large plots is necessary to confirm these initial, but anticipated observations.

Community Ecology and Managing Insect Resistance to *B.t.k* Cotton

In many USA cotton production regions, cotton is grown in close proximity, a mosaic, with corn, soybean, sorghum, vegetables, and other crops. Polyphagous insect species often utilize several crops and population growth of a pest on one crop is often dependent upon management actions in another crop. The seasonal buildup of massive populations of sweet potato whitefly (*Bemisia tabaci*) as a result of favorable cropping sequences of multiple crops in a given geographic region (Watson *et al.* 1992) illustrates the importance of crop and community ecology in insect management. These relationships are often poorly understood and warrant additional research.

Community ecology issues associated with managing the development of insect populations resistance to endotoxin proteins are of particular interest. Some insect species like soybean looper occur in soybean and cotton. The soybean looper is effectively controlled in soybean with foliar applications of *B. thuringiensis* and has limited pest status in cotton. However, soybean looper only reaches pest status on soybean in areas where soybean is grown in close proximity to cotton, presumably because the female moths utilize cotton as a source of nectar with significant increases in fecundity (Burleigh 1972). *B.t.k.*-cotton could provide a mechanism to suppress population growth of soybean looper. It could also provide a source of selection for resistant genotypes that would decrease the effective life of foliar applications of *B. thuringiensis* on soybean. Deployment strategies for *B.t.k.*-cotton that include refugia as a component of resistance management for tobacco budworm should also limit the selection for endotoxin resistance in the soybean looper. The extent of soybean looper reproduction in cotton is unknown, and although the moths use cotton as a source of nectar, most oviposition probably occurs in soybean. This example is presented to illustrate the importance of community ecology to effective management of polyphagous insect pests.

The development of transgenic corn expressing endotoxin proteins of *B. thuringiensis* would also create several social and biological questions relative to management of insect resistance to the endotoxins in the numerous species of polyphagous insects (European corn borer, fall armyworm, cotton bollworm) inhabiting both crops within the same cropping region. As with the soybean looper, the suppressive action of transgenic plants can enhance the selection for resistant genotypes (Gould 1988), but it can also provide a highly effective population suppression mechanism. These ecological relationships need additional examination. It is important to note that the impact of insect control activities on multiple cropping systems within an area is not unique to *B.t.k.*-cotton. The same concerns should be expressed for all insecticides targeted at polyphagous insect species on most agronomic crops.

The potential impact of pest resistance on the long-term utility of *B.t.k.*-cotton is a significant issue (McGaughey and Whalon 1992). Any insect control method that provides high levels of control is likely to provide significant selection for the evolution of resistant genotypes. The value of *B.t.k.*- cotton for managing crop pest injury is high and warrants protection from resistance problems. Private (Fischoff 1992) and public sector (McGaughey and Whalon 1992) scientists are addressing these issues. It is important to note that the continuous, constant expression of insecticidal activity by *B.t.k.* plants may make *B.t.k.* cotton an ideal theoretical technology for resistance management. Some problems with the development of insecticide- resistant pest populations are associated with the decaying of the active ingredient on the plant and thus the selective killing of susceptible genotypes at low doses. The continuous, high-dose expression of insecticidal activity by *B.t.k.*-cotton would avoid the influence of insecticidal decay on selection for resistance in susceptible pest populations.

Relationships with Boll Weevil Control and Management of Other Insect Pests

The ability of *B.t.k.*-cotton to protect the plant from insect injury without disrupting natural control of insects compliments the goals of several contemporary programs designed to eliminate or manage the boll weevil (*Anthonomus grandis grandis*) in USA cotton. USDA entomologists in the Rio Grande Valley of Texas are developing a biological control program to control boll weevils that is dependent upon *B.t.k.*-cotton becoming available to control the tobacco budworm-bollworm complex rather than insecticides (Summy *et al.* 1993). The program is using a small wasp that attacks and kills the boll weevil larva. The wasp is very susceptible to insecticides used to control the tobacco budworm-bollworm complex. Combining *B.t.k.*-cotton with this program would control three major pests of cotton (boll weevil, tobacco budworm, bollworm) without conventional insecticides. The reduction in insecticide usage would be increased over that estimated for deployment of *B.t.k.*-cotton alone. The ideal addition to this program would be a plant bug resistant cotton variety to eliminate almost all insecticide usage. Plant bug resistant varieties are grown in several areas of Texas (Masud *et al.* 1990, Ring *et al.* 1993b).

The Boll Weevil Eradication Project has successfully removed boll weevil as a major pest of cotton in much of the Southeast. The program will expand into the Mid-south and Southwest in the near future. Successful removal of the boll weevil as a pest of cotton would further reduce the need for insecticide applications and expand opportunities for non-insecticidal control measures in conjunction with *B.t.k.*-cotton. Most of the insecticide applications made to cotton in the Mid-south and Southwest are targeted at two pests, the boll weevil and the tobacco budworm-bollworm complex. The boll weevil is, perhaps, the most important key pest of cotton because its presence in a management system triggers control actions early in the season. The early season applications often reduce densities of parasites

and predators and set the stage for subsequent insect pest outbreaks. Removal of the boll weevil coupled with an ecological sound method of managing the tobacco budworm-bollworm complex would offer opportunities for management of cotton insects not previously possible.

Although *B.t.k.*-cotton and boll weevil eradication will have a major impact on insecticide use in cotton, they will not eliminate the need for insecticides. The sucking pests of cotton (mirids, aphid, whiteflies, thrips, etc.) will not be directly affected by the *B.t.k.*-cotton insecticidal proteins, nor will the eradication project eliminate the need for control of sucking pests. In fact, some pests previously suppressed by chemical insecticides directed at tobacco budworm-bollworm and/or boll weevil may emerge as being more important. In the Southeast, this happened when boll weevil sprays were eliminated by the eradication effort and stinkbugs became more common as a pest of cotton (Barbour *et al.* 1988). *B.t.k.*-cotton will not eliminate the need for crop monitoring and management by professional scouts. Trained professionals must be available to note the changes in the pest complex and implement appropriate plant protection measures. They must also be relied upon to integrate *B.t.k.*-cotton into an overall insect management and crop production scheme.

Implications of *B.t.k.* Cotton Introduction on Current IPM Programs in Different Geographic Regions of the USA

Based on the Beltwide Cotton Conference estimates (Head 1990, 1991, 1992) summarized in Tables 1, 2, and 3, and assuming that *B.t.k.*-cotton would effectively remove the crop loss and control costs of all lepidopterous pests of cotton, introduction of *B.t.k.*-cotton on all USA cotton acreage would reduce total losses to cotton arthropod pests by 42.6% or $312 million. However, this estimate assumes that *B.t.k.*-cotton is highly active on all lepidopteran pests of cotton. It is not equally effective on all lepidopteran pests and additional research is necessary to measure the crop protection provided by *B.t.k.*-cotton against lepidopteran pests less susceptible to endotoxin proteins (e.g. armyworm species). If the assumptions regarding effectiveness were reduced to the savings associated only with tobacco budworm-bollworm, a conservative assumption based on confirmed efficacy of *B.t.k.* cotton in field experiments, total annual losses would be reduced 35.3% or $258 million.

The benefits of *B.t.k.*-cotton vary with the production capabilities and pest spectrums of the different geographic regions of cotton production in the USA. Considering the acreage involved and average annual costs of control plus crop loss, the total annual cost of all lepidopterous pests (including tobacco budworm-bollworm) to cotton producers in the Southeast, Mid-south, Southwest, and West is $70.78 million, $150.19 million, $56.25 million, and $34.75 million (Table 2), respectively. Similar total costs for the tobacco budworm-bollworm complex alone are $54.08 million, $145.83 million, $55.05 million, and $3.64 million, respectively, for the Southeast, Mid-south, Southwest, and West (Table 2). These data illustrate the importance of the tobacco budworm-bollworm complex in the Southeast, Mid-south and Southwest, and the importance of pink bollworm in the West.

Opportunities for expanding cotton IPM with the introduction of *B.t.k.*-cotton vary among and within (Table 3) each production region. The following provides a prospectus of the potential impact of *B.t.k.*-cotton on regional cotton IPM programs in the Southeast, Mid-south, Southwest, and West.

Southeast

The Southeast region of the USA Cotton Belt is one of the areas that has a high potential for benefiting from the utilization of transgenic *B.t.k.*- cotton. This area historically has the highest populations of tobacco budworm-bollworm complex extending over a longer period of time than anywhere else in the Cotton Belt. As a result the Southeast receives high inputs of insecticide.

During the last few years, the average crop loss to the tobacco budworm-bollworm complex in the Southeast ranged from 1.3% in South Alabama to a high of 6.8% in North Carolina (Table 3). This represents the preponderance of all loss from lepidopterous insect pests. Insecticide use on cotton in the Southeast, likewise, is targeted primarily at the tobacco budworm-bollworm complex with a low of 1.4 applications per season in Virginia to 5.7 applications in Florida, compared to 1.4 to 6.0 for all lepidopterous pests in the same regions, respectively. These data are an average of 1990 - 1992 estimates and may be somewhat low in comparison to historical averages. Prior to the Boll Weevil Eradication Program, applications in the Southeast ranged from about 4 in the areas of lowest infestations to as many as 12 in the more heavily infested areas. A substantial portion of the reduction in insecticide use can be directly attributed to the lack of disruption of the natural enemy complex historically associated with insecticidal control of the boll weevil.

Cotton acreage in the Southeast is increasing rapidly as a result of the success of the Boll Weevil Eradication Program. Growers have found that the crop can be grown with a wider margin of profit than many alternative crops. This is due largely to a reduction in insecticide input for boll weevil. However, high inputs of insecticide are still required for control of the tobacco budworm-bollworm complex. If the impact of this pest complex could be dramatically reduced or eliminated with the use of *B.t.k.*-cotton, additional acreage might be placed into cotton production in the Southeast.

The indirect impact of *B.t.k.*-cotton on secondary, non-lepidopterous pests may also allow for a reduction in insecticide applications. The natural enemy complex for aphids, *Lygus* spp. and whiteflies are typically destroyed by insecticide applications targeted at other pests. If these disruptive applications are reduced the natural enemy complex may be allowed to regulate secondary pest populations. Biological control is a recognized important component of cotton aphid control in most of the Southeast with fewer insecticide applications being required for their control than in other regions (Head 1990, 1991, 1992). The benefits of

reduced insecticide use can be extended to management of other pests. In 1992, fields in south Georgia that had not been treated with insecticide were found to have a high rate of parasitization (up to 90%) in sweet potato and bandedwinged whitefly populations. Fields that had been treated with insecticide did not benefit from the whitefly parasitoids, and populations of the pest reached high densities with subsequent crop loss (Herzog, unpublished data).

The development of resistance to endotoxin is an obvious concern once commercialization and widespread utilization of *B.t.k.*- cotton has occurred. The Southeast may not be as likely to be affected from this problem as other areas of the Cotton Belt. Historically, resistance to insecticide classes have shown up in other areas much earlier and at a much higher magnitude than in the Southeast. It is believed that the reason for this is because of the tremendous diversity in agricultural enterprises that may be found in the Southeast. There are numerous crops that are grown that do not have the intensity of insecticide inputs associated with cotton. These crops provide refugia for untreated tobacco budworm-bollworm populations and thus delay resistance buildup.

Mid-south

The Mid-south is one of the major targets for marketing of *B.t.k.*-cotton because of the large acreage typically treated several times annually with insecticide for control of tobacco budworm-bollworm. This pest complex costs Mid-south growers ca. $145 million each year in insecticide costs and crop loss. The intensity of the pest pressure varies within the region. The % crop loss due to tobacco budworm-bollworm complex ranges from 0.74% in Tennessee to 4.98% in Louisiana. Tennessee growers apply an average of 0.74 applications of insecticide per acre for tobacco budworm-bollworm control. Louisiana growers apply an average of 4.8 applications per acre.

Other than the tobacco budworm-bollworm complex, Mid-south cotton insect problems are dominated by the presence of boll weevil, a complex of mirids (particularly the tarnished plant bug, *Lygus lineolaris*), thrips, and aphids. Introduction of *B.t.k.*-cotton is likely to reduce total insecticide inputs in the Mid-south, but the extent of the reduction is unclear. Cotton will continue to need infurrow insecticides for thrips control. Foliar applications of insecticide will also be required for boll weevil and mirids during the early season. Introduction of *B.t.k.*-cotton may reduce the need for some insecticide applications against aphids because they are largely an insecticide-induced pest problem. Traditional applications of insecticide for tobacco budworm-bollworm also provide some suppression of other pests, particularly mirids in the Mid-south. Removal of the insecticide treatments for tobacco budworm-bollworm would likely result in an increase in applications for some other pests, probably tarnished plant bug. Based on current observations and limited data, it appears that *B.t.k.*-cotton may result in a reduction of 2-4 insecticide applications per year across most of the cotton in the Mid-south depending upon the development of secondary pest problems.

Recent problems with insecticide resistant populations of tobacco budworm and cotton aphids are threatening profitable cotton production in some areas of the Mid-south (Luttrell 1994). Growers in the Mississippi Delta spent more than $100 per acre during 1992 for cotton insect control. Because of these problems, Mid-south growers will readily adopt *B.t.k.*-cotton when it is commercially available, and insecticide use should decline as the technology is deployed.

Interestingly, the planned commercial release of *B.t.k.*-cotton coincides with the westward movement of the Boll Weevil Eradication Program into the Mid-south. The vast majority of all insecticide use on Mid-south cotton is targeted at the tobacco budworm-bollworm complex and the boll weevil. Simultaneous introduction of *B.t.k.*-cotton and implementation of boll weevil eradication efforts offers a historical opportunity to dramatically reduce the use of conventional insecticides on Mid-south cotton. Mirid pests, especially the tarnished plant bug, are likely to emerge as the most important key pests of cotton. Research should be initiated immediately to develop appropriate management strategies for the sucking pests of cotton assuming insecticide applications for tobacco budworm-bollworm and boll weevil will be dramatically reduced.

Southwest

The Southwest cotton production region of New Mexico, Oklahoma and Texas harvested cotton from an average of 5.2 million acres per year during the 1990-1992 period (Table 1). The Southwest has yields ranging from less than one-half bale per acre on arid dryland cotton, to three bales per acre on irrigated river bottom land. The tobacco budworm-bollworm complex costs Southwest growers ca. $55 million each year in insecticide costs and crop loss (Table 2). The % crop loss in Texas due to the tobacco budworm-bollworm complex ranges from 0.67 in Texas District 10 to 7.96 in Texas District 13. District 13 is a high input irrigated area known as the Winter Garden because of its winter production of numerous vegetable crops. It is a green island surrounded by arid desert and brushland. District 13 also has the highest number of insecticide applications, 6.17, for the tobacco budworm-bollworm complex whereas Texas District 1 has the lowest, 0.43. The intensity of injury for this complex varies across the region and between years.

