03-155-01p

syngenta

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June 3, 2003

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Re: Application for the Determination of Non-Regulated Status for Lepidopteran Insect Protected VIP3A Cotton Transformation Event COT102

Dear Ms. Bech:

Enclosed please find two original and four photocopies of the above referenced Petition. Syngenta requests that cotton event COT102, and any progeny derived from crosses of event COT102 with conventional cotton varieties, and any progeny derived from crosses of event COT102 with transgenic cotton varieties that have also received a determination of non-regulated status, no longer be considered regulated under 7 CFR Part 340.

If you have any questions regarding this submission, please do not hesitate to contact me.

Sincerely,

Lori Artim
Regulatory Affairs Manager

Enclosures

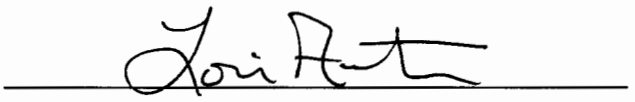
cc: Jeff Stein, Director of Regulatory Affairs, Syngenta Seeds, Inc.

Petition for the Determination of Non-Regulated Status:

Lepidopteran Insect Protected VIP3A Cotton Transformation Event COT102

The undersigned submits this petition under 7 CFR 340.6 to Request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by:



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Date:

June 3, 2003

Contains No Confidential Business Information

Summary

Syngenta Seeds, Inc. has developed a new transgenic cotton line engineered for broad-spectrum lepidopteran insect resistance. This line produces a pesticidal protein, which has activity against several lepidopteran species including, but not limited to, *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), *Pseudoplusia includens* (soybean looper), *Trichoplusia ni* (cabbage looper), and *Bucculatrix thurberiella* (cotton leaf perforator).

Event COT102 was produced via *Agrobacterium*-mediated transformation of *Gossypium hirsutum* L. cultivar Coker 312 and has been transformed with two genes, *vip3A(a)* and *aph4*. The insecticidal gene *vip3A(a)* was derived from *Bacillus thuringiensis* strain AB88. This gene is under the regulatory control of the actin-2 promoter derived from *Arabidopsis*, which confers expression of VIP3A protein throughout the plant. The selectable marker gene *aph4*, encoding the enzyme hygromycin-B phosphotransferase, was originally derived from *E. coli* and is under the regulatory control of the ubiquitin-3 promoter from *Arabidopsis*. Expression of the *aph4* gene product allows for growth of transformed plant cells on artificial growth medium containing hygromycin B.

The pesticidal protein VIP3A is a member of a class of recently discovered insecticidal proteins that are naturally produced by *Bacillus thuringiensis* (Bt), a gram-positive bacterium commonly found in soils. Bt strains are characterized by their production of parasporal crystalline inclusions during sporulation. These crystals contain one or more δ-endotoxin proteins (Cry proteins), each of which is highly specific in its toxicity to certain lepidopteran, coleopteran, or dipteran insect larvae upon ingestion of small quantities. The high specificity of Cry proteins is the basis for their utility as targeted pest control agents and their demonstrated safety when used either as formulated microbial products or as plant-incorporated protectants. Unlike the Cry proteins, VIP3A and other "vegetative insecticidal proteins" (VIPs) are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. Bt cultures continue to produce VIP3A during stationary phase. Unlike the thermostabile non-proteinaceous β-exotoxin secreted by some Bt strains, VIP3A protein is thermolabile.

Other than its demonstrated insecticidal activity, VIP3A is not known to have any other biological or catalytic function. Although VIP3A protein shares no homology with known Cry proteins, extensive testing has established that VIP3A is similarly very specific in its activity demonstrating toxicity only to the larvae of certain lepidopteran species. Furthermore, Syngenta scientists have shown that VIP3A apparently targets a different receptor than CryI proteins in sensitive species and therefore represents a potential tool in the prevention or management of pest resistance.

Event COT102 derived cotton plants have been evaluated across the U.S. cotton belt during the 2000, 2001, and 2002 growing seasons in fifty-one separate field trials carried out in twelve states. Event COT102 provides excellent insect protection throughout the season and results in significantly higher lint yields. Plants derived from event COT102 have been agronomically evaluated and fall within the normal range of variability observed in traditional cotton varieties for all parameters measured. No unintended effects with regards to observed plant pest

characteristics have been observed in any of the field trials. The lack of any significant environmental impact of the Bt family of proteins has been demonstrated in microbial products and in existing plant-incorporated protectants. Additionally, in all cases where VIP3A was tested for impact to non-target organisms, no adverse affects were determined and the no observable effect concentration (NOEC) greatly exceeded the maximum environmental concentration, indicating minimal risk to non-target organisms.

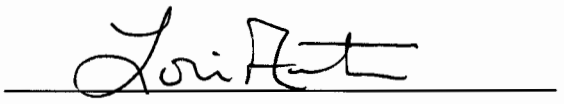
The environmental consequences of pollen flow from genetically enhanced cotton products has recently been reviewed by EPA in its environmental reassessment of the registered *B.t.* plant-incorporated protectants and summarized its findings in the "Biopesticides Registration Action Document" for these products. In that reassessment of Bt cotton, EPA reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories, and concluded that the possibility for gene transfer exists only in limited geographic locations where wild or feral cotton relatives exist, *i.e.* in Florida, Hawaii, and the Caribbean. Additionally, USDA/APHIS has made this same determination under its statutory authority. These conclusions made with respect to commercial Bt cotton (producing a Cry1Ac endotoxin) are also applicable to VIP3A Bt cotton. Accordingly, the same geographical restrictions (*e.g.,* no commercial plantings in Hawaii and south Florida) that are currently in effect for Cry1Ac Bt cotton are expected to be applicable to VIP3A cotton.

Transgenic insect resistant crop plants have proven to be an important new tool for modern day agriculture for use in integrated pest management programs. The technology allows the crop plant to deliver its own means of protection against insect attack. The result is a very specific and directed biological control method that is environmentally sound and that has proven to reduce the need for manual and chemical inputs from the grower. This, in turn, can have additional environmental and consumer benefits by (1) reducing risks associated with environmental spills or misapplications of chemical insecticides, (2) eliminating unwanted effects on beneficial insect populations (which are often times susceptible to conventional chemical applications); these beneficial insects can, in turn, further reduce the reliance upon chemical means of pest control, (3) reducing the consumption of fossil fuels required to deliver chemical inputs by machinery, and (4) contributing to the availability of a more reliable, high-quality, and plentiful source of food and feed.

The data provided in this request demonstrate that event COT102 does not represent a plant pest risk. Syngenta requests that cotton event COT102, and any progeny derived from crosses of event COT102 with conventional cotton varieties, and any progeny derived from crosses of event COT102 with transgenic cotton varieties that have also received a determination of non-regulated status, no longer be considered regulated under 7 CFR Part 340.

Certification

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



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Abbreviations Used in this Petition

aadA	Adenylytransferase gene
Act2	Promoter isolated from *Arabidopsis* actin-2 gene
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service
APN	Aminopeptidase N
APH4	Selecable marker protein hygromycin B phosphotransferase
aph4	Gene in event COT102 encoding the selectable marker protein APH4
BBMV	Brush border membrane vescicles
Bp	Base pair
Bt	*Bacillus thuringiensis*
Bwt	Body weight
CFR	Code of Federal Regulations
ColE1	*E.coli* origin of replication
CPFA	Cyclopropenoid fatty acids
Cry	Crystal protein delta endotoxins
DNA	Deoxyribonucleic acid
DT_{50}	Time to dissipation of 50% of the initial bioactivity
EEC	Estimated exposure concentration
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organism
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HPLC	High Performance Liquid Chromatography
IRM	Insect resistance management
Kb	Kilobase
kDa	Kilo Dalton
LB	Left border
LC_{50}	50% lethal concentration
LC_{90}	90% lethal concentration
LD_{50}	50% lethal dose
LOQ	Level of quantitation
NOEC	No observable effect concentration
NOEL	No observable effect level
nos	Nopaline synthase terminator
nptII	Gene encoding the neomycin phosphotransferase enzyme
MOE	Margins of exposure
OECD	Organization for Economic Cooperation and Development
pCOT1	Plasmid employed to create event COT102
PCR	Polymerase chain reaction
PPQ	Plant Protection and Quarantine
psi	Pounds per square inch
RB	Right border
RepA	Bacterial replication protein

SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOP	Standard operating procedure
spec	Spectinomycin resistance gene
T0	First generation transgenic
T1	Second generation transgenic
T-DNA	Transfer DNA
Ti-plasmid	Tumor inducing plasmid
Ubq3	Ubiquitin-3 gene isolated from Arabidopsis.
Ubq3int	Promoter plus the first intron isolated from *Arabidopsis* ubiquitin-3 gene.
USDA	United States Department of Agriculture
US EPA	United States Environmental Protection Agency
VIP3A	Insecticidal protein produced in event COT102
vip3A(a)	Gene in event COT102 encoding insecticidal protein VIP3A
VIPs	Vegetative insecticidal proteins
VS1	*Agrobacterium* origin of replication
WHO	World Health Organism

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Chapter 1

REQUEST FOR THE DETERMINATION OF NON-REGULATED STATUS OF COTTON EVENT COT102 PRODUCING THE VIP3A INSECT CONTROL PROTEIN DERIVED FROM BACILLUS THURINGIENSIS STRAIN AB88

A. Background Information

The transgenic cotton line described herein (COT102) has been engineered for broad-spectrum lepidopteran insect resistance. This line has been transformed with a gene that produces a pesticidal protein, which has activity against several lepidopteran species including *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), *Pseudoplusia includens* (soybean looper), and *Trichoplusia ni* (cabbage looper). The pesticidal active ingredient that is the subject of this request is the insect control protein VIP3A that occurs naturally in *Bacillus thuringiensis* strain AB88. The plasmid pCOT1 has been employed to insert a synthetic version of *vip3A(a)* and selectable marker genes into cotton plants.

1) VIP3A and the Vegetative Insecticidal Proteins of *Bacillus thuringiensis*

VIP3A is a member of a class of recently discovered insecticidal proteins that are naturally produced by *Bacillus thuringiensis* (Bt), a gram-positive bacterium commonly found in soils (Estruch *et al.,* 1996; Warren *et al.*, 1996). Bt strains are characterized by their production of parasporal crystalline inclusions during sporulation. These crystals contain one or more δ-endotoxin proteins (Cry proteins), each of which is highly specific in its toxicity to certain lepidopteran, coleopteran, or dipteran insect larvae upon ingestion of small quantities (van Frankenhuyzen and Nystrom, 2002). The high specificity of Cry proteins is the basis for their utility as targeted pest control agents and their demonstrated safety when used either as formulated microbial products or as plant-incorporated protectants. Unlike the Cry proteins, VIP3A and other "vegetative insecticidal proteins" (VIPs) are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. Bt cultures continue to produce VIP3A during stationary phase and sporulation (Estruch *et al.,* 1996). Unlike the thermostabile non-proteinaceous β-exotoxin secreted by some Bt strains, VIP3A protein is thermolabile (Estruch *et al.,* 1996; Barreto *et al.,* 1999)

Other than its demonstrated insecticidal activity, VIP3A is not known to have any other biological or catalytic function. Although VIP3A protein shares no homology with known Cry proteins, extensive testing has established that VIP3A is similarly very specific in its activity, demonstrating toxicity only to the larvae of certain lepidopteran species. Because certain lepidopteran pests [*e.g. Agrotis ipsilon* (black cutworm), *Spodoptera frugiperda* (fall armyworm), *S. exigua* (beet armyworm)] are more sensitive to VIP3A than to the Cry1Ab and Cry1Ac proteins currently used in some commercial transgenic plants (van Frankenhuyzen and Nystrom, 2002), VIP3A protein offers advantages for control of key crop pests. Further, because

VIP3A apparently targets a different receptor than Cry proteins in sensitive species (see section entitled 'Mode of Action of VIP3A Protein'), it represents a potential tool in the prevention or management of pest resistance to Cry proteins.

2) Source of the *vip3A(a)* Gene

The VIP3A protein expressed in event COT102 is produced from a synthetic *vip3A(a)* gene encoding a polypeptide of 789 amino acids (*ca.* 89,000 molecular weight). The entire coding region of the *vip3A(a)* gene was synthesized to accommodate the preferred codon usage for corn. (Murray *et al.*, 1989.) The native *vip3A(a)* gene (GenBank accession number L48811) was cloned from Bt strain AB88, originally isolated from sour milk. The synthetic *vip3A(a)* gene used in plant transformations encodes the identical amino acid sequence as the native *vip3A(a)* gene, with the exception of a single amino acid difference at position 284; the native gene encodes lysine, whereas the synthetic gene encodes glutamine at this position.[1]

Genes with *ca.* 99% homology to the *vip3A(a)* gene [*e.g., vip3A(b)*] have been cloned from Bt strains isolated from different sources, including mossy pine cones and soil (Estruch *et al.*, 1996; Selvapandiyan *et al.*, 2001). Using a hybridization method, Estruch *et al.* (1996) screened a collection of 463 *Bacillus* strains and determined that 15% of them contained *vip3A*-like genes. Using a PCR-based method, Rice (1999) screened a collection of 125 Bt strains isolated from soils and grain dust and determined that 23% of the strains (29 strains representing *ca.* 20 serovars) contained *vip3A*-like genes. Similarly, Guttmann and Ellar (2000) screened 17 Bt strains by a PCR method and found that six had *vip3A*-like genes, while *vip3A* sequences were not detected in strains of two other *Bacillus* species tested (three *B. cereus* strains and three *B. anthracis* strains). Based on current information, *vip3A* or *vip3A*-like genes appear to occur commonly in Bt strains from a variety of sources; their occurrence in non-Bt strains has not been documented to date.

3) Insect Specificity

Extensive testing indicates that VIP3A is a lepidopteran-specific toxin that displays activity against several crop pests of economic importance in the US and/or elsewhere. Table 1.1 lists the lepidopteran species that have demonstrated sensitivity to VIP3A protein preparations and/or VIP3A-expressing plants. Table 1.2 lists the insensitive lepidopteran species that have been identified to date, based on direct laboratory feeding studies.

Table 1.3 lists the non-lepidopteran invertebrate species, representing nine different Orders that have been tested to date in direct laboratory feeding studies. These species demonstrated no

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[1] This amino acid difference arose because the native *vip3A(a)* sequence as published by Estruch *et al.* (1996) incorrectly represented amino acid 284 as glutamine, whereas subsequent re-sequencing of the gene, using improved methodology, indicated that lysine was encoded at this position. This discovery occurred after the synthetic gene, encoding the same amino acid sequence as the original published sequence, had been made and used in plant transformations. Therefore, the single amino acid difference between the VIP3A protein encoded by the *correct* native codon sequence and that encoded by the synthetic gene was inadvertent. The substitution of glutamine for lysine is a conservative one; both are polar amino acids with a molecular weight of 146. The single amino acid difference has had no apparent effect on VIP3A protein function.

sensitivity towards VIP3A protein derived from VIP3A maize plants (*e.g.,* pollen, leaf protein extracts) or from microbial preparations. These studies support a conclusion that VIP3A is a specific toxin with no direct biological activity outside the order Lepidoptera.

4) Mode of Action of VIP3A Protein

As VIP3A is one of a novel class of insecticidal proteins, Syngenta scientists are actively generating information relating to its mode of action. The investigative strategy has been to conduct similar studies to those previously done with the Bt Cry1A δ-endotoxins.

Cry1 toxins, the most studied of Bt endotoxins, are solubilized in the alkaline pH of the lepidopteran midgut and activated by midgut proteases. In sensitive larvae, the activated toxin then binds to specific receptor(s) located on the epithelial cell brush border membranes. After binding, the toxin is integrated into the midgut membrane to form pores, which result in ion imbalances and insect death.

VIP3A toxin is also proteolytically activated to a toxin core in the lepidopteran larval midgut and forms pores in the gut membranes of sensitive species, a mechanism that appears to correlate with its toxicity. However, VIP3A has been shown to have significantly different receptor binding properties and pore forming properties than does Cry1Ab protein, indicating that VIP3A has a different target and mode of action than the δ-endotoxins in the Cry1 family. Summarized below are the VIP3A mechanistic studies that have been concluded to date (Lee *et al.,* 2002).

1. Activation by lepidopteran gut proteases

Proteolytic activation of VIP3A was investigated with trypsin and lepidopteran gut juice treatments. The full-length *ca.* 89 kDa VIP3A protein is rapidly converted into a *ca.* 60 kDa relatively stable toxin core by both trypsin and gut juice extracts. In the case of the Cry1A toxins, the full-length *ca.* 130 kDa protoxin is proteolytically activated to a *ca.* 65 kDa toxin core. Proteolytic activation of VIP3A was assessed by *in vitro* and *in vivo* methods in susceptible insects such as *Manduca sexta*, *Heliothis virescens, Helicoverpa zea, Agrotis ipsilon,* and *Spodoptera frugiperda,* as well as in non-susceptible insects such as *Ostrinia nubilalis,* and found to be similar. These data indicate that the proteolytic activation process is not a key factor in insect specificity.

2. Competition binding assays

VIP3A binding properties have been assessed by competition binding assays in which either ^{125}I- or biotin-labeled *ca.* 60 kDa VIP3A toxin was challenged with unlabeled *ca.* 60 kDa VIP3A toxin. Using brush border membrane vesicles (BBMVs) prepared from lepidopteran larval midguts, the VIP3A toxin showed competitive binding with all BBMVs tested, including those of the VIP3A-sensitive species *M. sexta*, *H. virescens*, and *H. zea*, as well as the relatively insensitive species, *O. nubilalis*. In quantitative binding assays VIP3A showed less binding (low binding site concentration) in *O. nubilalis* as compared to *M. sexta.* Although VIP3A showed competitive binding to *M. sexta* BBMVs, its binding affinity and

binding site concentration were lower compared to Cry1Ab toxin. VIP3A and Cry1Ab showed similar biological activity toward *M. sexta*.

Heterologous competition binding assays were also performed to examine the binding site relationship. Binding of either ^{125}I- or biotin-labeled activated Cry1Ab toxin was not inhibited by unlabeled activated VIP3A toxin, and binding of labeled VIP3A toxin was not inhibited by unlabeled Cry1Ab toxin in *M. sexta*, indicating that Cry1Ab and VIP3A toxins do not share the same binding sites.

3. Receptor binding studies

Additional *in vitro* assays were conducted to evaluate binding of activated VIP3A toxin to a 120 kDa aminopeptidase N (APN), which is known as a Cry1Ab receptor (Masson *et al.*, 1995). APN was purified from *M. sexta* BBMVs using HPLC ion exchange and size exclusion columns. Blotting experiments showed that activated VIP3A had no measurable binding to APN, while Cry1Ab showed strong binding. *M. sexta* BBMV ligand blotting assays also revealed that activated VIP3A did not bind to a 210 kDa cadherin-like glycoprotein, also shown to be a putative receptor for Cry1Ab (Vadlamudi *et al.*, 1995). A 20 kDa fragment of the cadherin-like molecule, known as a Cry1Ab binding domain, was cloned and expressed. Cry1Ab showed strong binding to the cadherin ectodomain, but activated VIP3A did not show measurable binding. Instead, activated VIP3A toxin showed binding to three proteins (*ca.* 80 kDa, 100 kDa, and >200 kDa), all of which are distinctly different in size from the Cry1Ab binding proteins. Similarly, activated VIP3A did not bind to the Cry1Ab binding protein APN in either *H. virescens* or *H. zea*. The described binding data strongly suggest that VIP3A and Cry1Ab do not share a common receptor.

4. Pore formation in larval midguts and planar lipid bilayer membranes

The ability of VIP3A to form pores was examined by (1) voltage clamping experiments with isolated insect midguts (Harvey and Wolfersberger, 1979) and (2) planar lipid bilayer assays (Finkelstein, 1974). Voltage clamping assays with *M. sexta* midgut tissue indicated that activated VIP3A toxin could form pores. However, the pore forming response occurred at least 10 times slower than that with Cry1Ab toxin. The full-length VIP3A toxin did not form pores even at very high concentrations. When larval midguts of the non-susceptible butterfly *Danaus plexippus* (monarch butterfly) were tested in voltage clamping experiments, activated VIP3A showed no response (no pore formation); however, Cry1Ab toxin produced a very fast response (pore formation). A direct correlation was observed between pore formation and biological activity.

