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SAFETY, COMPOSITIONAL AND NUTRITIONAL ASPECTS OF BOLLGARD™ COTTON LINE 531: CONCLUSION BASED ON STUDIES AND INFORMATION EVALUATED ACCORDING TO FDA'S POLICY ON FOODS FROM NEW PLANT VARIETIES

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Abbreviations Used in this Summary of the Safety, Compositional and Nutritional Aspects of Bollgard™ Cotton Line 531

aad	Gene for 3"(9)-O-aminoglycoside adenylyltransferase
AAD	3"(9)-O-aminoglycoside adenylyltransferase
APHIS	Animal and Plant Health Inspection Service
ATP	Adenosine triphosphate
bp	Base pairs
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
C	Centigrade
C312	Coker cotton variety 312
cal/g	Calories per gram
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
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
g	gram
IPM	Integrated Pest Management
Kb	Kilobase pairs
lbs	Pounds
Line 531	Bollgard™ Cotton Line 531
M	Million
mL	milliliter
mg	milligram
NOS 3'	Poly A termination signal sequence 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
7S 3'	Poly A termination signal sequence for *cryIA(c)*
SOP	Standard Operating Procedure
T-DNA	Transfer-DNA
TBW	Tobacco budworm
µg	microgram
USDA	United States Department of Agriculture
UV	Ultraviolet

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INFORMATION TO SUPPORT THE HUMAN FOOD/ANIMAL FEED SAFETY OF BOLLGARD™ COTTON LINE 531

I. INTRODUCTION

A. Application of FDA Food Policy

In its May 29, 1992 statement of policy concerning "Foods Derived from New Plant Varieties," ("Food Policy" or the "Policy"), the Food and Drug Administration ("FDA") provided guidance for determining whether a new plant variety developed with the aid of new genetic techniques is as safe and nutritious as its parental variety, (See 57 Fed. Reg. 22965). The Policy is structured around decision trees that are designed to establish whether the new plant variety is materially different in composition, safety or any relevant parameter from its parental variety. The Agricultural Group of Monsanto Company has carefully followed the guidance in the Policy to assess whether cotton modified by the addition of genes producing a lepidopteran control protein derived from *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) and the selectable marker neomycin phosphotransferase II (NPTII) are materially different from cotton currently being marketed. To ensure as thorough an assessment as possible, Monsanto has conducted numerous compositional studies and accessed all existing relevant data and information. Upon qualitatively and quantitatively evaluating all of the data available, we have been able to ensure that there are no biologically important compositional differences between Bollgard™ Cotton Line 531 (Line 531) and its parental variety Coker 312 (C312). In a few instances a small, but statistically significant, difference was noted. Where the differences exist, the values or effects are well within established ranges documented and reported in the scientific literature for cotton. This assessment is summarized in this submission in a manner consistent with the Policy. The conclusions are straightforward: except for resistance to lepidopteran caterpillars and the presence of the selectable marker protein, Line 531 is not materially different from and is as safe and nutritious as cotton varieties now marketed.

B. The *B.t.k.* Protein and the Selectable Marker

The use of the *B.t.k.* protein in Line 531 is regulated by EPA as a plant pesticide. The NPTII protein has no insecticidal effect and is identical to the NPTII protein which is an approved food additive processing aid (FDA, 1994) for use in cotton, tomato and oilseed rape and by EPA as a plant-pesticide inert ingredient in or on all agricultural commodities (EPA, 1994). As noted above, our extensive studies and information collection establish that the presence of these proteins and the process used to produce the new plants result in no material difference between Line 531 and cotton grown commercially today.

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The FDA Policy discusses at some length the jurisdictional issues presented upon federal review and consideration of plants modified to express pesticidal substances, (See 57 Fed. Reg. 22965, 23004-5). The discussion recognizes not only the legal basis for one agency assuming priority of review and decision making on aspects of an issue but also the efficiency inherent in such a process. In fact, FDA's Policy is premised on the practical application of existing statutory provisions in an effort to minimize duplication in the regulation of genetically engineered crops. In particular, the Policy is designed to eliminate repetitive expenditure of federal resources on the same issues and to focus only on those issues ripe for a decision. This reflects sound regulatory and public health decision making.

The effect of this policy is to allocate the safety review of aspects of Line 531 between FDA and EPA. The safety of the *B.t.k.* protein is being thoroughly evaluated by EPA on the basis of scientific and empirical information submitted to that agency by Monsanto. It is anticipated that evaluation of our submission by the EPA, will shortly result in the registration of the *B.t.k.* protein as a plant pesticide and its exemption from the requirement of the tolerance governing such use. The regulatory and scientific decisions by the EPA, will establish the safety of the *B.t.k.* protein in Line 531. All other issues that pertain to the food and feed safety of cotton fall within the compass of the FDA's review and are addressed at length in this submission.

In accordance with this policy, Monsanto will seek the following approvals before commercializing Line 531:

- A Determination from USDA/APHIS that Line 531, and all progenies derived from crosses between Line 531 and other cotton cultivars, is no longer a regulated article according to 7CFR §340.6 (USDA, 1994). This petition was submitted on November 5, 1994.

- Regulatory approval from the EPA of the *B.t.k.* insecticidal protein as expressed in Line 531 under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). This petition (#4F4331) was submitted on February 15, 1994. In addition, this same petition requests that EPA establish an exemption from the requirement of a tolerance for the *B.t.k.* insecticidal protein, under section 408 of the Federal Food Drug and Cosmetic Act (FFDCA).

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.

C. Consultations with FDA

The submissions to the EPA and USDA fully support the environmental and non-target safety of this cotton line and the proteins produced. Therefore, we will conclude our consultations with FDA on the safety and wholesomeness of the non-pesticidal components of Line 531, under the Statement of Policy "Foods Derived From New Plant Varieties", published in the Federal Register May 29, 1992 (FDA, 1992). We have held consultations with FDA, starting in 1990, to define and discuss studies to assess the composition and safety of cotton and other plant varieties. The concepts and approaches we have employed are derived from and consistent with the guidance presented in the flow charts found in the FDA Food Policy (FDA, 1992). For each question, we have developed answers based on extensive studies or analyses. The thoroughness and detail of these studies are unprecedented for the typical introduction of foods or feeds from a new plant variety. Our data and findings in every case have led us to the conclusion of "no concern", as described in the relevant sections of the following summary. Under these circumstances, following the Agency's Food Policy, the data have provided us with a basis for concluding that Line 531 is as safe and nutritious as its non-transformed parental variety, C312.

II. RATIONALE FOR THE DEVELOPMENT OF LINE 531

A. Rationale for the Development of Insect Resistant Cotton

Cotton is the leading plant fiber crop produced in the world and the most important in the United States. Cotton production in the United States is located primarily in the tier of 15 southern states stretching from North Carolina to California, with approximately 13 M acres grown. 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 with approximately $[redacted] spent annually for chemical control, ([redacted] 1993).

Monsanto, through genetic modification, has developed cotton plants which control lepidopteran insect pests by producing the insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) (Perlak *et al.* 1990; Perlak *et al.*, 1991). The protein produced by insect resistant cotton line 531 is essentially identical (99.4% identical) to a *B.t.k.* protein found in nature (Adang *et al.*, 1985) and comparable to the *B.t.k.* protein present in commercial microbial formulations (EPA MRID# 43145201), registered with the EPA and exempt from the requirement of a tolerance since 1957.

The *B.t.k.* HD-73 and NPTII proteins are produced as a small fraction of the total weight of a cotton plant and cotton seed, (1.56 and 0.86 µg/gram fresh weight for cotton leaf and seed tissue respectively for *B.t.k.* HD-73 and approximately 3.14 and 2.45 µg/gram fresh weight for leaf and seed tissue respectively for NPTII) (EPA MRID# 43168701). Environmental fate studies have shown that the *B.t.k.* HD-73 protein is rapidly degraded in the soil and does not persist in the environment (EPA MRID# 43145215). In

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addition, the risk of an uncontrolled introduction of the genes introduced into this cotton variety to the environment, through hybridization or outcrossing to a native species resulting in a new weed variety, is virtually non-existent on the mainland of the United States as there are no wild relatives with which it can cross exist in cultivated areas of the U.S. (USDA, 1994). Finally, agronomic evaluations consisting of plant vigor, growth habit characteristics, and general disease susceptibility, have shown Line 531 to be equivalent to the parental C312 variety (USDA, 1994).

In support of Monsanto's request to the EPA for the registration and exemption from the requirement of a tolerance of the *B.t.k.* protein as a plant pesticide, studies demonstrating the safety of this protein to nontarget organisms and the environment were conducted. These studies demonstrated that the protein has a limited spectrum of insecticidal activity, with no deleterious effect on beneficial insects, mammals or birds (EPA MRID# 43145205 through EPA MRID# 43145213; EPA, 1988). These results fully confirm the findings of similar studies conducted with commercially available microbial *B.t.k.* formulations.

Results from three years of field experiments conducted throughout the primary cotton growing regions have demonstrated effective control of the targeted lepidopteran insect pests, comparable or superior to weekly application of chemical insecticides (USDA, 1994). Use of these insect resistant cotton plants will lead to a substantial reduction in chemical insecticides, enhance biological control of other insect pests and support implementation of other pest management strategies for cotton pests not susceptible to the *B.t.k.* protein; e.g. cotton boll weevil, lygus bugs, fleahoppers, spider mites, thrips and aphids.

The commercialization of Line 531 (and any progenies derived from crosses between Line 531 and traditional cotton varieties), following receipt of all required approvals, 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 method 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

Lepidopteran insects are the main pest problem on most of the 13 million acres of cotton produced in the United States, but 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 $[redacted] is 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 is also 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 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 Integrated Pest Management (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.

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

Introduction

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* subsp. *kurstaki* (*B.t.k.*).

- The *nptII* gene which encodes the selectable marker enzyme neomycin phosphotransferase II (NPTII), was needed to identify transformed cells that potentially 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 lack of any expression was confirmed.

These genes were stably inserted into the genome of cotton using the *Agrobacterium tumefaciens* mediated transformation utilizing a single border binary transformation vector, PV-GHBK04 (EPA MRID# 43145201).

A. The 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 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 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 Line 531 being 99.4% identical to the naturally occurring *B.t.k.* HD-73 protein.

The *cryIA(c)* gene sequence, as introduced into Line 531, is shown in Appendix I, Figure 1. The corresponding amino acid sequence is shown in Appendix I, Figure 2.

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 encoded by the *nptII* gene, 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 Line 531 is shown in Appendix I, Figure 3. The corresponding amino acid sequence is shown in Appendix I, Figure 4.

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 in 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 Line 531 is shown in Appendix I, Figure 5. The corresponding amino acid sequence is shown in Appendix I, Figure 6.