In New Mexico and Oklahoma the % crop loss due to the tobacco budworm-bollworm complex ranges from 3.14 to 2.00, respectively. New Mexico growers apply an average of 0.63 applications of insecticide per acre and Oklahoma growers apply 1.10 applications per acre for tobacco budworm-bollworm control. Most of the % crop loss and number of applications for all Lepidoptera in the Southwest region are due to the tobacco budworm-bollworm complex. However, in some Texas Districts beet armyworms and fall armyworms occur occasionally in densities requiring treatment. In New Mexico and Texas District 1 the pink bollworm is a frequent pest requiring insecticidal control.

The other primary pests of Texas and Oklahoma cotton are the boll weevil and a complex of mirids (tarnished plant bug, lygus bugs, and cotton fleahopper) that vary in severity across the region. Some areas such as the High Plains of Texas, Districts 1, 2, and 3, and New Mexico are free of boll weevil infestations or experience only infrequent sub-economic infestations.

Introduction of *B.t.k.*-cotton should reduce insecticide use dramatically in some areas particularly where boll weevils are not pests such as New Mexico and Texas Districts 1, 2, and 3. Throughout areas of Texas two different insecticides (pyrethroids for tobacco budworm-bollworm and organophosphorus insecticides for boll weevil) are applied simultaneously to control the tobacco budworm-bollworm complex and boll weevils. However in other areas growers use pyrethroids or methyl parathion (an organophosphorus insecticide) alone to simultaneously control both pests. In those areas where two insecticides are used, it is expected that with the introduction of *B.t.k.*-cottons a reduction in insecticide usage and a savings in dollars will occur.

In recent years insecticide-resistant tobacco budworm (particularly to pyrethroids) have been reported in several Texas Districts where insecticide usage is high, such as District 13. Moreover where multiple late season applications of pyrethroids are used frequently, outbreaks of spider mites or aphids occur which may require additional insecticide applications. Introduction of *B.t.k.*-cotton could relieve developing pyrethroid resistance problems, reduce secondary outbreaks of spider mites and aphids, and reduce insecticide applications in many cotton producing areas of the Southwest. Across the Southwest region reductions could range from 0 to 8 applications with an estimated average reduction of 1 application on 5.2 million acres per year. However the extent of the reduction is unclear.

West

Cotton pest control strategies in the desert Southwest (Arizona and Southern California) changed dramatically in 1966. This resulted from the spread of the pink bollworm, *Pectinophora gossypiella* (Saunders) across the entire cotton-producing area of Arizona and southern California. Once established in these areas the pink bollworm required routine scheduled insecticide applications in order to maintain it below economic thresholds. Initially growers were applying up to 20 or more insecticide applications in order to control this insect alone. Once research established sound economic thresholds and more precision in timing of applications, this number was reduced to a more reasonable level. The numbers vary from area to area, generally associated with elevation, e.g., in eastern Arizona the average number is 4-5, in central Arizona 6-8, and western Arizona and southern California 9-12 applications are generally required.

Were it not for the need to control pink bollworm (excluding the present problem with the sweet potato whitefly) much less insecticidal control would be needed, and then, on a non-scheduled basis.

A related impact of the pink bollworm in the desert Southwest has been the continual threat to the vast acreage of cotton in the San Joaquin Valley of California. High infestations in the Imperial Valley provide the source for wind movement of moths into the San Joaquin Valley. This initiated an annual multi-million-dollar, sterile-moth release program in the San Joaquin Valley in an effort to prevent establishment of the pink bollworm there.

Indirect costs associated with the pink bollworm problem have been those resulting from secondary pest outbreaks following scheduled applications for this key pests.

The biology and seasonal history of the pink bollworm make it ideally suited for management with *B.t.k.*-cotton. The pink bollworm over-winters as diapausing larvae in the cotton field where they were produced. Spring moth emergence occurs over an extended period of time with a large proportion of the moths emerging and dying prior to the production of susceptible cotton fruit (squares). Therefore, the key to the initiation of a new years infestation is the coincidence of susceptible fruit and last-emerging moths. This coupled with high winter mortality, results in a fairly low population level to start the new infestation. A high level of mortality during this first generation would probably preclude the subsequent development of populations to damaging levels for the rest of the season.

Without the need for scheduled applications of insecticides for pink bollworm control, a great deal of flexibility would be possible with the remainder of the pest complex in western cotton. For example, biological and cultural control methods could be used for management of other pests such as lygus bugs, *Heliothis* spp., beet armyworm and cotton leafperforator.

There are indications of low-level resident populations of pink bollworm in the southern end of the San Joaquin Valley. If this persists and spreads, scheduled applications of foliar sprays will be required in order to prevent serious loss. This would almost surely result in serious wide-spread outbreaks of spider mites and certain other Lepidoptera such as bollworm and cabbage looper.

By eliminating the pink bollworm as an in-season pest requiring scheduled insecticide applications, a number of benefits would ensue which would re-establish western cotton production as a profitable enterprise. In addition to the overall improvement in the management of all other pests, and more economically at that, yields would probably move upward towards the levels obtained in pre-pink bollworm days.

Summary and Overall Prospectus of *B.t.k.* Cotton

Results of published experiments with Monsanto's *B.t.k.* cottons indicate that these transgenic cottons exhibit a high level of efficacy against tobacco budworm and pink bollworm, and that bollworm is effectively controlled in field environments by these cottons. We believe that *B.t.k.*-cotton offers opportunities for improved pest control, and that the technology will be actively sought by growers for control of the lepidopterous pests of cotton susceptible to endotoxin proteins. Control of other lepidopterous pests, such as armyworm species which are less susceptible to endotoxin proteins, is possible but sufficient data are not available to suggest that *B.t.k.*-cotton will eliminate insecticide treatments for these more tolerant pests.

Transgenic technology provides an innovative, unique mechanism to deliver an insecticide selectively to target pest species. This selective activity will not disrupt populations of beneficial insects as is the case with traditional control measures that use broad-spectrum, nerve-toxin insecticides. Opportunities for expanded use of biological control should develop as the intensity of insecticide use is reduced with expanded implementation of *B.t.k.*-cotton.

Major economic and management advantages of *B.t.k.*-cotton are associated with its potential to reduce the use of traditional insecticides and increase yields of USA cotton. Reduced insecticide use with *B.t.k.*-cotton is likely, but the extent of reduction is difficult to predict because of the dynamic, interrelationships among cotton pest and beneficial arthropods. If *B.t.k.*-cotton effectively reduced all insecticide inputs for lepidopterous pests of cotton in the USA without altering control inputs for other pests, cotton growers would save $312 million in control costs and crop damage. If the reduction in insecticide use and crop loss is limited to that due to the tobacco budworm-bollworm complex, growers would still save $258 million. These estimates do not consider the added cost of *B.t.k.*-cotton seed to the farmer.

The development of *B.t.k.*-cotton provides a unique opportunity to manage lepidopterous pests of cotton with a highly efficacious, environmentally safe control measure. The technology couples the efficacy of an effective insecticide with the environmental advantages of host plant resistance. This technology should serve as the foundation for historical improvements in cotton IPM programs over the next 5 to 10 years.

Table 1. Production and insect control characteristics of different geographic regions of cotton production in the USA.

Characteristic	Geographic Region Southeast	Mid-south	Southwest	West
Acres Harvested X1000	1408	3794	5208	1456
Yield in Bales Per Acre	1.30	1.50	0.77	2.53
% Crop Loss to *Heliothis* and *Helicoverpa*	3.4	2.7	1.4	0.1
% Crop Loss to Lepidoptera	4.5	2.7	1.4	1.0
% Crop Loss to All Insects	6.8	6.7	5.2	6.0
No. Insecticide Applic./Acre for *Heliothis* and *Helicoverpa*	3.5	3.0	0.8	0.2
No. Insecticide Applic./Acre for Lepidoptera	4.1	3.1	0.8	1.6
No. Insecticide Applic./Acre for All Insects	6.6	7.4	2.5	3.9
$ Spent/Acre for Control of *Heliothis* and *Helicoverpa*	24.14	25.45	7.16	1.71
$ Spent/Acre for Control of Lepidoptera	31.16	26.60	7.39	15.89
$ Spent/Acre for Control of All Insects	49.42	48.04	18.59	49.44

* Calculated as an average of annual estimates published by the Beltwide Cotton Conferences (Head 1990, 1991, 1992).

** Estimates of % crop loss, number of insecticide applications per acre, and $ spent per acre for Lepidoptera include similar estimates for *Heliothis* and *Helicoverpa* (tobacco budworm and bollworm). Estimates for all insects include those for Lepidoptera.

Table 2. Total annual expenditures for cotton insect control in different geographic regions of the USA (average of 1990-1992 data).

	Total Annual Estimated Amount X 1,000,000				
	Southeast	Mid-south	Southwest	West	Entire USA
Estimates for Heliothis-Helicoverpa Complex					
No. of Acre Applications	4.93	11.27	4.17	0.06	20.42
$ Expended for Control Costs	33.98	96.56	37.29	2.49	170.32
$ Crop Loss Above Control Costs	20.10	49.27	17.76	1.15	88.29
$ Total Loss	54.08	145.83	55.05	3.64	258.61
Estimates for All Lepidoptera					
No. of Acre Applications	5.74	11.61	4.32	2.27	23.94
$ Expended for Control Costs	43.87	100.92	38.49	23.14	206.42
$ Crop Loss Above Control Costs	6.91	49.27	17.76	11.61	105.56
$ Total Loss	70.78	150.19	56.25	34.75	311.98
Estimates for All Insects					
No. of Acre Applications	9.32	28.23	11.18	9.83	51.55
$ Expended for Control Costs	69.58	182.26	96.82	71.98	420.65
$ Crop Loss Above Control Costs	41.67	127.51	68.63	73.36	311.16
$ Total Loss	111.25	309.77	165.45	145.34	731.81

* Calculated from annual estimates published by the Beltwide Cotton Conference (Head 1990, 1991, 1992). Dollar values for yield loss assumed that each bale weighed 480 pounds and that cotton was valued at $0.65 per pound. Seed values were not included.

** Estimates for All Lepidoptera include estimates for *Heliothis-Helicoverpa* (tobacco budworm-bollworm). Estimates for All Insects include estimates for All Lepidoptera.

Table 3. Cotton crop losses and control expenditures for *Heliothis-Helicoverpa* (HEL), all Lepidoptera (LEP), and all arthropod pests (ALL) within major geographic production regions of the USA.*

	% Crop Loss Due To			No. Insecticide Applic./Acre For			$ Spent/Acre For Control Of		
	HEL	LEP†	ALL†	HEL	LEP	ALL	HEL	LEP	ALL
SOUTHEAST REGION									
Alabama-Central	1.67	3.07	7.60	3.67	4.00	9.80	18.25	9.42	43.34
Alabama-North	2.23	2.32	9.84	1.80	1.80	5.57	9.55	9.55	17.39
Alabama-South	1.30	3.42	5.90	4.30	5.03	7.77	21.60	33.80	40.30
Florida	5.37	6.01	6.22	5.73	6.00	6.57	48.19	51.88	57.91
Georgia	2.58	4.68	5.34	4.43	5.93	9.07	28.85	43.80	83.29
North Carolina	6.84	7.45	8.00	2.97	3.03	3.93	27.62	29.71	41.95
South Carolina	2.34	3.02	4.76	3.77	4.13	5.57	25.92	30.92	39.53
Virginia	5.56	5.56	6.23	1.40	1.40	2.07	16.22	16.22	22.87
MID-SOUTH REGION									
Arkansas-North	1.15	1.15	2.27	1.15	2.20	5.17	17.88	17.88	36.09
Arkansas-South	2.65	2.65	4.21	2.65	4.43	7.27	41.42	41.78	60.69
Louisiana	4.98	4.99	8.33	4.80	4.98	10.20	38.52	38.88	58.35
Mississippi-Delta	3.50	3.57	6.61	3.50	4.33	10.16	38.35	42.47	72.43
Mississippi-Hill	1.85	1.98	7.18	1.85	2.27	9.33	14.93	16.66	45.90
Missouri	1.44	1.44	8.87	1.44	0.23	2.37	1.97	1.97	22.79
Tennessee	0.74	0.74	10.19	0.74	0.33	2.93	1.97	1.97	13.92
SOUTHWEST REGION									
New Mexico	3.14	3.89	11.21	0.63	0.83	2.30	6.42	8.20	20.92
Oklahoma	2.00	2.00	4.36	1.10	1.10	3.53	10.95	10.95	25.59
Texas-Dist. 1	3.14	3.14	5.23	0.43	0.43	1.47	4.20	4.20	9.02
Texas-Dist. 2	0.98	0.98	4.60	0.80	0.80	1.97	7.79	7.79	16.30
Texas-Dist. 3	1.25	1.26	5.00	0.50	0.53	1.77	4.63	5.03	12.05
Texas-Dist. 4	1.50	1.50	6.41	0.50	0.50	3.33	3.10	3.10	14.15
Texas-Dist. 5&9	1.73	1.73	8.45	0.93	0.93	3.97	10.06	10.07	31.61
Texas-Dist. 6	1.44	1.86	5.47	0.60	0.80	1.93	4.50	5.80	15.59
Texas-Dist. 7	4.29	4.29	11.21	1.37	1.37	4.17	10.27	10.27	26.60
Texas-Dist. 8	5.71	5.71	23.83	1.13	1.13	7.03	11.40	11.40	40.88
Texas-Dist. 10	0.67	0.67	1.53	1.97	1.97	3.03	12.59	12.59	18.22
Texas-Dist. 11	1.36	1.36	4.62	1.10	1.10	5.40	7.18	7.18	19.31
Texas-Dist. 12	1.48	1.59	7.01	1.06	1.17	5.00	9.37	10.47	43.29
Texas-Dist. 13	7.96	7.96	29.22	6.17	6.17	20.30	63.71	63.71	149.03
Texas-Dist. 14	1.12	1.23	6.30	0.63	0.63	4.83	4.92	4.92	27.31
WEST REGION									
Arizona	0.11	2.23	6.33	0.43	4.57	9.70	4.33	45.77	113.94
California	0.13	0.43	5.93	0.03	0.20	1.37	0.47	2.30	21.63

* Calculated from annual estimates published by the Beltwide Cotton Conference (Head 1990, 1991, 1992).

† Estimates for LEP include estimates for HEL. Estimates for ALL include estimates for LEP.