To examine the ability of VIP3A to form ion channels in various membrane systems, a synthetic planar lipid bilayer system was employed. Syngenta scientists documented that activated Cry1Ab formed ion channels in this system with the most common open state at 750 to 800 pS conductance, whereas activated VIP3A consistently showed channels that opened to about 300 pS conductance. While both Cry1Ab and VIP3A share similar properties in terms of open state stability, voltage-independence, and general cation

selectivity, activated VIP3A channels indicated a notable difference in cation specificity. Whereas Cry1Ab channels clearly exhibited some preference for potassium over sodium ions, channels formed by VIP3A indicated virtually no specificity between these ions. Taken together with the voltage clamping data described herein, these data support the interpretation that activated VIP3A can form ion channels that play a role in VIP3A toxicity to susceptible insects. Furthermore, the ion channels formed by VIP3A clearly differ from those formed by Cry1Ab, based on their *in vitro* responses.

5) Presence of VIP3A-Like Proteins in Formulated Microbial Bt Products

Syngenta Seeds examined several commercial lepidopteran-active formulations of Bt-based microbial insecticides for the presence of VIP3A protein (Syngenta Seeds, unpublished data). The products examined were all US EPA-registered formulations exempt from food and feed tolerance requirements. ELISAs were conducted using protein A-purified polyclonal rabbit and immunoaffinity-purified goat antibodies specific for VIP3A protein. All eight Bt products evaluated contained quantifiable (*ca.* 0.4 - 32 μg/g sample) material that cross-reacted with the VIP3A antibody. In some formulations, sufficient immunoreactive material was present to visualize by SDS-PAGE on an 8% polyacrylamide gel followed by western blot analysis using polyclonal goat anti-VIP3A antibody. The products Dipel®, Javelin® and Condor® insecticides, for example, were observed to contain immunoreactive proteins of comparable molecular weight (*ca.* 89,000) as VIP3A protein.

In a preliminary screening program, Baretto *et al.* (1999) determined that the supernatants of two Bt strains with high activity against *Spodoptera frugiperda* (fall armyworm) larvae had heat-labile proteins of comparable molecular weight as VIP3A. Recently, Donovan *et al.* (2001) demonstrated that VIP3A protein is partially responsible for the insecticidal activity of Bt subsp. *kurstaki* strain HD1 toward *Agrotis ipsilon* (black cutworm) and *Spodoptera exigua* (beet armyworm). Strain HD1 is used in registered microbial insecticide products. The investigators modified the wild-type HD1 strain to replace the *vip3A* gene with a *vip3A* allele containing a 'knock-out' deletion mutation. Compared with the wild-type HD1 strain, the strain lacking a functional *vip3A* gene was one-fourth as toxic to *A. ipsilon* larvae and less than one-tenth as toxic to *S. exigua* larvae. When streptomycin was included in the *S. exigua* diet to inhibit the germination of spores or the growth of Bt after ingestion by the insect, the toxicity of the modified HD1 strain was *ca.* half that of the wild-type HD1 strain. Addition of HD1 spores increased the toxicity of purified Cry1 protein more than 600-fold against *S. exigua*, whereas addition of spores from the *vip3A*-deleted HD1 strain increased toxicity of Cry1 protein *ca.* 10-fold. These results strongly suggest that an important component of Bt insecticidal activity against *S. exigua* is the synthesis of VIP3A protein by Bt cells after ingestion of spores and crystal proteins by insect larvae. It is possible that VIP3A, or related proteins, contribute to the lepidopteran toxicity and pathogenicity of many Bt strains and to what has been described as the insecticidal "spore effect" that is not attributable to δ-endotoxins (Donovan *et al.*, 2001).

Since VIP3A (or a very similar protein, based on size and/or immunoreactivity) appears to be present in registered biological insecticide products used on food crops, including fresh market produce, it is conceivable that small quantities of VIP3A protein are present in the food supply.

Additionally, because Bt, the native source of VIP3A protein, is found on plants and in soils, trace amounts of VIP3A protein may be present on raw agricultural commodities that have not been treated with microbial insecticides.

B. Rationale for the Development of VIP3A Cotton

Syngenta scientists have developed the described genetically improved line of cotton that produces the VIP3A insect control protein that occurs naturally in *Bacillus thuringiensis* strain AB88. This protein is a member of a class of insecticidal proteins known as Vegetative Insecticidal Proteins (VIP, Estruch *et al.* 1996). The VIP3A protein exhibits insecticidal activity against several lepidopteran species including, *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), *Pseudoplusia includens* (soybean looper), and *Trichoplusia ni* (cabbage looper).

Other than its demonstrated insecticidal activity, VIP3A is not known to have any other biological or catalytic function. Although VIP3A protein shares no homology with known Cry proteins, extensive testing has established that VIP3A is similarly very specific in its activity, demonstrating toxicity only to the larvae of certain lepidopteran species. Because certain lepidopteran pests [*e.g. Agrotis ipsilon* (black cutworm), *Spodoptera frugiperda* (fall armyworm), *S. exigua* (beet armyworm)] are more sensitive to VIP3A than to the Cry1Ab and Cry1Ac proteins currently used in some commercial transgenic plants (van Frankenhuyzen and Nystrom, 2002), VIP3A protein offers advantages for control of key crop pests. Further, because VIP3A apparently targets a different receptor than Cry proteins in sensitive species (see Mode of Action of VIP3A Protein), it represents a potential tool in the prevention or management of pest resistance to Cry proteins.

Event COT102 was produced via disarmed *Agrobacterium* mediated transformation of cotton cultivar Coker 312. The genetic components of the transformation vector and resulting molecular characterization of the event are described in detail in Chapter 3 of this Petition. Event COT102 contains a single copy of the Arabidopsis actin-2 promoter, which regulates the expression of the *Bacillus thuringiensis vip3A(a)* gene, followed by the *Agrobacterium tumefaciens* nopaline synthase terminator and the Arabidopsis ubiquitin-3 promoter, which regulates the expression of the *E. coli aph4* gene, followed by the *Agrobacterium tumefaciens* nopaline synthase terminator. The *aph4* gene (hygromycin B phoshotransferase) (Waldron 1997; Kaster *et al.*, 1983) encodes a phosphotransferase enzyme (an aminocyclitol phosphotransferase) that catalyzes the phosphorylation of hygromycin and some related aminoglycosides. The *aph4* gene, when transformed into some plants cells, allows growth and, hence, selection of the transformed cells in the presence of hygromycin (GenBank Accession No. CAA85741). The donor organisms of the genetic components in event COT102, *Bacillus thuringiensis*, *Escherichia coli*, and *Arabidopsis thaliania* are commonly found in the environment and are not considered plant pests. The terminator from the nopaline synthase gene (nos) from *Agrobacterium tumefaciens* should not be considered a plant pest risk or deleterious to the environment because the genetic sequences do not code for a protein or result in any trait that presents a plant pest or environmental risk.

Field tests of event COT102 derived cotton lines have been performed in 2000, 2001, and 2002 in the United States in order to evaluate their agronomic performance in different geographic regions of the cotton belt and to introgress the insecticidal gene into a variety of cotton germplasm. Results of these trials indicate no detectable adverse environmental impact. The lack of any significant environmental impact of the Bt family of proteins has been demonstrated in microbial products (see above) and in existing plant-incorporated protectants. Additionally, in all cases where VIP3A was tested for impact to non-target organisms, no adverse affects were determined and the no observable effect concentration (NOEC) greatly exceeded the maximum environmental concentration, indicating minimal risk to non-target organisms (see Chapter 7 of this Petition).

The environmental consequences of pollen flow from genetically enhanced cotton products has recently been reviewed by EPA in its environmental reassessment of the registered *B.t.* plant-incorporated protectants and summarized its findings in the "Biopesticides Registration Action Document" for these products (US EPA, 2001). In that reassessment of Bt cotton, EPA reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories, and concluded that the possibility for gene transfer exists only in limited geographic locations where wild or feral cotton relatives exist, *i.e.* in Florida, Hawaii, and the Caribbean. Additionally, USDA/APHIS has made this same determination under its statutory authority. These conclusions made with respect to commercial Bt cotton (producing a Cry1Ac endotoxin) are also applicable to VIP3A Bt cotton. Accordingly, the same geographical restrictions (*e.g.,* no commercial plantings in Hawaii and south Florida) that are currently in effect for Cry1Ac Bt cotton are expected to be applicable to VIP3A cotton.

Transgenic insect resistant crop plants have proven to be an important new tool for modern day agriculture for use in integrated pest management programs. The technology allows the crop plant to deliver its own means of protection against insect attack. The result is a very specific and directed biological control method that is environmentally sound and that has proven to reduce the need for manual and chemical inputs from the grower. This, in turn, can have additional environmental and consumer benefits by (1) reducing risks associated with environmental spills or misapplications of chemical insecticides, (2) eliminating unwanted effects on beneficial insect populations (which are often times susceptible to conventional chemical applications); these beneficial insects can, in turn, further reduce the reliance upon chemical means of pest control, (3) reducing the consumption of fossil fuels required to deliver chemical inputs by machinery, and (4) contributing to the availability of a more reliable, high-quality, and plentiful source of food and feed.

Additionally, the introduction of an insect control cotton line expressing a broad spectrum lepidopteran toxin with a different mode of action as compared to already approved lines will greatly aid in the delay of potential insect resistance and assist in the preservation of this important technology.

Before commercializing VIP3A cotton event COT102 in the US, the following actions with US regulatory agencies will be completed.

1. Substances that are pesticides as defined under Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. Section 136(u) are subject to the regulatory authority of the Environmental Protection Agency. A request for commercial registration of VIP3A as a plant-incorporated protectant was submitted to EPA in December 2002. Pursuant to Section 408(d) of the Federal Food Drug and Cosmetic Act (FFDCA), requests for exemption from the requirement of tolerances for VIP3A and APH4 were submitted to the EPA along with the commercial registration.

2. VIP3A cotton event COT102 falls within the scope of the Food and Drug Administration's 1992 Statement of Policy: Foods Derived from New Plant Varieties, including genetically engineered varieties. Syngenta intends to conduct a consultation with FDA in 2003.

3. Under regulations administered by the Animal and Plant Health Inspection Service (APHIS) of USDA (7 CFR 340), event COT102 is currently considered a regulated article. Syngenta is now requesting a determination of non-regulated status for this cotton event and all progenies derived from crosses between this line and other cotton lines.

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Table 1.1. Lepidopteran species shown or reported to be sensitive to VIP3A protein

Genus and species	Family	Common name	References
Agrotis ipsilon[1]	Noctuidae	Black cutworm	Estruch *et al.*, 1996; Yu *et al.*, 1997; Donovan *et al.*, 2001; Privalle, 2002a.
Anticarsia gemmatalis	Noctuidae	Velvetbean caterpillar	Syngenta, unpublished data
Chilo partellus[2]	Pyralidae	Spotted stem borer	Selvapandiyan *et al.*, 2001
Chilo suppressalis	Pyralidae	Rice striped stem borer	Syngenta, unpublished data
Cnaphalocrocis medinalis	Pyralidae	Rice leaffolder	Syngenta, unpublished data
Cochylis hospes	Cochylidae	Banded sunflower moth	Syngenta, unpublished data
Diatraea grandiosella	Pyralidae	Southwestern corn borer	Syngenta, unpublished data
Diatraea saccharalis	Pyralidae	Sugarcane borer	Syngenta, unpublished data
Helicoverpa armigera	Noctuidae	Cotton bollworm	Syngenta, unpublished data Liao *et al.*, 2002
Helicoverpa punctigera	Noctuidae	Native budworm	Syngenta, unpublished data Liao *et al.*, 2002
Helicoverpa zea	Noctuidae	Corn earworm	Estruch *et al.*, 1996; Liao *et al.*, 2002
Heliothis virescens	Noctuidae	Tobacco budworm	Estruch *et al.*, 1996; Donovan *et al.*, 2001; Liao *et al.*, 2002
Homoeosoma electellum	Pyralidae	Sunflower moth	Syngenta, unpublished data
Manduca sexta	Sphingidae	Tobacco hornworm	Syngenta, unpublished data
Ostrinia furnacalis	Pyralidae	Asian corn borer	Syngenta, unpublished data
Pectinophora gossypiella	Gelechiidae	Pink bollworm	Syngenta, unpublished data
Phthorimea operculella[2]	Gelechiidae	Potato tuber moth	Selvapandiyan *et al.*, 2001
Pseudoplusia includens	Noctuidae	Soybean looper	Syngenta, unpublished data
Scirpophaga incertulas	Pyralidae	Yellow stem borer	Syngenta, unpublished data
Sesamia nonagrioides	Noctuidae	Mediterranean corn borer	Syngenta, unpublished data
Spodoptera exigua	Noctuidae	Beet armyworm	Estruch *et al.*, 1996; Donovan *et al.*, 2001
Spodoptera frugiperda	Noctuidae	Fall armyworm	Estruch *et al.*, 1996; Yu *et al.*, 1997; Barreto *et al.*, 1999; Privalle, 2002a.
Spodoptera littoralis	Noctuidae	Egyptian cotton leafworm	Syngenta, unpublished data
Spodoptera litura[2]	Noctuidae	Cluster caterpillar	Selvapandiyan *et al.*, 2001
Spodoptera ornithogalli	Noctuidae	Yellow striped armyworm	Syngenta, unpublished data
Trichoplusia ni	Noctuidae	Cabbage looper	Syngenta, unpublished data; Donovan *et al.*, 2001

[1] Reported to be relatively inactive in this species by Selvapandiyan *et al.* (2001) using a VIP3A protein (GenBank Accession No. Y17158) that appears to be 99% homologous to the VIP3A protein used in the Syngenta studies described in this data volume.
[2] VIP3A activity spectrum appears to be somewhat different for the VIP3A protein used in the studies by Selvapandiyan *et al.* (2001); see footnote 1 and Table 1.2.

Table 1.2. Lepidopteran species shown to be insensitive to VIP3A protein

Genus and species	Family	Common name	References
Danaus plexippus	Danaidae	Monarch butterfly	Syngenta, unpublished data
Hyphantria cunea	Arctiidae	Fall webworm	Syngenta, unpublished data
Ostrinia nubilalis	Pyralidae	European corn borer	Estruch *et al.*, 1996; Yu *et al.*, 1997; Privalle, 2002a.
Plodia interpunctella	Pyralidae	Indian meal moth	Syngenta, unpublished data
Plutella xylostella[1]	Plutellidae	Diamondback moth	Privalle, 2002a.

[1] Selvapandiyan *et al.* (2001) reported that a VIP3A protein from a local Bt isolate had activity in this species. This VIP3A protein (GenBank Accession No. Y17158) appears to be 99% homologous to the VIP3A protein used in the studies described in this data volume.

Table 1.3. Non-lepidopteran invertebrate species insensitive to the VIP3A protein *via* direct exposure in laboratory studies

Scientific Name	Common name	References
INSECTS		
Order/*Genus* + *species*		
Coleoptera		
Coleomegilla maculata	Pink spotted ladybeetle	Teixeira, 2002a
Diabrotica longicornis	Northern corn rootworm	Syngenta, unpublished data
Diabrotica undecimpunctata	Southern corn rootworm	Syngenta, unpublished data
Diabrotica virgifera	Western corn rootworm	Warren, 1997
Leptinotarsa decemlineata	Colorado potato beetle	Warren, 1997
Popillia japonica	Japanese beetle	Syngenta, unpublished data
Tenebrio molitor	Yellow meal worm	Warren, 1997
Anthonomus grandis	Boll weevil	Syngenta, unpublished data
Diptera		
Culex pipiens	Northern house mosquito	Warren, 1997
Drosophila melanogaster	Fruit fly	Warren, 1997
Musca domestica	House fly	Syngenta, unpublished data
Homoptera		
Myzus persicae	Green peach aphid	Syngenta, unpublished data
Hymenoptera		
Apis mellifera	Honeybee	Maggi, 2002.
Isotomidae		
Folsomia candida	Springtail (collembola)	Privalle, 2002b.
Neuroptera		
Chrysoperla carnea	Green lacewing	Teixeira, 2002b
Thysanoptera		
Frankliniella occidentalis	Western flower thrip	Syngenta, unpublished data
NON-INSECT SPECIES		
Phylum/Subphylum/*Genus* + *species*		
Annelida		
Eisenia foetida	Earthworm	Teixeira, 2002c
Arthropoda/Crustacea		
Daphnia magna	Water flea	Putt, 2002

Chapter 2

THE COTTON FAMILY

The following was excerpted from the USDA Agricultural Biotechnology website (http://www.aphis.usda.gov/ppq/biotech/cotton.html) detailing the biology of cotton, authored by James Lackey, Ph.D., Botanist, USDA, APHIS, PPQ Biotechnology Permits, email: jlackey@aphis.usda.gov

A. Cotton as a Crop

Four species of the genus *Gossypium* are known as cotton, which is grown primarily for the seed hairs that are made into textiles. Cotton is predominant as a textile fiber because the mature dry hairs twist in such a way that they can be spun into fine, strong threads. Other products, such as cottonseed oil, meal, and cotton linters are by-products of fiber production. Cotton, a perennial plant cultivated as an annual, is grown in the United States mostly in areas from Virginia southward and westward to California in an area often referred to as the Cotton Belt (McGregor 1976).

B. Taxonomy of Cotton

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

C. Genetics of Cotton

At least seven complete sets of genes, designated A, B, C, D, E, F, and G, are found in the genus (Endrizzi 1984). Diploid species (2n=26) are found on all continents, and a few are of some agricultural importance. The A genome is restricted in diploids to two species (G. arboreum and G. herbaceum) of the Old World. The D genome is restricted in diploids to some species of the New World, such as G. thurberi. By far the most important agricultural cottons are G. hirsutum and G. barbadense. These are both allotetraploids of New World origin and presumably resulted from an ancient cross between Old World A genomes and New World D genomes. How and when the original crosses occurred is speculative. Euploids of these plants have 52 somatic chromosomes and are frequently designated as AADD. Four additional New World allotetraploids occur in the genus, including G. tomentosum, the native of Hawaii. G. tomentosum has been crossed with G. hirsutum in breeding programs. The New World allotetraploids are peculiar in the genus because the species, at least in their wild forms, grow near the ocean as invaders in the constantly disturbed habitats of strand and associated environs. It is from these "weedy" or invader species that the cultivated cottons developed (Fryxell 1979).

D. Pollination of Cotton

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

E. Weediness of Cotton

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

EPA recently concluded its environmental reassessment of the registered *B.t.* plant-incorporated protectants and summarized its findings in the "Biopesticides Registration Action Document" for these products (US EPA, 2001). In its reassessment of Bt cotton, EPA reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories, and concluded that the possibility for gene transfer exists only in limited geographic locations where wild or feral cotton relatives exist, *i.e.* in Florida, Hawaii, and the Caribbean. In addition, the USDA/APHIS has made this same determination under its statutory authority. These conclusions, made with respect to commercial Bt cotton (producing a Cry1Ac endotoxin), are also applicable to VIP3A Bt cotton. Accordingly, the same geographical restrictions (*e.g.,* no commercial plantings in Hawaii and south Florida) that are currently in effect for Cry1Ac Bt cotton are expected to be applicable to VIP3A cotton.

F. Modes of Gene Escape in Cotton

Genetic material of G. hirsutum may escape from a planting site by vegetative material, by seed, or by pollen. Vegetative propagation is not a common mechanism by which cotton reproduces. Movement of genetic material by pollen is possible only to those plants with the proper chromosomal type, in this instance only to those allotetraploids with AADD genomes. In the United States, this group would include only the cultivated species G. hirsutum, G. barbadense, and the wild species G. tomentosum. G. thurberi, the native diploid from Arizona

with a DD genome, is not a suitable recipient. Movement to G. hirsutum and G. barbadense is possible if suitable insect pollinators are present and if there is a short distance from transgenic plants to recipient plants. Physical barriers, intermediate pollinator-attractive plants, and other temporal or biological impediments would reduce the potential for pollen movement. Movement of genetic material to G. tomentosum is less well documented. The plants are chromosomally compatible with G. hirsutum, but there is some doubt as to the possibility for pollination. The flowers of G. tomentosum seem to be pollinated by moths, not bees, and the flowers are receptive at night, not in the day. Both these factors would seem to minimize the possibility of cross-pollination. However, Fryxell (1979) reports that G. tomentosum may be losing its genetic identity from introgression hybridization of cultivated cottons by unknown means.