B. 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 (E35S/*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).

C. *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

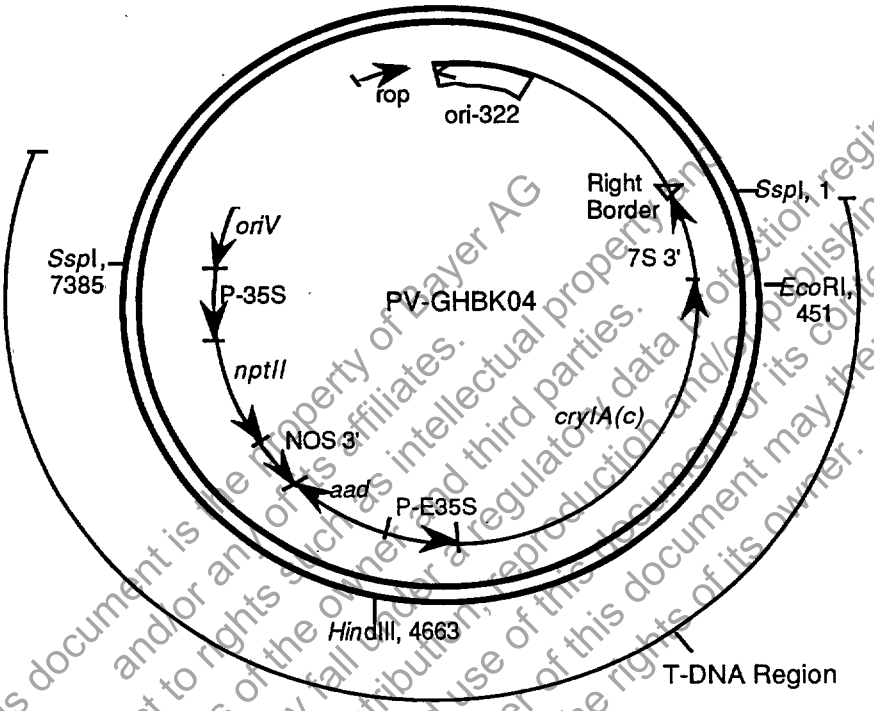

Figure III-1. Plasmid map of the 11.4 Kb binary vector PV-GHBK04 used to produce Bollgard™ Cotton Line 531. Restriction sites and their locations in bp, utilized during Southern analyses are shown. The T-DNA region is marked and the right border is denoted by an open triangle.

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.

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 insecticidal activity.

D. Description of a Genetic Element Contained in PV-GHBK04 but Absent from Line 531

The *ori322* region is present on the plasmid PV-GHBK04, but was not transferred and, hence, not present in the genome of Line 531. The *ori322* region is a 1.8 Kb segment of pBR322 (contained on a 3.0 Kb *SalI* to *PvuI* 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 Line 531 was demonstrated by Southern blot analyses.

E. Genetic Analysis

1. Insert number, copy number and insert integrity

As described in Part III-C, 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 (C312) and 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-2A, lane 7 shows that upon digestion of the DNA from 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-3A and III-4A, lane 7). The 0.7 Kb fragment did not hybridize to either the *cryIA(c)* or *nptII* probes (Figures III-3A and III-4A, lane 7) but did hybridize to the *oriV* probe (Figure III-5A, 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-3A, lane 7) but not the *nptII* probe, (Figure III-4A, 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 encodes the carboxy-terminal non-insecticidally active portion of the protein (Geiser, *et al.*, 1986), therefore, the active portion of the *B.t.k.* protein is not encoded by the second copy. In addition, the promoter for the *cryIA(c)* gene is not present, therefore, it is improbable that any protein would be produced from the second copy. The sizes of the fragments generated from the DNA isolated from Line 531 and cleaved with *Ssp*I are schematically illustrated in Figures III-2B through III-5B.

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-2A, lane 10, three fragments were released: the 4.2 Kb fragment which hybridized to the *cryIA(c)* gene probe (Figure III-3A, lane 10), a 3.6 Kb border fragment which hybridized to the *nptII* and *oriV* probes (Figures III-4A and III-5A,

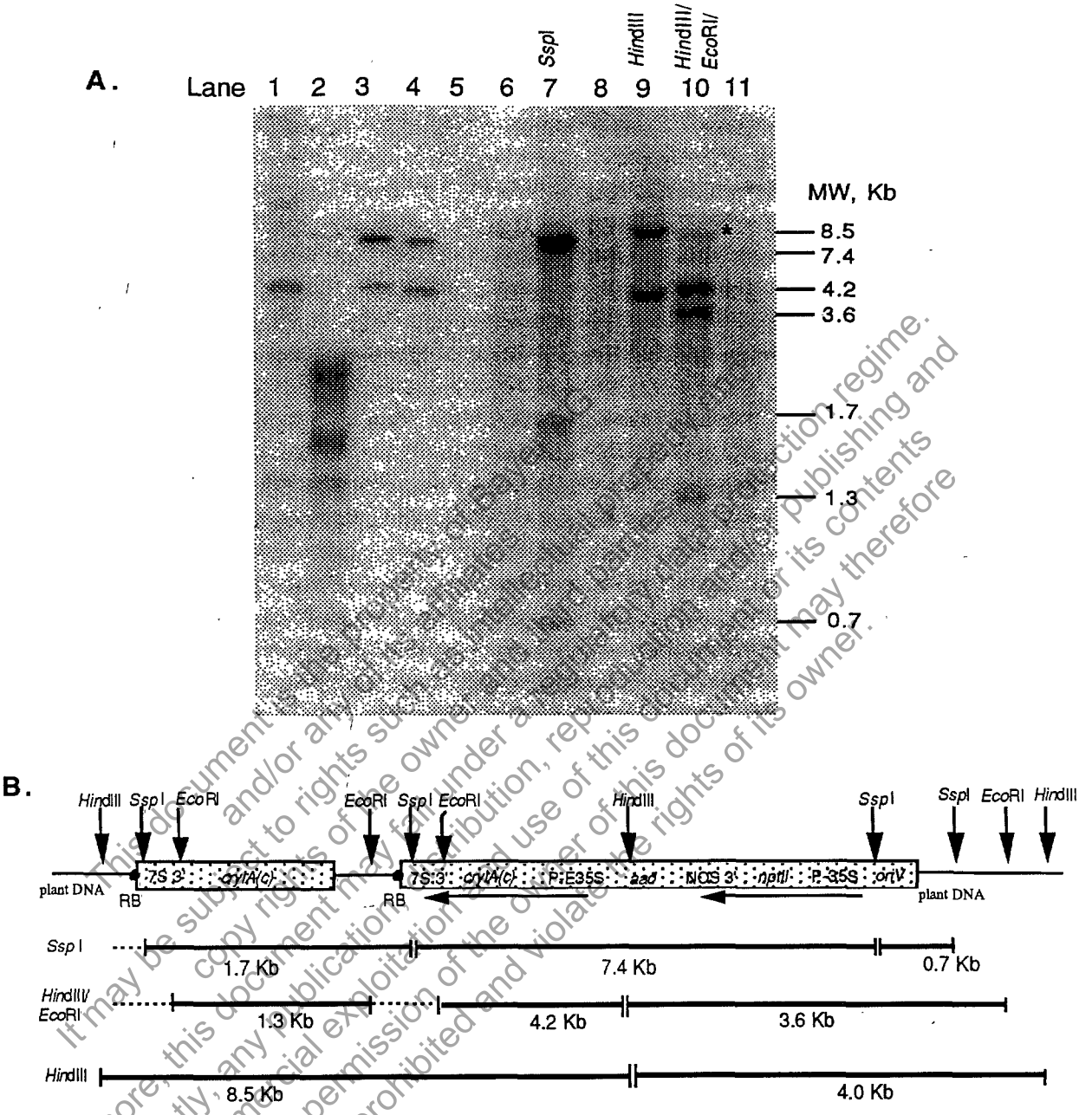

Figure III-2. Southern blot analysis using PV-GHBK04 as the probe. **A.** Southern blot analysis of DNA from line 531 using the entire plasmid, PB-GHBK04, as a probe. Lanes 1 and 2 are molecular weight standards and lanes 3 and 4 are plasmid PV-GHBK04 cleaved with the restriction enzymes *Hin*dIII and *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).

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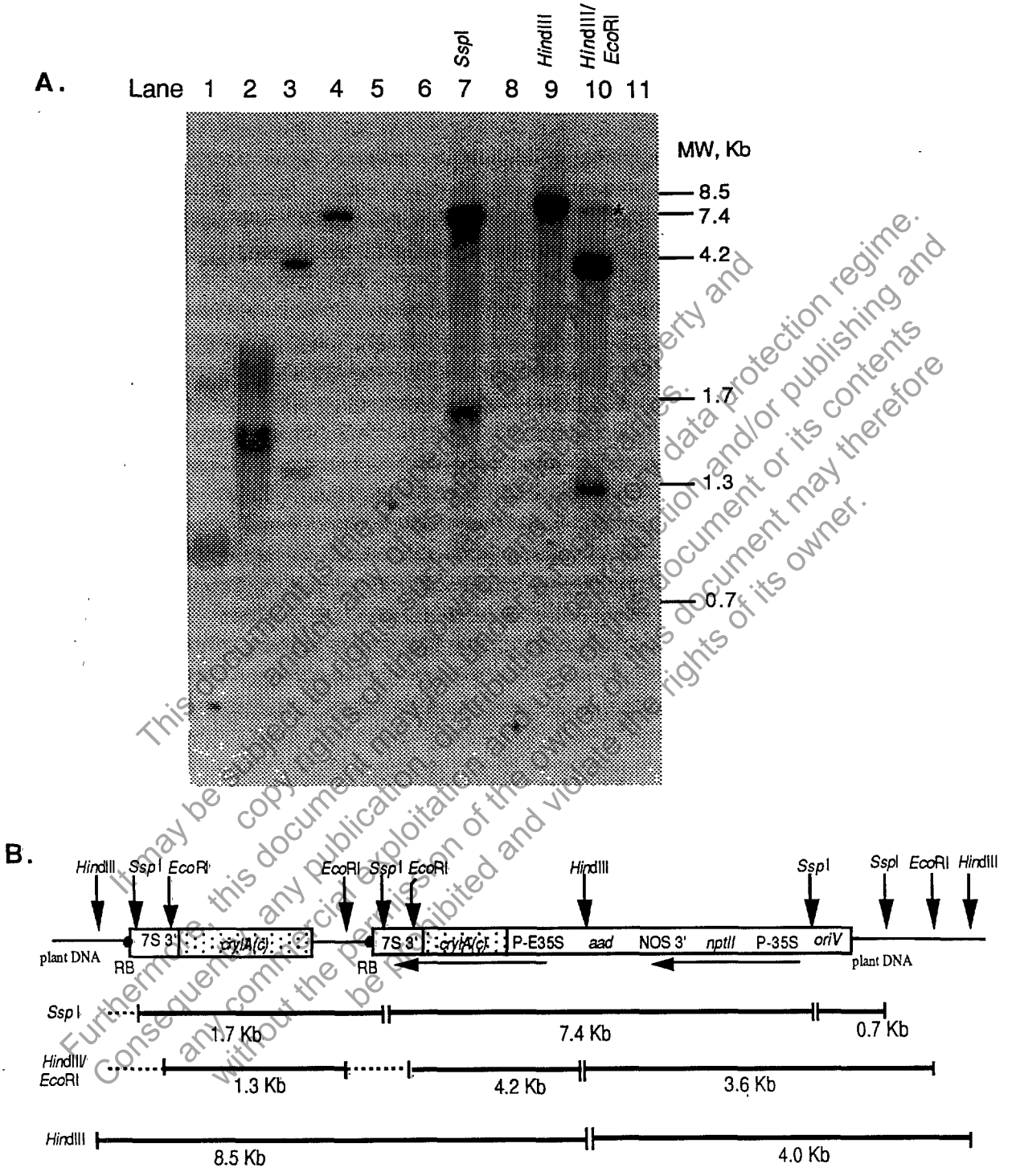

Figure III-3. Southern blot analysis using *cryIA(c)* as the probe. A. The same Southern blot from Figure 2, the plasmid probe removed, and reprobed with the *cryIA(c)* gene. Lane designations are the same as in Figure III-2. *Indicates a partial digestion. **B.** Schematic illustration of the T-DNA 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-2B.