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Appendix II

Economic Impacts of *B.t.k.* Insect Resistant Cotton

Economic Impacts of *B.t.k.* Insect Resistant Cotton

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Abstract

The introduction of genetically engineered plants which are designed to control insects without the use of chemicals will have significant impacts on the profitability of some farmers and agribusinesses. *B.t.k.* cotton, created to control Lepidoptera infestations, will allow cotton growers to eliminate some conventional insecticide applications, and thus reduce pesticide expenses. Based on available cost and acreage data and assumptions concerning the portion of current cotton acres that would be converted to *B.t.k.* cotton, it is estimated that cotton producers could save over $77 million per year on insect control costs by adopting *B.t.k.* cotton. As the *B.t.k.* cotton seed market develops and grows during the adoption period, the demand for conventional cotton seed and some insecticides will decrease.

Introduction

In recent years, public concern about the use of some agricultural chemicals has increased in the United States. Frequently, legal action was taken to force the EPA to ban or severely restrict the use of particular pesticides. Economic studies have been conducted to examine the likely impacts from such restrictive pesticide regulations. Taylor *et al.* (1991) developed a regional model and concluded that agricultural income in the South would be negatively impacted by more restrictive pesticide regulations. Richardson *et al.* (1991) 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. It is possible that genetically engineered plants which are designed to control insects without the use of chemicals will be able to offset some of the negative impacts from increased pesticide regulations.

B.t.k. cotton is designed to control Lepidoptera infestations, eliminating the need to control these pests with conventional insecticide applications. Revenue-related factors such as lint yields and quality characteristics are expected to be similar under both conventional and *B.t.k.* cotton production systems. However, per-acre production costs of *B.t.k.* cotton are expected to be impacted due to the reduction in insecticide use and the substitution of *B.t.k.* cotton seed for conventional cotton seed. Growers who adopt *B.t.k.* cotton will simply substitute *B.t.k.* cotton seed for conventional cotton seed and certain types of insecticides. Thus, the added cost of the *B.t.k.* cotton seed must be compared with the savings obtained from eliminating conventional seed and some insecticides.

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, it is possible to speculate on the direction of change in these variables. For instance, pesticide regulations in the U.S. will likely become more restrictive over time. Reductions in insecticide use without *B.t.k.* insect resistant cotton will cause cotton yields to decline, farm profits to decline, and acres devoted to cotton production to decline, especially in those regions where insecticide use is an integral production practice. A scenario which allows for the introduction of *B.t.k.* cotton results in a very different forecast. Reductions in insecticide 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 adoption by large farms over that of 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 *B.t.k.* cotton is not expected to be related to farm size; i.e., small and large farms will have the same per-acre costs and benefits from the adoption of *B.t.k.* cotton, and thus will likely have equal adoption rates.

Economic Impacts

The introduction of *B.t.k.* insect resistant cotton will provide cotton growers with a choice of either maintaining or altering their current production practices. Each cotton grower will need to evaluate the profit potential of *B.t.k.* cotton relative to that of conventional cotton. Due to different Lepidopteran insect population pressures across the country, it is expected that some growers will be able to increase profits by adopting *B.t.k.* cotton, while 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 *B.t.k.* seed, conventional seed, and some insecticides will shift over time as the *B.t.k.* 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; i.e, to allow the market to achieve a new equilibrium position. Directional impacts on price and quantity from shifts in supply and demand may be summarized as follows:

Type of Shift	Impact on Price	Impact on Quantity
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 *B.t.k.* cotton seed (used for planting) market will exhibit growth during the first few years after introduction. Participants will gather information during this early stage of the adoption period. There will be much uncertainty in supply and demand, generating an environment in which price discovery will evolve over time. As the *B.t.k.* cotton seed market matures over time, a more stable supply-demand relationship should develop.

Cotton growers who decide to adopt *B.t.k.* cotton will replace conventional cotton seed with *B.t.k.* cotton seed. Seed companies will retain some of the *B.t.k.* cotton seed produced with the current year's *B.t.k.* cotton crop and make it available to growers for production of the next year's *B.t.k.* cotton crop. Thus, the supply of *B.t.k.* cotton seed is expected to increase during the first few years. As the *B.t.k.* 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 *B.t.k.* cotton.

Growers who use *B.t.k.* cotton seed will be able to reduce their applications of chemical insecticides that are used to control Lepidoptera infestations. Thus, a decrease in the demand for these types of insecticides will occur, causing a decrease in both the quantity and price of certain insecticides. In some regions of the country, a common practice is for cotton growers to hire custom applicators (either ground rigs or aerial sprays) to apply some insecticides and other chemicals. Therefore, in conjunction with the decline in insecticide use, there will also be a decrease in the demand for custom applicators in these regions.

Cotton insect scouts and consultants are often hired by cotton growers to help make management decisions throughout the growing season. It is expected that growers who adopt *B.t.k.* cotton will still utilize scouts and consultants for various kinds of insect problems. Therefore, the impact of the introduction of *B.t.k.* cotton on scouts and consultants is expected to be minor.

The economic impacts on cotton growers who adopt *B.t.k.* cotton could be significant. Elimination of certain pesticides will reduce a grower's insecticide cost and application cost. However, *B.t.k.* 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 *B.t.k.* cotton seed, the profits from *B.t.k.* cotton production must be greater than the profits from conventional cotton production. Thus, to assure adoption of *B.t.k.* cotton, the increased expense of *B.t.k.* cotton seed must be more than offset by the savings from reduced insecticide use. Supply and demand relationships in related markets will adjust over time until an equilibrium position exists between *B.t.k.* cotton and conventional cotton. It is expected that growers who adopt *B.t.k.* cotton will exhibit an increase in profitability.

In some regions of the country, cotton production is unprofitable due to high insecticide costs, and thus acreage is not allocated to cotton in these regions. The introduction of *B.t.k.* cotton, which will have lower insecticide costs, could allow cotton production to become profitable in these regions, allowing the acreage devoted to cotton production to increase. If an increase in cotton acreage and thus the supply of cotton occurs, the price of cotton should decrease, leading to lower wholesale and retail prices of cotton-related products.

Insect Control Cost Reductions

An estimate of the insect control cost reductions due to adoption of *B.t.k.* cotton provides an indication of the potential benefits that cotton producers may expect to achieve. The United States was divided into four regions based on differing insect problems and control practices. The regions were defined as follows: 1) Southeast - Alabama, Georgia, Florida, South Carolina, and North Carolina; 2) Delta - Louisiana, Mississippi, and Arkansas; 3) Coastal Bend of Texas - Districts 10 through 14; 4) West - Arizona. Insects considered were bollworms and budworms in all four regions and leaf perforators and pink bollworms in the West. Although *B.t.k.* cotton may provide some level of control on other insects, the economic impacts would be small relative to the impacts on these major pests.

Results presented here were derived from data compiled by Head for the years 1990-1992. The per-acre costs of controlling major susceptible Lepidopteran insects for each region are presented in Table 1. The acres of cotton that were harvested are reported in Table 2. The estimates of the reduction in insect control costs due to the introduction and adoption of *B.t.k.* cotton are presented in Table 3.

Insect control cost per acre varied across regions and years (Table 1). The three-year average ranged from a low of $10.05 per acre in the Coastal Bend of Texas to a high of $42.97 per acre in the West. Variation in cost per acre from year to year is expected due to the fluctuations in insect populations.

Cotton acres also varied somewhat over years (Table 2). The three-year average is considered to be representative of the average cotton acreage over the near future.

If it is assumed that *B.t.k.* cotton will be used on one-half of all cotton acres in a region, then the potential reduction in insect control costs for a region may be determined. Based on available cost and acreage data and assumptions concerning the portion of acres using *B.t.k.* cotton, it is estimated that cotton producers could save over $77 million per year (on average) on insect control costs by adopting *B.t.k.* cotton. The Delta region could expect an impact of over $46 million. Some portion of the cost savings will have to be used to offset the expected higher seed cost. Also, it is expected that some other insect control practices could change with the adoption of *B.t.k.* cotton. The economic impacts (whether positive or negative) of these changes are expected to be relatively small compared to the cost reductions presented in Table 3.

Conclusions

The adoption rate of *B.t.k.* insect resistant cotton will be influenced by economic factors. Cotton growers will evaluate the profit potential of *B.t.k.* cotton relative to that of conventional cotton. Due to varying Lepidopteran insect populations in different regions of the country, some growers will be able to increase profits by adopting *B.t.k.* cotton, while 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 *B.t.k.* cotton seed market grows, it is expected that the markets for conventional cotton seed and some insecticides will exhibit a decline in demand.

Table 1. Insect control cost per acre by region and year[1]

Region	1990	1991	1992	avg.
	----------($/acre)----------			
Southeast	24.63	26.38	21.89	24.30
Delta	26.48	19.53	48.69	31.57
Coastal Bend	7.67	6.51	15.96	10.05
West	78.50	37.00	13.40	42.97

1 Insects are the bollworm, budworm, leaf perforator, and pink bollworm.

Table 2. Cotton acres harvested by region and year

Region	1990	1991	1992	avg.
	----------(thousand acres)----------			
Southeast	1,127.5	1,546.0	1,534.0	1,402.5
Delta	2,702.6	2,966.4	3,185.0	2,951.3
Coastal Bend	787.3	1,051.1	787.3	875.2
West	460.0	450.0	410.0	440.0

Table 3. Insect control cost savings by region[1]

Region	*B.t.k.* Acres	Insect Control Cost	Reduction in Cost
	thousand	$/acre	million $
Southeast	701.3	24.30	17.04
Delta	1,475.7	31.57	46.59
Coastal Bend	437.6	10.05	4.40
West	220.0	42.97	9.45
Total	2,834.5	27.33	77.48

1 Assuming that one-half of all cotton acres are converted to *B.t.k.* cotton.

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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:

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

APPROVED BY:

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Chief, Section 1
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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].

Approximate distance from test (ft)	Location									
	A	B		C		D	E		F		G
	%	%	S.D.+	%	S.D.	%	%	S.D.	%	S.D.	%
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

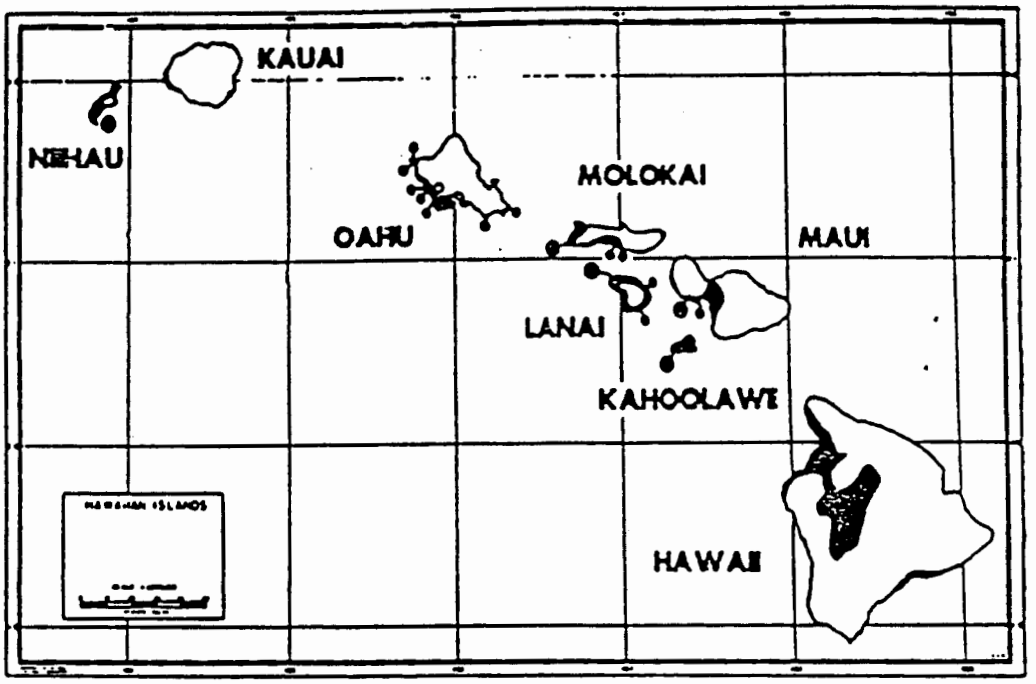

Figure 1. Geographic range of *Gossypium tomentosum* Nutt. in the Hawaiian Islands (1963). Solid circles indicate collection sites; those enclosed in rings represent sites of former collections unchecked during the present study. The open circle indicates site of hybrid populations. Shaded areas correspond 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

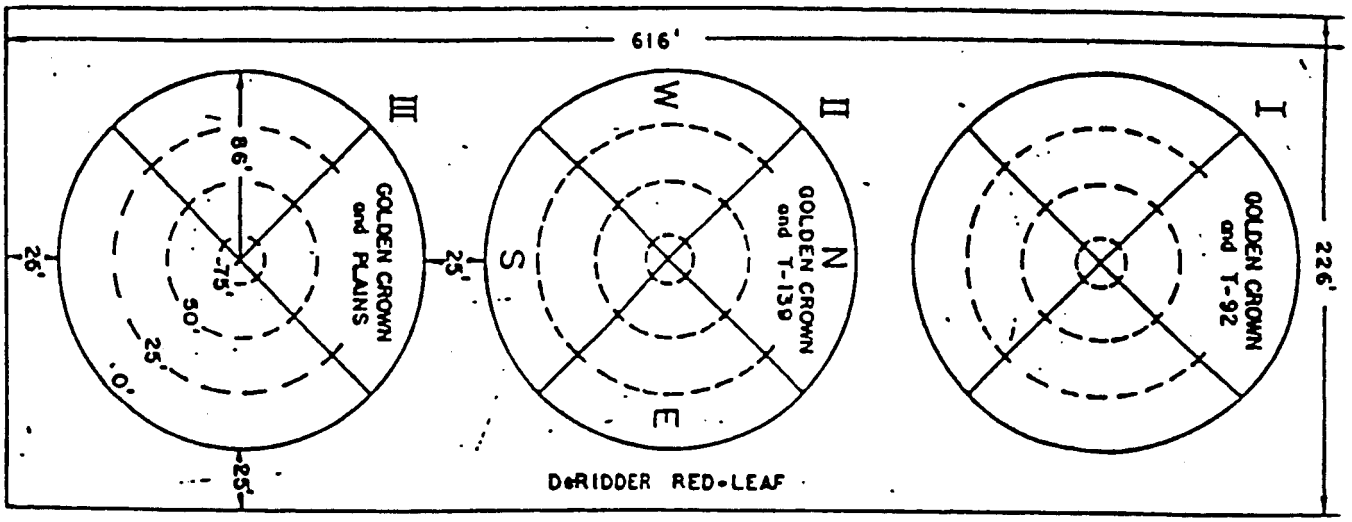

Figure 1.-Diagram of field lay-out of natural crossing experiment. The circles were planted in alternate rows of Golden Crown and green-leaf varieties. The area outside the circles was planted with DeRidder, a red-leaf cotton. Simpson, D.M. and E.N. Duncan, 1956. p. (306)

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

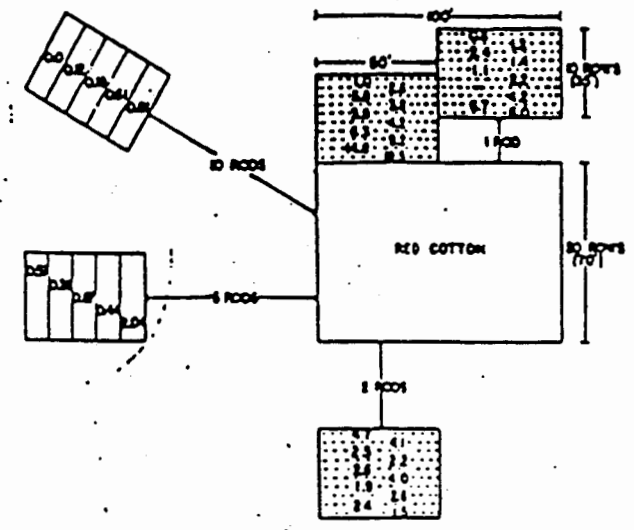

Figure 1.-Arrangement of the blocks of red and green cotton grown in 1951 near Lake Carl Blackwell, Okla. The five smaller blocks were planted to normal green cotton. Percentages of hybrids resulting from natural crossing are indicated for each row in the blocks at 0, 1, and 2 rods, and for 10 foot sections 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		1		2	
	Total	%Hybrids	Total	%Hybrids	total	%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		10	
	Total	%Hybrids	Total	%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

1993 *Bt* COTTON FIELD RELEASES
(USDA PERMIT#93-011-05)
FINAL REPORT
April 11, 1994

Eric M. Johnson
Monsanto Co.