G. Characteristics of the Non-transformed Cultivar

Event COT102 was produced via disarmed non-pathogenic *Agrobacterium* transformation of the parental cotton cultivar Coker 312. This cotton line was released by the Coker Pedigreed Seed Company in 1974, and the variety is currently owned by the SeedCo Corporation of Lubbock, TX. Coker 312 is not considered a highly desirable cotton cultivar with today's grower; however, it is quite amenable to modern tissue culture techniques and is considered desirable for molecular transformation purposes. Even though Coker 312 is not widely planted, it is still considered a viable commercial cultivar and, therefore, an acceptable genetic background for the purposes of agronomic performance evaluations.

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Chapter 3

MOLECULAR ANALYSIS OF EVENT COT102

A. Description of the Transformation System

Event COT102 was produced via *Agrobacterium*-mediated transformation of *Gossypium hirsutum* L. cultivar Coker 312 (Tinland and Hohn, 1995; Trolinder *et al.*, 1988a, Trolinder *et al.*, 1988b), using the transformation vector pCOT1 (Figure 3.1). Event COT102 has been transformed with two genes, *vip3A(a)* and *aph4*. The insecticidal gene *vip3A(a)* was derived from *Bacillus thuringiensis* strain AB88 (Estruch *et al.*, 1996). This gene is under the regulatory control of the actin-2 promoter (An *et al*, 1996) derived from *Arabidopsis*, which confers expression of VIP3A protein throughout the plant. Expression of the *vip3A* gene confers resistance towards a number of insect pests of cotton. The selectable marker gene *aph4*, encoding the enzyme hygromycin-B phosphotransferase, was originally derived from *E. coli* and is under the regulatory control of the ubiquitin-3 promoter from *Arabidopsis* (Norris SR *et al*, 1993). Expression of the *aph4* gene product allows for growth of transformed plant cells on artificial growth medium containing hygromycin B.

Transformation was carried out by incubating Agrobacterium cells, harboring the transformation vector pCOT1 (Figure 3.1), with cotton hypocotyl tissue and subsequent plating of that tissue onto synthetic culture medium containing hygromycin B. Plants were regenerated and individually analysed for the presence of the *vip3A(a) gene* by PCR and for insecticidal bioactivity. The selected T_0 transformed plants were self-pollinated to produce T_1 seed, and a single homozygous plant designated event COT102 was selected from the T_1 generation for further breeding. Table 3.1 indicates the genetic lineage of even COT102 and analysis carried out.

Table 3.1. Event COT102 Genetic Lineage

Generation	Method Produced	Analysis
T_0	Primary transformant in Coker 312 background	
T_1	Result of self-pollinated T_0	Homozygous chosen and designated event COT102.
T_2	Result of self-pollinated T_1	2000 field evaluations.
T_3	Result of self-pollinated T_2	2001 field evaluations.
T_4	Result of self-pollinated T_3	2002 field evaluations; fiber quality analysis, cottonseed compositional analysis; molecular characterization.
T_5	Result of self-pollinated T_4	2003 field evaluations
F_1	Result of T_1 generation back-cross to Coker 312	Mendelian inheritance analysis
BC_1F_1	Result of F_1 generation back-cross	

	to non-transgenic commercial germplasm	
BC_1F_2	Result of self-pollinated BC_1F_1	Mendelian inheritance analysis
BC_2F_1	Result of BC_1F_1 generation back-cross to non-transgenic commercial germplasm	Mendelian inheritance analysis
BC_2F_2	Result of self-pollinated BC_2F_1	Mendelian inheritance analysis
BC_3F_1	Result of BC_2F_1 generation back-cross to non-transgenic commercial germplasm	Mendelian inheritance analysis

B. Donor Genes and Regulatory Sequences

The binary Agrobacterium transformation vector, pCOT1, (Figure 3.1), was constructed using state of the art molecular biology techniques. Only those genetic elements within the left and right border regions are efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions are generally not. (Table 3.2) The vector also contains well-characterized DNA elements required for selection and replication of the plasmid in bacteria (Table 3.2).

The *Agrobacterium* strain GV3101 containing the tumor inducing (Ti) plasmid pMP90 was used to transform Coker 312 cotton tissue in event COT102. The Ti plasmid has had its tumor inducing genes removed by homologous recombination and replaced with a gentamycin gene that allows for selection and maintenance of the strain. The plasmid provides the genes and virulence functions essential for T-DNA transfer (Koncz, and Schell, 1986).

Table 3.2. Summary of DNA sequences included in pCOT1. (See Figure 3.1)

Genetic Element	Base pair range (11801 bp plasmid)	Introduced during transformation
Left border region	11732-11801 bp and 1-60 bp	Partial Transfer
Left border	1-25 bp	Partial Transfer
Intervening Sequence	61-107 bp	Yes
nos terminator	108-362 bp	Yes
Synthetic Linker	363-394 bp	Yes
aph4 gene	395-1420 bp	Yes
Synthetic Linker	1421-1451 bp	Yes
ubiquitin-3 promoter	1452- 3172 bp	Yes
Synthetic Linker	3173-3222 bp	Yes
actin-2 promoter	3223-4630 bp	Yes
Synthetic Linker	4631-4639 bp	Yes
vip3A(a) gene	4640-7009 bp	Yes
Synthetic Linker	7010-7033 bp	Yes
nos terminator	7034-7288 bp	Yes
Intervening Sequence	7289-7449 bp	Yes
Right border region	7450-7615 bp	Partial Transfer
Right border	7494-7518 bp	Partial Transfer
Vector Backbone	7519-7630 bp	No
ColE1	7631-8437 bp	No
Vector Backbone	8438-9114 bp	No
VS1ori	9115-9519 bp	No
Vector Backbone	9520-9561 bp	No
RepA	9562-10635 bp	No
Vector Backbone	10636-10663 bp	No
Spectinomycin	10664-11452 bp	No
Vector Backbone	11453-11731 bp	No

C. Descriptions of the Functional Genetic Components of pCOT1

1. Insecticidal Principal:

The Actin promoter (Act2): Promoter from the actin-2 gene of *Arabidopsis thaliana* (promoter sequence includes first exon and intron from the non-translated leader sequence of the actin-2 gene) (An *et al*, 1996). This promoter is 1407 bp in length. In event COT102 cotton plants, the Act2 promoter confers constituitive expression of *vip3A(a)*. (Accession number U41998)

The *vip3A(a)* gene: Synthetic version of the *vip3A(a)* gene from *Bacillus thuringiensis* strain AB88 (Estruch *et al.*, 1996). The entire coding region of the *vip3A(a)* gene (2369bp) was synthesized to accommodate the preferred codon usage for corn. (Murray *et al.*, 1989) The

codon adjustments did not result in any changes in the amino acid sequence to the protein. (Figure 3.11) (Accession number AAC37036)

Nos terminator: Nos terminator from the *Agrobacterium tumefaciens* nopaline synthase gene (Bevan *et al.*, 1983). The function of this 254 bp sequence is to terminate transcription and to provide a polyadenylation site. This sequence does not encode a protein.

2. Selectable Marker

The Ubiquitin promoter (Ubq3int): Promoter plus the first intron from the ubiquitin-3 gene of *Arabidopsis thaliana* (Norris *et al*, 1993). This promoter is 1720 bp in length. In COT102, the Ubq3int promoter confers constitutive expression of the *aph4* gene. (Genbank Accession number U29159)

The *aph4* gene: The *aph4* gene (1025bp), derived from *E. coli*, encodes the enzyme hygromycin B phosphotransferase (Figure 3.12) that catalyzes the phosphorylation of hygromycin . (Waldron, 1997; Kaster *et al.*, 1983) (GenBank Accession No. CAA85741)

Nos terminator: Nos terminator from the *Agrobacterium tumefaciens* nopaline synthase gene (Bevan *et al.*, 1983). The function of this 254 bp sequence is to terminate transcription and to provide a polyadenylation site. This sequence does not encode a protein.

3. *Agrobacterium* Binary Vector Components:

The transformation vector containing the sequences above also harbors the following elements, which are not introduced into the plant cell during the transformation process.

Spectinomycin resistance gene: Streptomycin adenylyltransferase (Spec.) *aadA* (789 bp) from Tn7 (GenBank Accession Number X03043). The gene encodes a protein that confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker.

VS1 origin: Consensus sequence origin of replication and partitioning region (405 bp) from plasmid pVS1 of Pseudomonas (Itoh *et al.* 1984); similar to GenBank Accession Number U10487. Serves as origin of replication in *Agrobacterium tumefaciens* host.

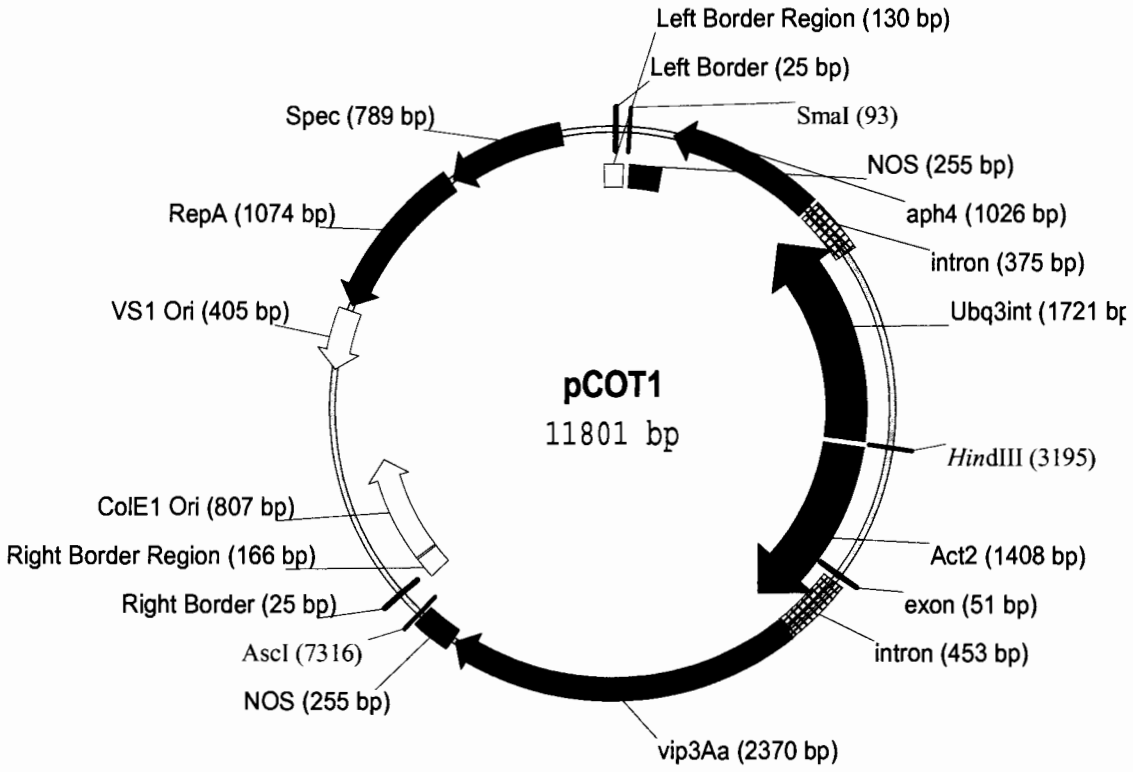
RepA: pVS1 replication protein (1074 bp) from Pseudomonas, part of the minimal pVS1 replicon used in gram-negative plant-associated bacteria. (Genbank accession number AAD21676)

ColE1 origin: Origin of replication (807 bp) that permits replication of pCOT1 in *E. coli.*

Left border region/ Left Border (LB): Left border region from *Agrobacterium tumefaciens* nopaline Ti-plasmid (130 bp) contains the left border inverted repeat (25bp) which is required for the transfer of the T-DNA into the plant cell. (Zambryski *et al.*, 1980) EMBL no: J01825.

Right border region/ Right Border (RB): Right border region from *Agrobacterium tumefaciens* nopaline Ti-plasmid (166 bp) contains the right border inverted repeat (25bp) which is required for the transfer of the T-DNA into the plant cell. (Yadav et al., 1982) EMBL no: J01826, V00087.

Figure 3.1. pCOT1 Plasmid Map



D. Genetic Analysis

Traditional molecular techniques have been employed to analyze the inserted DNA in event COT102. Southern blot analysis was used to determine the insert copy number (number of pCOT1 T-DNA insertions within the cotton genome), intactness of both the *vip3A(a)* and *aph4* coding regions, intactness of both the *vip3A(a)* and *aph4* expression cassettes, and to assess whether vector backbone sequences were introduced during the transformation process. Southern blots were individually probed with the following genetic elements: *vip3A(a),* , *aph4* , actin-2 promoter, ubiquitin-3 promoter, *Spec* gene, RepA gene, VS1 ori, and ColE1 ori. All of the probes used for Southern analysis were ^{32}P random prime labeled.

The data provided below (Table 3.3) indicates that event COT102 contains a single intact T-DNA insert from pCOT1. Data also indicates absence of any vector sequences other than the DNA within the T-DNA borders.

Table 3.3. Summary of the Molecular Characterization of Cotton Event COT102

Analysis	Result
# of inserts	1
# copies of *vip3A(a)* and *aph4*, actin-2 promoter, ubiquitin-3 promoter	1
VIP3 expression cassette	Intact
APH4 expression cassette	Intact
Vector backbone sequences	Not detected

1. Southern Analysis for *vip3A(a)* expression cassette:

a.) Analysis of *vip3A(a)* probed Southerns (Figures 3.2)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid control DNA were digested with restriction endonucleases, processed by gel electrophoresis, transferred by blotting to nylon membranes, and probed with a full-length *vip3A(a)* specific probe (2370 bp). (Figure 3.2c) To examine whether the T-DNA present in COT102 is full-length, the DNAs were digested with SmaI and AscI restriction endonucleases, which recognize single sites at the 5' and 3' ends of the insert DNA, respectively. The single ~7.2 kb band (Figure 3.2a, lane 3) corresponds to the expected size of the T-DNA from pCOT1. Lanes 5, 7, 8, and 9, positive controls for the hybridization, demonstrated similar sized bands using three increasing concentrations of the plasmid pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312.

To demonstrate the intactness of the *vip3A(a)* functional genetic unit, event COT102 and control DNA samples were digested with HindIII and AscI restriction endonucleases, which recognize single sites at the 5' end of the actin-2 promoter and the 3'-end of the nos terminator, respectively. The single ~4.0 Kb band (Figure 3.2b, lane 1) corresponds to the expected size of the intact actin-2 promoter, the *vip3A(a)*

gene , and the nos terminator fragment. Lanes 3, 5, 6, and 7 are positive controls for the hybridization, which give equal sized bands using three increasing concentrations of the plasmid pCOT1 DNA, as well as when the plasmid is introduced into genomic control DNA, Coker 312.

The copy number of the *vip3A(a)* gene was examined by digesting event Cot102 DNA with HindIII, which has a single restriction site within the COT102 insert. For each copy of the *vip3A* gene, a single hybridizing band is expected to be present. The single band (Figure 3.2b, lane 9) indicates that there is only one copy of the *vip3A(a)* gene present

Data presented in Figures 3.2a and 3.2b demonstrate that the T-DNA insert from pCOT1 is intact, the actin-2 promoter, *vip3A(a)* gene, and nos terminator cassette is intact, and that there is a single copy of the *vip3A(a)* gene in COT102.

b.) Analysis of *actin-2 promoter* probed Southern (Figure 3.3)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid control DNA were digested with restriction endonucleases, processed by gel electrophoresis, transferred by blotting to nylon membranes, and probed with a Actin-2 specific probe (1354 bp; Figure 3.3b) To demonstrate the intactness of the *vip3A(a)* functional genetic unit the DNA was digested with HindIII and AscI, which recognize single sites at the 5' end of the actin-2 promoter and the 3'-end of the nos terminator, respectively. The single ~4.0 Kb band present (Figure 3.3a, lane 3) corresponds to the expected size of the actin 2-promoter, *vip3A(a)* gene and the nos terminator portion of the T-DNA insert. Lanes 5, 7, 8, and 9 are positive controls for the hybridization, which gave equal sized bands using three increasing concentrations of the plasmid pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312.

COT102 DNA was also digested with HindIII which cuts once within the COT102 insert to examine the number of copies of the actin-2 promoter that are present. The single band (Figure 3.3a. lane 11) indicates that there is one copy of the actin-2 promoter present.

Data presented in Figure 3.3a demonstrates that the actin-2 promoter, the *vip3A(a)* gene, and the nos terminator are intact and contiguous, and that there is a single copy of the actin-2 promoter in event COT102.

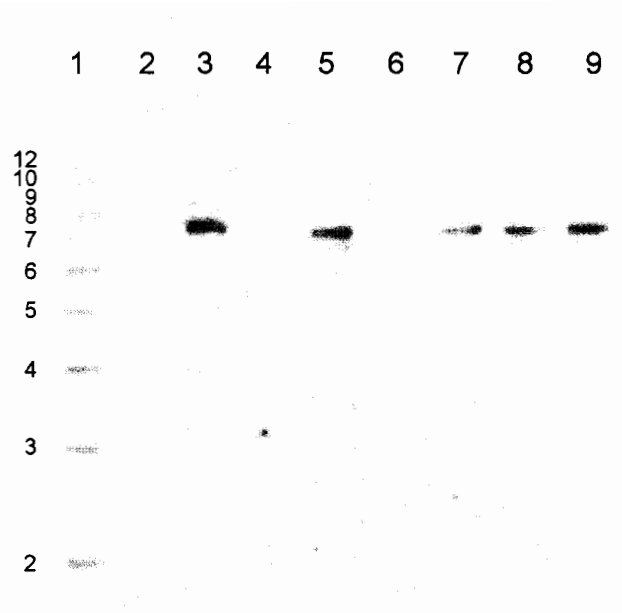


Figure 3.2a. Southern blot analysis of event COT102: *vip3A(a)* probe
COT102 genomic DNA and controls isolated from leaf tissue were digested with the indicated restriction enzymes. The resulting blots were probed with a ^{32}P-labeled *vip3A(a)* specific probe (2370 bp). Lane designations are as follows:

Lane 1: 1 Kb molecular weight marker (Stratagene)
Lane 2: blank
Lane 3: 7.5µg COT 102 genomic DNA digested with SmaI and AscI
Lane 4: 7.5µg Coker 312 genomic DNA digested with SmaI and AscI
Lane 5: 7.5µg Coker 312 genomic DNA + 5.2 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 6: blank
Lane 7: 5.2 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 8: 10.4 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 9: 26 pg pCOT1 plasmid DNA digested with SmaI and AscI

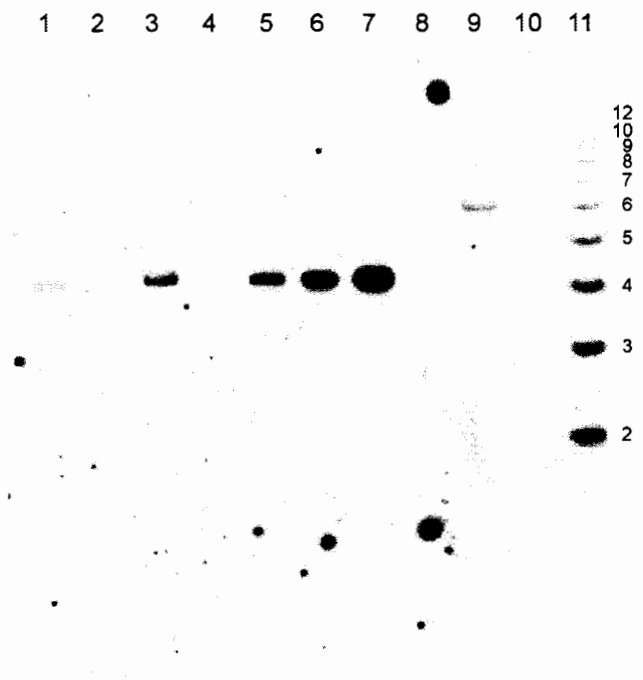
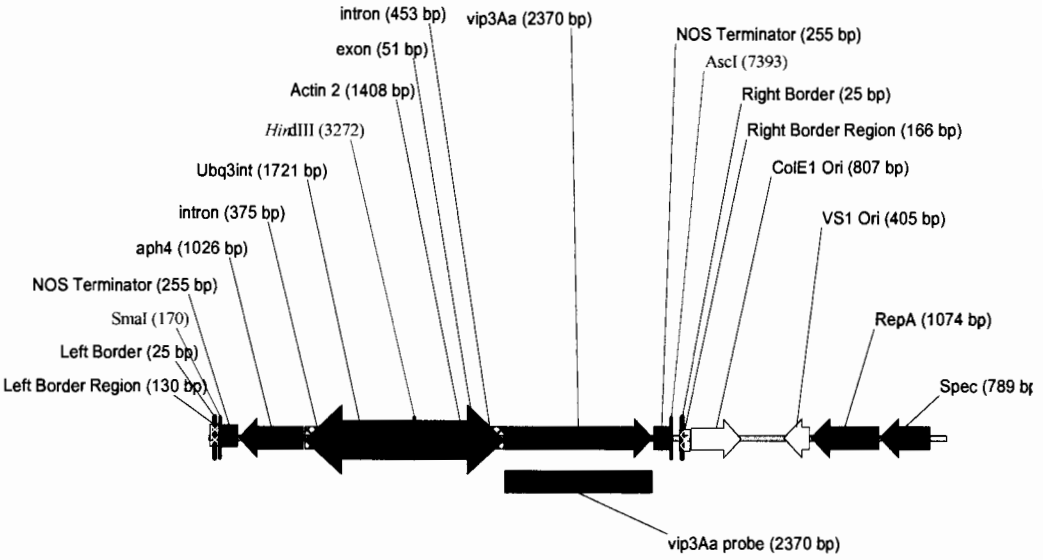