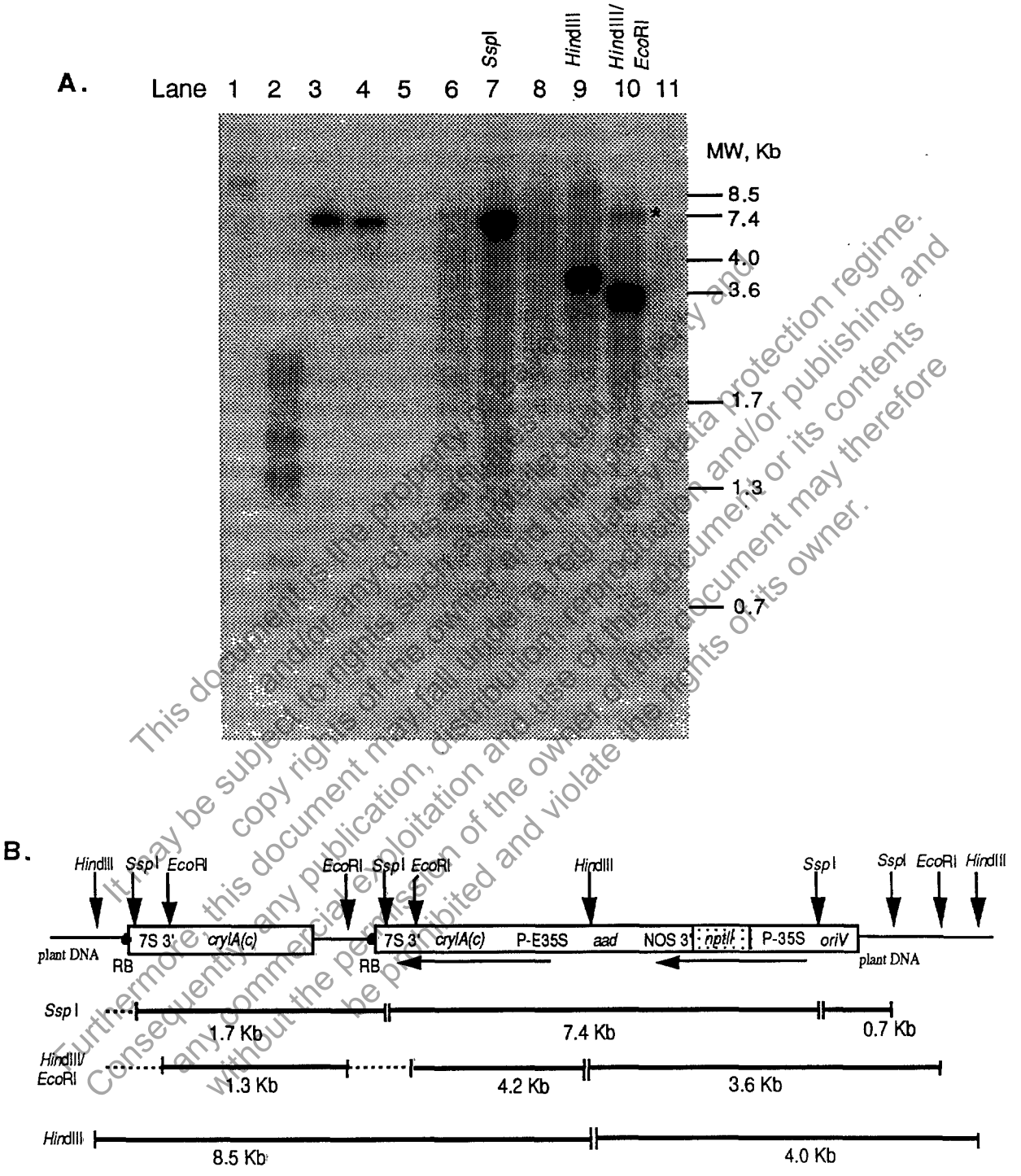

Figure III-4. Southern blot analysis using *nptII* as the probe. A. The same Southern blot from Figure III-2, the plasmid probe removed, and reprobed with the *nptII* probe. Lane designations are the same as in Figure III-2. *Indicates a partial digestion. **B.** Schematic illustration of the T-DNA insertion 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-2B.

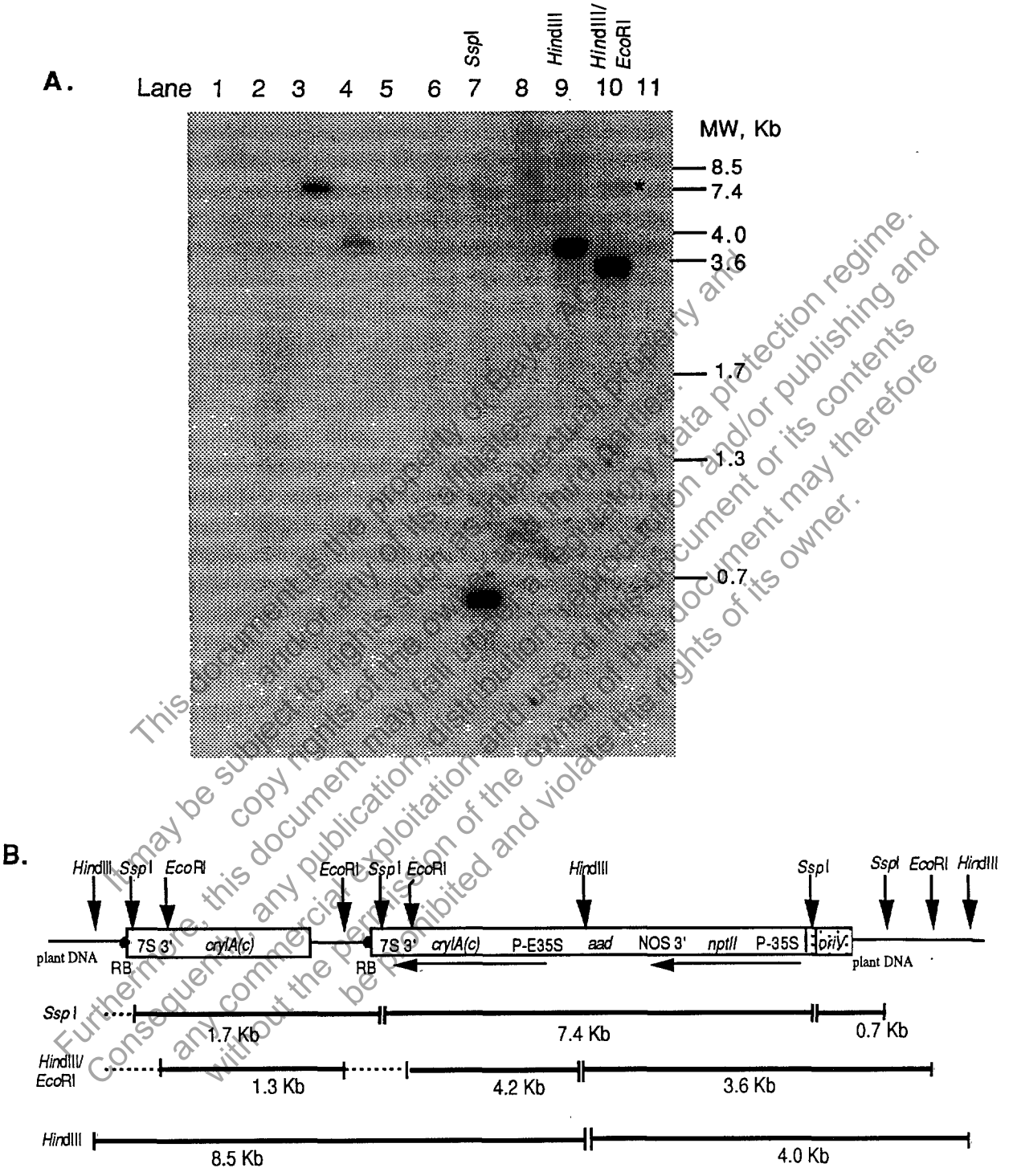

Figure III-5. Southern blot analysis using *oriV* as the probe. A. The same Southern blot from Figure III-2, the plasmid probe removed, and reprobed with an *oriV* probe. Lane designations are the same as in Figure III-2. *Indicates a partial digestion. **B.** Schematic illustration of the T-DNA insertion 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-2B.

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lanes 10, respectively) and a third band of approximately 1.3 Kb which also hybridized to the *cryIA(c)* gene probe (Figure III-3A, 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-2A through III-5A, 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 a 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-2B through III-5B.

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-2A, lane 9. The 4.0 Kb fragment hybridized to the *nptII* and *oriV* probes, Figures III-4A and III-5A, lane 9, while the

8.5 Kb fragment hybridized only to the *crylA(c)* probe, Figure III-3A, 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 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 *crylA(c)* gene, Figure III-3A, 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 *crylA(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 *crylA(c)* gene on a single fragment. The sizes of the fragments generated from the *Hin*dIII digestion is schematically illustrated in Figures III-2B through III-5B.

Further evidence that the two T-DNA inserts are tightly linked was provided from the analysis of commercial lines that were crossed with 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 Line 531. One T-DNA insert, of approximately 8.2 Kb in size, contains a full length *crylA(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 *crylA(c)* gene that cannot be insecticidally active since it does not contain the insecticidally active 5' region of the *crylA(c)* gene. The two inserts were shown to be linked and this is supported by segregation data from commercial backcrossed lines.

F. 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 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 *crylA(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 Line 531. The *crylA(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 Line 531 have been elucidated based on the nucleotide sequences.

- The *B.t.k.* protein produced in 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 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.