The purpose of this field release was to test cotton genetically-modified to contain the gene from *Bacillus thuringiensis* var. *kurstaki(B.t.k.)* that encodes its insect control protein. The cotton was tested at twenty one sites by twenty two different cooperators (listed below).

Sites and cooperators	Cotton Lines Tested
Loxley Alabama site	531, 757, 931, 1076, 1172, 1195
[CBI DELETED]	
Prattville Alabama site	1076
[CBI DELETED]	
Casa Grande Arizona site	Not Planted
[CBI DELETED]	
Maricopa Arizona site #1	531, 1076
[CBI DELETED]	
Maricopa Arizona site #2	531, 757. 1076, 1172
[CBI DELETED]	

Sites and cooperators	Cotton Lines Tested
Yuma Arizona site	531, 1076
[CBI DELETED]	
Wabbaseka Arkansas site	531, 757, 931, 1076, 1172, 1578, 1626, 1849, 1888, 2020
[CBI DELETED]	
Wilmot Arkansas site	1076
[CBI DELETED]	
Shafter California site	757, 931, 1076, 1172
[CBI DELETED]	
Tifton Georgia site	531, 757, 1076, 1172, 1578, 1849, 2020
[CBI DELETED]	
Bossier City Louisiana site	531, 757, 931, 1076, 1172
[CBI DELETED]	

Sites and cooperators	Cotton Lines Tested
St. Joseph Louisiana site	1076
[CBI DELETED]	
Chatham Mississippi site	1076
[CBI DELETED]	
Morgan City Mississippi site	081, 757
[CBI DELETED]	
Scott Mississippi site	531, 757, 931, 1076, 1172, 1578 1626, 1849, 1888, 2020
[CBI DELETED]	
Starkville Mississippi site #1	531, 757, 931, 1076, 1172, 1195 1578, 1849, 1888, 2020
[CBI DELETED]	
Starkville Mississippi site #2	757
[CBI DELETED]	

Sites and cooperators	Cotton Lines Tested
Florence South Carolina site	1076
[CBI DELETED]	
Grand Junction Tennessee site	1076
[CBI DELETED]	
Corpus Christi Texas site	1076
[CBI DELETED]	
Halfway Texas site	531
[CBI DELETED]	
Sinton Texas site	531, 757, 931, 1076, 1172, 1195
[CBI DELETED]	

Genotypes:

This field release included the following genotypes:

- Derivatives of Coker 312 homozygous for PV-GHBK01,PV-GHBK02,PV-GHBK03, PV-GHBK04 and PV-GHBK07
- Coker 312 controls.

Schedule of major operations:

May-Jun	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
May-Jun	Seed planted
Aug-Dec	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 in the fall for volunteer plants. continued monitoring for volunteers will continue until the end of the 1994 cropping cycle in this area. All volunteer plants observed will be destroyed by hand weeding, cultivation, or with chemical sprays.

Plant growth and general observations:

The transgenic plants did deviate from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to *B.t.k.* 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 *B.t.k.* 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 *B.t.k.* 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:
The expression of the *B.t.k.* gene was measured through insect control. Excellent insect control was observed at all sites with several different insects including cotton bollworm, tobacco budworm, and European corn borer.

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.USDA#93-011-02

Individual Site Information

Loxley, AL site

Planted - June 2, 1993
Harvested - October 26, 1993

Field Monitoring for Weediness Characteristics
Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on 20 plants on June 28, 1993

Number of days from planting to flowering (75% of plants have initiated)
No differences were observed between the transformed and non-transformed. Observation made on 25 plants on June 19, 1993.

Number of flowers or bolls per plant
No differences were observed between the transformed and non-transformed. Observation made on 25 plants on September 7, 1993.

Monitoring for Plant Growth Characteristics
The trial was observed on June 28, July 26, August 23, September 20 and October 11. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

The trial was observed on June 28, July 26, August 23, September 20 and October 11. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

The trial was observed on June 28, July 26, August 23, September 20 and October 11. No differences in the susceptibility of the plants to diseases were observed.

Prattville, AL site

Planted - May 17, 1993
Harvested - October 15 and October 25, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on May 27, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 13, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on August 2, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 21, July 9, July 20, July 30, August 9, August 20, August 31 and September 10. No differences in plant vigor, leaf morphology plant height and other characteristics were observed. On , September 15 and September 17 it was reported that the plants seemed to be shedding their leaves prematurely. At first this was suspected to be due to the heavy boll load, as the leaf loss was more pronounced in the *B.t.k.* plots. Later laboratory analyses of the plants revealed that the leaf loss was due to a potassium deficiency.

Field Monitoring for Insect Susceptibility

Approximately 300 plants within the trial were observed on June 21, July 9, July 20, July 30, August 9, August 20, August 31, September 10, September 15 and September 17. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 21, July 9, July 20, July 30, August 9, August 20, August 31, September 10, September 15 and September 17. No differences in the susceptibility of the plants to diseases were observed.

Maricopa, Az site

Planted - May 18, 1993
Harvested -December 6 - 8, 1993

Field Monitoring for Weediness Characteristics
Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on June 7, 1993

Number of days from planting to flowering (75% of plants have initiated)
No differences were observed between the transformed and non-transformed. Observation made on all plants on July 15, 1993.

Number of flowers or bolls per plant
No differences were observed between the transformed and non-transformed. Observation made on all plants on September 16, 1993.

Monitoring for Plant Growth Characteristics
All plants within the trial were observed on June 7, June 30, July 15, August 3, September 16, October 5, November 2 and December 7. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on May 26, June 7, June 30, July 15, July 29, August 3, August 13, September 16, October 5 and November 2. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted that both the transgenic and non-transgenic cotton plants were equally susceptible to the sweetpotato whitefly. This is expected as the *B.t.k.* protein does not have activity against the sweet potato whitefly.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 30, July 15, August 3, September 16, October 5, November 2 and December 7. No differences in the susceptibility of the plants to diseases were observed.

Yuma, Az site (Field 1)

Planted - May 18, 1993
Harvested -November 22, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on May 24, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Monitoring for Plant Growth Characteristics

These observations were not recorded.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on May 20, May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13 and July 16. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted on June 23 that both the transgenic and non-transgenic cotton plants were equally infested with Armyworm, leaf hoppers, miners and Lygus. This is not surprising as the *B.t.k.* protein present in these plants is not considered active against these pests.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on May 20, May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13 and July 16. No differences in the susceptibility of the plants to diseases were observed.

Yuma, Az site (Field 2)

Planted - May 18, 1993
Harvested -November 22, 1993

Field Monitoring for Weediness Characteristics

Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on June 7, 1993

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13, July 16 and July 20. No differences in plant vigor, leaf morphology plant height and other characteristics, other than expected varietal differences were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on May 24, May 28, June 7, June 11, June 23, June 30, July 7, July 8, July 13, and July 16. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted on June 23 that both the transgenic and non-transgenic cotton plants were equally infested with Armyworm, leaf hoppers, miners and Lygus. On July 7, all plants were observed as having a high infestation of SPWF. This is not surprising as the *B.t.k.* protein present in these plants is not considered active against these pests.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on May 24, May 28, June 7, June 11, June 23, June 30, July 7, July 8 and July 20. No differences in the susceptibility of the plants to diseases were observed.

Wabbaseka, AR site (Breeding Nursery)

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

Field Monitoring for Weediness Characteristics

Some dormancy was observed in the transgenic seeds that had been harvested immediately before planting. This is normal because cottonseed has a dormant factor which breaks down over time. Transgenic seeds harvested 6 months earlier emerged at the same time as the control (May 26, 1993).

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

Some plants of line 931 were later in flowering than the non-transgenic parent line (July 20 1993).

Number of flowers or bolls per plant

Boll set on the transgenic lines was better due to insect control (August 26, 1993).

Monitoring for Plant Growth Characteristics

The following observations were made:

June 17 - all looked similar
July 20 - Some of the line 931 plants were later in blooming.
August 16 - there is variation in boll size and maturity but this is probably due to genetic variation which is much greater in the transgenic versus the non-transgenic.
September 20 - Variation exists in plant height, maturity and boll size but is no more than expected in segregating populations. 931 and 1172 appear to be later maturing than 1075 and 757.
October 13 - transgenic lines had much more genetic variation because of the early generation populations.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 17, July 20, August 16, September 20 and October 13. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. It was noted on July 20 and August 16 that aphids and boll weevils were present throughout the plot. This is not surprising as the *B.t.k.* protein present in these plants is not considered active against these pests. On October 13 it was noted that the non-transgenic plants showed greater boll damage than the non-transgenic plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 17, July 20, August 16, September 20 and October 13. No differences in the susceptibility of the plants to diseases were observed.

Wabbaseka, AR site (Breeding Nursery)

Planted - May 19, 1993
Harvested -not harvested, destroyed September 9, 1993

Field Monitoring for Weediness Characteristics
No significant differences in plant emergence was observed on May 25.

Number of days from planting to flowering (75% of plants have initiated)
The time from planting to flowering was 63 to 70 days for all plants.

Number of flowers or bolls per plant
All plants were reported to have similar fruiting with the transgenic plants having longer peduncles.

Monitoring for Plant Growth Characteristics
All plants were observed on June 24, July 2, July 21, July 28, July 30 and August 3. It was reported that some difference in general appearance such as long peduncles and perhaps a slightly different growth rate in the transgenics.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on July 2, July 8, July 9, July 14, July 19, July 26, July 27, August 2, August 6 and August 10. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Approximately 50 plants within the trial were observed on June 4, July 8, July 14, July 19, July 26, August 2 and August 10. No differences in the susceptibility of the plants to diseases were observed.

Shafter, CA site

Planted - May 24, 1993
Harvested -November 11, 1993

Field Monitoring for Weediness Characteristics

Due to the very late planting of the transgenic cotton, it was very difficult to compare growth habits. However, no unusual characteristics were observed .

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

This information was not recorded.

Number of flowers or bolls per plant

This information was not recorded.

Monitoring for Plant Growth Characteristics

On June 1 the plants were observed and noted that they were much delayed in growth due to the late planting which made this comparison difficult to make.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 1 and October 1, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants. The field had very light insect pressure.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 1 and October 1, 1993. No differences in the susceptibility of the plants to diseases were observed. The field had a very light incidence of disease.

Tifton, GA site

Planted - May 21, 1993
Harvested -October 28, 1993

Field Monitoring for Weediness Characteristics

There was better emergence and seedling vigor in transgenic plans than in the non-transgenic plants, but differences were not significant (June 1)

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 20, 1993.

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 1, June 8, June 15, June 22, June 29, July 6, July 13, July 20, July 27, August 3, August 10, August 17, August 24, August 31, September 7, September 14, September 21, September 28 and October 5. No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 1, June 8, June 15, June 22, June 29, July 6, July 13, July 20, July 27, August 3, August 10, August 17, August 24, August 31, September 7, September 14, September 21, September 28 and October 5. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 1, June 8, June 15, June 22, June 29, July 6, July 13, July 20, July 27, August 3, August 10, August 17, August 24, August 31, September 7, September 14, September 21, September 28 and October 5. No differences in the susceptibility of the plants to diseases were observed.

Bossier City, LA site

Planted - May 18, 1993
Harvested -October 15, 1993

Field Monitoring for Weediness Characteristics

No differences were observed in emergence and seedling vigor between the transgenic and non-transgenic plants (May 27).

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

No differences were observed between the transformed and non-transformed. Observation made on all plants on July 22, 1993.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed. Observation made on all plants on September 3, 1993.

Monitoring for Plant Growth Characteristics

The following observations were recorded:

June 18 - No differences in plant vigor, leaf morphology plant height and other characteristics were observed.

July 14 - Saw 4 - 5 plants with silvered leaves, asymmetrical and usually one or more lobes on leaves appeared malformed. All plants small but within normal size range. This observation is limited to line 1076.

August 11 and September 14 - Same as on July 14.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 18, July 14, August 11 and September 14. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 18, July 14, August 11 and September 14. No differences in the susceptibility of the plants to diseases were observed.

St. Joseph, LA site

Planted - May 17, 1993
Harvested -August 27, September 3 and September 27

Field Monitoring for Weediness Characteristics
Germination of the *B.t.k.* Cotton was determined to be equivalent to the non-transformed Coker 312. Observation made on all plants on June 18, 1993

Number of days from planting to flowering (75% of plants have initiated)
No differences were observed between the transformed and non-transformed. Observation made on all plants on June 18, 1993.

Number of flowers or bolls per plant
No differences were observed between the transformed and non-transformed.

Monitoring for Plant Growth Characteristics
All plants within the trial were observed on June 18, July 13, August 2 and August 27. No differences in plant vigor, leaf morphology plant height and other characteristics, other than expected varietal differences were observed.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 18, July 13 and August 2. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 18, July 13, August 2 and August 27. No differences in the susceptibility of the plants to diseases were observed.