Figure 3.2b. Southern blot analysis of event COT102: *vip3A(a)* probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blots were probed with a ^{32}P-labeled *vip3A(a)* specific probe (2370 bp). Lane designations are as follows:

Lane 1: 7.5µg COT 102 Genomic DNA digested with HindIII and AscI
Lane 2: 7.5µg Coker 312 genomic DNA digested with HindIII and AscI
Lane 3: 7.5µg Coker 312 genomic DNA + 3.0 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 4: blank
Lane 5: 3.0 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 6: 6.0 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 7: 15.0 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 8: blank
Lane 9: 7.5µg COT 102 genomic DNA digested with HindIII
Lane 10: blank
Lane 11: 1 Kb molecular weight marker (Stratagene)

Figure 3.2c. Linear map of pCOT1 with the *vip3A(a)* probe



pCOT1 with vip3A(a) probe

11801 bp

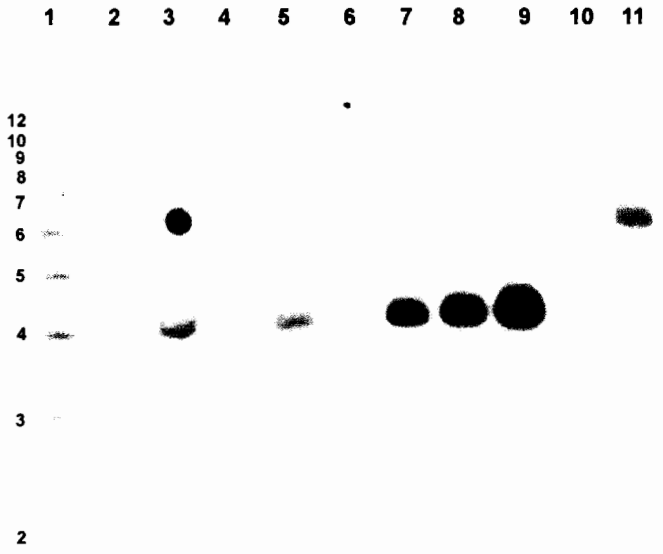
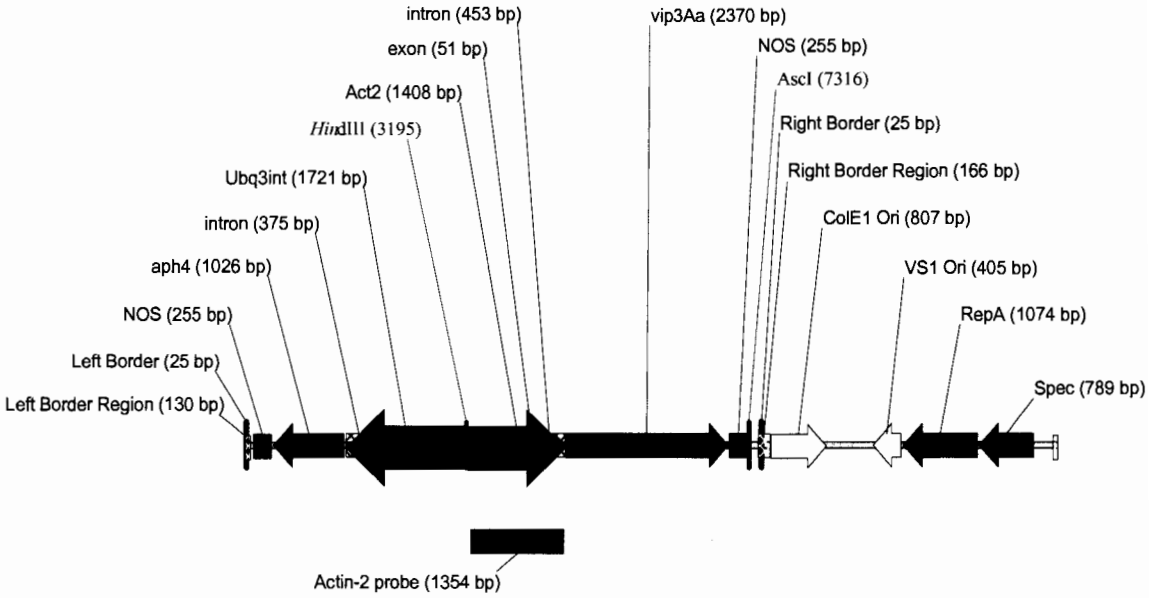


Figure 3.3a. Southern blot analysis of event COT102: actin-2 promoter probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled actin-2 promoter specific probe (1354 bp). Lane designations are as follows:

Lane 1: 1Kb molecular weight marker (Stratagene)
Lane 2: Blank
Lane 3: 7.5 µg COT 102 Genomic DNA digested with HindIII and AscI
Lane 4: 7.5 µg Coker 312 genomic DNA digested with HindIII and AscI
Lane 5: 7.5 µg Coker 312 genomic DNA + 5.2 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 6: blank
Lane 7: 5.2 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 8: 10.4 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 9: 26 pg pCOT1 plasmid DNA digested with HindIII and AscI
Lane 10: blank
Lane 11: 7.5 µg COT 102 Genomic DNA digested with HindIII

Figure 3.3b. Linear map of pCOT1with Actin-2 probe



2.) Southern Analysis for *aph4* expression cassette

a.) Analysis of *aph4* probed Southern (Figure 3.4)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid DNA were digested with restriction endonucleases and following gel electrophoresis were probed with a full-length *aph4* gene specific probe (1026 bp). (Figure 3.4b) To examine whether the T-DNA present in COT102 is full-length, the DNAs were digested with SmaI and AscI restriction endonucleases, which recognize single sites at the 5' and 3' ends of the insert DNA, respectively. The single ~7.2 kb band (Figure 3.4a, lane 3) corresponds to the expected size of the T-DNA from pCOT1. Lanes 5, 7, 8, and 9 are positive controls for the hybridization, which gave identical bands with three increasing concentrations of the plasmid pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312.

To demonstrate intactness of the *aph*4 functional genetic unit, event COT102 and control DNA was digested with SmaI and HindIII restriction endonucleases, which recognize single sites at the 5' end of the ubiquitin-3 promoter and the 3' end of the nos terminator, respectively. The single ~3.0 kb band (Figure 3.4a, lane 11) corresponds to the expected size of the contiguous ubiquitin-3 promoter, *aph4* gene and nos terminator portion of the COT102 T-DNA. Lanes 13, 15, 16, and 17 are positive controls for the hybridization, which gave equal sized bands using three increasing concentrations of the plasmid pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312.

COT102 DNA was also digested with HindIII which cuts once within the COT102 insert to examine the number of copies of the *aph4* gene that are present. The single band in lane 19 (Figure 3.4) indicates that there is only one copy of the *aph4* gene present in the COT102 genome.

Data presented in Figure 3.3a demonstrates that the T-DNA insert from pCOT1 is intact, the ubiquitin-3 promoter, the *aph4* gene, and nos terminator are contiguous, and there is a single copy of the *aph4* gene in event COT102.

b.) Analysis of ubiquitin-3 promoter probed Southern (Figure 3.5)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid DNA were digested with restriction endonucleases and following gel electrophoresis were probed with a ubiquitin-3 promoter specific probe (1678 bp). (Figure 3.5b)

To demonstrate intactness of the *aph*4 functional genetic unit, event COT102 and control DNA was digested with SmaI and HindIII restriction endonucleases, which recognize single sites at the 5' end of the ubiquitin-3 promoter and the 3' end of the nos terminator, respectively. The single ~3.0 kb band produced (Figure 3.5a, lane 11) corresponds to the expected size of the contiguous ubiquitin-3 promoter, *aph4* gene and nos terminator portion of the COT102 T-DNA. Lanes 5, 7, 8, and 9 are positive

controls for the hybridization, which gave equal sized bands using three increasing concentrations of the plasmid pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312.

COT102 DNA was also digested with HindIII which cuts once within the COT102 insert to examine the number of copies of the ubiquitin-3 promoter that are present. The single band (Figure 3.5a, lane 11) indicates that there is only one copy of the ubiquitin-3 promoter present.

Data presented in Figure 3.5a demonstrates that the ubiquitin-3 promoter the *aph4* gene, and the nos terminator are intact and continuous, and that there is a single copy of the ubiquitin-3 promoter in event COT102.

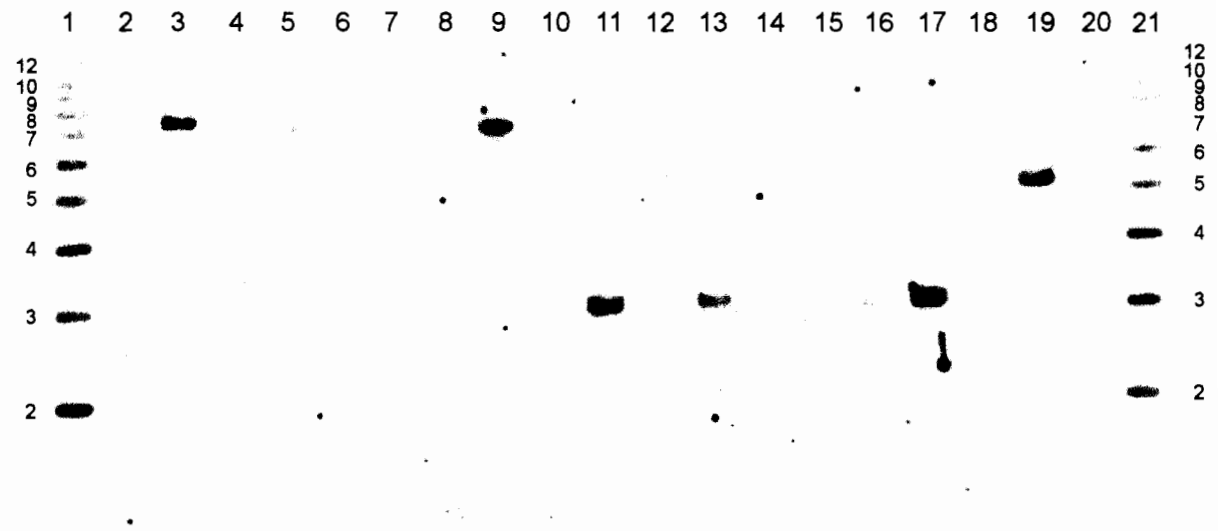
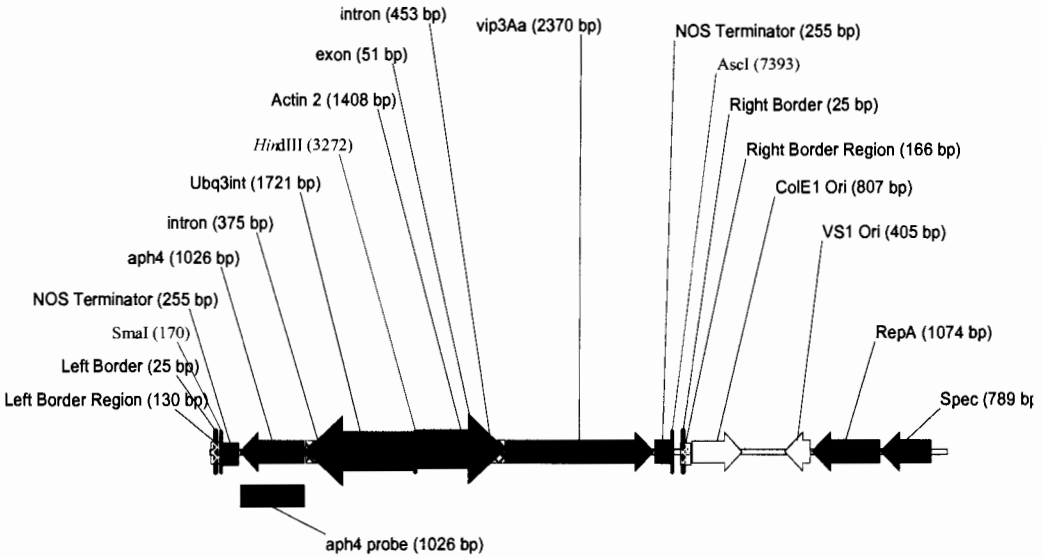


Figure 3.4a. Southern blot analysis of event COT102: *aph4* probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled *aph4* specific probe (1026 bp). Lane designations are as follows:

Lane 1: 1 Kb molecular weight marker (Stratagene)
Lane 2: blank
Lane 3: 7.5 µg COT102 genomic DNA digested with SmaI and AscI
Lane 4: 7.5 µg Coker 312 genomic DNA digested with SmaI and AscI
Lane 5: 7.5 µg Coker 312 genomic DNA + 5.2 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 6: blank
Lane 7: 5.2 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 8: 10.4 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 9: 26 pg pCOT1 plasmid DNA digested with SmaI and AscI
Lane 10: blank
Lane 11: 7.5 µg COT 102 genomic DNA digested with HindIII and SmaI
Lane 12: 7.5 µg Coker 312 genomic DNA digested with HindIII and SmaI
Lane 13: 7.5 µg Coker 312 genomic DNA + 3.0 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 14: blank
Lane 15: 3.0 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 16: 6.0 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 17: 15.0 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 18: blank
Lane 19: 7.5 µg COT 102 genomic DNA digested with HindIII
Lane 20: blank
Lane 21: 1 Kb molecular weight marker (Stratagene)

Figure 3.4b. Linear map of pCOT1 with *aph4* probe



pCOT1 with aph4 probe

11801 bp

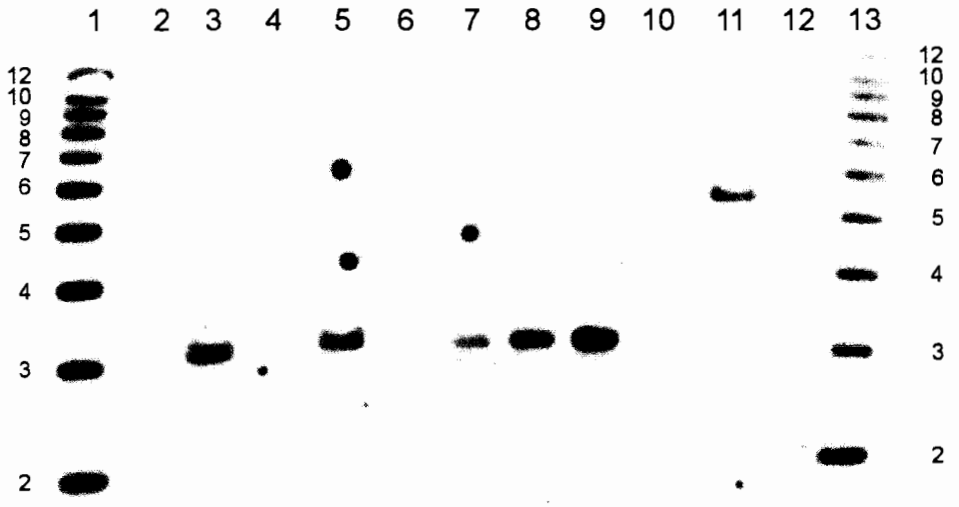
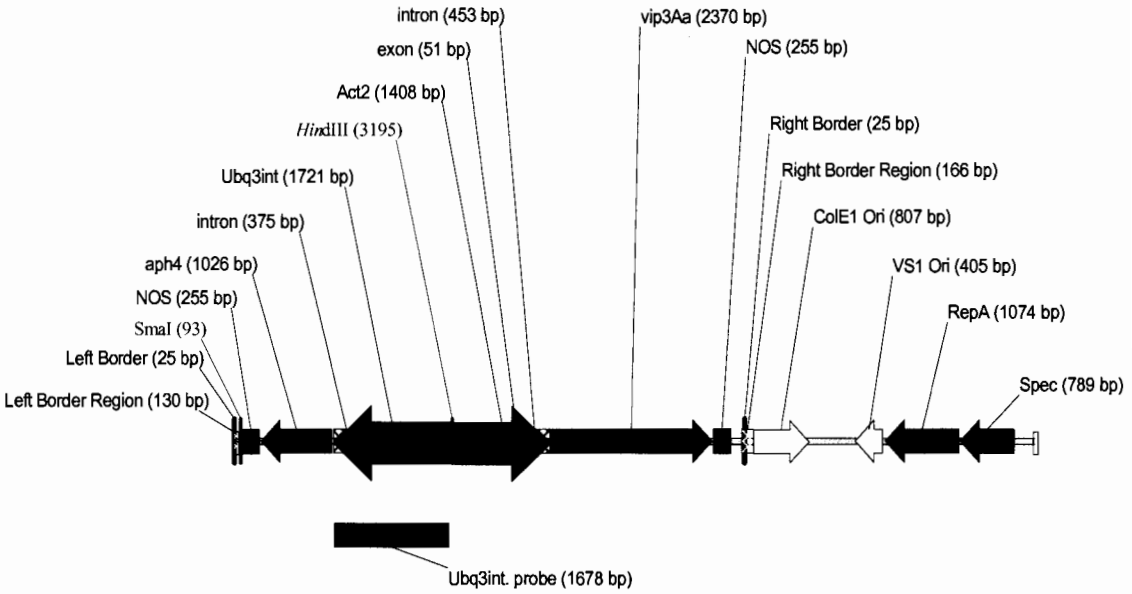


Figure 3.5a. Southern blot analysis of event COT102: ubiquitin-3 promoter probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled ubiquitin-3 promoter specific probe (1678 bp). Lane designations are as follows:

Lane 1: 1Kb molecular weight marker (Stratagene)
Lane 2: Blank
Lane 3: 7.5 µg COT 102 genomic DNA digested with HindIII and SmaI
Lane 4: 7.5 µg Coker 312 genomic DNA digested with HindIII and SmaI
Lane 5: 7.5 µg Coker 312 genomic DNA + 5.2 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 6: blank
Lane 7: 5.2 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 8: 10.4 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 9: 26 pg pCOT1 plasmid DNA digested with HindIII and SmaI
Lane 10: blank
Lane 11: 7.5 µg COT 102 Genomic DNA digested with HindIII
Lane 12: blank
Lane 13: 1 Kb molecular weight marker (Stratagene)

Figure 3.5b. Linear map of pCOT1 with Ubiquitin-3 promoter (Ubq3int) probe



3. Southern Analysis for the Vector Backbone Components

a.) Spectinomycin Gene (Figure 3.6)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid control DNA were digested with the restriction endonuclease HindIII and following gel electrophoresis were probed with a spectinomycin gene specific probe (720 bp), (Figure 3.6b). The result (Figure 3.6a, lane 3) indicates that there are no intact or partial copies of the spectinomycin gene present in COT102. Lanes 5, 7, 8, and 9 are positive controls for hybridization indicating that the probe readily detected the spectinomycin gene present in the transformation vector, pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312. The bands in these lanes are approximately 11.8 Kb, which is expected for the linear form of pCOT1.

b.) VS1 Ori (Figure 3.7)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid control DNA were digested with the restriction endonuclease HindIII and following gel electrophoresis were probed with a VS1 Ori specific probe (396 bp), (Figure 3.7b) The result (Figure 3.7a, lane 3) indicates that there are no intact or partial copies of the VS1 Ori present in COT102. The bands in lanes 5, 7, 8, and 9 are positive controls for hybridization indicating that the probe readily detected the VS1 Ori present in the transformation vector, pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312. The bands in these lanes are approximately 11.8 Kb, which is expected for the linear form of pCOT1.

c.) RepA (Figure 3.8)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid control DNA were digested with the restriction endonuclease HindIII and following gel electrophoresis were probed with a full-length RepA specific probe (1074 bp), (Figure 3.8b) The result (Figure 3.8a, lane 3) indicates that there are no intact or partial copies of the RepA gene present in COT102. The bands in lanes 5, 7, 8, and 9 are positive controls for hybridization indicating that the probe readily detected the RepA gene in the plasmid DNA, pCOT1, as well as when the plasmid is introduced into genomic control DNA, Coker 312. The bands in these lanes are approximately 11.8 Kb, which is expected for the linear form of pCOT1.

d.) ColE 1 Ori (Figure 3.9)

Event COT102 genomic DNA, non-transgenic Coker 312 genomic DNA, and pCOT1 plasmid control DNA were digested with the restriction endonuclease HindIII and following gel electrophoresis were probed with a ColE1 Ori specific probe (783 bp),(Figure 3.9b) The result (Figure 3.9a, lane 3) indicates that there are no intact or partial copies of the ColE1 Ori sequence present in COT102. The bands in lanes 5, 7, 8, and 9 are positive controls for hybridization indicating that the probe readily detected the ColE 1 Ori sequence in the plasmid DNA, pCOT1, as well as when the

plasmid is introduced into genomic control DNA, Coker 312. The bands in these lanes are approximately 11.8 Kb, which is expected for the linear form of pCOT1.