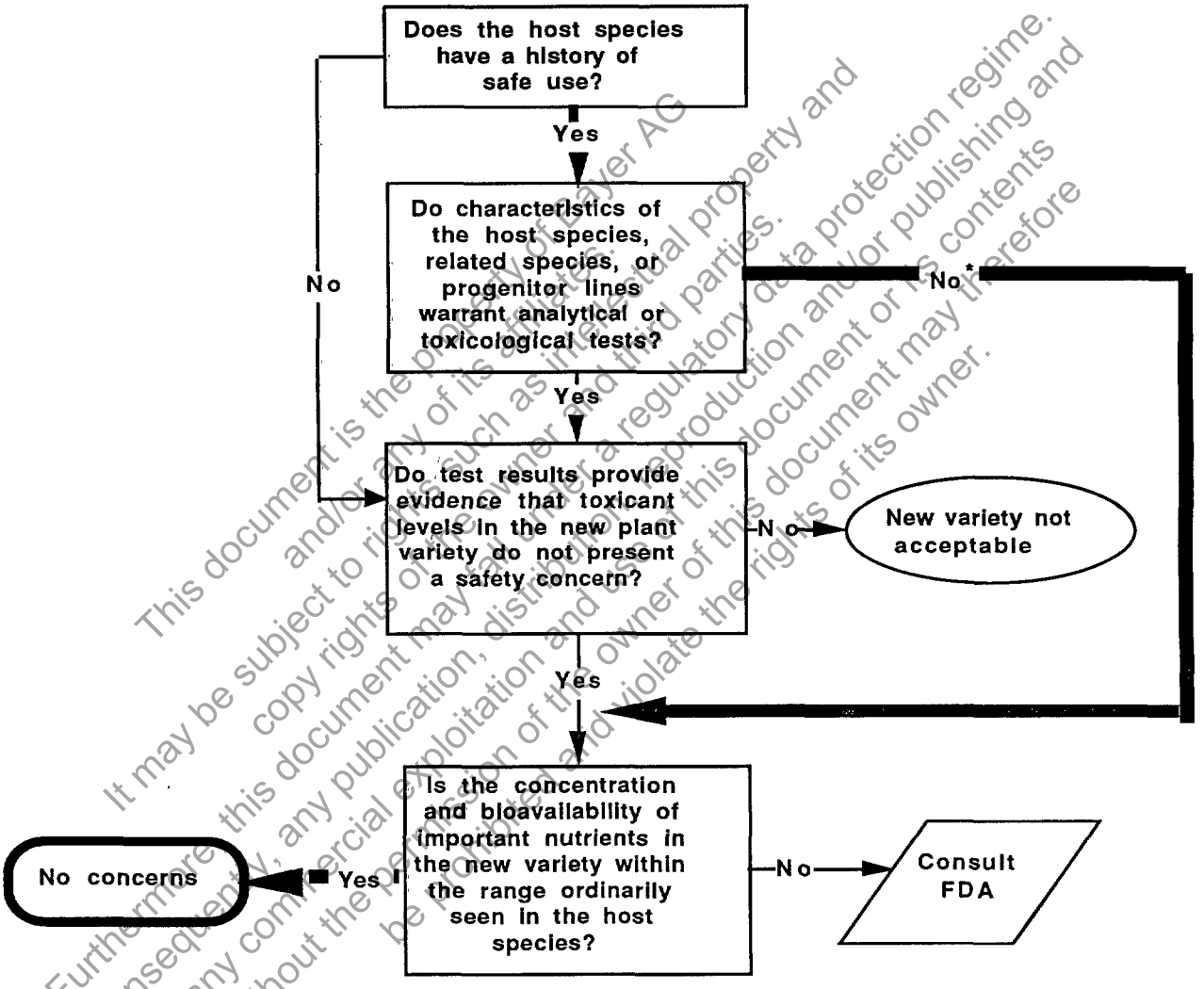
- The NPTII enzyme expressed in Line 531 functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation. It has no pesticidal activity and is not known to be toxic to any species.

- The *aad* gene, present in 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 Line 531.

IV. SAFETY ASSESSMENT OF THE NEW COTTON VARIETY

The flow charts presented in sections VII.D through VII.F of the FDA Food Policy, were utilized to organize the following summary of the studies conducted on Line 531 and other information which demonstrate the substantial equivalence of Line 531 as compared to the parental line C312 and other cotton varieties commercially grown. Background information regarding the development of insect resistant cotton, including a description of the insect control protein which confers the lepidopteran insect resistance phenotype, precedes these data.

Figure IV-1. Safety assessment of new varieties: the host plant (taken from FDA Food Policy, Figure 2). The pathway leading to "no concerns" for Bollgard™ Cotton Line 531 is highlighted with bold arrows.



* New cotton varieties are not typically subjected to extensive analytical or feeding tests. However, compositional analyses to verify levels of nutrients and antinutrients, as well as feeding studies to ensure the wholesomeness of Line 531, were performed as discussed in the Food Policy.

A. Safety Assessment of New Varieties: the Host Plant.

1. Safety Assessment of the Host Plant, Cotton

Cotton, the host plant, is the leading plant fiber crop produced in the world. Both cottonseed oil and cotton fiber, in the form of processed cotton linters, are routinely used for food products and have a history of safe use that is well documented. Therefore, the characteristics of cotton and Coker 312, the specific progenitor variety, do not warrant analytical or toxicological tests. Typically, cotton breeders make genetic crosses to generate new cultivars with enhanced commercial value. They evaluate new varieties based on lint and seed yield, quality, disease and pest resistance. The following is provided as additional background information on the production and consumption of cotton.

a. Cotton Production and Use

Cotton is grown worldwide, typically in arid regions of the tropical or sub-tropical areas (Niles and Feaster, 1984). It is grown primarily for the value of the fiber with cotton seed being a by-product. Cotton fiber accounts for almost 50% of the world textile fibers, with approximately 80% of the cotton fiber market made up of apparel and household products (Anonymous, 1979). Short fibers, known as linters, are a major source of cellulose for chemical and food uses. The fiber consists primarily of cellulose (>99%) (Cottonseed and Its Products, 1989). Based on the composition of fiber and the extensive processing used to process fiber prior to food use, fiber used for food is not expected to contain any detectable genetic material or protein. Cotton seed is used to produce oil for human food applications, and meal used for cattle feed.

b. Human/Animal Consumption

Cotton seed is processed into four major products as follows: oil, meal, hulls, and linters. Processing of cotton seed typically yields (by weight): 16% oil, 45% meal, 9% linters, and 26% hulls, with 4% lost during processing (Cherry and Leffler, 1984). Cotton seed oil is a premium quality oil that is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil. Cotton seed meal is principally sold as feed for livestock (Cottonseed and Its Products, 1989). According to a letter from [redacted] (Manager of Research & Education Services of the National Cottonseed Products Association), a 1987 survey indicated that approximately 92.9% of the cotton seed meal is used for cattle feed.

Cotton seed meal is not currently used for human consumption in the United States (Morgan, 1990; Cottonseed Oil, 1990). The presence of gossypol and cyclopropenoid fatty acids in cotton seed also limits its use as a protein supplement in animal feed except for cattle, which are unaffected by these components. Inactivation or removal of these components during processing enables the use of some cotton seed meal for catfish, poultry and swine, accounting for most of the remaining 7.1% of the cotton seed meal from above.

Cotton seed is highly processed during the production of oil and meal. After the majority of the fiber is removed at the cotton gin, a significant amount of fuzzy fiber remains associated with the seed. These short fibers (linters) are removed from the seed during delinting. After extensive processing at alkaline pH and high temperatures, the linters can be used as a high fiber dietary product, and food uses include casings for bologna, sausages, frankfurters, and to improve viscosity in products such as toothpaste, ice cream, and salad dressings (Cottonseed And Its Products, 1989).

Once the lint is removed from the seed, the hulls are cut and separated from the seed. After hulling, the cotton seed is flaked by a rolling process to facilitate oil removal. Prior to oil extraction, the flakes are heated to: (i) break down the cell walls; (ii) reduce the viscosity of the oil; (iii) coagulate the protein; (iv) inactivate proteins and kill any microbial contamination; (v) detoxify gossypol by the combination of heat and moisture; and (vi) fix certain phosphatides in the cake to minimize refining losses.

After cooking, oil is typically removed from the meal by direct solvent extraction with hexane. The material left after the extraction of the crude cotton seed oil is the cotton seed meal. The gossypol levels after extraction are reduced by approximately half. Crude cotton seed oil is further processed, depending on the end use of the product. A winterization step is added to produce cooking oil, whereas for solid shortening, a hydrogenation step is added to transform the liquid oil into a solid fat. Further processing (refining) for all the uses of cotton seed oil includes deodorization and bleaching. Deodorization greatly reduces the cyclopropenoid fatty acid content of the oil due to extreme pH and temperature conditions (Cottonseed Oil, 1990). The resulting oil contains no detectable protein (EPA MRID# 43168701).

2. Characteristics of the Non-transformed Cultivar

The altered genetic material which is the subject of this application is the Coker 312 cultivar of cotton (*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 commercialize Line 531, 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, Line 531 generated with a Coker 312 background is acceptable from an agronomic perspective for testing purposes.

3. Compositional Analysis of Line 531

Note: A description of the methods utilized to obtain the data reported as part of this summary are found in Appendix II of this summary. A complete description of these studies is found in EPA MRID# 43168701.

Six field sites were selected at which the Line 531 and C312 were grown, and subsequently used in the analyses summarized below. The six sites selected were: Starkville, Mississippi; Bossier City, Louisiana; College Station, Texas; Tifton, Georgia; Maricopa, Arizona; and Loxley, Alabama. Each is located in a major cotton growing region of the United States and is representative of various local growing practices where insect resistant cotton varieties are suitable as a commercial product. These locations provided a variety of environmental conditions and insect pressures from agronomically important insect pests. Insect resistant cotton and the control cotton variety were grown under the same conditions at each location. Agronomic practices and conditions for each sites were monitored and recorded.

The levels of the major components (protein, oil, carbohydrate, moisture, ash and calories) were shown to be compositionally equivalent in the field grown cottonseed from Line 531 and the C312 (Table IV-1). There were no significant differences in the total lipids between cottonseed from Line 531 and the C312 control (Table IV-2). Minor, but statistically significant differences were observed between Line 531 and the C312 control for three of the eleven individual fatty acids (Table IV-3). However, all these fell within the published ranges for commercial cotton varieties and, therefore, are assumed to 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 between the Line 531 and the C312 control at any of the six locations where the cotton was grown (Table IV-4). 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 Line 531 and the C312 control (Table IV-4).

The four primary aflatoxins commonly found in cottonseed were undetectable at a sensitivity of 1 part per billion for the Line 531 at all six sites and for the C312 control at five of the six sites (Table IV-5). The sample of the C312 control seed grown at the Arizona field site showed relatively high aflatoxin contamination.

Table IV-1. Proximate Analysis of Cottonseed from Line 531 and Control Line, C312, Grown under Field Conditions[1,2]

Component	Coker 312	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. The numbers in parentheses indicate the standard deviation of the mean. Means were compared using the paired t-test.

Table IV-2. The Level of Lipids in Cottonseed from Line 531 and Coker 312 Grown under Field Conditions[1]

Line Number	mean[2]	Range
C312	39.21	35.4 - 43.2
Line 531	39.97	34.1 - 45.3

1 Lipid levels were determined for cottonseed and are expressed as the percent of total lipid compared to the lyophilized dry weight of the cotton meal.

2 Mean percent lipid across all 6 sites. Comparison of the means were performed by pairing values within each site; the value for C312 was subtracted from the value for 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 IV-3. Levels of Major Fatty Acids in Seed from Line 531 and Control Cotton Line C312 Grown under Field Conditions[1]

Fatty Acid	Published Range/Mean	Line No.	mean[2]	Range
14:0	(0.64-1.30)[5]	312	0.90	0.7 - 1.1
		531	0.77*	0.6 - 0.9
16:0	(22.18-27.76)[5]	312	24.01	20.9 - 27.9
		531	24.65	22.7 - 26.6
16:1	(0.56-0.82)[4]	312	0.62	0.5 - 0.7
		531	0.57	0.5 - 0.7
17:0		312	0.15	0.1 - 0.2
		531	0.20	0.1 - 0.3
18:0	(2.14-3.23)[5]	312	2.25	2.0 - 2.6
		531	2.58*	2.4 - 2.7
18:1	(13.95-21.16)[5]	312	15.54	14.9 - 16.7
		531	16.77*	15.0 - 18.4
18:2	(45.84-57.83)[5]	312	51.33	45.0 - 55.0
		531	48.60	44.1 - 52.4
18:3	(0.23)[5]	312	0.18	0.1 - 0.2
		531	0.20	0.2 - 0.2
20:0	(0.41)[5]	312	0.22	0.2 - 0.3
		531	0.23	0.2 - 0.3
22:0		312	0.12	0.1 - 0.2
		531	0.13	0.1 - 0.2
24:0	(0.18)[5]	312	0.03	nd - 0.1
		531	0.02	nd - 0.1

1 Values are expressed as % of the total lipid.

2 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.