Catham, MS site

Planted - June 7, 1993
Harvested -October 27 and 28, 1993

Field Monitoring for Weediness Characteristics

Two plots with non-transgenic plants have a poor stand. Appears to be the result of non-uniform irrigation in this area. Too much water (June 30, 1993).

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

Coker 312 plants were observed to have blooms on July 23, ,line 1076 had no blooms. Line 1076 had a later fruit setting and the cause was not determined but did not appear to be early insect damage (July 23, 1993).

Number of flowers or bolls per plant

Coker 312 appears to have more and larger bolls than line 1076. Does not appear to be insect related (August 20, 1993).

Monitoring for Plant Growth Characteristics

All plants within the trial were observed on June 30, July 23 and August 20. The following observations were recorded:
June 30 - No differences in plant vigor, leaf morphology plant height and other characteristics were observed.
July 23 - non-transgenic had blooms while line 1076 hod no blooms.
August 20 - line 1076 had fewer and smaller bolls than the non-transgenic Coker 312.

Field Monitoring for Insect Susceptibility

All plants within the trial were observed on June 30, July 23 and August 20. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

All plants within the trial were observed on June 30, July 23 and August 20. No differences in the susceptibility of the plants to diseases were observed.

Morgan City, MS site

Planted - June 2, 1993
Harvested -November 11, 1993

Field Monitoring for Weediness Characteristics

One Hundred of each line were compared and no differences were observed between the transgenic and non-transgenic (June 15, 1993).

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

One Hundred of each line were compared and no differences were observed between the transgenic and non-transgenic (August 10, 1993).

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics between Coker 312 and line 757 were observed throughout the growing season.

Field Monitoring for Insect Susceptibility

There was a tendency to have a higher population of *Lygus* spp. in the line 757 plot versus the Coker 312 plot.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to diseases to the Coker 312 and line 757 were observed throughout the growing season.

Scott, MS site

Planted - May 21 and May 27, 1993
Harvested -

Field Monitoring for Weediness Characteristics

This information was not recorded.

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

This information was not recorded.

Number of flowers or bolls per plant

This information was not recorded.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics were observed between the transgenic and non-transgenic lines.

Field Monitoring for Insect Susceptibility

No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to any disease were observed between the transgenic and non-transgenic lines.

Volunteers

No volunteer plants were observed on January 17, 1994.

Scott, MS site

Planted - May 13 and May 19, 1993
Harvested -September 20, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic lines.

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

No differences were observed between the transgenic and non-transgenic lines.

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics were observed between the transgenic and non-transgenic lines.

Field Monitoring for Insect Susceptibility

No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to any disease were observed between the transgenic and non-transgenic lines.

Florence, SC site

Planted - May 25, 1993
Harvested - October 21, 1993

Field Monitoring for Weediness Characteristics

Stand counts on June 9 indicated that the germination percentage was slightly higher for the transgenic plants when compared to the non-transgenic plants

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

Transgenic plants bloomed later than the non-transgenic plants (July 16, 1993).

Number of flowers or bolls per plant

Transgenic plants had higher numbers of bolls than did the non-transgenic plants. On July 30, bolls per 100 plants were 613 for non-transgenic treated, 548 for non-transgenic not treated and 833, 730, 695, 825 and 710 for treatments 1,2,3,4 and 6 respectively.

Monitoring for Plant Growth Characteristics

On June 21, the transgenic plants were smaller than the non-transgenic plants. Plant height was 8.9 inches compare to 9.7 inches.

Field Monitoring for Insect Susceptibility

The entire plot was observed on July 6, July 22, August 3 and August 4. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

No differences in the susceptibility to any disease were observed between the transgenic and non-transgenic lines on June 25 and August 3.

Grand Junction, TN site

Planted - May 21, 1993
Harvested - October 25 and November 11, 1993

Field Monitoring for Weediness Characteristics

More than 50 plants were observed on June 10, 1993 and no differences were observed between the transgenic and non-transgenic.

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

More than 50 plants were observed on July 16, 1993 and no differences were observed between the transgenic and non-transgenic.

Number of flowers or bolls per plant

More than 50 plants were observed on July 30, 1993 and no differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics between Coker 312 and line 1076 were observed throughout the growing season. Observations were taken on June 10, June 22, July 2, July 16, July 30, August 16 and August 26, 1993.

Field Monitoring for Insect Susceptibility

Observations were taken on June 10, June 22, July 2, July 16, July 30, August 16 and August 26, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 10, June 22, July 2, July 16, July 30, August 16 and August 26, 1993. No differences in the susceptibility to diseases to the Coker 312 and line 1076 were observed throughout the growing season.

Corpus Christi, TX site

Planted - May 18, 1993
Harvested - N/A

This site was lost to excessive rains following planting. All plants were reported as dead by August 1993.

Monitoring for Volunteers

Following termination of this trial, the field has been monitored for volunteers. Observations were taken on 9/15, 10/13, 11/17/1993, 1/7, and 3/8/1994. NO volunteer cotton plants were ever observed at the plot site.

Halfway TX site

Planted - May 19, 1993
Harvested - November 23, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic.

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

No differences were observed between the transgenic and non-transgenic.

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

No differences in plant growth characteristics between Coker 312 and line 531 were observed throughout the growing season. Observations were taken on June 14, June 25, July 15, August 10, August 26, September 13, October 11, October 28 and November 11, 1993.

Field Monitoring for Insect Susceptibility

Observations were taken on June 14, June 25, July 15, August 10, August 26, September 13, October 11, October 28 and November 11, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 14, June 25, July 15, August 10, August 26, September 13, October 11, October 28 and November 11, 1993. No differences in the susceptibility to diseases to the Coker 312 and line 531 were observed throughout the growing season.

Sinton TX site - Efficacy Trial

Planted - May 17, 1993
Harvested - September 17 and 20, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic.

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

Line 1076 was rated approximately 6 days slower to develop and mature than was Coker 312.

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines.

Monitoring for Plant Growth Characteristics

Observations were taken on June 8, July 2, July 29 and August 26, 1993. The following observations were recorded:

Line 1076 appeared slightly shorter and slower to develop than Coker 312 (July 2).

Line 1076 was later in flowering than Coker 312 (July 29).

Line 1076 has smaller bolls which opened more slowly than Coker 312 (August 26).

Field Monitoring for Insect Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences in the susceptibility to diseases to the Coker 312 and the transgenic lines tested were observed throughout the growing season.

Field Monitoring for Volunteers

The filed was monitered for volunteers on March 8, 1994. None were observed.

Sinton TX site - Gene Evaluation Trial

Planted - May 17, 1993
Harvested - September 17 and 20, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transgenic and non-transgenic (May 28).

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

Lines 931, 1076, 1172 and 1195 were slower to produce and develop flower buds versus the Coker 312. This was possibly a function of more fruit on the *B.t.k.* lines than on Coker 312 (July 15, 1993).

Number of flowers or bolls per plant

No differences were observed between the transgenic and non-transgenic lines (August 26, 1993).

Monitoring for Plant Growth Characteristics

Observations were taken on June 8, July 2, July 29 and August 26, 1993. The following observations were recorded:

Line 931 appears shorter and with less mainstem nodes than Coker 312 (July 2).
Line 931 is still shorter in plant height. Lines 931, 1076, 1172 and 1195 all flowered later than Coker 312 (July 29).
Lines 931, 1076 and 1172 have slower boll opening than Coker 312 (August 26).

Field Monitoring for Insect Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insects infesting the plants.

Field Monitoring for Disease Susceptibility

Observations were taken on June 8, July 2, July 29 and August 26, 1993. No differences in the susceptibility to diseases to the Coker 312 and the transgenic lines tested were observed throughout the growing season.

Field Monitoring for Volunteers

The filed was monitored for volunteers on; October 13, November 17, 1993 and January 7, February 8 and March 8, 1994. None were observed. In December 1993, some volunteers emerged and were destroyed by disking.

1993 *Bt* COTTON FIELD RELEASES
(USDA PERMIT#93-056-05)
FINAL REPORT

Frank S. Serdy
Monsanto Co.

The purpose of this field release was to establish a demonstration plot of the insect resistant cotton, (modified to contain the gene from *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) that encodes its insect control protein), at the Asgrow Research Farm, located near Queenstown, MD. This plot, along with other examples of transgenic plants were then shown to interested individuals in the Washington area.

Primary Cooperator: [CBI DELETED]

Address of the site: [CBI DELETED]
Queenstown, MD 21658
[CBI DELETED]

The cotton line 1076 containing Vector # PV-GHBK04 was planted along with the non-transgenic parental variety Coker 312.

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.

June 2, 1993 - Seed planted

July through September - Plot used for demonstration purposes.

November 11, 1993 - Plot destroyed by disking

Summary of Observations

Plant growth and general observations:

The transgenic plants did deviate from the agronomic standard exhibited by Coker 312. The variation is random in its expression with no correlation to *B.t.k.* 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 *B.t.k.* cotton was planted more than 1/4 mile away from any other cotton in the area, hence, border rows were not used to surround the plot. Based on the knowledge of pollen movement from cotton and the lack of related plants in the area to receive the pollen, we consider that there is no probability of out-crossing as a result of this trial.

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:
The expression of the *B.t.k.* gene was measured through insect control. Excellent insect control was observed when the plants were inoculated with eggs of predatory lepidopteran insects.

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

Planted - June 2, 1993
Plot Destroyed - November 11, 1993

Field Monitoring for Weediness Characteristics

No differences were observed between the transformed and non-transformed.

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

No differences were observed between the transformed and non-transformed.

Number of flowers or bolls per plant

No differences were observed between the transformed and non-transformed.

Monitoring for Plant Growth Characteristics

All of the 40 plants in the trial were observed on July 1, August 1, September 1 and November 1. No differences in plant vigor, leaf morphology plant height and other characteristics observed.

Field Monitoring for Insect Susceptibility

All of the 40 plants in the trial were observed on July 1, August 1, September 1 and November 1. No differences were observed between the transformed and non-transformed plants in the incidence of non-target insect infestation of the plants.

Field Monitoring for Disease Susceptibility

All of the 40 plants in the trial were observed on July 1, August 1, September 1 and November 1. No differences in infection were observed between the transformed and non-transformed plants.

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 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

Field trial sites were chosen to represent the major cotton growing areas of the United States, where Insect Resistant Cotton may be expected to be a commercial product. Locations in Texas and Arizona represented 'plains' type cotton culture, and locations in Mississippi, Georgia, Louisiana and Alabama were chosen for typical 'Southern cotton' environmental conditions. These locations provided a variety of environmental conditions and insect pressure from agronomically important pests. Insect resistant and control cotton lines were produced under the same conditions at each location. The six field sites were as follows: Starkville, Mississippi; Bossier City, Louisiana; West Sinton, Texas; Tifton, Georgia; Maricopa, Arizona; and Loxley, Alabama.

There were two types of plots established at each field location: 'efficacy' and 'isolated.'

Historically the field trials for collection of samples for expression analysis were conducted simultaneously with evaluation of insecticidal efficacy (a separate Monsanto study). The 'efficacy' plots were arranged in a random, split-block design incorporating both test and control lines in close proximity. One half of the plot area received a routine application of pyrethroid insecticides ('sprayed' plots); the other plots, not receiving pyrethroid insecticide treatments were called 'unsprayed' plots.

The split-block design was not optimal for sampling or for preventing out-crossing of traits among lines. To enable sampling and harvest of non-outcrossed seed, a second plot type was incorporated into this study and was designated as 'isolated'. The 'isolated' plots were designed to minimize outcrossing of the genes among lines and were physically separated by 8 buffer rows from the efficacy plots and other test lines. Since the 'isolated' plots were not evaluated for insecticidal efficacy, they were treated with chemical insecticides to maximize yield (similar to control plots and 'sprayed' efficacy plots).

To enable sampling of young leaf and seed tissues from all lines at all six sites, samples of young leaf and seedcotton from individual bolls were taken from both the 'efficacy' and 'isolated' plots for expression analysis. Tissue samples for this study were drawn from unsprayed sub-plots of replicates 1, 3, and 5 of the 'efficacy' plots and from three sampling areas designated within the 'isolated' plots. Bulk seedcotton was harvested only from the 'isolated' plots since these would be used in composition/quality and safety assessment studies.

Because of seed supply and land limitations, 'isolated' plots for each line were not established at all sites. Bollgard™ Cotton lines 531, 757, and 1076 were planted in 'isolated' plots at four field sites. No 'isolated' plots were planted at the Texas site. Therefore, three or six samples of leaf and seed were analyzed for expression, depending upon the site. Field locations and the lines grown at each site are shown below:

	'Efficacy' Plots						'Isolated' Plots				
LINE #	1076	1172	1195	531	931	757	1076	1172	757	531	931
Mississippi	X	X	X	X	X	X	X	X			
Georgia	X	X		X		X	X	X	X	X	
Louisana	X	X	X	X	X	X	X	X	X	X	X
Texas	X	X	X	X	X	X					
Arizona	X	X		X		X			X	X	
Alabama	X	X	X	X	X	X	X	X	X	X	X

Expression levels of the *B.t.k.* HD-73 and NPTII proteins were measured young leaf tissue, seed, leaf tissue sampled throughout the cotton growing season at West Sinton, Texas, and whole, mature cotton plants collected at West Sinton, Texas. Analysis for AAD was only performed for the young leaf and seed samples. Since none was detected in either of these tissues, no analysis for AAD was performed for leaves harvested throughout the season or in whole plants.

Compositional analysis of the important cottonseed components (protein, oil, carbohydrate, ash, moisture and calories), as well at the composition of individual fatty acids and natural toxicants (gossypol, cyclopropenoid fatty acids and aflatoxin) present in Bollgard™ Cotton Lines 757 and 1076 were compared to the Coker 312 parental control to verify that the genetic engineering process did not alter these important seed components. Samples of cottonseed were obtained from the isolated plots at the field sites. One sample per site was analyzed (four samples for the Bollgard™ Cotton Lines 757 and 1076, and five samples of the control cotton line, C312.)

Cottonseed from Bollgard™ Cotton Lines 757 and 1076 collected at four of the locations was pooled and processed to commercially representative fractions to compare the processing and processed fractions (particularly the toasted meal and refined oil). Control cottonseed was collected at five of the field sites and pooled for processing. In addition, the levels of the *B.t.k.* HD-73 and NPTII proteins in the processed fractions were determined to facilitate exposure assessment of these proteins in human food and animal feed.

The following is a summary of the methods used to analyze these plant fractions.