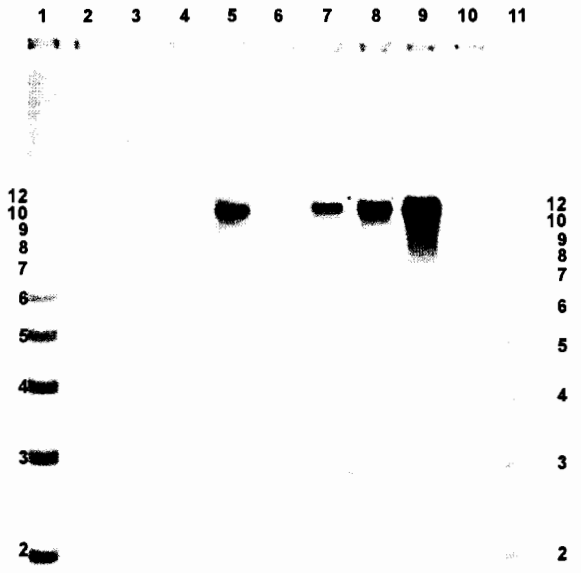
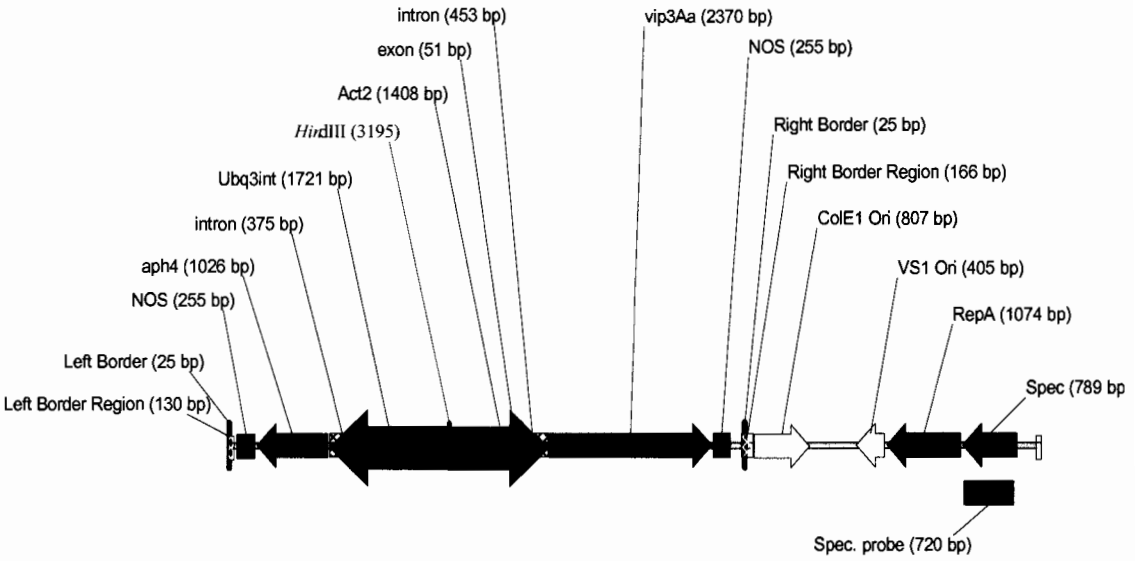


Figure 3.6a. Southern blot analysis of event COT102: Spectinomycin probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled spectinomycin gene specific probe (720 bp). Lane designations are as follows:

Lane 1: 1 Kb Lane Marker (Stratagene)
Lane 2: blank
Lane 3: 7.5 μg COT 102 genomic DNA digested with HindIII
Lane 4: 7.5 μg Coker 312 genomic DNA digested with HindIII
Lane 5: 7.5 μg Coker 312 genomic DNA + 8.3 pg of pCOT1 plasmid DNA digested with HindIII
Lane 6: blank
Lane 7: 8.3 pg pCOT1 plasmid DNA digested with HindIII
Lane 8: 16.6 pg pCOT1 plasmid DNA digested with HindIII
Lane 9: 41.5 pg pCOT1 plasmid DNA digested with HindIII
Lane 10: blank
Lane 11: 1 Kb Lane Marker (Stratagene)

Figure 3.6b. Linear map of pCOT1 with Spectinomycin probe



pCOT1 with Spec probe

11801 bp

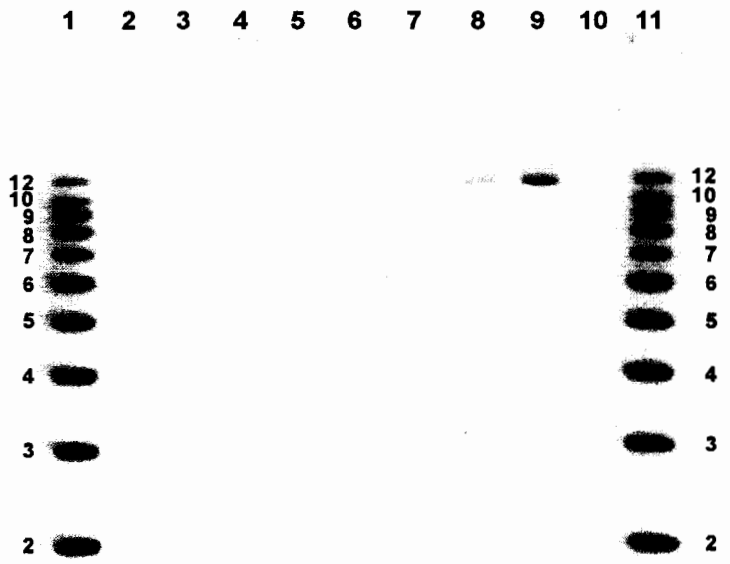
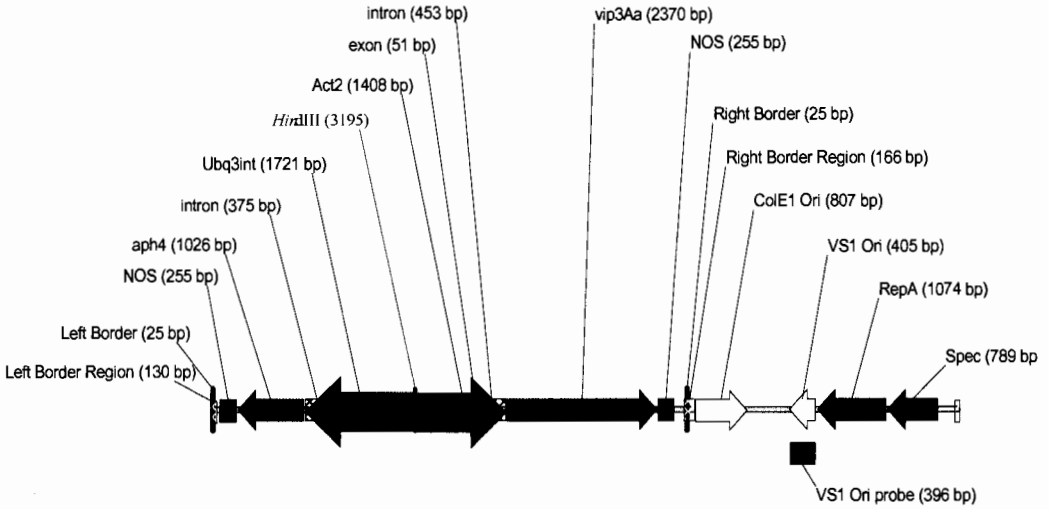


Figure 3.7a. Southern blot analysis of event COT102: VS1 Ori probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled VS1 ori specific probe (396 bp). Lane designations are as follows:

Lane 1: 1 Kb Lane Marker (Stratagene)
Lane 2: blank
Lane 3: 7.5 µg COT 102 genomic DNA digested with HindIII
Lane 4: 7.5 µg Coker 312 genomic DNA digested with HindIII
Lane 5: 7.5 µg Coker 312 genomic DNA + a 8.3 pg of pCOT1 plasmid DNA digested with HindIII
Lane 6: blank
Lane 7: 8.3 pg pCOT1 plasmid DNA digested with HindIII
Lane 8: 16.6 pg pCOT1 plasmid DNA digested with HindIII
Lane 9: 41.5 pg pCOT1 plasmid DNA digested with HindIII
Lane 10: blank
Lane 11: 1 Kb Lane Marker (Stratagene)

Figure 3.7b. Linear map of pCOT1 with VS1 Ori probe



pCOT1 with VS1 Ori probe

11801 bp

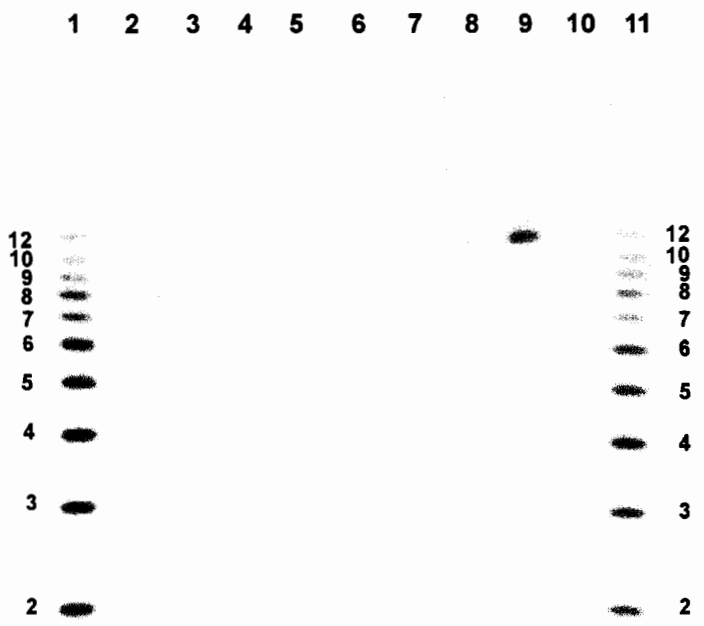
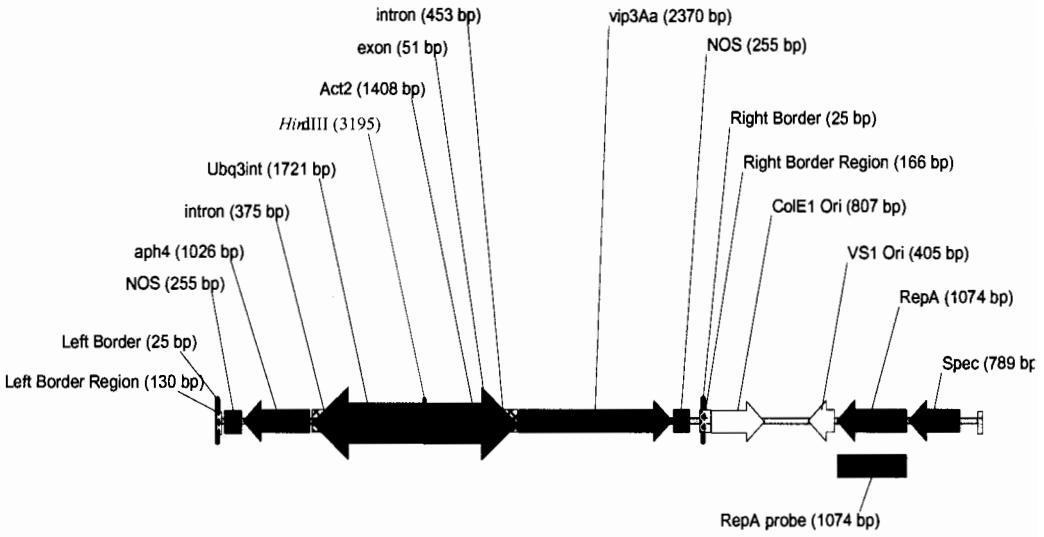


Figure 3.8a. Southern blot analysis of event COT102: *RepA* probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled Rep A specific probe (1074 bp). Lane designations are as follows:

Lane 1: 1 Kb Lane Marker (Stratagene)
Lane 2: blank
Lane 3: 7.5 µg COT 102 genomic DNA digested with HindIII
Lane 4: 7.5 µg Coker 312 genomic DNA digested with HindIII
Lane 5: 7.5 µg Coker 312 genomic DNA + 8.3 pg of pCOT1 plasmid DNA digested with HindIII
Lane 6: blank
Lane 7: 8.3 pg pCOT1 plasmid DNA digested with HindIII
Lane 8: 16.6 pg pCOT1 plasmid DNA digested with HindIII
Lane 9: 41.5 pg pCOT1 plasmid DNA digested with HindIII
Lane 10: blank
Lane 11: 1 Kb Lane Marker (Stratagene)

Figure 3.8b. Linear map of pCOT1 with RepA probe



pCOT1 with RepA probe

11801 bp

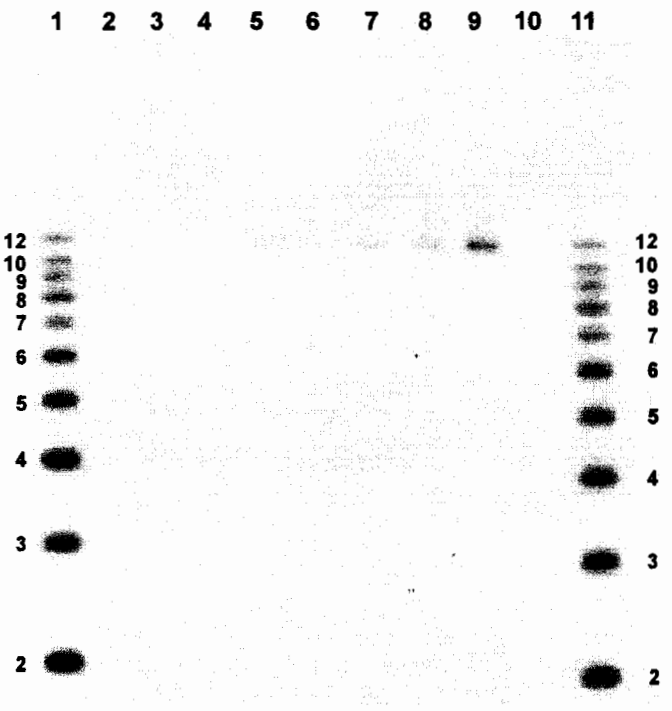
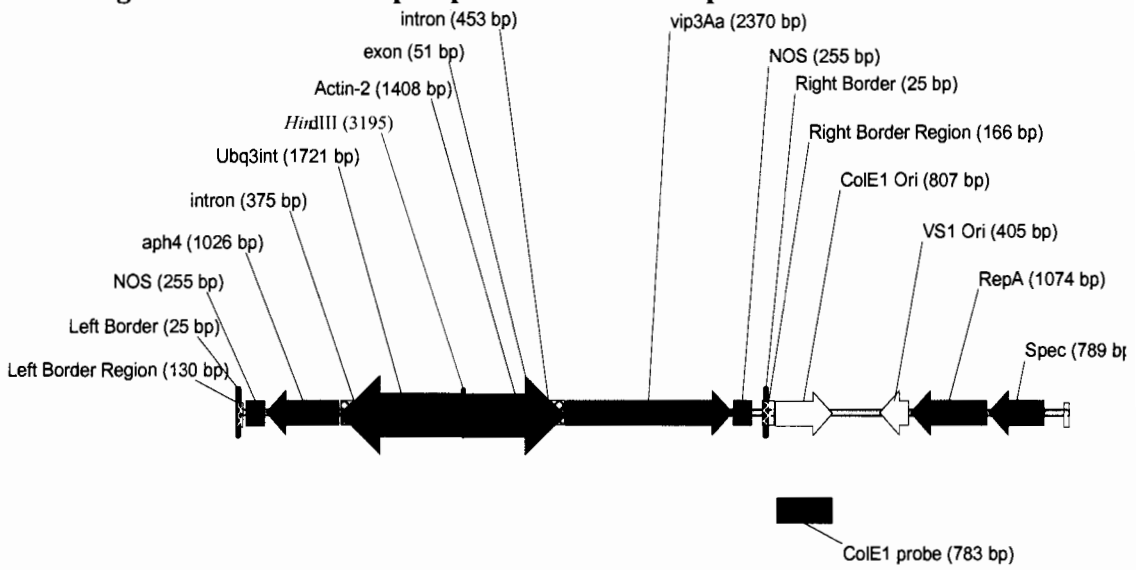


Figure 3.9a. Southern blot analysis of event COT102: ColE 1 Ori probe
COT102 genomic DNA and controls were digested with the indicated restriction enzymes. The resulting blot was probed with a ^{32}P-labeled ColE1 ori specific probe (783 bp). Lane designations are as follows:

Lane 1: 1 Kb Lane Marker (Stratagene)
Lane 2: blank
Lane 3: 7.5 µg COT 102 genomic DNA digested with HindIII
Lane 4: 7.5 µg Coker 312 genomic DNA digested with HindIII
Lane 5: 7.5 µg Coker 312 genomic DNA + 8.3 pg of pCOT1 plasmid DNA digested with HindIII
Lane 6: blank
Lane 7: 8.3 pg pCOT1 plasmid DNA digested with HindIII
Lane 8: 16.6 pg pCOT1 plasmid DNA digested with HindIII
Lane 9: 41.5 pg pCOT1 plasmid DNA digested with HindIII
Lane 10: blank
Lane 11: 1 Kb Lane Marker (Stratagene)

Figure 3.9b. Linear map of pCOT1 with ColE1 probe



pCOT1 with ColE1 probe

11801 bp

E. Mendelian Inheritance

The genetic stability of the insert contained within event COT102 was analyzed utilizing COT102 plants representing five breeding generations. Individual plants of the generations indicated (Figure 3.10) (F_1, BC_1F_2, BC_2F_1, BC_2F_2, and BC_3F_1) were tested for expression of the VIP3 protein via qualitative enzyme-linked immunosorbent assay (ELISA). Results are shown in Table 3.4.

All generations segregated as expected for a single insertion site. The F_1 progeny were produced from a homozygous population and, as expected, all expressed the VIP3A protein. All four of the other generations tested produced the expected results with regards to expression of the VIP3 protein. The Chi square analysis of the results indicated the critical value[1] to reject the hypothesis at the 5% level with one degree of freedom is 3.84 (Strickberger, 1976). A value less than 3.84 indicates that the inheritance of the *vip3A(a)* gene is behaving in a Mendelian fashion. The single insertion site remains stably inserted over multiple generations in selfed and back-crossed generations.

[1] Values greater than the critical values would only occur by chance one time out of twenty if the hypothesis is correct.