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 IV-4. The Level of Toxicants in Cottonseed from Line 531 and Coker 312 Grown under Field Conditions[1]

Toxicant	Published Range	Line No.	Mean[5]	Range
Gossypol[2]	(0.39 - 1.7)[2]	312	1.33	1.13 - 1.46
		531	1.28	1.09 - 1.49
C-19[3]	(0.2 - 0.8)[4]	312	0.27	0.2 - 0.3
		531	0.23	0.2 - 0.3
Sterculic[3]	(0.3 - 0.7)[4]	312	0.58	0.5 - 0.8
	(0.3 - 0.5)[6]	531	0.75	0.5 - 1.5
Malvalic[3]	(< 0.1 - 1.9)[4]	312	0.30	0.1 - 0.5
	(0.7 - 1.5)[6]	531	0.32	0.1 - 0.5

1 Seed were collected and analyzed from six field test sites.
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., 1986.
5 Mean value across six field sites. Means were compared using the paired t-test.
6 Phelps *et al.*, 1965.

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. Line 531 controlled the pink bollworm present at the Arizona site. As a result, no aflatoxins were detected in Line 531 while they were detected in the C312 controls. This result provides additional evidence of the effective control of the target insect by Line 531.

Compositional data showed that Line 531 and the C312 control are not significantly altered in any characteristic except for the aflatoxin in cotton from Arizona, which established an additional benefit that insect resistant cotton will have on feed safety of cottonseed from Line 531.

Table IV-5. The Level of Aflatoxins in Cottonseed from Line 531 and Coker 312 Grown under Field Conditions[1]

Aflatoxin[2]	Line No.	Site Number/Location 701 MS	702 LA	703 TX	704 GA	705 AZ	706 AL	Mean
B1	312	nd	nd	nd	nd	92.7	nd	n/a[3]
	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 Aflatoxin levels were determined for cottonseed from field sites 701 through 706 which refer to: Starkville, MS; Bossier City, LA; College Station, TX; Tifton, GA; Maricopa, AZ; and Loxley, AL, respectively.

2 Reported as parts per billion (ppb) based upon fresh weight of the seed; nd = not detected (less than 1 ppb);

3 n/a = mean not appropriate.

4. Cottonseed Processing

Yields of the processed cottonseed fractions (linters, linter motes, delinted seed, hulls, kernels, toasted meal, crude oil and refined oil) were comparable for both the Line 531 and the C312 control and comparable to the means and ranges previously reported for processed cottonseed fractions from other cotton cultivars (Table IV-6). The levels of total and free gossypol in the raw cottonseed kernels, toasted meal and refined oil were comparable for both Line 531 and C312 (Table IV-6). 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 more than 18% in the toasted meal for both lines. These data establish that cottonseed from Line 531 processes comparably to cottonseed from the Coker 312 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.

Table IV-6. 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††		

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.

5. Protein in Cottonseed Oil

Refined oil from both Line 531 and C312 showed no detectable protein at a sensitivity of 1.3 ppm of total protein. This supports previous reports which also concluded that there was no protein in cottonseed oil.

6. *B.t.k.* and NPTII Proteins in Cotton Lint

The *B.t.k.* protein was not detected in raw cotton fiber, cleaned cotton fiber, or cleaned linters using a very sensitive insect bioassay with a sensitivity of 1 ng per ml of diet. An extremely low level of the *B.t.k.* protein was detected in raw cotton linters. The linters are the portion of the fiber adjoining the seed coat; since the raw linters could contain a small amount of hull material and we know that the hulls contain the protein, this would account for detection of the low level of *B.t.k.* protein in raw linters. Processing the linters removed these trace levels of this protein. The same result, nothing detected, was obtained when the lint and linters fractions were tested using a harsh protein extraction and western blot analyses (Monsanto Unpublished Data).

7. Four Week Rat Feeding Study with Cotton Seed from Line 531

The wholesomeness of the cotton seed derived from Line 531 was comparable to that of the C312 control when fed raw to rats for 4 weeks.

Ten male and female rats were fed rodent chow with a diet containing 5 and 10% (wt/wt) of raw, ground cotton seed from either Line 531 or the C312 control for 4 weeks. There was no mortality in the study and all animals appeared healthy. Food consumption was decreased slightly for rats of both sexes fed 10% Line 531 relative to the parental control during the study; the reduction was statistically significant for females during the first study week (Table IV-7). Diets containing Line 531 appeared to be slightly less palatable than the parental line (C312) at a 10% dietary incorporation rate. Food consumption at the 5% dietary incorporation was similar for rats in either group.

Body weights and cumulative body weight gains were comparable for male and female rats fed 5% of either line of cottonseed (Table IV-7). At the 10% dietary incorporation rate, body weight gain (males) and body weight and body weight gain (females) was slightly, but statistically significantly lower during the first week of the study for rats fed Line 531 cottonseed meal. For the remainder of the study, there were no statistically significant differences in body weight or body weight gain for male or female rats of either group. By the end of the study, Line 531 female body weights exceeded that of parental controls while body weight for Line 531 males remained slightly lower than controls. The transient statistically significant reductions in body weight and/or body weight gain are probably related to slightly reduced palatability of Line 531 diets at the 10% incorporation rate, since this difference disappeared after the first week of the study (Table IV-7).

There were no treatment related findings at necropsy and organ weights (liver, kidney, testes) were comparable for the control and test animals.

In summary, for most of the study, there were no statistically significant differences in body weight, cumulative body weight gain, food consumption or absolute and relative organ weights between rats fed either cottonseed line at dietary concentrations of approximately 5 and 10%. Reductions in body weight and/or body weight gain in male and females rats fed 10% Line 531 cottonseed at week 1 are probably related to reduced palatability of this diet. Since there were no other substantive changes in the biological parameters measured, it was concluded that the wholesomeness of unprocessed cottonseed meal from Line 531 was comparable to the parental line when fed to rats.

Table IV-7 Body Weight gms (Cumulative Body Weight Gain, gm)

Week	Malest† 5%	Malest† 10%	Femalest† 5%	Femalest† 10%
		Line C312		
1	231 (49.8)	226 (45.7)	181 (20.6)	182 (20.9)
2	293 (111.6)	286 (105.2)	205 (45.2)	196 (35.5)
3	348 (166.5)	335 (154.0)	223 (62.4)	214 (53.2)
4	382 (200.6)	365 (184.5)	235 (74.8)	223 (62.0)
		Line 531		
1	227 (45.8)	218 (37.3*)	178 (17.0)	173* (12.9*)
2	296 (115.1)	279 (97.7)	201 (40.7)	198 (38.2)
3	355 (173.5)	326 (145.6)	215 (54.9)	217 (57.1)
4	383 (202.0)	346 (165.5)	227 (66.6)	225 (65.1)
		Food Consumption (gm/day)		
		Line C312		
1	23.3	21.4	16.5	17.1
2	28.3	26.8	18.2	18.0
3	29.6	28.2	17.6	19.0
4	29.1	28.6	18.6	19.4
		Line 531		
1	23.5	21.5	16.0	14.5*
2	27.6	25.8	17.9	17.0
3	29.6	27.9	17.2	18.0
4	29.8	27.9	18.2	18.5

† 10 rats for each sex and treatment were tested
* $p \leq 0.05$

8. Conclusions

The Food Policy recommends that key compositional components of genetically modified plant varieties be assessed prior to commercial introduction. Monsanto has, therefore, performed extensive analytical studies to compare the compositional quality of Line 531 to the parental variety, C312.

- The composition of cottonseed (proximate analysis, lipid content, fatty acid composition) of the Line 531 is comparable to the C312 control and to published ranges for other cotton varieties.

- Levels of the three toxicants (gossypol, cyclopropenoid fatty acids and aflatoxin) for Line 531 is comparable to C312, with the exception that Line 531 controls pink bollworm and drastically reduced the aflatoxin level in Line 531 compared to C312 at the Arizona site, which was the only site with pink bollworm damage. Reduction in aflatoxin levels provides an important safety benefit for cottonseed use in animal feed.

- Cottonseed from Line 531 processed comparably to the C312 control, with comparable reductions in the levels of gossypol in the processed meal for both lines. As expected, there was no gossypol in refined cottonseed oil.

- The wholesomeness of unprocessed cottonseed meal from Line 531 was comparable to the parental line when fed to rats.

The absence of unexpected or unintended effects due to the presence of the *B.t.k.* and *nptII* genes in Line 531 is demonstrated by:

1. The soil microorganism *Bacillus thuringiensis* subsp. *kurstaki* and the insect control protein it produces are ubiquitous in nature, have a history of safe use and have been extensively studied (EPA, 1988).

2. The NPTII enzyme expressed in Line 531 has been approved as a processing aid food additive by FDA (FDA, 1994). 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).

3. The recipient organism, cotton, has a history of safe use.

4. The extensive compositional analysis of the Line 531 compared to the Coker 312 control showed no differences when compared to each other or published historical data.