Samples

Representative plant tissue samples were collected at various times during the growing season from Bollgard™ Cotton Lines 757 and 1076 and from the Coker 312 control. These samples included representative samples of the first true leaves, young leaves sampled approximately each month after the first true leaf samples were obtained (Texas site only), mature whole plants sampled just prior to harvest at one location (Texas), analytical seed samples and bulk seed samples (collected and pooled across replicates at four or five locations). Nectar and pollen from these lines was collected from cotton plants grown in the greenhouse.

Protein Extraction from Cotton Leaf Tissue

For analyses, each leaf sample (containing four leaves) was mixed, sampled and extracted in a single vessel, according to SOP # BtC-PRO-019. Briefly, frozen leaves, as shipped from the field, were crushed to a course powder and mixed while in the sample container bag on dry ice. Frozen tissue was weighed and cold Tris-Borate (T-B) extraction buffer added to a final ratio of approximately 1 mg leaf tissue/80µL buffer (1:80). The T-B extraction buffer is 100 mM Tris-HCL, pH 7.5, 10mM sodium borate, 0.05% (v/v) Tween-20, 5mM $MgCl_2$, 0.2% (w/v) L-ascorbate. The tissue was extracted with a Polytron PT3000 tissue homogenizer (Brinkman, Inc. Westbury, NY) equipped with a PTA 10TS generator for 1 minute at approximately 22,000 rpm and immediately placed on ice. Insoluble material was removed by centrifugation at approximately 10,000 x g for approximately 10 minutes at approximately 4°C. The supernatant was removed, aliquoted and used as the "cotton leaf extract" in further analyses. Aliquots of leaf extract were stored at approximately -80°C until analyzed.

Protein Extraction from Cotton Seed Tissue

Five cotton seeds were weighed from each sample of delinted seed (analytical seed samples) and extracted in a single vessel, according to SOP # BtC-PRO-019. The seeds were individually cracked, placed in a plastic tube, and cold T-B extraction buffer (described above) added to a final ratio of approximately 1 mg seed tissue/40µL buffer (1:40). The seeds were

homogenized with a Polytron PT3000 tissue homogenizer (Brinkman, Inc., Westbury, NY) equipped with a PTA 10TS generator using four bursts of approximately 15 seconds, allowing cooling and settling of the tissue to occur between bursts; after extraction the homogenate was immediately placed on ice. The homogenate was clarified by centrifugation at approximately 10,000 x g for approximately 10 minutes at approximately 4°C. The supernatant was removed, aliquoted and used as the "cotton seed extract" in further analyses. Aliquots of leaf extract were stored at approximately -80°C until analyzed.

Protein Analysis

Crude protein content in the toasted meal fractions from processing was measured by Kjeldahl analysis (AOAC official method 976.06, 1990) according to SOP at the Delta Branch Experiment Station in Stoneville, Mississippi.

Total protein in tissue extracts was measured by the method of Bradford (1976) using the microtiter plate application of the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). the procedure (SOP #BtC-PRO-015) was validated, showing acceptable variability and appropriateness for evaluating total protein in cotton tissue extracts. Bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO) dissolved in T-B extraction buffer, was chosen as the appropriate standard by comparing protein assay results to amino acid composition of the same extracts (Rogan, *et al.*, 1992).

Quantitation of the levels of B.t.k. HD-73, NPTII and AAD proteins

The amount of *B.t.k.* HD-73, NPTII and AAD proteins in the extracts prepared from cotton leaf and seed samples were determined by validated Enzyme-Linked Immuno-Sorbent methods (ELISAs). Each ELISA was shown to be sensitive to the specific protein analyzed. The accuracy, precision and ruggedness of each of these assays was assessed. Spike-and-recovery and extraction efficiencies for each of the proteins measured in each of the matrices was evaluated for young leaf and seed tissue, for young leaves over the season and for whole plants. Stability of these proteins in the respective cotton tissue matrices was assessed and all assays were performed within the known limits of stability for each protein.

For *B.t.k.* HD-73, the full length protein expressed in the respective tissue was treated with trypsin to convert this protein to the trypsin-resistant core, which was then quantitated in the validated ELISA. Trypsinization was required to accurately estimate the amount of *B.t.k.* HD-73 protein present in these tissues.

Validated computer systems and software were used for data collection and reduction. Statistical analyses were performed as described in each of the attached reports.

Western Blot Analyses

Western blot analysis was completed according to SOP # BtC-PRO-002, a procedure similar to that described by Matsudaira (1987). Briefly, acrylamide gels from SDS-PAGE were equilibrated in the same buffer used for electrolution (transfer). Proteins were transferred out of the acrylamide gel onto nitrocellulose membrane. Additional protein binding sites on the membrane were blocked using 3% bovine serum albumin (BSA) in Tris-HCl (pH 8.0)/saline/Tween-20 buffer (TBST). The blots were incubated with a 1:1500 dilution (in TBST/1% BSA) of F204 antibody (bleed 9) specific for the HD-73 protein followed by incubation with goat anti-rabbit antibody-alkaline phosphatase conjugate (Promega Corp, Madison, WI). Protein bands bound by antibody were visualized using the NBT/BCIP colorimetric substrate system (Promega, Corp., Madison, WI). Levels of the *B.t.k.* HD-73 protein were quantitated by comparison to standards spiked into the same matrix and contained on the same blot.

Compositional analysis of cottonseed

The levels of protein, fat, ash, carbohydrates, calories and moisture (proximate analysis) were determined for cottonseed obtained from each site and each line (the seed were pooled across plots at each field test site). Tocopherol was evaluated in the refined oil. The analyses were conducted at Hazelton Laboratories, Madison, WI. The analytical methods utilized are as follows:

Protein (N x 6.25)

Official Methods of Analyses (1990), 15th Edition, Method 955.04C, 979.09, AOAC, Arlington, Virginia, (Modified).

The Kjeldahl method for Organic Nitrogen, R.B. Bradstreet, Academic Press, New York, New York (1965)

Quantitative Inorganic Analysis, Kelthoff and Aandell (1948), Revised Edition.

Fat

Official Methods of Analysis (1990), 15th Edition, Method 960.39, AOAC, Arlington, Virginia, (Modified).

Ash

Official Methods of Analysis (1990), 15th Edition, Method 923.03, AOAC, Arlington, Virginia, (Modified).

Carbohydrates

The total carbohydrate level is determined by difference after the percentages of protein, moisture, ash and fat are known. % Carbohydrates = 100% - (% protein + % fat + % ash + % moisture)

Calories

The total calories in the proteins, carbohydrates and fats of various food and feed types have been determined by bomb calorimetry and feeding studies. The 4 cal/g (protein), 9 cal/g (fat) and 4 cal/g (carbohydrate) factors are averages of the values derived from these tests.

Moisture, 100 Degree Vacuum Oven.

Seed samples were placed in a vacuum oven at 100°C and dried to a constant weight (approximately 5 hours). Official Methods of Analysis (1990), 15th Edition, Method 926.08, 925.09, AOAC, Arlington, Virginia, (Modified).

Aflatoxin

Proceeding of the 3rd International Congress of Food Science and Technology, Pages 705-711 (Modified).

Determination by High Performance Liquid Chromatography: Journal of Assoc. Official Analytical Chemist, Volume 71, No.1, 26.052-26.060 (1988) (Modified).

Determination by One Dimensional Thin Layer Chromatography: Journal Assoc. Official Analytical Chemist, Volume 71, No.1, 26.031 (1988) (Modified).

Determination by Two Dimensional Thin Layer Chromatography: Journal Assoc. Official Analytical Chemist, Volume 71, No.1, 26.074 (1988) (Modified).

The levels of aflatoxins B_1, B_2, G_1 and G_2 were determined for each line from each of the six field test sites, and calculated according to OP-AC 103.

Alpha-tocopherol

Oil samples were saponified to release the tocopherols, which were then extracted with organic solvent, follwed by quantitation by HPLC using a silica column and fluorescence detection.

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Cottonseed processing

Seed cotton was ginned and pooled (by line) across sites where isolated plots were established. The composited samples were used as a source of seed cotton for processing. Cottonseed was processed at the Food Protein Research & Development Center at Texas A&M University using a solvent extraction method, according to SOP# 8.27 R02, "Small- Scale Processing of Glanded Cotton to Bind Gossypol", SOP# 8.33 R01, "Small-Scale Toasting of Meal", and SOP# 8.1 R04, "Small Scale Processing of Cottonseed". The processing procedure used for this experiment was a scaled down version of the commercial procedure. The *B.t.k.* HD-73 and NPTII content in the cottonseed meal before and after processing was estimated by ELISA and western blot analysis. The proximate composition of the toasted meal and the free and total gossypol levels in the raw and processed cottonseed meal was assessed.

Preparation of Seed Kernel Material for Gossypol and Fatty Acid Analyses

Cottonseed were dehulled with a Bauer Mill and the kernels separated from the hulls by hand. The kernels were ground on dry ice using a stainless steel Wiley mill and passage through a 10 mesh screen. Duplicate samples of ground kernel, weighing approximately 3 grams each, were placed in glass vials, one set used for gossypol analysis, the second for fatty acid analysis.

Moisture Determination for Gossypol and Fatty Acid Analysis

Percent moisture in each samples of the kernel material was determined by weight difference before and after lyophilization. Samples were lyophilized in tared flasks to remove all water and obtain a true dry weight to the nearest 0.1 mg.

Measurement of Free and Total Gossypol Levels

Free and total gossypol levels were measured in the cottonseed kernel (prior to processing), toasted cottonseed meal (processed), and refined cottonseed oil at the USDA-ARS Southern Crop Research Laboratory, College Station, Texas. Evaluation of free gossypol levels was completed using high performance liquid chromatography (HPLC) according to the procedure described by Stipanovic, *et al.*, 1988 and A.O.C.S. Official Method Ba 7-58. Total gossypol levels (corrected for moisture) were measured spectrophotometrically using aniline as a complexing agent (Pons, *et al.*, 1958 and A.O.C.S. Official Method Ba 8-78).

Quantitation of Fatty Acid Levels (cottonseed and refined oil)

Lipids were extracted using a double Bligh and Dyer procedure (Bligh and Dwyer, 1959), as recently described by Wood (1991).

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, 1968a). The free fatty acids were converted quantitatively to phenacyl derivatives according to the procedure of Wood and Lee (1983).

Approximately 400 µg of the phenacyl derivatives were analyzed by high performance liquid chromatography (HPLC) according to the procedure used to examine the fatty acids of cottonseed (Wood, 1986a and 1986b). Peak elution order and peak shape were monitored by a strip recorder. 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. Peak area for each fatty acid is directly proportional to the percent of each fatty acid contained in total lipid.

Tobacco budworm bioassays.

Tobacco budworm diet incorporation assays (SOP #BUG-PRO-022-02) were used to assess the insecticidal activity/spectrum of the *B.t.k.* HD-73 protein. Insecticidal activities were estimated in terms of EC_{50} values. EC_{50} is the concentration of *B.t.k.* HD-73 protein that is required to reduce the weight of the treated tobacco budworm larvae to 50% of the untreated larvae.

Insect feeding assay

The biological activity of purified and seed-expressed CryIA(c) protein was evaluated using a pinto bean-based (PB) insect diet incorporation assay (Reese et al. 1972, MacIntosh et al. 1990). *H. virescens* were obtained from the USDA-ARS, Stoneville, MS. Liquid agar-based pinto bean diet with 20% of the water omitted (24 mL) was added to 6 mL samples of test liquid (distilled water containing doses of the test, reference, or control substance). Treated diet was blended using a Vortex mixer, poured into 96-well insect assay trays, and allowed to cool and harden. One first instar *H. virescens* larva was added to each well. Apparently healthy, motile TBW larvae were impartially assigned to treatments. Wells were covered with Mylar® plastic and ventilated with a single insect pin hole. Assays were incubated at 28 ± 2°C and evaluated after 7 days.

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Appendix VII

Analyses of Allelochemicals in Transgenic Cottons and Controls: 1993

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Pages 504-505

93MONCHE.WK1

LEAVES

ENTRY	FLAV	ANTHOCYN	GOSS	TANNIN
1421	0.51	0.11	0.159	3.667
1445	0.56	0.11	0.135	3.838
1698	0.59	0.10	0.136	4.268
C312	0.58	0.11	0.144	3.710
F-VALUE	ns	ns	ns	ns
LSD 0.05	0.06	0.02	0.051	1.229

Data from Study 93-01-30-01
Study 93-454-706. Sample received cool but not frozen on 9/22/93. Analysis by Helen at Mississippi State location
JMJ 4/14/94

LEAVES

ENTRY	FLAV	ANTHOCYN	GOSS	TANNIN
COK312	0.80	0.34	0.143	17.106
DES119	0.64	0.28	0.123	14.562
M1076	0.75	0.28	0.119	11.053
M1172	0.70	0.23	0.106	11.445
M1195	0.78	0.26	0.126	13.304
M531	0.72	0.31	0.120	11.852
M757	0.70	0.26	0.111	11.199
M931	0.74	0.26	0.116	12.306
F-VALUE	*	*	ns	**
LSD 0.05	0.08	0.06	0.030	2.113

SQUARE

ENTRY	FLAV	ANTHOCYN	GOSS	TANNIN
COK312	0.39	0.11	0.294	14.885
DES119	0.37	0.11	0.325	15.066
M1076	0.38	0.11	0.258	12.534
M1172	0.35	0.11	0.262	13.233
M1195	0.41	0.12	0.303	14.824
M531	0.38	0.12	0.291	12.246
M757	0.36	0.13	0.280	11.590
M931	0.36	0.11	0.271	12.377
F-VALUE	**	*	**	**
LSD 0.05	0.03	0.01	0.031	1.297