Figure 3.10. Progeny Map of Event COT102 Generations Used for Genetic Stability Testing (generations underlined were evaluated)

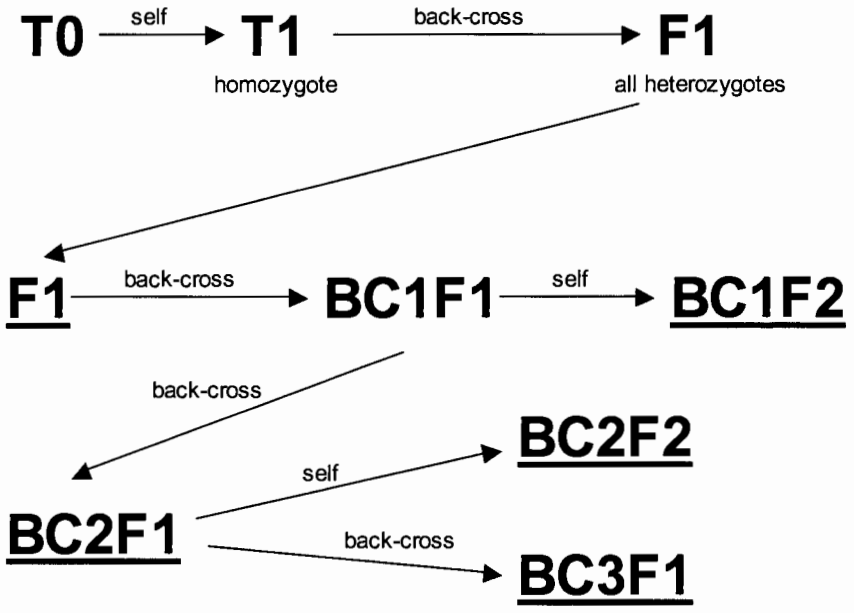


Table 3.4: Segregation Data of Progeny of Event COT102

Generation	Expected Positive	Expected Negative	Observed[1] Positive	Observed[1] Negative	Chi Square
F_1 (1:0)	122	0	122	0	
BC_1F_2 (3:1)	82.5	27.5	85	25	0.1939[2]
BC_2F_1 (1:1)	54.5	54.5	47	62	1.7982[2]
BC_2F_2 (3:1)	36	12	33	15	0.5277[2]
BC_3F_1 (1:1)	24	24	26	22	0.1875[2]

1: Based on a VIP3A qualitative ELISA
2: Not significant at p=0.05.

F. Conclusions

The molecular data presented in this chapter indicates that event COT102 has a single intact insertion of the transgene from the pCOT1 vector in its intended molecular arrangement. This has been demonstrated via Southern analysis. The Southern analysis also demonstrates that the genetic elements of the vector backbone are not present in event COT102.

Additionally event COT102 has been shown to segregate in a Mendelian fashion for a single insert.

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Figure 3.11: VIP3A Amino Acid Sequence (789 amino acids)

```
  1  MNKNNTKLST RALPSFIDYF NGIYGFATGI KDIMNMIFKT
 41  DTGGDLTLDE ILKNQQLLND ISGKLDGVNG SLNDLIAQGN
 81  LNTELSKEIL KIANEQNQVL NDVNNKLDAI NTMLRVYLPK
121  ITSMLSDVMK QNYALSLQIE YLSKQLQEIS DKLDIINVNV
161  LINSTLTEIT PAYQRIKYVN EKFEELTFAT ETSSKVKKDG
201  SPADILDELS ELTELAKSVT QNDVDGFEFY LNTFHDVMVG
241  NNLFGRSALK TASELITKEN VKTSGSEVGN VYNFLIVLTA
281  LQAQAFLTLT TCRKLLGLAD IDYTSIMNEH LNKEKEEFRV
321  NILPTLSNTF SNPNYAKVKG SDEDAKMIVE AKPGHALIGF
361  EISNDSITVL KVYEAKLKQN YQVDKDSLSE VIYGDMDKLL
401  CPDQSEQIYY TNNIVFPNEY VITKIDFTKK MKTLRYEVTA
441  NFYDSSTGEI DLNKKKVESS EAEYRTLSAN DDGVYMPLGV
481  ISETFLTPIN GFGLQADENS RLITLTCKSY LRELLLATDL
521  SNKETKLIVP PSGFISNIVE NGSIEEDNLE PWKANNKNAY
561  VDHTGGVNGT KALYVHKDGG ISQFIGDKLK PKTEYVIQYT
601  VKGKPSIHLK DENTGYIHYE DTNNNLEDYQ TINKRFTTGT
641  DLKGVYLILK SQNGDEAWGD NFIILEISPS EKLLSPELIN
681  TNNWTSTGST NISGNTLTLY QGGRGILKQN LQLDSFSTYR
721  VYFSVSGDAN VRIRNSREVL FEKRYMSGAK DVSEMFTTKF
761  EKDNFYIELS QGNNLYGGPI VHFYDVSIK
```