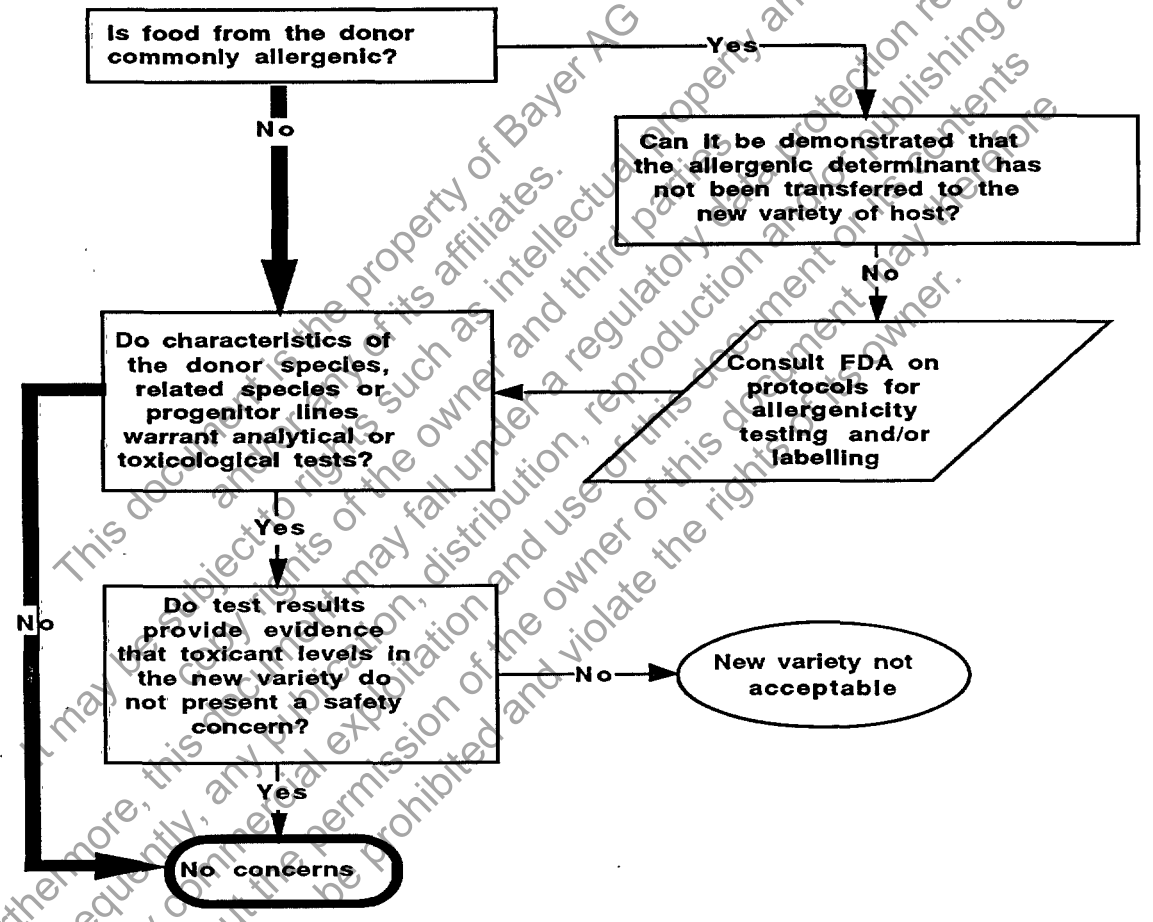
5. The wholesomeness of the unprocessed cottonseed meal from Line 531 was equivalent to that of C312 when fed to rats.

6. THe *aad* gene present in Line 531 does not encode the AAD protein.

Based upon these data and information we have reached the conclusion of "No Concerns" as listed on Figure IV-1.

B. Safety Assessment of New Varieties: the Donors

Figure IV-2. Safety assessment of new varieties: the donor (taken from FDA Food Policy Figure 3). The pathway leading to "no concern" for Bollgard™ Cotton Line 531.



000217

1. Donor Organisms

The safety of the donor organisms of the *B.t.k.* and *nptII* genes, was considered. These organisms are not commonly used directly as a food or feed source, however are ubiquitous in nature and are likely present as contaminates on the food and feed consumed. In addition, *E. coli*, is present in the digestive systems of humans and animals. In response to the question posed in Figure IV-2, " Safety Assessment of New Varieties: the Donors", the following discussion considers the safety of the donor organisms.

Bacillus thuringiensis subsp. *kurstaki*, which produces the *B.t.k.* insect control protein, is the basis of microbial formulations commercially available for lepidopteran insect control for over 30 years (EPA, 1988). Based on the available scientific data, EPA and other regulatory agencies, worldwide, have determined that use of registered *B.t.k.* products pose no significant risks to human health, non-target organisms or the environment. The protein produced by Line 531 is nearly identical (>99.4%) to that found in nature and in commercial *B.t.k.* formulations. The results of Monsanto sponsored studies submitted to the Environmental Protection Agency on February 15, 1994 (EPA Pesticide Petition 4F4331), supporting the registration of the insect control protein as a plant pesticide and an exemption from the requirement of a tolerance, fully confirm the safety of this protein.

The *nptII* gene and its product, the NPTII enzyme, were isolated from kanamycin resistant bacteria that contained the Tn*5* transposon (Berg *et al.*, 1975) and has been used as a selectable marker in *E. coli* (Rao and Rogers, 1979), animal and human cells (Blaese, 1993) and plants (Fraley *et al.*, 1983). The NPTII protein, which has no insecticidal effect, is ubiquitous in the environment and found in microbes present on food and within the human digestive system. The safety of the NPTII protein has been reviewed and discussed broadly because of its wide use as a selectable marker for plant transformation (Nap *et al.*, 1992; Flavell *et al.*, 1992; WHO, 1993; Fuchs *et al.*, 1993a; Fuchs *et al.*, 1993b). In addition, the use of this protein was approved as a processing aid food additive in tomato, canola and cotton, as requested by Calgene, Inc. (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).

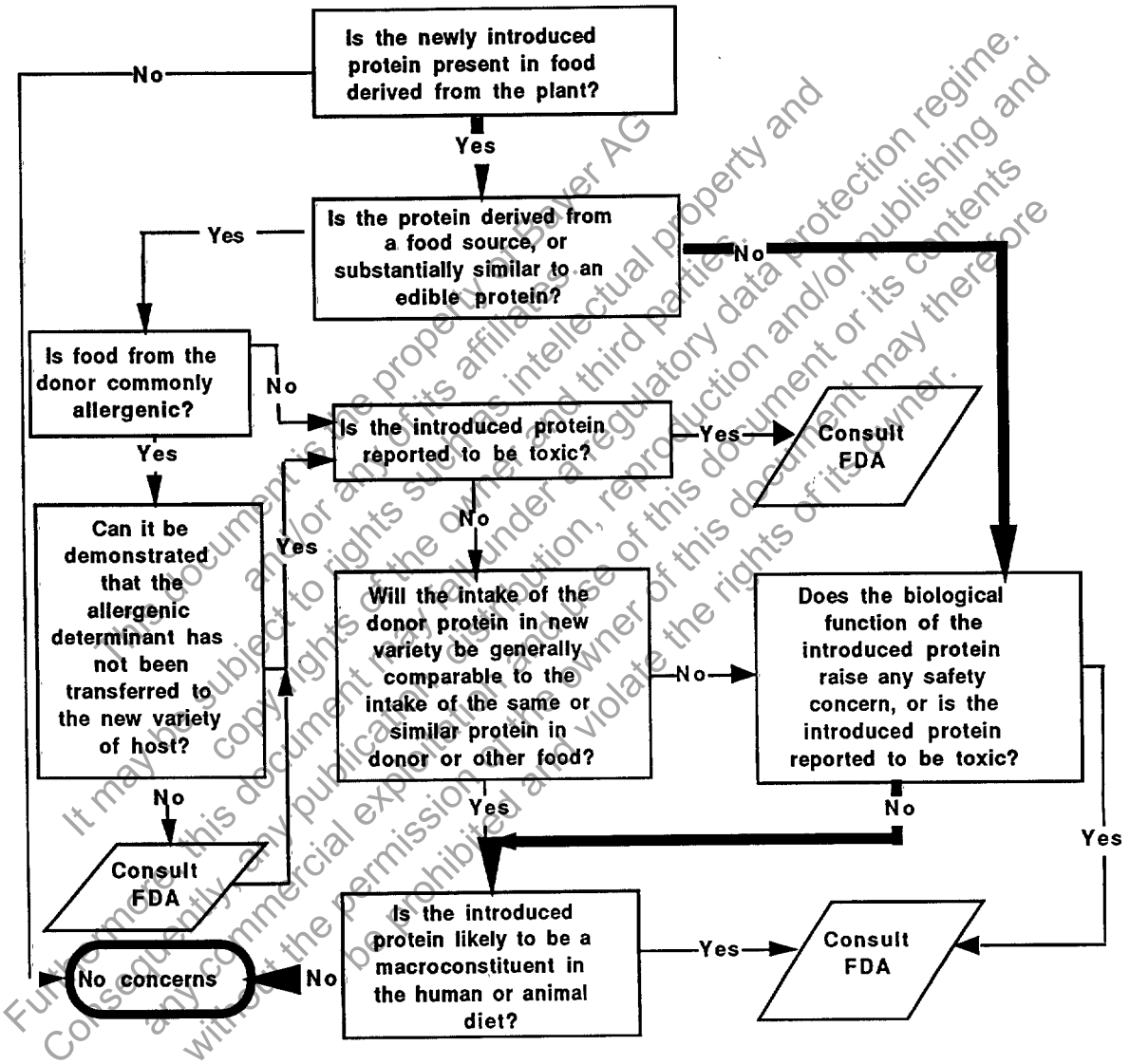
In conclusion, the characteristics of the donor organisms, *Bacillus thuringiensis* subsp. *kurstaki* and *E. coli* , do not warrant analytical or toxicological tests for the following reasons:

- Only the specific sequenced genes encoding the *B.t.k.* insect control protein and the NPTII enzyme were transferred to the host organism, cotton.
- The safety of these proteins is well established and have previously been approved by EPA and FDA for use in or on foods and feeds.
- Humans are not expected to be exposed to either the genes or expressed proteins. The only cotton fractions known to be used for human food are the oil and fiber, neither of these fractions contain either the genes or proteins.

These facts led us to the conclusion of "no concern" for the source of the donor genes as listed on Figure IV-2.

C. Safety Assessment of New Varieties: Proteins Introduced from Donor(s)

Figure IV-3. Safety assessment of new varieties: proteins introduced from donor (taken from FDA Food Policy Figure 4). The pathway leading to "no concerns" for Bollgard™ Cotton Line 531 is highlighted with bold arrows.



000219

FDA's Statement of Policy "Foods Derived From New Plant Varieties", published in the Federal Register May 29, 1992 (FDA, 1992) and the proposed Plant Pesticide Policy of the Environmental Protection Agency (EPA, 1992), agree that, under FIFRA, the EPA will have regulatory oversight for plant pesticides and selectable markers used in the plant transformation process for the purpose of identifying the cells containing the pesticidal gene. Monsanto has consulted with and submitted studies to the EPA supporting the registration of the *B.t.k* HD-73 insect control protein as a plant pesticide and its exemption from the requirement of a tolerance.

Data has also been submitted to the United States Department of Agriculture requesting a Determination of Non-regulated Status for Line 531 (USDA, 1994). These submissions fully support the environmental and non-target safety of this cotton variety and the proteins produced.

1. Allergenic Properties of the Expressed Proteins.

Introduction of insect-protected cotton varieties does not pose an increased risk of allergies. Cottonseed oil and cotton fiber are the only cotton products used for human food (National Cottonseed Products Association, 1989). Analysis of cottonseed oil and cotton fiber derived from both the Coker 312 control and the insect-protected cotton lines confirmed that there is no detectable protein in either cottonseed oil (EPA MRID# 43168701) or cotton fiber (Monsanto, unpublished). Therefore, there will be no significant human consumption of the *B.t.k.* HD-73 and NPTII proteins introduced into these insect-protected varieties. Furthermore, direct food challenge of individuals allergic to proteins contained in the respective meal derived from oil seed crops (e.g. soybean, peanut and sunflower), with the oil from these respective crops has established that refined oil does not elicit an allergenic response (Bush *et al.*, 1985; Halsey *et al.*, 1986; Taylor *et al.*, 1981). This is consistent with the lack of detectable protein in the oil (Tattrie and Yaguchi, 1973). This information provides a strong basis to conclude that insect-protected cotton varieties pose no significant allergenic concerns.