Samples from Mississippi State location. JMJ 4/14/94

1993 EXP 1511 CHEMICAL ANALYSIS

ENTRY	ENTRY#	REP	TISSUE	PLOT	GOSSY	TANNIN	FLAVON	ANTHO
M 531	1	1	SQUARE	4809	0.328	12.653	0.378	0.152
M 531	1	2	SQUARE	4841	0.306	12.764	0.396	0.128
M 531	1	3	SQUARE	4893	0.226	13.454	0.347	0.104
M 531	1	4	SQUARE	4917	0.289	12.035	0.383	0.116
M 531	1	5	SQUARE	4941	0.285	11.115	0.388	0.128
M 531	1	6	SQUARE	4985	0.310	11.456	0.400	0.116
M 757	2	1	SQUARE	4813	0.310	12.781	0.357	0.137
M 757	2	2	SQUARE	4861	0.258	14.011	0.326	0.118
M 757	2	3	SQUARE	4873	0.286	11.440	0.368	0.135
M 757	2	4	SQUARE	4909	0.261	10.586	0.334	0.116
M 757	2	5	SQUARE	4937	0.297	11.052	0.393	0.144
M 757	2	6	SQUARE	4997	0.269	9.667	0.364	0.109
M 931	3	1	SQUARE	4817	0.264	13.368	0.322	0.112
M 931	3	2	SQUARE	4853	0.237	12.453	0.336	0.096
M 931	3	3	SQUARE	4881	0.249	14.186	0.380	0.104
M 931	3	4	SQUARE	4921	0.287	12.193	0.354	0.112
M 931	3	5	SQUARE	4953	0.297	11.471	0.375	0.113
M 931	3	6	SQUARE	4973	0.292	10.591	0.386	0.101
M 1076	4	1	SQUARE	4821	0.268	12.617	0.364	0.115
M 1076	4	2	SQUARE	4849	0.249	13.952	0.403	0.133
M 1076	4	3	SQUARE	4885	0.235	16.141	0.362	0.106
M 1076	4	4	SQUARE	4913	0.256	11.340	0.367	0.105
M 1076	4	5	SQUARE	4949	0.242	9.872	0.388	0.103
M 1076	4	6	SQUARE	4977	0.295	11.280	0.410	0.116
M 1172	5	1	SQUARE	4825	0.246	15.032	0.344	0.114
M 1172	5	2	SQUARE	4869	0.230	13.239	0.334	0.091
M 1172	5	3	SQUARE	4889	0.263	15.513	0.339	0.111
M 1172	5	4	SQUARE	4905	0.269	12.759	0.350	0.109
M 1172	5	5	SQUARE	4945	0.246	10.488	0.359	0.108
M 1172	5	6	SQUARE	4981	0.319	12.368	0.354	0.104
M 1195	6	1	SQUARE	4829	0.290	17.973	0.414	0.107
M 1195	6	2	SQUARE	4845	0.249	16.297	0.408	0.123
M 1195	6	3	SQUARE	4897	0.318	16.256	0.370	0.114
M 1195	6	4	SQUARE	4933	0.271	12.218	0.364	0.112
M 1195	6	5	SQUARE	4965	0.321	12.088	0.452	0.129
M 1195	6	6	SQUARE	4969	0.370	14.110	0.443	0.147
COK 312	7	1	SQUARE	4833	0.291	17.196	0.397	0.123
COK 312	7	2	SQUARE	4857	0.343	16.323	0.425	0.132
COK 312	7	3	SQUARE	4877	0.285	17.361	0.364	0.108
COK 312	7	4	SQUARE	4925	0.276	14.875	0.411	0.108
COK 312	7	5	SQUARE	4957	0.282	11.463	0.404	0.107
COK 312	7	6	SQUARE	4989	0.285	12.091	0.365	0.096
DES 119	8	1	SQUARE	4837	0.327	18.261	0.394	0.118
DES 119	8	2	SQUARE	4865	0.291	15.560	0.343	0.115
DES 119	8	3	SQUARE	4901	0.295	18.026	0.364	0.112
DES 119	8	4	SQUARE	4929	0.335	12.707	0.395	0.114
DES 119	8	5	SQUARE	4961	0.320	12.183	0.352	0.103
DES 119	8	6	SQUARE	4993	0.381	13.657	0.350	0.118

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New Products Division - Regulatory Sciences

SAMPLE HANDLING FORM
SOP Reference: GEN-POL-002 OR GEN-FLD-005

Study #: 93 01 30 01 Experiment #: 93-454-706

	Monsanto Sample # for Monsanto Use only	Plot #	Sample Description (e.g. PLANT PART & LINE, NUMBER OF SAMPLES, WGT., ETC.)	Collection Date	Collectors Initials	Rec'd (✓)	Comments
1.	1	1002	1421	9/21	JE	✓	
2.	2	106	Coker 312	9/21	JE	✓	
3.	3	501	1698	9/21	JE	✓	
4.	4	1101	1698	9/21	JE	✓	
5.	5	903	1445	9/21	JE	✓	
6.	6	202	1421	9/21	JE	✓	
7.	7	301	Coker 312	9/21	JE	✓	
8.	8	201	1698	9/21	JE	✓	
9.	9	1103	1445	9/21	JE	✓	
10.	10	402	1421	9/21	JE	✓	
11.	11	406	Coker 312	9/21	JE	✓	
12.	12	303	1445	9/21	JE	✓	
13.							
14.							
15.							
16.							
17.							
18.							
19.							
20.							

Interim Storage: On Ice

Number of Shipping Containers: 1 Method of Shipment: UPS/Next day

Shipping Conditions (✓ one): ☐ Frozen on Dry Ice ☐ Cool on Ice ☐ Ambient Temp.

☑ Other (please specify): Cool on dry ice

Samples Transferred by: Signature [signature] Date and Time: 9/21/93 · 11:

The following should be completed by Recipient:

Samples Received and Checked by [CBI DELETED] Date and Time: 9/22/93 4:45

Condition of Samples upon receipt: Cool Unit not frozen

Interim Storage:

Distribution of Copies: WHITE COPY Included with Shipment, recipient sends finalized version to Study Director
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New Products Division - Regulatory Sciences

SAMPLE HANDLING FORM
SOP Reference: GEN-POL-002 OR GEN-FLD-0

Study #: 93 01 30 01 Experiment #: 93-454-706

	Monsanto Sample # for Monsanto Use only	Plot #	Sample Description (e.g. PLANT PART & LOC., NUMBER OF SAMPLES, WGT., ETC.)	Collection Date	Collectors Initials	Rc'd (✓)	Comments
1.	1	1002	1421	9/21	JC	✓	
2.	2	106	Cobra 312	9/21	JC	✓	
3.	3	501	1698	9/21	JC	✓	
4.	4	1101	1698	9/21	JC	✓	
5.	5	903	1445	9/21	JC	✓	
6.	6	202	1421	9/21	JC	✓	
7.	7	301	Cobra 312	9/21	JC	✓	
8.	8	201	1698	9/21	JC	✓	
9.	9	1103	1445	9/21	JC	✓	
10.	10	402	1421	9/21	JC	✓	
11.	11	406	Cobra 312	9/21	JC	✓	
12.	12	303	1445	9/21	JC	✓	
13.							
15.							
16.							
17.							
18.							
19.							
20.							

Interim Storage: On Ice

Number of Shipping Containers: 1 Method of Shipment: UPS/Next day

Shipping Conditions (✓ one): ☐ Frozen on Dry Ice ☐ Cool on Ice ☐ Ambient Temp.

☑ Other (please specify): Cool on dry ice

Samples Transferred by: [signature] Date and Time: 7/21/93
Signature

The following should be completed by Recipient:

Samples Received and Checked by: [CBI DELETED Date and Time: 9/23/93 4

Condition of Samples upon receipt: Cool but not frozen

Interim Storage:

Distribution of Copies: WHITE COPY Included with Shipment, recipient sends finalized version to Study Director
YELLOW COPY Included with Shipment, Recipient Copy
PINK COPY Shipper Copy

ENTRY	ENTRY#	REP	TISSUE	PLOT	GOSSY	TANNIN	FLAVON	ANTHO
M 531	1	1	LEAVES	4809	0.106	10.299	0.583	0.303
M 531	1	2	LEAVES	4841	0.125	13.053	0.855	0.405
M 531	1	3	LEAVES	4893	0.109	9.757	0.587	0.200
M 531	1	4	LEAVES	4917	0.116	12.710	0.714	0.262
M 531	1	5	LEAVES	4941	0.146	12.604	0.809	0.381
M 531	1	6	LEAVES	4985	0.118	12.690	0.761	0.315
M 757	2	1	LEAVES	4813	0.118	8.136	0.535	0.174
M 757	2	2	LEAVES	4861	0.100	12.471	0.697	0.214
M 757	2	3	LEAVES	4873	0.095	12.425	0.759	0.310
M 757	2	4	LEAVES	4909	0.120	12.811	0.730	0.313
M 757	2	5	LEAVES	4937	0.124	11.502	0.802	0.334
M 757	2	6	LEAVES	4997	0.106	9.849	0.658	0.213
M 931	3	1	LEAVES	4817	0.144	12.171	0.686	0.252
M 931	3	2	LEAVES	4853	0.129	12.873	0.698	0.220
M 931	3	3	LEAVES	4881	0.081	12.310	0.856	0.337
M 931	3	4	LEAVES	4921	0.104	13.739	0.766	0.264
M 931	3	5	LEAVES	4953	0.118	11.060	0.738	0.226
M 931	3	6	LEAVES	4973	0.118	11.684	0.688	0.237
M 1076	4	1	LEAVES	4821	0.116	9.800	0.698	0.285
M 1076	4	2	LEAVES	4849	0.101	11.232	0.839	0.342
M 1076	4	3	LEAVES	4885	0.094	10.705	0.685	0.231
M 1076	4	4	LEAVES	4913	0.086	12.339	0.831	0.319
M 1076	4	5	LEAVES	4949	0.169	11.207	0.733	0.252
M 1076	4	6	LEAVES	4977	0.145	11.035	0.687	0.259
M 1172	5	1	LEAVES	4825	0.115	13.349	0.766	0.263
M 1172	5	2	LEAVES	4869	0.092	10.121	0.645	0.187
M 1172	5	3	LEAVES	4889	0.114	12.356	0.653	0.213
M 1172	5	4	LEAVES	4905	0.089	12.235	0.656	0.264
M 1172	5	5	LEAVES	4945	0.092	9.147	0.681	0.203
M 1172	5	6	LEAVES	4981	0.135	11.461	0.800	0.261
M 1195	6	1	LEAVES	4829	0.106	12.515	0.739	0.230
M 1195	6	2	LEAVES	4845	0.177	9.367	0.800	0.260
M 1195	6	3	LEAVES	4897	0.084	18.707	0.758	0.276
M 1195	6	4	LEAVES	4933	0.138	14.975	0.817	0.270
M 1195	6	5	LEAVES	4965	0.132	13.902	0.776	0.273
M 1195	6	6	LEAVES	4969	0.118	10.355	0.799	0.234
COK 312	7	1	LEAVES	4833	0.184	18.468	0.752	0.361
COK 312	7	2	LEAVES	4857	0.185	17.808	0.905	0.456
COK 312	7	3	LEAVES	4877	0.089	16.450	0.789	0.312
COK 312	7	4	LEAVES	4925	0.136	17.237	0.714	0.255
COK 312	7	5	LEAVES	4957	0.109	18.768	0.819	0.356
COK 312	7	6	LEAVES	4989	0.157	13.907	0.795	0.324
DES 119	8	1	LEAVES	4837	0.158	17.043	0.637	0.279
DES 119	8	2	LEAVES	4865	0.102	15.647	0.696	0.320
DES 119	8	3	LEAVES	4901	0.128	13.683	0.590	0.244
DES 119	8	4	LEAVES	4929	0.113	15.750	0.733	0.322
DES 119	8	5	LEAVES	4961	0.080	13.158	0.607	0.275
DES 119	8	6	LEAVES	4993	0.157	12.090	0.563	0.247

1993 RT LINES CHEMICAL ANALYSIS

ENTRY#	REP	TISSUE	PLOT	GOSSY	TANNIN	FLAVON	ANTHO
1421	1	LEAVES	202	0.151	2.880	0.446	0.117
1421	2	LEAVES	402	0.166	3.697	0.514	0.107
1421	3	LEAVES	1002	0.160	4.423	0.575	0.118
1445	1	LEAVES	303	0.141	3.548	0.560	0.110
1445	2	LEAVES	903	0.136	3.560	0.520	0.103
1445	3	LEAVES	1103	0.129	4.407	0.603	0.129
1698	1	LEAVES	201	0.175	4.945	0.578	0.117
1698	2	LEAVES	501	0.135	3.219	0.594	0.093
1698	3	LEAVES	1101	0.098	4.639	0.594	0.100
C312	1	LEAVES	106	0.116	4.191	0.572	0.113
C312	2	LEAVES	301	0.169	3.147	0.576	0.097
C312	3	LEAVES	406	0.148	3.792	0.603	0.120

Samples for RT analysis are from Study 93-01-30-01, Study 93-45-4-706. Samples were received at Mississippi State location on 9/22/93. They were cool but not frozen.

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Appendix VIII

Comparison of the *B.t.k.* HD-73 Protein Expressed by Insect Resistant Cotton with Commercially Available Microbial Pesticides Containing *B.t.* Proteins

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Appendix IX

Management of Insect Pests with Insect Resistant Plants: Recommended Approaches

MANAGEMENT OF INSECT PESTS WITH INSECT RESISTANT PLANTS: RECOMMENDED APPROACHES

Monsanto Agricultural Group
St. Louis, MO

Abstract

Insect resistant corn, cotton, and potatoes, which exhibit a high level of protection to damage and yield loss by lepidopteran pests (cotton and corn) and the Colorado potato beetle (potatoes) have been developed through the expression of *B.t.* genes in plants. Monsanto has developed recommended approaches to utilize these plants to maximize the utility and durability of these new insect control products. These approaches are being tested and will be optimized in the field prior to commercial introduction of insect resistant crops.

Introduction

Insect resistant crops represent an important new management tool to control crop damage and loss due to insect pests. These plants offer significant benefits to the grower, the consumer and the environment. Insect resistance has been developed through the expression of genes that produce insecticidal proteins from *Bacillus thuringiensis* (*B.t.*) in the cells of the plants. The particular genes being developed by Monsanto for cotton and corn are derived from the *B.t. kurstaki* strain, and for potatoes from *B.t. tenebrionis*. These proteins are the basis of several commercially available microbial insecticides, which have been demonstrated as highly selective for insects, with no activity against other types of living organisms such as mammals, fish, birds or non-insect invertebrates (earthworms, spiders, etc.) (EPA, 1991; EPA, 1988). In addition, these proteins show a remarkable insect specificity (MacIntosh *et al.*, 1990). The *B.t.* genes developed for cotton and corn produce proteins that are active only against certain lepidopteran larvae with no activity against other orders of insects. Importantly, this activity spectrum overlaps with several important pests of these crops which include the tobacco budworm, cotton bollworm or corn earworm, European corn borer, pink bollworm and several others such as cabbage looper, salt marsh caterpillar and cotton leaf perforator. Likewise, the *B.t.t.* gene developed for potatoes produces a protein active only against the Colorado potato beetle (CPB). Because these control agents are proteins, they have been found to break down rapidly in the environment and in mammalian digestive systems (Monsanto, 1993; Monsanto, 1994).

The use of insect resistant plants will provide important benefits to growers, society and the environment (McGaughey and Whalon, 1992; Gasser and Fraley, 1989; Gould, 1988). First and foremost, these plants offer an alternative to chemical insecticides currently used to control susceptible insect pests with efficacy equal to or better than that of current control methods. The use of insect resistant cotton, corn and potatoes will significantly reduce the application of chemical insecticides directed at these pests. The reduction of insecticide use will have direct benefits to the grower, such as less time and effort spent on insect control and reduced exposure to chemical insecticides.