Figure 3.12: APH4 Amino Acid Sequence (341 amino acids)

```
  1  MKKPELTATS VEKFLIEKFD SVSDLMQLSE GEESRAFSFD
 41  VGGRGYVLRV NSCADGFYKD RYVYRHFASA ALPIPEVLDI
 81  GAFSESLTYC ISRRAQGVTL QDLPETELPA VLQPVAEAMD
121  AIAAADLSQT SGFGPFGPQG IGQYTTWRDF ICAIADPHVY
161  HWQTVMDDTV SASVAQALDE LMLWAEDCPE VRHLVHADFG
201  SNNVLTDNGR ITAVIDWSEA MFGDSQYEVA NIFFWRPWLA
241  CMEQQTRYFE RRHPELAGSP RLRAYMLRIG LDQLYQSLVD
281  GNFDDAAWAQ GRCDAIVRSG AGTVGRTQIA RRSAAVWTDG
321  CVEVLADSGN RRPSTRPRAK E
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Chapter 4

AGRONOMIC PERFORMANCE

A. Introduction

The agronomic performance and insect efficacy of cotton event COT102, expressing the VIP3A insecticidal protein, has been evaluated in multiple locations throughout the U.S. cotton belt from 2000 through 2003. In addition, event COT102 has been evaluated in field trials conducted in Argentina, China, Australia, South Africa, Costa Rica, and Vietnam. Table 4.1 lists the approved USDA Notifications for environmental release of cotton event COT102. Termination reports have been submitted as indicated for completed trials. Trials planned and in progress in the 2003 growing season are also included.

Table 4.1. USDA Notifications for Event COT102 Environmental Release

USDA Permit Number	# Sites Approved for Environmental Release	States:Counties	Termination Report Status
00-122-04n	1	TX:Lubbock	Submitted
00-301-03n	3	HI, TX:Victoria, Lubbock	Submitted
01-039-07n	4	AZ:Maricopa, GA:Mitchell, TN:Shelby, MS:Stoneville	
01-082-05n	5	AZ:Pinal, GA:Tift, MS:Bolivar, SC;Darlington, TX:Haskell	Submitted
01-078-20n	5	AZ:Maricopa, GA:Lee, MS:Stoneville, TN:Shelby, TX:Lubbock	Submitted
01-109-01n	1	MS:Washington	Submitted
01-131-04n	1	CA:Kern	Submitted
01-192-09n	1	TX:Haskell	Permit not used
01-303-02n	1	MS:Washington	Permit not used
02-063-06n	3	CA:Fresno, MS: Washington, TX:Victoria	Submitted
02-063-08n	6	AZ:Pinal, GA:Tift, MS:Bolivar,Washington, SC:Darlington, TX:Haskell	Submitted
02-063-09n	1	AZ:Pinal	Submitted
02-063-10n	5	AZ:Pinal, GA:Mitchell, MS:Washington, TN:Shelby, TX:Lubbock	Submitted
02-072-15n	6	AL:Autaga, GA:Whitfield, LA:Franklin, Bossier, MS:Oktibbeha, TX:Nueces	Submitted
02-072-17n	6	AL:Henry, GA:Brooks, NC:Franklin, LA:Franklin, TX:Fort Bend, McLennan	Submitted
02-086-15n	1	MS:Washington	Submitted
02-086-14n	6	AR:Jackson, CA:Tulare, FL:Indian River,, MS:Washington (2 locations), NC:Martin	Submitted

USDA Permit Number	# Sites Approved for Environmental Release	States:Counties	Termination Report Status
02-093-02n	2	AZ:Yuma, Pinal	Submitted
02-100-01n	1	AR:Drew	Submitted
02-105-08n	1	CA:Kern	Submitted
02-108-04n	2	AZ:Yuma, Pinal	Submitted
02-113-04n	1	TX:Hale	Submitted
02-206-04n	1	HI	Permit not used
03-062-01n	8	TX: Harris City, Fort Bend, McLennon (2 locations), Nueces, Tom Green, Williamson, Victoria	Trials in progress
03-104-02n	22	AL: Henry, Autauga; AR: Jackson; AZ: Yuma; CA: Fresno, Tulare; FL: Indian River, GA: Brooks, Tift; LA: Bossier, Franklin; MS: Washington, Oktibbeha, NC: Franklin, Wayne, Martin, Nash; SC: Barnwell; TN: Hardenan; TX: Lubbock	Trials in progress
03-126-02n	2	MS: Washington, Bolivar	Trials in progress
03-112-02n	6	AZ: Pinal, GA: Tift; MS: Washington, Bolivar; SC: Darlington; TX: Haskell	Trials in progress

B. Insect Efficacy Evaluations

The VIP3A expressing cotton event, COT102, has been evaluated across the U.S. cotton belt to determine its activity against various cotton insect pests. Event COT102 derived cotton plants provide excellent control of numerous lepidopteran cotton pests including *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), *Pseudoplusia includens* (soybean looper), *Trichoplusia ni* (cabbage looper), and *Bucculatrix thurberiella* (cotton leaf perforator).

Reported here are representative results of field studies designed to assess the efficacy and spectrum of control obtained from VIP3A expressing cotton plants derived from event COT102.

1) Materials and Methods

Both Syngenta personnel and independent University cooperators conducted a total of 18 field studies in the states of Texas, Louisiana, Mississippi, Arkansas, Alabama, Georgia, North Carolina, Arizona, and California. In most locations, plots consisted of eight rows 30 ft in length, which were replicated four times in a randomized complete block design. VIP3A cotton event COT102 and its non-transgenic parent, Coker 312, were evaluated in side-by-side comparisons with no additional insecticide applications made for lepidopteran control. All other non-lepidopteran insect pests were managed on an "as needed" basis with narrow spectrum insecticides. Lepidopteran insect populations and their damage to cotton structures were monitored throughout the growing season. Sampling regimes varied across locations. In most cases, percent infestation and percent damage in terminals, squares, flowers, bloom

tags, and bolls were estimated by sampling 25 to 50 structures per plot per assessment date. Species composition (tobacco budworm versus cotton bollworm) was estimated in each location at various time intervals during the growing season. Yield was estimated by harvesting the center four rows of each plot. Data are presented as cumulative numbers over the course of the season. Data were subjected to ANOVA, and means were separated according to Student-Newman-Keuls (P= 0.05).

2) Efficacy Results

I. Tobacco budworm (*H. virescens*) and Cotton bollworm (*H. zea*) (Heliothine Complex)

Due to the difficulty in distinguishing the difference between egg masses and larvae of *H. virescens* and *H. zea*, species composition was determined at each trial location through (1) the collection of larvae in the field and subsequent rearing to adult stage, (2) adult moth traps, or (3)Heli-ID kit (Agdia, Inc.). Species composition is reported by location (Table 4.2). Results indicate that some locations consisted of nearly complete bollworm populations for most of the season, and some locations reported a mixture of the Heliothine complex, with several shifting from bollworm early in the growing season to predominantly budworm during late season. In addition, insect pressure and duration of moth flights varied widely from one location to the next.

Table 4.2: Species composition of the Heliothine complex

Location	USDA Notification	*H. virescens*	*H. zea*	Sampling Period	Efficacy Assessment
Waco, TX	02-072-17n	0%	100%	Season Long	7/06/02 to 8/20/02
Winnsboro, LA(1)	02-072-15n	5%	95%	7/30/02	7/12/02 to 8/26/02
		45%	55%	8/14/02	
		98%	2%	8/22/02	
Leland, MS	02-086-14n	67%	33%	7/27/02	6/30/02 to 9/05/02
		50%	50%	8/11/02	
		71%	29%	8/22/02	
		86%	14%	8/29/02	
		88%	12%	9/05/02	
Beasley, TX	02-072-17n	0%	100%	6/03/02	6/11/02 to 8/27/02

Location	USDA Notification	*H. virescens*	*H. zea*	Sampling Period	Efficacy Assessment
		28%	72%	6/29/02	
		45%	55%	7/09/02	
		96%	4%	7/29/02	
Quitman, GA	02-072-17n	0%	100%	7/04/02	7/01/02 to 8/15/02
		60%	40%	7/24/02	
		93%	7%	8/22/02	
Newport, AR	02-086-14n	2%	79%	Season Long	7/31/02 to 8/26/02
Headland, AL	02-072-17n	0%	100%	Season Long	7/03/02 to 9/12/02
Winnsboro, LA(2)	02-072-17n	50%	50%	7/08/02	7/10/02 to 9/12/02
		0%	100%	7/21/02	
		0%	100%	8/07/02	
		71%	29%	9/07/02	
		67%	33%	9/18/02	
Corpus Christi, TX	02-072-15n	80%	20%	Early/mid-season	7/15/02 to 8/22/02
		100%	0%	Late season	
Jamesville, NC	02-086-14n	0%	100%	Season Long	7/31/02 to 8/19/02

Terminal Infestation and Damage

Across all the locations in 2002, there were no noted differences between event COT102 and the Coker 312 non-transgenic line with respect to numbers of eggs observed (Table 4.3). This data indicates no ovipositional preference by moths of the Heliothine complex for either COT102 or the non-transgenic Coker 312. Averaged across locations, the cumulative percent of terminals with at least one egg were 12.4% and 11.2% for event COT102 and Coker 312, respectively. However, event COT102 did significantly impact the survival of the developing larvae. The cumulative percent larval infestation observed in COT102 terminals was significantly lower compared to Coker 312 in five of the six locations reported (Table 4.4).

Percent terminal infestation ranged from 0% to 4.3% in COT102 compared to 1.4% to 34.5% in Coker 312. Averaged across the six locations, there were 6.3 times more larvae observed in Coker 312 terminals than in event COT102. In addition, VIP3A protein significantly reduced the level of damage to cotton terminals compared to Coker 312 (Table 4.4). Cumulative percent damaged terminals ranged from 5% to 28.5% in COT102 compared to 18% to 71.5% in Coker 312.

Table 4.3. Percent infestation based on the cumulative number of Heliothine eggs observed on cotton terminals.

Location	COT102	Coker 312
Winnsboro, LA(1)	21.0 a	18.8 a
Newport, AR	7.6 a	4.8 a
Beasley, TX	13.9 a	13.8 a
Headland, AL	8.9 a	9.3 a
Winnsboro, LA(2)	10.8 a	9.8 a
Average Across Locations	**12.4%**	**11.2%**

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

Table 4.4. Cumulative percent Heliothine infestation and damage on terminals.

	Cumulative Percent Infestation		Cumulative Percent Damage	
Location	**COT102**	**Coker 312**	**COT102**	**Coker 312**
Winnsboro, LA(1)	4.3 b	11.2 a	10.9 b	18.1 a
Waco, TX	3.5 b	34.5 a	5.0 b	41.5 a
Newport, AR	3.5 b	9.5 a	28.5 b	44.5 a
Headland, AL	0.0 b	5.6 a	--	--
Winnsboro, LA (2)	0.2 a	1.4 a	--	--
Corpus Christi, TX	0.0 b	10.0 a	10.0 b	71.5 a
Range	**0%-4.3%**	**1.4%-34.5%**	**5.0%-28.5%**	**18.1%-71.5%**
Average Across	**1.9**	**12.0**	**13.6**	**43.9**

Location				
Fold difference	**6.3 X less**		**3.2 X less**	

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

Square Infestation and Damage

Percent Heliothine larvae infestation observed in squares was significantly reduced in COT102 compared with Coker 312 (Table 4.5). Eight of the nine locations reported significantly lower percent square infestation in COT102 (0% to 6.0%) compared to Coker 312 (2.5% to 34.0%). Very low insect pressure in the Tift Co., GA location did not allow for statistical separation of the treatments. Averaged across all locations, Coker 312 plots contained 6.9 times more larvae than COT102. Percent damaged squares were significantly lower in COT102 than Coker 312 in all locations (Table 4.5). Cumulative percent damaged squares ranged from 0% to 12.2% and 6.2% to 69.7% for COT102 and Coker 312, respectively. Averaged across all locations, percent damaged squares in COT102 were 7.0 times lower than in Coker 312.

Table 4.5. Cumulative percent Heliothine infestation and damage on squares.

	Cumulative Percent Infestation		**Cumulative Percent Damage**	
Location	**COT102**	**Coker 312**	**COT102**	**Coker 312**
Winnsboro, LA(1)	0.7 b	4.7 a	2.9 b	14.7 a
Waco, TX	6.0 b	34.0 a	12.2 b	69.7 a
Newport, AR	0.0 b	4.0 a	5.2 b	20.3 a
Beasley, TX	0.6 b	6.9 a	2.0 b	23.4 a
Leland, MS	1.7 b	6.4 a	3.0 b	22.3 a
Headland, AL	--	--	0.0 b	6.2 a
Quitman, GA	--	--	4.1 b	35.4 a
Winnsboro, LA(2)	1.0 b	5.1 a	1.7 b	13.5 a
Corpus Christi, TX	2.0 b	18.5 a	1.2 b	10.7 a
Jamesville, NC	1.5 b	11.0 a	4.0 b	35.6 a
Tifton, GA[1]	0.0 a	2.5 a	--	--
Range	**0%-6.0%**	**2.5%-34.0%**	**0%-12.2%**	**6.2%-69.7%**

Average Across Location	**1.5**	**10.3**	**3.6**	**25.2**
Fold difference	**6.9 X less**		**7.0 X less**	

1 USDA Notification # 02-072-15n; Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

Flower Infestation and Damage

VIP3A protein expression in flowers resulted in a significant reduction in the percent larval infestation observed in this structure across most locations (Table 4.6). Cumulative percent flower infestation ranged from 1.5% to 20.7% in COT102 compared with 6.4% to 45% in Coker 312. Averaged across all locations, Coker 312 exhibited 3.0 times more larvae on flowers than COT102. In addition, COT102 had significantly lower flower damage compared with Coker 312 (Table 4.6). Cumulative percent damage flower ranged from 1.6% to 9.3% in COT102 compared with 14.4% to 64.0% in Coker 312. Averaged across all locations, percent damaged flowers in COT102 were 6.7 times lower than in Coker 312.

Table 4.6. Cumulative percent Heliothine infestation and damage on flowers.

	Cumulative Percent Infestation		**Cumulative Percent Damage**	
Location	**COT102**	**Coker 312**	**COT102**	**Coker 312**
Winnsboro, LA(1)	2.5 b	7.7 a	3.6 b	14.4 a
Waco, TX	20.7 b	45.0 a	9.3 b	44.0 a
Newport, AR	1.7 b	7.5 a	3.0 b	15.3 a
Beasley, TX	1.7 b	11.0 a	4.9 b	17.4 a
Leland, MS	1.7 b	6.4 a	1.6 b	16.6 a
Quitman, GA	--	--	5.3 b	36.2 a
Winnsboro, LA(2)	2.8 a	10.6 a	3.1 b	15.9 a
Jamesville, NC	1.5 b	11.0 a	2.6 b	64.0 a
Range	**1.5%-20.7%**	**6.4%-45.0%**	**1.6%-9.3%**	**14.4%-64.0%**
Average Across Location	**4.7%**	**14.2%**	**4.2%**	**28.0%**
Fold difference	**3.0 X less**		**6.7 X less**	

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

Bloom Tag Infestation and Damage

In regards to VIP3A protein expression in bloom tags, also referred to as stuck blooms, and in the apical portion of bolls covered by bloom tags, there were significantly fewer larvae and reduced damage observed in COT102 plots compared with Coker 312 (Table 4.7). Cumulative percent bloom tag infestation ranged from 1.4% to 2.2% and 10.9% to 15.1% for COT102 and Coker 312, respectively. In addition, COT102 plots had significantly lower cumulative percent damage to apical areas of bolls covered by bloom tags (1.6% to 3.5%) compared with Coker 312 plots (20.0% to 35.9%).

Table 4.7 Cumulative percent Heliothine infestation on bloom tags and damage to apical portions of bolls covered by bloom tags.

	Cumulative Percent Infestation		**Cumulative Percent Damage**	
Location	**COT102**	**Coker 312**	**COT102**	**Coker 312**
Beasley, TX	1.4 b	15.1 a	3.5 b	35.9 a
Winnsboro, LA(2)	2.2 b	10.9 a	1.6 b	20.0 a
Range	**1.4%-2.2%**	**10.9%-15.1%**	**1.6%-3.5%**	**20.0%-35.9%**
Average Across Location	**1.8%**	**13%**	**2.6%**	**28.0%**
Fold difference	**7.2 X less**		**10.8 X less**	

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

Boll Infestation and Damage

The cumulative percent larval infestation observed in COT102 bolls was significantly lower compared to Coker 312 in all locations reported (Table 4.8). Percent boll infestation ranged from 0.4% to 3.0% in COT102 plots compared to 3.1% to 41.5% in Coker 312. Averaged across the six locations, there were 9.2 times more larvae observed in Coker 312 bolls than in COT102 bolls. Percent damaged bolls were significantly lower in COT102 than Coker 312 at all locations (Table 4.8). Cumulative percent damaged bolls ranged from 0.6% to 8.2% and 3.2% to 66.5% for COT102 and Coker 312, respectively. Averaged across all locations, percent damaged bolls in COT102 plots were 6.8 times lower than in Coker 312.

Table 4.8. Cumulative percent Heliothine infestation and damage on bolls.

	Cumulative Percent Infestation		Cumulative Percent Damage	
Location	**COT102**	**Coker 312**	**COT102**	**Coker 312**
Winnsboro, LA(1)	2.5 b	6.5 a	4.8	21.8 a
Waco, TX	2.0 b	41.5 a	5.7	52.7 a
Newport, AR	0.4 b	3.1 a	2.8	18.1 a
Beasley, TX	0.8 b	9.6 a	5.5	33.9 a
Leland, MS	--	--	4.4	13.5 a
Headland, AL	--	--	0.6	3.2 a
Quitman, GA	--	--	6.5	49.2 a
Winnsboro, LA(2)	0.7 b	5.3 a	1.8	16.1 a
Jamesville, NC	3.0 b	22.0 a	8.2	66.5 a
Range	**0.4%-3.0%**	**3.1%-41.5%**	**0.6%-8.2%**	**3.2%-66.5%**
Average Across Location	**1.6%**	**14.7%**	**4.5%**	**30.6%**
Fold difference	**9.2 X less**		**6.8 X less**	

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

II. Beet armyworm (*Spodoptera exigua)*

COT102 efficacy toward beet armyworm was assessed at five locations (Table 4.9). Overall, beet armyworm pressure was low to absent in most locations in 2002. In those locations, COT102 plots had significantly lower numbers of surviving larvae compared with the non-transgenic Coker 312 plots. Although assessment methods varied across locations, numbers of beet armyworm larvae were reduced from 89.3% to 100% in the COT102 plots as compared with Coker 312.

Table 4.9. Levels of beet armyworm larvae infesting COT102 and Coker 312 cotton.

	Locations and Sampling Unit				
	Corpus Christi, TX No. larvae/10 leaves	Newport, AR No. larvae/12 row ft	Leland, MS No. larvae/12 row ft	Quitman, GA No. larvae/60 fruit	Beasley, TX No. hits/16 rows
COT102	0.0 b	0.6 b	1.0 b	1.2 b	1.4 b
Coker 312	1.5 a	5.6 a	10.5 a	23.7 a	15.1 a
Percent Reduction	100 %	89.3 %	90.5 %	95.0 %	90.7 %

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

III. Soybean Looper

COT102 efficacy toward soybean looper was assessed at three locations (Table 4.10). Overall, soybean looper pressure was light to moderate in those locations. COT102 significantly reduced the number of soybean looper larvae compared with Coker 312 in two of the three locations. Even though assessment methods varied among locations, COT102 resulted in a reduction in larval numbers ranging from 60% to 97%.

Table 4.10. Levels of soybean looper larvae infesting COT102 and Coker 312 cotton.

	Locations and Sampling Unit		
	Winnsboro, LA No. larvae/2 sweeps	Newport, AR No. larvae/row ft	Leland, MS No. larvae/row ft
COT102	2.8 b	0.2 a	0.2 b
Coker 312	11.8 a	0.5 a	6.9 a
Percent Reduction	76.3 %	60 %	97.1 %

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

IV. Pink Bollworm (*Pectinophora gossypiella*)

The efficacy of event COT102 towards pink bollworm (PBW) was evaluated in field trials performed at the University of Arizona in 2002 (USDA Notification # 02-063-09n). Bolls of COT102 (n = 72) and non-transgenic Coker 312 (n = 80) were individually infested with egg

masses (~100 eggs/boll). The number of entry holes per boll was assessed, and 5-9 days later bolls were dissected to determine the number of surviving larvae.

Bolls from both COT102 and Coker 312 suffered from a large number of larval entry holes (504 and 503, respectively). Upon dissection, 276 live L3 PBW larvae were identified from 75 of the Coker 312 bolls. In contrast, only a single boll of COT102 harbored (2) larvae.

V. Cabbage Looper (*Trichoplusia ni*)

Event COT102, Coker 312, and a commercial variety "Maxxa" were planted in Shafter California on May 24, 2002 (USDA Notification # 02-105-08n). Each plot consisted of two 60 ft rows spaced 38 inches apart that were replicated four times in a randomized complete block design. Cabbage looper damage reached economic thresholds in the Maxxa and Coker 312 plots in late July. Plots were visually evaluated for incidence and severity of looper damage on leaves on Aug 4. Thirty plants per plot were visually scored for presence of damage and percent of leaf damaged on the main stem leaf on the fifth node from the top of the plant. COT102 plots showed significantly less damage than either Coker 312 or Maxxa (Table 4.11).

Table 4.11. Incidence and severity of cabbage looper damage.

Variety	Incidence	Severity*
	% plants	
Maxxa	98.00	26.00
COT102	36.00	2.00
Coker 312	96.00	25.00
CV	20.6	19.0
LSD	22.00	5.00
PROB. ENTRY	0.002	0.000
R-SQUARED	0.884	0.963
* Estimated % of leaf damaged		

VI. Cotton Leaf Perforator

Activity of VIP3A protein in COT102 on cotton leaf perforator was assessed at a single location (Corpus Christi, TX) in 2002. COT102 significantly reduced the numbers of larvae per leaf (0.2) compared with Coker 312 (3.0). Similarly, leaf damage observed in COT102 (3.2%) was significantly less than that observed in Coker 312 (48.7%).

3) Insect Efficacy Conclusions

Whether tested against discrete bollworm populations or a bollworm/budworm pest complex, event COT102 provided effective season long control of these pests. The expression of

VIP3A insecticidal protein in event COT102 derived cotton plants effectively reduced the level of damage to squares, flowers, and bolls by an average of 85% as compared to Coker 312, indicating robust insecticidal protein expression throughout the plant structures critical to yield and those that are commonly attacked by the Heliothine complex. In addition, VIP3A expression in the apical portion of bolls covered by bloom tags resulted in a 91% reduction in damage compared to Coker 312. Although limited field data is available on the efficacy of VIP3A cotton against other lepidopteran pests, results presented here indicate that event COT102 also exhibits good control of pink bollworm, beet armyworm, soybean looper and cotton leaf perforator. The excellent efficacy exhibited by event COT102 against the Heliothine complex and broad spectrum of activity against other lepidopteran pests makes it a very attractive tool for the control of key cotton pests. In addition, VIP3A represents a novel insecticidal protein with a different mode of action from delta-endotoxin proteins that are currently marketed. These attributes enable VIP3A expressing cotton to have a unique fit into integrated pest management systems in cotton, providing a valuable tool for resistance management strategies for all Bt derived insecticidal proteins.

C. Morphological and Agronomic Characteristics

The VIP3A expressing cotton event, COT102, has been evaluated across the U.S. cotton belt during the 2000, 2001, and 2002 growing seasons in fifty-one separate field trials carried out in twelve states. In most of those field trials both insect efficacy and agronomic performance were evaluated. Detailed quantitative agronomic measurements were recorded at five locations during the 2001 growing season and at fifteen locations during 2002. Parameters considered included the typical measurements taken in traditional plant breeding including indicators of general plant growth and morphology, reproductive traits, productivity, and fiber quality. Experienced agronomists, breeders, and field scientists performed the agronomic performance evaluations. Few significant differences have been noted in agronomic performance when event COT102 was compared to its non-transgenic parental counterpart Coker 312. Where differences were detected, those differences were not consistent across locations and could otherwise be attributed to differences in lepidopteran insect damage incurred by the lines or seed source variability. Most notably, event COT102 produces a significant yield increase resulting from substantial insect protection.

Two slightly different trial protocols have been used by different investigators in the evaluation of agronomic performance of event COT102.

1) Trial Protocol Method #1:

The field trial protocol #1 included eight rows by thirty-feet plots, four replications, and two treatments (COT102 and the non-transformed parent cultivar Coker 312) conducted at ten locations in the states of Alabama, Arkansas, Georgia, Louisiana, Mississippi, North Carolina, and Texas from which quantitative data was collected (USDA Notification #'s 02-072-15n, 02-072-17n, and 02-086-14n). A randomised complete block plot design was employed. Field locations were selected to represent a diversity of climate-soils. Cottonseed of both event COT102 and Coker 312 was produced in the field during the 2001 growing season at multiple locations in the U.S. Seed quality varied by production location, and germination frequency ranged from 33.3% to 68.8% for COT102 and 37.5% to 60.4% for Coker 312. Agronomic evaluations included measurement of emergence, plant height, number of nodes, and node of first fruiting branch. Data was collected from each plot generating a mean of the four replications. Height to node ratios were calculated and subsequently reported here. Yield evaluations are also reported as seed cotton per acre measurement.

Results:

I. Plant Stand

Plant stand counts were recorded at seedling (approximately 30 days after planting) and at early bloom stage (approximately 60 days after planting) at each of the ten