If the *B.t.k.* and NPTII proteins introduced into the insect-protected variety were consumed, these proteins have a long history of safe use and do not share the biochemical properties common to known allergenic proteins. The NPTII protein was recently approved by the FDA as a processing aid food additive for tomato, cotton and canola (Food and Drug Administration, 1994). This approval included an assessment of potential allergenic concerns for the NPTII protein. Therefore, this assessment will focus only on the *B.t.k.* protein. The *B.t.k.* HD-73 protein expressed in these insect-protected cotton plants is comparable to the *B.t.k.* protein contained in microbial formulations that have been used safely commercially for almost 30 years (EPA MRID# 43145201; Lüthy *et al.*, 1982). These microbial formulations have been used on a wide variety of crops, including fresh produce like lettuce and tomato, with no reported allergenic responses, establishing a sound basis for the lack of allergenic concern for the *B.t.k.* HD-73 protein.

Large quantities of a vast variety of proteins are consumed in diets each day. Rarely do any of these tens of thousands of proteins elicit an allergenic response (Taylor, 1992). Although there are no predictive assays available to definitively predict the allergenic potential of proteins (Food and Drug Administration, 1992), the biochemical profile of

the *B.t.k.* protein provides a basis for allergenic assessment when compared with known protein allergens. Allergenic proteins are often, though not always, glycosylated proteins between 10,000 and 70,000 daltons in size. Moreover, protein allergens must be stable to the peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response. Another significant factor contributing to the allergenicity of proteins is their high concentration in foods that elicit an allergenic response (Taylor, 1992; Taylor *et al.* 1987; and Taylor *et al.*, 1992).

Although the full length *B.t.k.* HD-73 protein is proteolytically cleaved to a 67 kd protein that fits within the size range of known allergens, neither of these forms of the *B.t.k.* protein possess any of the other characteristics common to protein allergens. The biological activity of the *B.t.k.* HD-73 protein was lost upon the processing/toasting procedure used to remove cottonseed oil (EPA MRID# 43168701), however, a portion of the *B.t.k.* HD-73 protein is still present as detected by western blot analysis of the processed material. These data indicate that the tertiary structure was altered and the protein was converted to a non-functional, denatured molecule during the processing procedure, as expected, since proteins are typically labile to high temperatures.

More importantly, the *B.t.k.* HD-73 protein was shown to be very labile to digestion by the proteases present in the mammalian digestive system, minimizing any potential for this protein to be absorbed by the intestinal mucosa, if consumed. *In vitro*, simulated mammalian gastric and intestinal systems digestive mixtures were established and used to assess the susceptibility of *B.t.k.* HD-73 protein to proteolytic digestion. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopeia (1989), a frequently cited reference for *in vitro* digestion. *In vitro* studies with simulated digestive solutions are widely used as models of animal digestion. They have been used to investigate the digestibility of plant proteins (Nielson, 1988; Marquez and Lajolo, 1981), animal proteins (Zikakis *et al.*, 1977) and food additives (Tilch and Elias, 1984); to assess protein quality (Akeson and Stahmann, 1964); to study digestion in pigs and poultry (Fuller, 1991); to measure tablet dissolution rates to monitor biodegradation for pharmaceutical applications (Alam *et al.*, 1980); and to investigate the controlled-release of experimental pharmaceuticals (Doherty *et al.*, 1991).

The data from the simulated digestion experiments demonstrated a half-life for *B.t.k.* HD-73 of less than 30 seconds in the gastric system (EPA MRID# 43145214). As expected, in the intestinal system, the full length *B.t.k.* HD-73 protein was rapidly converted to the trypsin-resistant core, which was not further degraded. To put the rapid degradation of the *B.t.k.* HD-73 protein in the simulated gastric system into perspective, solid food has been estimated to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes (Sleisenger and Fordtran, 1989). Therefore, any *B.t.k.* HD-73 protein consumed would be rapidly degraded in the gastric system.

Since most protein allergens are glycosylated, the *B.t.k.* HD-73 protein, as purified from the seed of insect-protected cotton, was examined for glycosylation. No glycosylation was observed (EPA MRID# 43145202). This result was expected since enzymatic glycosylation in plants requires passage through the rough endoplasmic

reticulum and Golgi bodies (Taiz and Zeiger, 1991). This transport requires specific targeting sequences on the protein, which were not engineered into the *B.t.k.* HD-73 gene and, hence, the resulting *B.t.k.* HD-73 protein. The *B.t.k.* HD-73 protein contained no targeting sequence. Because there is no glycosylation of the *B.t.k.* HD-73 protein, it does not fit the profile of common allergenic proteins.

The full length *B.t.k.* HD-73 protein shows no significant homology to any known protein allergen ([redacted] 1994). *B.t.k.* HD-73 showed no significant homology to any of the 121 amino acid sequences reported for allergens (using allergen as the key word) in the three current protein data bases (Genpept, Pir protein and Swissprot databases). There was no greater homology of the native full length *B.t.k.* HD-73 protein to any of the 121 amino acid sequences for the allergenic proteins than for a scrambled sequence of the same amino acids that comprise the *B.t.k.* HD-73 protein.

Finally, most allergens are present as major protein components in the specific food. This is true for the allergens in milk (Baldo, 1984; Lebenthal, 1975; Taylor, 1986; Taylor *et al.*, 1987), soybean (Shibasaki *et al.*, 1980; Burks *et al.*, 1988; Pedersen and Djurtoft, 1989), peanuts (Barnett *et al.*, 1983; Sachs *et al.*, 1981; Barnett and Howden, 1986; Kemp *et al.*, 1985), etc. In contrast to this generality for common allergenic proteins, the *B.t.k.* HD-73 protein is present in cottonseed at low levels, approximately <0.002% of fresh weight of the cottonseed and approximately <0.001% of the total protein, using an estimate that cottonseed contains approximately 20% protein (EPA MRID# 43168701). Furthermore, there is no significant consumption of cottonseed meal in the human diet. The low levels of the *B.t.k.* HD-73 protein in cottonseed, combined with the lack of consumption of cotton products (except for cottonseed oil which is free of protein), and the thermal and digestive lability of this protein relative to that for known food allergens establishes an extremely low probability of the *B.t.k.* HD-73 protein being consumed, being absorbed via the intestinal mucosa during consumption and triggering production of antibodies including the IgE antibodies responsible for allergenicity.

In summary, the data and analyses described above and summarized in Table IV-8 support the conclusion that there will be no or extremely minimal consumption of the *B.t.k.* HD-73 protein, that the *B.t.k.* HD-73 protein does not possess the characteristics of known protein allergens and the *B.t.k.* HD-73 protein shows no significant homology to allergenic proteins that have been characterized.

This information, coupled with the extremely rapid digestion of this protein under *in vitro* digestive conditions that mimic human digestion, established that, using the best methodology available today, there is no reason to believe that the *B.t.k.* HD-73 protein should pose any significant allergenic risks for consumption of the products generated from insect-protected cotton plants.

Table IV-8. Characteristics of Known Allergenic Proteins[a]

Characteristic	Allergens	*B.t.k.* HD-73
Molecular wt 10-70 kdal	yes	yes
Glycosylated	yes[b]	no
Stable to digestion	yes	no
Stable to processing	yes	no
Similar to known allergens	-	no
Prevalent protein in food	yes	no

[a] As described in Taylor (1992) and Taylor *et al.* (1987)
[b] Typically but not absolutely.

Based upon these data and information we have reached the conclusion of "No Concerns" as listed on Figure IV-3.

V. CONCLUSION FOR THE SAFETY ASSESSMENT OF BOLLGARD™ COTTON LINE 531

Bollgard™ Cotton Line 531 (Line 531), is not materially different from cotton varieties now being sold in any meaningful way except for the ability to resist feeding by lepidopteran insect pests. The results of extensive analyses demonstrated that the levels of the important cotton seed components (protein, oil, carbohydrate, fiber and moisture) in Line 531 are comparable to the Coker 312 control variety or are within established ranges for commercial cotton varieties. The levels of fatty acids are also comparable to the Coker 312 control. Natural toxicants were also measured and comparisons again showed no material difference when compared to the control, except that the level of aflatoxin was decreased, as expected, due indirectly to the control of a targeted insect pest (pink bollworm), which provides a benefit for the use of this cotton variety. The wholesomeness of Line 531 was also shown to be comparable to Coker 312 in a 28 day rat feeding study.

Two additional proteins are present in Line 531 at very low levels. The *B.t.k.* insect control protein is the basis of microbial formulations commercially available for lepidopteran pest control for over 30 years in the United States and the NPTII protein is ubiquitous in the environment and found in microbes present on food and within the human digestive system. The safety of both proteins has been well documented and the results of extensive analyses sponsored by Monsanto and submitted to the EPA fully confirm these findings. The NPTII protein has also recently been approved as a food

additive in cotton by FDA, and exempted as a pesticide formulation inert by EPA. These data lead to a conclusion of "no concerns" for every criterion in the flow charts outlined in the Food Policy. Cotton modified to be resistant to lepidopteran insect pests is not materially different in composition, safety, wholesomeness or any relevant parameter from cotton now grown, marketed and consumed. Sales and consumption of cotton seed derived from this variety would be fully consistent with the Agency's Food Policy, the Federal Food Drug and Cosmetic Act, and current practices for the development and introduction of new cotton varieties.