Insect resistant crops are also likely to produce secondary benefits in pest control as an indirect result of the reduction in use of chemical insecticides. Chemical insecticides like pyrethroids are relatively non-specific and have the effect of killing beneficial predatory and parasitic insects (Roush and Tingey, 1993; Van den Bosch and Stern, 1962). Because the *B.t.* proteins produced by insect resistant plants are not active against these beneficial insects, populations have been shown to rise significantly in fields planted with insect resistant cotton and CPB resistant potatoes compared to nontransgenic cotton and potatoes treated with chemical insecticides (Feldman, *et al.*, 1993; Reed *et al.*, 1992; Stone *et al.*, 1992; Luttrell, pers. comm.). Preserving the beneficial insect population should enhance the biological control of both target pests and non-target pests such as mites, aphids, and leafhoppers, which increase as problems as their natural predators are removed. In addition, insect resistant cotton and corn and CPB resistant potatoes are equally capable of controlling target pest populations, which are beginning to lose their sensitivity to chemical insecticides (Everich, 1994; Stone and Sims, 1993), thus filling a need that is likely to grow in coming years.

The use of insect resistant plants will provide important benefits to growers, society and the environment. To achieve these benefits, it is important that insect resistant plant strategies be implemented and managed properly. In this respect, these plants are no different than any other pesticide. There are two aspects of this management. First, is the development of pest management techniques that allow the farmer to maximize the ability of these plants to control target pests. In essence, this is the development of a total insect management package that will be centered around a new tool, insect resistant cotton, corn or potatoes. Second, is the development of appropriate strategies to maximize the product durability and the utility of insect resistant crops. Part of this management program is the development and implementation of strategies targeted to prevent the development of insect resistance to the *B.t.* proteins produced by these plants. Because both management aspects can affect the way in which insect resistant plants are used by the grower, these two types of management, total pest management and insect resistance management, are interconnected.

Resistance management is not an issue particular to insect resistant plants, given the development of insect resistance to chemical insecticides. Monsanto scientists have addressed insect resistance for several years in laboratory and field studies and with outside collaborators we have examined nearly every suggestion that has been made for resistance management in insect resistant plants (Everich, 1994; Roush, 1994; Sachs, 1993; Stone and Sims, 1993). As the following discussion will demonstrate, promising strategies for resistance management for insect resistant plants are available and can be recommended. These strategies have been developed in consultation with an expert advisory panel established for each crop taking into account existing research and an understanding of crop production and agronomic practices. Consequently, these strategies may be specific for each crop and target pest. It is evident, however, that insect resistant plants offer some unique options in pest and resistance management that are not available with traditional pesticides.

Integrated Pest and Resistance Management with Insect Resistant Plants

As part of a package to provide economic control of insect loss and damage in cotton, corn, and potatoes, these insect resistant crops will provide a central focus around which other insect management practices will be applied. In many areas lepidopteran pests are the primary damaging insects of cotton and corn, so the use of these insect resistant plants to control these pests will be a major portion of total insect control. The primary pest in potato production is the CPB. Its control impacts the populations of other pests such as aphids and leafhoppers. By substituting genetically modified cotton, corn or potatoes for chemical pesticides directed at their target pests, a positive impact on overall insect management will result. Many of the details of pest management with insect resistant plants can only be determined by multi-year large scale field tests designed to incorporate these genetically modified crops into current production practices. Such field trials are in progress and are providing the data needed for developing a pest and resistance management program for these crops. These trials involve collaborations between Monsanto, HybriTech Seed International (a wholly owned subsidiary of Monsanto), seed company partners, and academic and extension entomologists. They are examining the impact of insect resistant plants on populations of beneficial and pest insects endemic to the crops and the impact on the use of conventional insecticides for controlling non-target pests (Feldman, *et al.*, 1993; Reed *et al.*, 1992; Stone *et al.*, 1992; Luttrell, pers. comm.), the establishment of the baseline susceptibility of our insect targets to *B.t.* protein (Stone and Sims, 1993; Everich, 1994; Luttrell, pers. comm.) and the impact of mixtures of resistant and non-resistant plants on yield loss (Roush, 1994).

Insect resistant cotton, corn and CPB resistant potatoes will be important additions to the available methods of controlling insect pests. The implementation of these plants is fully consistent with the goals of integrated pest management because:

a) the *B.t.* protein produced by the plants is insect specific, affecting only a few targeted pest species

b) the *B.t.* protein is active only against insects feeding on the plant and thus doing damage

c) use of the plants will reduce the application of chemical insecticides

d) use of the plants will preserve beneficial insects, which will enhance the biological control of non-target pests

Because pest and resistance management are interconnected, it is important to develop both of these approaches in tandem for each insect resistant crop.

Combination of Insect Resistant Plants with Chemical Insecticides

One aspect of the use of insect resistant plants for integrated pest management in corn, cotton, and potatoes is the continued use of chemical insecticides. Some insecticides will continue to be used in these crops for non-target pests. If possible, these insecticides need to be chosen so as to not negatively impact beneficial arthropods, which are integral in the biological control of non-susceptible species. The combination of insect resistant crops with chemical insecticides, while part of a total insect control package, is not a resistance management option for insect resistant plants per se. Chemical insecticides can reduce the population size of insects selected for resistance to *B.t.* but cannot alter the gene frequencies within this population (Roush, 1989). Alternatively, insect resistant plants should positively impact current chemical insecticides by helping slow resistance development and prolonging the life of these important agricultural chemicals.

Resistance Management for Insect Resistant Plants

As described above, part of managing the implementation of insect resistant plants is the design and implementation of appropriate strategies to delay or prevent the development of insect resistance to *B.t.* protein in cotton, corn or potatoes. Described below are approaches that will help manage resistance development in these crops. It is important to note that: 1) as insect resistance development is a biological phenomenon, the rate of development is difficult if not impossible to predict and consequently, the efficacy of a strategy to delay or prevent its development may be impossible to demonstrate; 2) because of the available technology, biology of the pest, and the production practices of the crop, implementation of these strategies will be dependent on the crop and the target pest; and 3) field research must be conducted to determine the practical implementation of these strategies within current crop production practices. These strategies have been recommended by several researchers (Gould, 1988; Stone *et al.*, 1991; McGaughey and Whalon, 1992) and are summarized briefly below and then expanded in greater detail in the next section.

Summary of Considered Resistance Management Strategies for Insect Resistant Cotton, Corn and Potatoes

- High dose expression of *B.t.* protein in plants to control insects heterozygous for resistance alleles.
- Refugia as hosts for sensitive insects provided through non-insect resistant plants or other non-modified hosts.
- Monitoring of insect populations for susceptibility to *B.t.* protein.
- Agronomic practices that minimize insect exposure to *B.t.* plants.
- Integrated pest management (as described above).
- Combination of multiple genes within the same cotton plant, both of which are active against targeted insects but with different sites/modes of action.
- Incorporation of host plant resistance traits into insect resistant cotton and corn as they are proven effective.
- Incorporation of novel proteins that provide effective control of targeted pests.

Details of Resistance Management Strategies

High Dose Expression

High dose expression for resistance management is based on three assumptions:

1) Resistance will most likely be controlled by one major locus with recessive esistance alleles McGaughey and Beeman, 1988; MacIntosh *et al.*, 1991; Sims and Stone, 1991).

2) Insects developing resistance to the *B.t.* protein will be rare initially and will almost always mate with susceptible insects giving rise to heterozygous progeny (Gould, 1986).

3) More than 95% of the heterozygous progeny will be disabled or killed by insect resistant plants with the same dose as the homozygous susceptible larvae.

The high dose expression strategy uses plant expression of *B.t.* protein in quantities sufficient to kill those insects heterozygous for resistance to *B.t.* (McGaughey and Whalon, 1992; Roush, 1989). This resistance strategy fits nicely with the fact that high dose expression is essential for commercial efficacy of CPB resistant potatoes and insect resistant cotton and corn

because of the range of sensitivity to the *B.t.* protein in corn and cotton insect targets (e.g., at least a 10-fold difference between tobacco budworm and European corn borer and cotton bollworm). High dose expression is also necessary to maintain consistent control across environments and genotypes. We plan to evaluate and develop the high dose expression strategy.

Refugia for Sensitive Insects

Refugia means providing a refuge for sensitive insects within a population so they will not be exposed to *B.t.* protein and not be selected for resistance. As a resistance management technique, refugia is based on the concept that control failure due to resistance is a population genetics phenomenon. Control failures are observed when the frequency of resistant insects in the population reaches a critical level. Refugia supply susceptible non-selected individuals to the general population. With adequate refugia, the frequency of resistance genes will be very low and spread only very slowly through the population. Refugia is an important component of our insect resistant crop resistance management strategies.

Refugia can be provided either within the crop or outside it. The refuge can also be planted specifically as such or exist naturally. In all of these approaches, the effectiveness of the refuge is based on those insects that survive on the refuge crop rather than its total acreage. This is an important point because, if the refuge is chemically treated, the refuge population is reduced and the amount of acreage required is increased. Examples of refugia that can be utilized are:

1) Refuge outside of the crop: Non-insect resistant cotton, corn or potatoes.

This type of refuge will exist in all the acres not covered by these insect resistant plants. This area will be substantial in the early years after introduction and could supply a sufficient refuge for several years. As insect resistant seed becomes more available and widely grown, this refuge will be reduced. Consequently, over time, reliance on non-insect resistant cotton, corn or potato fields for refugia may not be adequate.

2) Refuge outside of the crop: Non-modified crop hosts.

The European corn borer and the cotton bollworm or corn earworm have many non-corn or cotton hosts including other crops in all locations, which may provide an adequate refuge. The tobacco budworm and Colorado potato beetle have fewer alternatives and the pink bollworm has none. In some locations corn, cotton and potatoes may be the only host for at least one insect generation per season. The use of *B.t.* microbials or transgenic *B.t.* plants on other crops will also impact their utility as a refuge for insect resistant plants. This option must be evaluated carefully based on the crop, pest biology, and growing regions.

3) Refuge within the crop: Non-insect resistant plants.

In certain cases a likely solution is to provide an "in crop" refuge of non-insect resistant plants. For this in crop refuge, the choices are: a) random mixture of seed of insect resistant and non-resistant plants or b) non-insect resistant plants planted within the same field. The optimum refuge area required must be determined for each crop.

Mixed seed lines (*B.t.* and non-insect resistant seed within the same bag) have a certain appeal due to the "automatic" implementation. A possible problem with mixed seed arises from larvae that survive on a non-insect resistant plant and migrate to a modified plant where they may be less sensitive to *B.t.* protein because of their size. This could compromise insect control and increase selection pressure for resistance. The likelihood of this occurring is being investigated experimentally before this strategy is implemented.

There may also be economic and logistical problems if a mixed seed strategy is implemented. However, Monsanto, HybriTech and seed company partners are interested in determining the viability of the mixed seed approach. It is clear that field research is required to determine the percentage of non-insect resistant plants needed as a refuge, and what the impact of this percentage on over all yield, quality and seed company economics.

Another in-crop refuge could be non-insect resistant plants planted specifically by the farmer. Besides providing a refuge, such planting of separate indicator rows of non-insect resistant plants could potentially make scouting easier. Field research is needed to determine the optimum type of planting regime.

Agronomic Practices

Certain agronomic practices may need to be recommended for insect resistant plants. In particular, plow down dates to eliminate unnecessary insect exposure to *B.t.* protein from cotton regrowth or rotating CPB resistant potatoes with non-resistant potatoes may need to be recommended. The recommendation of these strategies will be determined on a regional basis, if necessary.

Monitoring Insect Resistance

Insect resistance monitoring is an important component of any insect resistance management strategy. A baseline frequency is in development. Resistance of major target pests to *B.t.* protein has not been detected in the field (Everich, 1994; Stone and Sims, 1993; Luttrell, pers. comm.). Baseline information should be collected on all *B.t.* products (engineered plants and *B.t.* microbials) to know when the frequency of resistant genotypes have increased within the population. This information must be developed on regional bases over several years so that susceptibility changes in populations can be identified and validated.

Pyramiding Traits

A set of strategies for the medium and long term focus on combining multiple insecticidal agents. The rationale is essentially the same for all of these: Expose the insects to two or more active agents with distinct modes of action at the same time, and the probability of any one insect being selected for resistance to both agents simultaneously is extremely low.

1) Combination with a Second Insect Resistance Gene

A second gene within the same plant possessing a different mode of action will significantly reduce the frequency of resistant individuals (Peferoen, 1992; Stone *et al.*, 1991; Van Rie, 1991). Population models indicate that other alternative uses of a second gene such as seed mixture or using single genes in rotation, may be as effective as two genes within the same plant (Gould, 1988; Gould 1986). Assuming initial gene frequencies for *B.t.* protein resistance are low, initial introduction of a product with a single *B.t.* gene should not negatively compromise a second gene because the single gene product will be planted on limited acres in the first few years. In the medium term the best choice of second gene is an unrelated *B.t.* gene. In the long term, the use of novel, non-*B.t.* insecticidal genes holds great promise. This area is under active research.

2) Combination with Host Plant Resistance Traits

This is a long term strategy to be implemented by seed companies or public breeders. Host plant resistance traits (HPR) used in combination with insect resistant cotton or corn need to be insecticidally effective and not negatively impact quality or yield. For example, Monsanto currently has funded research on HPR to help set direction on HPR traits that alone or in combination are useful in protecting the plant from lepidopteran insects in cotton (Sachs, 1993). Cotton seed companies are interested in incorporating these traits if they are effective and have no negative effects on yield or quality. Similar work is planned with insect resistant corn. This strategy may have limited application to potatoes, however, as there are few varieties available that provide adequate CPB control and have desirable yield and quality characteristics.

Summary

Insect resistant cotton, corn and potatoes will offer great benefits in overall insect control in these crops. These plants will be developed to fit within existing pest management practices. Research programs for each crop have been in place for several years and will continue. With proper management and implementation, the development of insect resistance to *B.t.* will not be a technical or commercial problem that will limit the value or efficacy of these products. Monsanto has developed a package of strategies that will help effectively manage the potential development of

insect resistance. The details of this program and its incorporation into existing pest management programs will be further developed and optimized in the field in the coming years.

Many aspects of the use of insect resistant plants in pest management and the implementation of resistance management strategies are unique to these products as compared to traditional chemical or microbial insecticides. For example, the use of refugia and the incorporation of multiple resistance traits through molecular biology or plant breeding are aspects that are ideally suited to insect resistant plants. This ability to utilize new methods in pest and resistance management make genetically modified insect resistant plants a critical component for successfully managing insect pests in the future.

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