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Appendix I

Figure 1. Nucleotide sequence of the *B.t.k.* HD-73 protein encoded by *cryIA(c)* in 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)*.

```
   1  ATGGACAACA ACCCAAACAT CAACGAATGC ATTCCATACA ACTGCTTGAG
  51  TAACCCAGAA GTTGAAGTAC TTGGTGGAGA ACGCATTGAA ACCGGTTACA
 101  CTCCCATCGA CATCTCCTTG TCCTTGACAC AGTTTCTGCT CAGCGAGTTC
 151  GTGCCAGGTG CTGGGTTCGT TCTCGGACTA GTTGACATCA TCTGGGGTAT
 201  CTTTGGTCCA TCTCAATGGG ATGCATTCCT GGTGCAAATT GAGCAGTTGA
 251  TCAACCAGAG GATCGAAGAG TTCGCCAGGA ACCAGGCCAT CTCTAGGTTG
 301  GAAGGATTGA GCAATCTCTA CCAAATCTAT GCAGAGAGCT TCAGAGAGTG
 351  GGAAGCCGAT CCTACTAACC CAGCTCTCCG CGAGGAAATG CGTATTCAAT
 401  TCAACGACAT GAACAGCGCC TTGACCACAG CTATCCCATT GTTCGCAGTC
 451  CAGAACTACC AAGTTCCTCT CTTGTCCGTG TACGTTCAAG CAGCTAATCT
 501  TCACCTCAGC GTGCTTCGAG ACGTTAGCGT GTTTGGGCAA AGGTGGGGAT
 551  TCGATGCTGC AACCATCAAT AGCCGTTACA ACGACCTTAC TAGGCTGATT
 601  GGAAACTACA CCGACCACGC TGTTCGTTGG TACAACACTG GCTTGGAGCG
 651  TGTCTGGGGT CCTGATTCTA GAGATTGGAT TAGATACAAC CAGTTCAGGA
 701  GAGAATTGAC CCTCACAGTT TTGGACATTG TGTCTCTCTT CCCGAACTAT
 751  GACTCCAGAA CCTACCCTAT CCGTACAGTG TCCCAACTTA CCAGAGAAAT
 801  CTATACTAAC CCAGTTCTTG AGAACTTCGA CGGTAGCTTC CGTGGTTCTG
 851  CCCAAGGTAT CGAAGGCTCC ATCAGGAGCC CACACTTGAT GGACATCTTG
 901  AACAGCATAA CTATCTACAC CGATGCTCAC AGAGGAGAGT ATTACTGGTC
 951  TGGACACCAG ATCATGGCCT CTCCAGTTGG ATTCAGCGGG CCCGAGTTTA
1001  CCTTTCCTCT CTATGGAACT ATGGGAAACG CCGCTCCACA ACAACGTATC
1051  GTTGCTCAAC TAGGTCAGGG TGTCTACAGA ACCTTGTCTT CCACCTTGTA
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1101 CAGAAGACCC TTCAATATCG GTATCAACAA CCAGCAACTT TCCGTTCTTG

1151 ACGGAACAGA GTTCGCCTAT GGAACCTCTT CTAACTTGCC ATCCGCTGTT

1201 TACAGAAAGA GCGGAACCGT TGATTCCTTG GACGAAATCC CACCACAGAA

1251 CAACAATGTG CCACCCAGGC AAGGATTCTC CCACAGGTTG AGCCACGTGT

1301 CCATGTTCCG TTCCGGATTC AGCAACAGTT CCGTGAGCAT CATCAGAGCT

1351 CCTATGTTCT CTTGGATACA CCGTAGTGCT GAGTTCAACA ACATCATCGC

1401 ATCCGATAGT ATTACTCAAA TCCCTGCAGT GAAGGGAAAC TTTCTCTTCA

1451 ACGGTTCTGT CATTTCAGGA CCAGGATTCA CTGGTGGAGA CCTCGTTAGA

1501 CTCAACAGCA GTGGAAATAA CATTCAGAAT AGAGGGTATA TTGAAGTTCC

1551 AATTCACTTC CCATCCACAT CTACCAGATA TAGAGTTCGT GTGAGGTATG

1601 CTTCTGTGAC CCCTATTCAC CTCAACGTTA ATTGGGGTAA TTCATCCATC

1651 TTCTCCAATA CAGTTCCAGC TACAGCTACC TCCTTGGATA ATCTCCAATC

1701 CAGCGATTTC GGTTACTTTG AAAGTGCCAA TGCTTTTACA TCTTCACTCG

1751 GTAACATCGT GGGTGTTAGA AACTTTAGTG GGACTGCAGG AGTGATTATC

1801 GACAGATTCG AGTTCATTCC AGTTACTGCA ACACTCGAGG CTGAGTACAA

1851 CCTTGAGAGA GCCCAGAAGG CTGTGAACGC CCTCTTTACC TCCACCAATC

1901 AGCTTGGCTT GAAAACTAAC GTTACTGACT ATCACATTGA CCAAGTGTCC

1951 AACTTGGTCA CCTACCTTAG CGATGAGTTC TGCCTCGACG AGAAGCGTGA

2001 ACTCTCCGAG AAAGTTAAAC ACGCCAAGCG TCTCAGCGAC GAGAGGAATC

2051 TCTTGCAAGA CTCCAACTTC AAAGACATCA ACAGGCAGCC AGAACGTGGT

2101 TGGGGTGGAA GCACCGGGAT CACCATCCAA GGAGGCGACG ATGTGTTCAA

2151 GGAGAACTAC GTCACCCTCT CCGGAACTTT CGACGAGTGC TACCCTACCT

2201 ACTTGTACCA GAAGATCGAT GAGTCCAAAC TCAAAGCCTT CACCAGGTAT

2251 CAACTTAGAG GCTACATCGA AGACAGCCAA GACCTTGAAA TCTACTCGAT

2301 CAGGTACAAT GCCAAGCACG AGACCGTGAA TGTCCCAGGT ACTGGTTCCC

2351 TCTGGCCACT TTCTGCCCAA TCTCCCATTG GGAAGTGTGG AGAGCCTAAC

2401 AGATGCGCTC CACACCTTGA GTGGAATCCT GACTTGGACT GCTCCTGCAG

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2451  GGATGGCGAG AAGTGTGCCC ACCATTCTCA TCACTTCTCC TTGGACATCG
2501  ATGTGGGATG TACTGACCTG AATGAGGACC TCGGAGTCTG GGTCATCTTC
2551  AAGATCAAGA CCCAAGACGG ACACGCAAGA CTTGGCAACC TTGAGTTTCT
2601  CGAAGAGAAA CCATTGGTCG GTGAAGCTCT CGCTCGTGTG AAGAGAGCAG
2651  AGAAGAAGTG GAGGGACAAA CGTGAGAAAC TCGAATGGGA AACTAACATC
2701  GTTTACAAGG AGGCCAAAGA GTCCGTGGAT GCTTTGTTCG TGAACTCCCA
2751  ATATGATCAG TTGCAAGCCG ACACCAACAT CGCCATGATC CACGCCGCAG
2801  ACAAACGTGT GCACAGCATT CGTGAGGCTT ACTTGCCTGA GTTGTCCGTG
2851  ATCCCTGGTG TGAACGCTGC CATCTTCGAG GAACTTGAGG GACGTATCTT
2901  TACCGCATTC TCCTTGTACG ATGCCAGAAA CGTCATCAAG AACGGTGACT
2951  TCAACAATGG CCTCAGCTGC TGGAATGTGA AAGGTCATGT GGACGTGGAG
3001  GAACAGAACA ATCAGCGTTC CGTCCTGGTT GTGCCTGAGT GGGAAGCTGA
3051  AGTGTCCCAA GAGGTTAGAG TCTGTCCAGG TAGAGGCTAC ATTCTCCGTG
3101  TGACCGCTTA CAAGGAGGGA TACGGTGAGG GTTGCGTGAC CATCCACGAG
3151  ATCGAGAACA ACACCGACGA GCTTAAGTTC TCCAACTGCG TCGAGGAAGA
3201  AATCTATCCC AACAACACCG TTACTTGCAA CGACTACACT GTGAATCAGG
3251  AAGAGTACGG AGGTGCCTAC ACTAGCCGTA ACAGAGGTTA CAACGAAGCT
3301  CCTTCCGTTC CTGCTGACTA TGCCTCCGTG TACGAGGAGA AATCCTACAC
3351  AGATGGCAGA CGTGAGAACC CTTGCGAGTT CAACAGAGGT TACAGGGACT
3401  ACACACCACT TCCAGTTGGC TATGTTACCA AGGAGCTTGA GTACTTTCCT
3451  GAGACCGACA AAGTGTGGAT CGAGATCGGT GAAACCGAGG GAACCTTCAT
3501  CGTGGACAGC GTGGAGCTTC TCTTGATGGA GGAA
```

Figure 2. Amino acid sequences for the *B.t.k.* HD-73 full length protein which is present in Line 531.

```
   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 3. Nucleotide sequence for the neomycin phosphotransferase II (*nptII*) gene present in Line 531.

```
   1  ATGATTGAAC AAGATGGATT GCACGCAGGT TCTCCGGCCG CTTGGGTGGA
  51  GAGGCTATTC GGCTATGACT GGGCACAACA GACAATCGGC TGCTCTGATG
 101  CCGCCGTGTT CCGGCTGTCA GCGCAGGGGC GCCCGGTTCT TTTTGTCAAG
 151  ACCGACCTGT CCGGTGCCCT GAATGAACTG CAGGACGAGG CAGCGCGGCT
 201  ATCGTGGCTG GCCACGACGG GCGTTCCTTG CGCAGCTGTG CTCGACGTTG
 251  TCACTGAAGC GGGAAGGGAC TGGCTGCTAT TGGGCGAAGT GCCGGGGCAG
 301  GATCTCCTGT CATCTCACCT TGCTCCTGCC GAGAAAGTAT CCATCATGGC
 351  TGATGCAATG CGGCGGCTGC ATACGCTTGA TCCGGCTACC TGCCCATTCG
 401  ACCACCAAGC GAAACATCGC ATCGAGCGAG CACGTACTCG GATGGAAGCC
 451  GGTCTTGTCG ATCAGGATGA TCTGGACGAA GAGCATCAGG GGCTCGCGCC
 501  AGCCGAACTG TTCGCCAGGC TCAAGGCGCG CATGCCCGAC GGCGAGGATC
 551  TCGTCGTGAC CCATGGCGAT GCCTGCTTGC CGAATATCAT GGTGGAAAAT
 601  GGCCGCTTTT CTGGATTCAT CGACTGTGGC CGGCTGGGTG TGGCGGACCG
 651  CTATCAGGAC ATAGCGTTGG CTACCCGTGA TATTGCTGAA GAGCTTGGCG
 701  GCGAATGGGC TGACCGCTTC CTCGTGCTTT ACGGTATCGC CGCTCCCGAT
 751  TCGCAGCGCA TCGCCTTCTA TCGCCTTCTT GACGAGTTCT TC
```


Figure 4. Amino acid sequence for neomycin phosphotransferase II (NPTII) protein present in Line 531.

```
   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 5. Nucleotide sequence for the aminoglycoside adenylyltransferase (*aad*) gene present in Line 531.

```
   1  ATGAGGGAAG CGGTGATCGC CGAAGTATCG ACTCAACTAT CAGAGGTAGT
  51  TGGCGTCATC GAGCGCCATC TCGAACCGAC GTTGCTGGCC GTACATTTGT
 101  ACGGCTCCGC AGTGGATGGC GGCCTGAAGC CACACAGTGA TATTGATTTG
 151  CTGGTTACGG TGACCGTAAG GCTTGATGAA ACAACGCGGC GAGCTTTGAT
 201  CAACGACCTT TTGGAAACTT CGGCTTCCCC TGGAGAGAGC GAGATTCTCC
 251  GCGCTGTAGA AGTCACCATT GTTGTGCACG ACGACATCAT TCCGTGGCGT
 301  TATCCAGCTA AGCGCGAACT GCAATTTGGA GAATGGCAGC GCAATGACAT
 351  TCTTGCAGGT ATCTTCGAGC CAGCCACGAT CGACATTGAT CTGGCTATCT
 401  TGCTGACAAA AGCAAGAGAA CATAGCGTTG CCTTGGTAGG TCCAGCGGCG
 451  GAGGAACTCT TTGATCCGGT TCCTGAACAG GATCTATTTG AGGCGCTAAA
 501  TGAAACCTTA ACGCTATGGA ACTCGCCGCC CGACTGGGCT GGCGATGAGC
 551  GAAATGTAGT GCTTACGTTG TCCCGCATTT GGTACAGCGC AGTAACCGGC
 601  AAAATCGCGC CGAAGGATGT CGCTGCCGAC TGGGCAATGG AGCGCCTGCC
 651  GGCCCAGTAT CAGCCCGTCA TACTTGAAGC TAGGCAGGCT TATCTTGGAC
 701  AAGAAGATCG CTTGGCCTCG CGCGCAGATC AGTTGGAAGA ATTTGTTCAC
 751  TACGTGAAAG GCGAGATCAC CAAGGTAGTC GGCAAA
```


Figure 6. Amino acid sequence for the aminoglycoside adenylyltransferase (*aad*) gene present in Line 531.

```
   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
```

Appendix II Methods

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 (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 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 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 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 plots at each location). these samples were used for quality analyses.

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/20 µL 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 seed 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). This 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 Assay 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).

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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 Dwyer 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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