locations (Table 4.12). Data were subjected to ANOVA, and means were separated according to Student-Newman-Keuls (P= 0.05). Statistically significant differences were noted in plant stand count at two of ten locations. No differences were noted at any of the other eight locations.

II. Plant Height to Node Ratio (HNR)

Plant height to node ratios were determined at early bloom stage and late bloom stage at each of the ten locations (Table 4.13). Data were subjected to ANOVA, and means were separated according to Student-Newman-Keuls (P= 0.05). Statistically significant differences were noted in height to node ratios at either the early or late time point at five of the ten locations. In every case where a difference was detected, Coker 312 plants were measured to be slightly taller that COT102 plants and can be attributed to general dissimilarity in vigor between COT102 and Coker 312 related to seed source and production conditions and sub-threshold levels of lepidopteran insects present in the test area causing fruit loss on the Coker 312 plants inducing those plants to compensate in accelerated growth. In either case significant differences in HNR were not consistent across locations or at the early bloom and late bloom stages at which measurements were recorded indicating normal variability within this biological system.

III. Node of the First Fruiting Branch

The node at which the first fruiting branch occurred was recorded at seven locations (Table 4.14). Data were subjected to ANOVA, and means were separated according to Student-Newman-Keuls (P= 0.05). No significant differences were noted at any location with regards to position of the first fruiting branch.

Table 4.12. Plant Stand Counts recorded at seedling (30 day) and early bloom stage (60 day)

	Headland AL		Newport AR		Quitman GA		Bossier City LA		Winnsboro LA (1)		Winnsboro LA (2)		Leland MS		Jamesville NC		Beasley TX		Waco TX	
	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
COT102	58.6a	57.3a	47.5a	55.4a	40.3a	39.0a	54.4a	58.5b	66.4b	66.4b	50.3a	59.5a	68.0b	67.5a	56.9a	55.7a	81.8a	97.8a	102.3a	105.8a
Coker 312	53.9a	52.8a	46.9a	53.5a	43.6a	42.6a	60.0a	64.3a	85.1a	85.1a	58.7a	58.9a	81.5a	81.5a	54.0a	52.8a	78.5a	101.3a	99.3a	107.3a
COT102 (TAN)	57.9a	56.4a	48.5a	58.4a	39.0a	38.1a	NT	NT	67.3b	67.3b	NT	NT	68.8b	71.5a	NT	NT	86.3a	103.8a	102.5a	106.0a
Coker 312 (TAN)	45.5a	43.8a	53.8a	60.8a	44.5a	43.9a	57.1a	57.8b	77.8ab	77.8ab	59.5a	57.4a	88.8a	82.0a	61.5a	60.2a	76.3a	95.5a	100.8a	104.3a

TAN = treated as needed for non-lepidopteran insect pests by the application of narrow spectrum insecticides. NT = not tested. Means followed by the same letter do not significantly differ (P=0.05, Student-Newman-Keuls).

Table 4.13. Height:Node Ratios; Measurements taken at early bloom and late bloom stages.

Treatment	Headland AL		Newport AR		Quitman GA		Bossier City LA		Winnsboro LA (1)		Winnsboro LA (2)		Leland MS		Jamesville NC		Beasley TX		Waco TX	
	E	L	E	L	E	L	E	L	E	L	E	L	E	L	E	L	E	L	E	L
COT102	2.0a	NT	1.7b	1.7a	1.6a	1.7b	1.5a	1.5a	2.0a	2.1a	1.8a	2.6a	1.4b	2.1a	1.37b	2.1a	2.1a	2.1b	2.4a	2.3a
Coker 312	2.0a	NT	1.8a	1.8a	1.6a	2.0a	1.6a	1.6a	2.0a	2.1a	1.9a	2.6a	1.5b	2.2a	1.43a	2.2a	2.3a	2.5a	2.4a	2.1a
COT102 (TAN)	2.0a	NT	1.5c	1.7ab	1.6a	1.7b	NT	NT	2.0a	2.0a	NT	NT	1.4b	2.3a	NT	NT	2.2a	2.1b	2.5a	2.3a
Coker 312 (TAN)	2.0a	NT	1.7b	1.7a	1.6a	1.8ab	1.6a	1.6a	2.0a	2.1a	1.8a	2.5a	1.6a	2.3a	1.45a	2.2a	2.2a	2.4a	2.4a	2.4a

E = early bloom stage; L = late bloom stage, NT = not tested. TAN = treated as needed for non-lepidopteran insect pests by the application of narrow spectrum insecticides. Means followed by the same letter do not significantly differ (P=0.05, Student-Newman-Keuls).

Table 4.14. Node of First Fruiting Branch

Treatment	Headland AL	Bossier City LA	Winnsboro LA (1)	Leland MS	Jamesville NC	Beasley TX	Waco TX
COT102	6.2a	5.5a	5.6a	5.7a	4.6a	5.2a	3.9a
Coker 312	6.1a	5.5a	5.7a	5.7a	4.6a	5.5a	3.9a
COT102 (TAN)	6.0a	NT	5.5a	5.6a	NT	5.4a	4.1a
Coker 312 (TAN)	6.1a	5.4a	5.5a	5.7a	4.5a	5.4a	4.0a

NT = not tested. TAN = treated as needed for non-lepidopteran insect pests by the application of narrow spectrum insecticides. Means followed by the same letter do not significantly differ (P=0.05, Student-Newman-Keuls).

IV. Yield Evaluations

Event COT102 yielded significantly more seed cotton than Coker 312 in eight of the ten locations reported (Table 4.15). Environmental conditions and/or lack of sufficient insect pressure were the factors mostly responsible for the inability to detect significant yield differences between COT102 and Coker 312 at the two Georgia locations. In the Brooks, GA location, prolonged rainfall prevented timely harvest of plots resulting in excessive lint drop. In the Tift Co., GA location, low insect pressure coupled by optimal growing conditions late in the growing season allowed Coker 312 plants to set a significant number of second and third position bolls, resulting in a compensatory effect. Event COT102 cotton yields ranged from 1,247 to 2,629 lb seed cotton per acre compared with 459 to 1,605 lb seed cotton per acre for Coker. Averaged across all locations, yields for COT102 and Coker were 1,991 and 1,024 lb, respectively. These differences represent an average increase of 967 lb of seed cotton per acre in the event COT102 line.

Table 4.15: Seed cotton yield expressed as pounds (lbs.) seed cotton/acre.

Location	COT102	Coker 312
Winnsboro, LA(1)	1378 a	975 b
Waco, TX	2210 a	459 b
Newport, AR	2138 a	1605 b
Beasley, TX	1912 a	635 b
Leland, MS	2629 a	1213 b
Headland, AL	1247 a	862 b
Quitman, GA	1646 a	1423 a
Winnsboro, LA(2)	2237 a	1306 b
Jamesville, NC	2526 a	742 b
Tifton, GA	1485 a	1425 a

Means within a row followed by the same letter do not differ significantly according to Student-Newman-Keuls (P = 0.05).

2) Trial Protocol Method #2:

The second field trial design included four row by forty-feet plots, six replications, and two treatments (COT102 and the non-transformed parent cultivar Coker 312) conducted

at five locations in the states of Mississippi, Georgia, Arizona, South Carolina, and Texas (USDA Notification #02-063-08n). A randomised complete block with replications down the row or completely randomised design was used. Field locations were selected to represent a diversity of climate-soils. COT102 cottonseed was produced in 2001 at the Hartsville, SC location, and Coker 312 seed produced at both Hartsville SC, and Maricopa AZ. The major categories of data collected were: in-season growth parameters, end-of-season box map, lint yield, and fiber quality. With the exception of fiber quality, data was collected from each plot generating a mean of the six replications. Table 4.15 indicates data collected in 2002.

Event COT102 was included in small event selections trials conducted in 2001 and similar measurements were taken. Due to very limited seed availability, the field trial design consisted of a single row (12 to 15 meters long) of COT102 and the non-transgenic parental line Coker 312. Trials were carried out at five locations (Mississippi, Texas, Arizona, South Carolina, and Georgia) (USDA Notification # 01-082-05n), and the limited data collected are included in the following analysis in addition to the 2002 data set when available.

Table 4.16. Data collected from Field Trial Evaluations

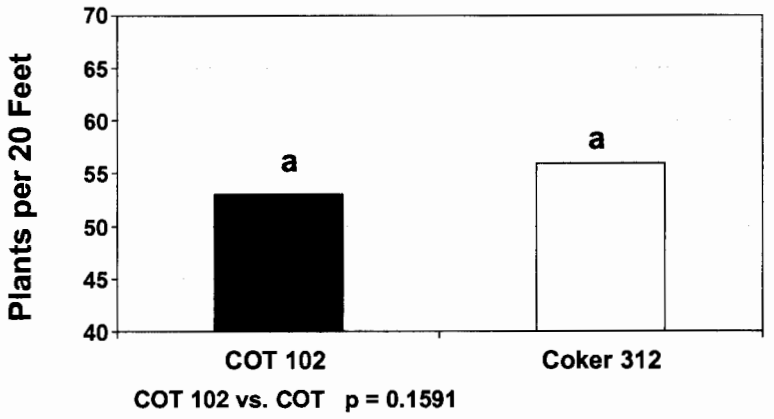
Data collected	Early Square	Early Bloom	Late Bloom	Harvest
Plant stand	X			
Plant height	X	X	X	X
Total number of fruiting branches	X	X	X	
Total number of nodes	X	X	X	X
Height to node ratio	X	X	X	X
Node number to 95% accumulation				X
Lint yield in pounds per acre from box map				X
Fiber quality analysis				X

Results:

I. Plant Stand

To evaluate germination, plant stand counts were measured from the center two rows of the four row plots (10 row feet) at approximately the first to second true leaf stage. Plant stand counts for COT102 and Coker 312 were not statistically different and were considered within the normal ranges for the Coker 312 variety at each test locations (Figure 4.1).

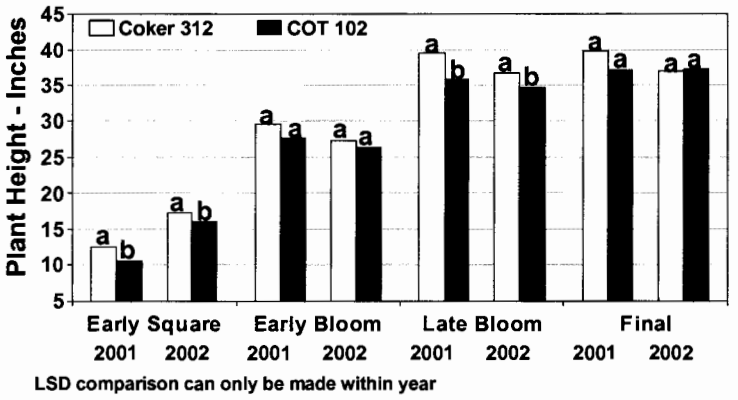
Figure 4.1. Plant Stand Counts, 2002



II. Plant Height

Plant height was measured at four growth stages during the growing season in each plot by measuring the height from the soil or cotyledons to the plant terminal in five plants for each plot (Figure 4.2). On two of the sampling dates Coker 312 was slightly taller than COT102, consistent with the 2001 data set, and those differences are most likely due to differences in seed quality between the COT102 and Coker 312 seed lots being produced at different locations. Those differences noted during late bloom stage were most likely attributable to poor fruit retention in the Coker 312 plants and therefore compensation in plant growth (i.e. height) due to lack of boll load. The difference noted at early square stage cannot be easily attributed to insect damage and may be a result of seed quality caused by the production of seed at difference locations. Plant height showed no significant difference at early bloom stage or end of season.

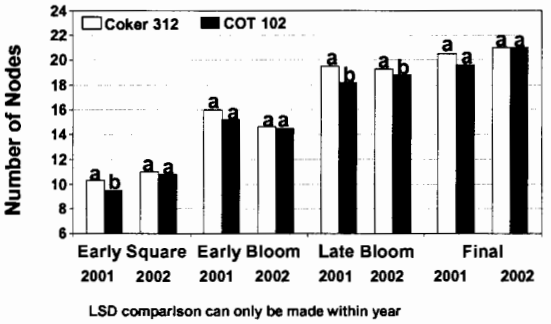
Figure 4.2. Plant Height 2001 & 2002



III. Total Number of Nodes

The total number of plant nodes was counted at four growth stages during the growing season. No differences were again noted at early bloom and end of season stages; however, small differences were measured at early square and late bloom stage (Figure 4.3). The early square stage differences were not consistent across years. Furthermore, the differences noted at late bloom stage are believed to be caused by significant lepidopteran insect damage to the Coker 312 plants causing a delay in maturity and late season vegetative growth.

Figure 4.3. Total Number of Nodes 2002 & 2001

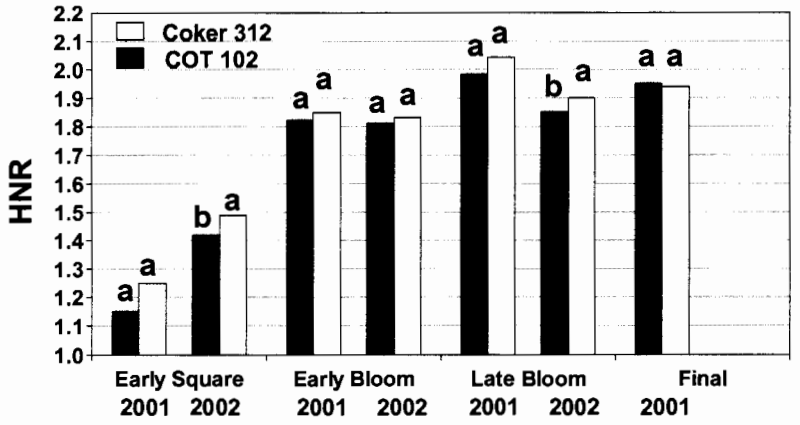


IV. Plant Height to Node Ratio (HNR)

The height to node ratio is reflective of several influences including but not limited to seed quality, phenotypic effects, and environmental impacts on plant vigor experienced during the life of the plant. With replicated and randomised plots, the seed quality and phenotypic components dominate the HNR. During the early square

period, the non-significant differences in total nodes, yet significant differences in plant height, result in a significant difference in HNR for 2002. The different seed sources for Coker 312 and COT102 could have accounted for the significant HNR values in 2002 since no difference was observed at the early bloom sampling date in either 2001 or 2002. Late bloom HNR differences most likely reflect favourable vegetative growth due to the reduced boll load on non-transgenic Coker 312 plants.

Figure 4.4. Height to Node Ratio
2001 & 2002

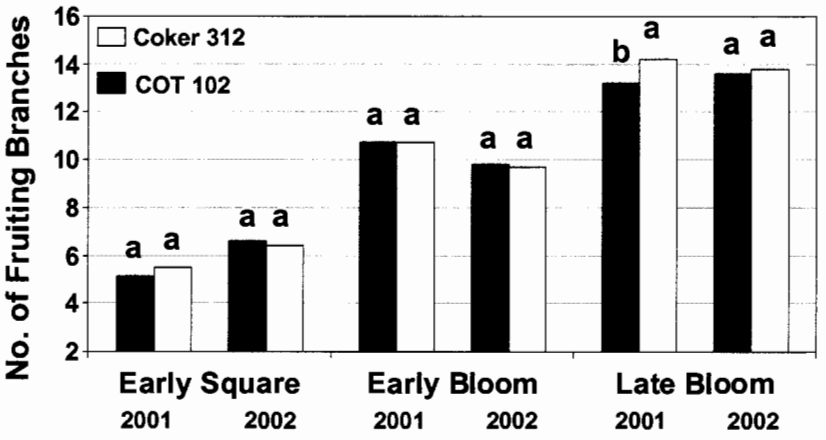


LSD comparison can only be made within year

V. Total Number of Fruiting Branches

The total number of fruiting branches were measured three times during the growing season (Figure 4.4) The only significant difference noted at late bloom stage in the 2001 data was attributed to insect pressure and a reduction in fruiting branches in the Coker 312 variety. An increase in fruiting branches and total nodes in the Coker 312 is expected at the end of the season, due to fruit damage and resulting delayed in cut-out (cut-out indicates the plant stage when blooms occur at the nodes near the terminal and fruit set is complete).

Figure 4.5. Number of Fruiting Branches 2001 & 2002



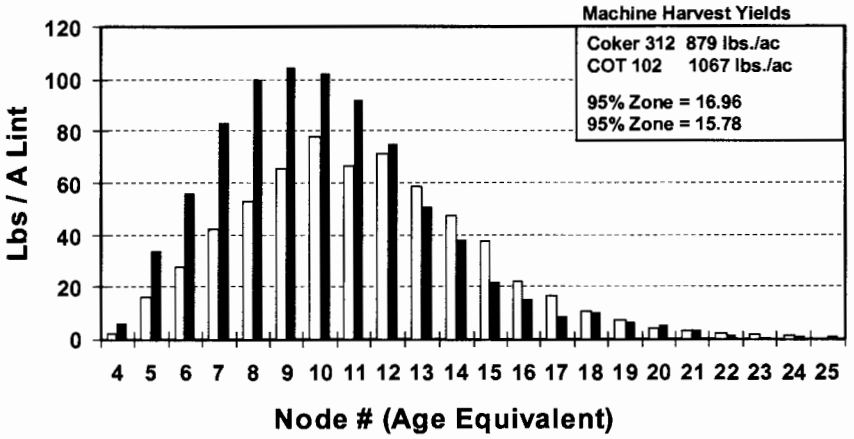
LSD comparison can only be made within year

VI. Box Mapping Data and Node to 95% Yield Accumulation

Cotton grows in an orderly and readily identifiable pattern of monopodial and sympodial nodes that develop in response to plant condition, weather, and pest injury. At each sympodial node exists a fruiting site that links each piece of fruit to a specific time period in the plants growth. End-of-season analysis of fruit size and presence at each sympodial node can be used to detect subtle differences between varieties with regards to plant condition or pest injury during specific time periods. Data was collected by accumulating bolls at each sympodial node on the plant for determination of seed and fiber yield. Box mapping data was collected independently at each location. Presented here are the analysis averaged over across locations.

Figure 4.7 indicates a well-balanced yield accumulation curve is observed for COT102. Starting low on the plant, yield accumulates rapidly, reaching a peak at nodes 8, 9 and 10, then declines symmetrically. Yield accumulation in Coker 312 was greatly reduced through node 11, and failed to fully compensate in the top of the plant. The effect seen with Coker 312 is typical of plants suffering from in season insect damage.

Figure 4.6. Yield Accumulation Over Locations, 2002



The nodes to the 95% zone represents the calculated main stem node (and age equivalent nodes) at which the box map yield accumulated 95% of the total box map yield. The 5-location mean indicates that COT102 accumulates 95% of its final yield in 16 nodes, as compared with Coker 312 that requires 17 nodes (Table 4.17). This difference is again a positive trait resulting from the insect protection incurred by event COT102.

Table 4.17. Nodes to the 95% Zone, 2002

Variety	Hartsville	Haskell	Maricopa	Tifton	Winterville	Over Locations
	Node #	Node #	Node #	Node #	Node #	Node #
Coker 312	16.12	15.91	20.52	15.21	12.91	16.96
COT 102	11.35	12.78	19.46	14.35	13.00	15.78

VIII. High Volume Instrument (HVI) Fiber Quality Analysis

Lint was retained from each of the five plots for fiber quality analysis. The typical HVI analysis includes measurements of micronaire, strength, length, uniformity

index, and elongation characteristics. The only significant quality difference noted between COT102 and Coker 312 occurred in the measurement of micronaire. This is not unexpected, since micronaire, being a measure of fiber maturity, is the parameter most influenced by the boll retention pattern. In general, crops that suffer early fruit injury from insects often compensate later in season. Those later developing, young bolls would result in fiber with reduced micronaire. No other significant differences in fiber quality parameters were detected between COT102 and Coker 312.

Figure 4.7. Micronaire

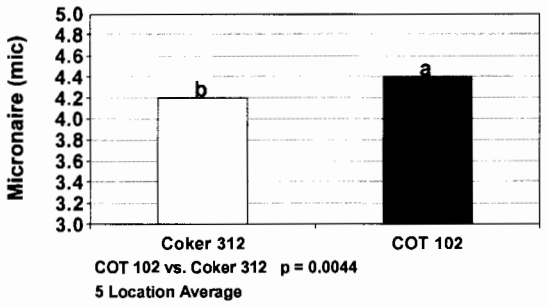


Figure 4.8. Length

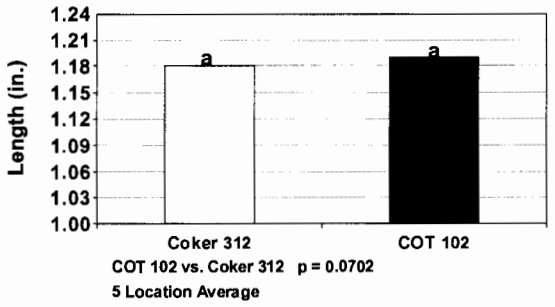


Figure 4.9. Strength

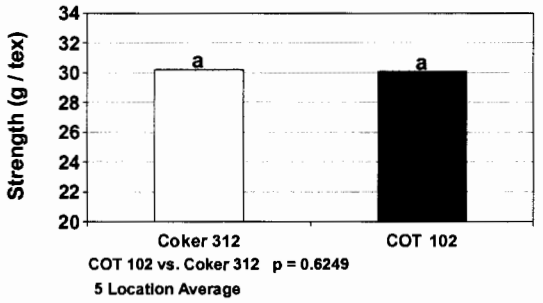


Figure 4.10. Uniformity Index

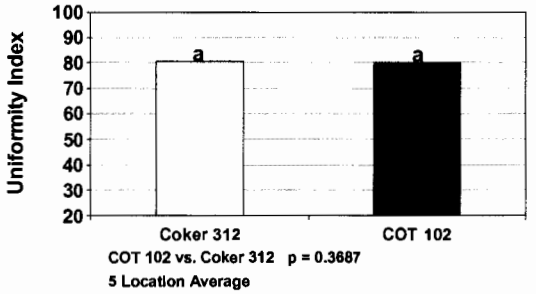
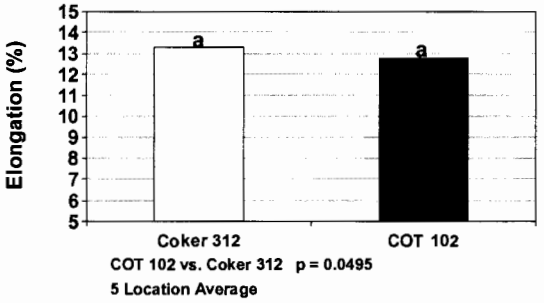


Figure 4.11. Elongation



3) Germination Assay

Germination and dormancy characteristics of cotton event COT102 seed were evaluated relative to five non-transgenic commercial varieties. The germination study was carried out by scientists at BioDiagnostics, Inc. using standard methods established by the Association of Official Seed Analysts using three temperature treatments. COT102

cottonseed was produced during the 2002 growing season in Arizona. Reference seed varieties were obtained from commercial seed stocks and included Deltapine 491, Deltapine Suregrow 747, Fibermax 989, Fibermax 958, and Fibermax 832. Four replicates each consisting of 100 seeds were tested per temperature. The assay was scored after 12 days. The response was scored by germinated seed, dead seed, hard seed, and firm swollen seed. The firm swollen seed were subsequently tested via the tetrazolium assay for viability and characterized as viable or non-viable firm swollen. The results (Table 4.18) indicate that the COT102 seed falls within or very close to the commercial reference range. Small differences may be attributed to the fact that event COT102 was in the Coker 312 genetic background which may show minor differences to current commercial germplasm. In summary, COT102 exhibited no significant effect in germination response to temperature.

Table 4.18. Germination Assay Results

Temp.	Variety[1]	Mean vhs[2] (Dormant) (%)	Mean germ[2] (%)	Mean vfms[2] (%)	Mean degen[2] (%)
10°C	COT 102	0.0	0	77.8	7.0
10°C	Ref. Range	(0-1.3)	(0-0)	(74.2-91.7)	(1.3-4.3)
20/30°C	COT102	0.0	82.5	0	17.5
20/30°C	Ref. Range	(0-0)	(89.5-98.5)	(0-0)	(1.5-17.3)
40°C	COT 102	0.0	71.0	0	20.0
40°C	Ref. Range	(0-0)	(61.8-96.5)	(0-0)	(3.5-40.8)

[1] There were 4 observations based on 100 seeds each for event COT102, in addition to 20 observations for reference varieties in each temperature regime.
[2] " vhs" = viable hard seed, "germ" = percent germinated seed, "vfms" = viable firm swollen seed, "degen" = degenerated seed

4) Disease and Pest Characteristics

Disease symptoms were evaluated during each of the event COT102 field trials planted in 2000, 2001, and 2002 (fifty-one locations). Event COT102 was planted with its non-transgenic parent cultivar, Coker 312 and compared for various fungal and bacterial diseases. Plots were visually inspected for symptoms such as leaf necrosis, wilting, spotted leaves, dampening off, or boll rot. No significant differences were noted between the non-transgenic Coker 312 and event COT102.

Non-target insect pest monitoring was also conducted during each of the field trials. Based on observations by professional breeders, agronomists, and entomologists taken throughout the growing season, there were no discernable differences in non-target pest damage incurred by event COT102 and non-transgenic Coker 312. In addition, non-target insect species were generally present at higher populations in the event COT102

and Coker 312 plots as compared to the sprayed treatments.

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5) Conclusions

In the agronomic evaluation of event COT102 during the 2001 and 2002 growing seasons, no commercially significant morphological deviations from the parent variety, Coker 312, were observed. Cotton plants were scrutinized for a multitude of traits under diverse environmental conditions. When differences were noted, those differences were not consistent across locations or over seasons indicating normal variability within a biological system. Additional differences were plainly attributable to lepidopteran insect pressure. When insect damage occurred to the control Coker 312 line, the plants often compensated with continued growth and delayed maturity. Event COT102 not only showed significant increases in yield accumulation under varying levels of insect pressure, but also matured earlier, both desirable traits.

Chapter 5

COMPOSITIONAL ANALYSIS OF COTTONSEED OF EVENT COT102

A. Cottonseed Nutritional Analysis

Cottonseed was evaluated by Covance Laboratories, Inc. (Madison, WI) in order to assess potential unintended effects on plant metabolism due to the transgene insertion or expression of the VIP3A or APH4 proteins. Forty-seven separate components were evaluated including proximates (moisture, fat, protein, fiber, ash), amino acids, fatty acids, minerals (copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), gossypol, and cyclopropenoid fatty acids. Cottonseed was collected over two years for the described analysis.

In 2001, cottonseed was produced at three locations (Lubbock TX, Leland MS, and Maricopa AZ) (USDA Notification #'s 01-078-20n, 01-303-02n and 01-078-20n, respectively). Plot size was limited due to seed availability and therefore, a single sample was collected at each location from both COT102 and the non-transgenic parental cultivar Coker 312. Each location was treated as a replicate for the purposes of statistical analysis. Means and standard deviations for COT102 and Coker 312 were calculated and the F-test was used to determine whether there were significant differences.

In 2002, cottonseed was produced at two locations (Leland, MS, and Visalia, CA) (USDA Notification #'s 02-063-06n, and 02-086-14n, respectively). At both locations the plot design consisted of a replicated (4 replicate samples), randomized block design. The data from 2002 was used to calculate means and standard deviations and the F-test was used to determine whether there were significant differences.

The 2001 and 2002 data are presented below (Tables 5.1 through 5.15). The 2002 data is presented with comparisons both within and across locations. Of the 47 measurements analyzed, there were few instances where the mean values for event COT102 were significantly different than those of the parental control, Coker 312. Moreover, those differences were not consistent across locations or over years. Furthermore, the values measured for both event COT102 and Coker 312 fell within the established range for cottonseed (USDA-APHIS, 2002), and are considered to be biologically and nutritionally insignificant. No pattern emerged that would indicate the noted differences were caused by the transgene insertion or expression of the novel proteins.

Table 5.1. 2001 Proximate Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Moisture	Fat	Protein	Fiber	Ash	Carbohydrate
		%	%	%	%	%	
2001	COT102	8.84	21.90	29.87	15.25	4.06	Nd
	Coker 312	9.27	22.12	29.34	15.81	4.21	
	SD	1.25	0.82	0.54	1.49	0.21	

Means do not differ significantly from the control ($p \leq 0.05$); Nd = not determined

Table 5.2. 2002 Proximate Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	Moisture	Fat	Protein	Fiber	Ash	Carbohydrate
			%	%	%	%	%	%
2002	CA	COT102	6.43	24.63	28.80	31.55	3.85	36.30
		Coker 312	6.68	23.58	28.68	32.93	3.85	37.23
		SD	0.30	2.30	1.27	1.85	0.25	1.87
	MS	COT102	6.88	21.20	29.23	36.15	4.03*	38.68
		Coker 312	7.28	21.83	29.43	32.93	4.35*	37.13
		SD	0.40	1.02	0.94	1.45	0.09	1.17

Means do not differ significantly from the control ($p \leq 0.05$) except where noted with a *.

Table 5.3. 2002 Proximate Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Moisture	Fat	Protein	Fiber	Ash	Carbohydrate
		%	%	%	%	%	%
2002	COT102	6.65	22.91	29.01	33.85	3.94	37.49
	Coker 312	6.98	22.70	29.05	32.93	4.10	37.18
	SD	0.35	1.78	1.12	1.66	0.19	1.56

Means do not differ significantly from the control ($p \leq 0.05$)

Table 5.4. 2001 Minerals Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	P	Ca	Na	Fe	Mg	Mn	K	Zn	Cu	Ch
		%	%	ppm	ppm	%	ppm	%	ppm	ppm	ppm
2001	COT102	0.64	0.11	969	82.1	0.33	13.6	0.81	30.3	9.1	<1
	Coker 312	0.68	0.12	929	81.7	0.34	13.6	0.82	31.6	9.4	<1
	SD	0.04	0.02	212	7.6	0.02	0.6	0.01	1.0	0.5	

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.5. 2002 Minerals Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	P	Ca	Na	Fe	Mg	Mn	K	Zn	Cu	Ch
			mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g
2002	CA	COT102	656	133	18.1*	4.17	379	1.16	1063	3.33	0.487	<0.200
		Coker 312	680	147	41.5*	4.38	378	1.23	1036	3.79	0.614	<0.200
		SD	87	7	9.6	0.32	30	0.09	55	0.43	0.065	
	MS	COT102	638*	126	23.8	5.16*	391*	1.23*	1103	3.14*	0.643	<0.200
		Coker 312	745*	119	14.6	5.92*	412*	1.31*	1153	3.57*	0.704	<0.200
		SD	13	5	10.3	0.24	2	0.03	40	0.17	0.030	

Means do not differ significantly from the control ($p \leq 0.05$) except where noted with a *.

Table 5.6. 2002 Minerals Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	P	Ca	Na	Fe	Mg	Mn	K	Zn	Cu	Ch
		mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g
2002	COT102	647	130	20.9	4.66*	385	1.19	1083	3.23*	0.565*	<0.200
	Coker 312	712	133	28.0	5.15*	395	1.27	1094	3.68*	0.659*	<0.200
	SD	62	6	9.9	0.28	21	0.07	48	0.33	0.050	

Means do not differ significantly from the control ($p \leq 0.05$) except where noted with a *.

Table 5.7. 2001 Fatty Acid Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
		%	%	%	%	%	%	%	%	%
2001	COT102	0.837	24.84	0.587	2.51	15.25	55.04	0.393	0.240	0.120
	Coker 312	0.813	24.47	0.570	2.51	15.51	54.94	0.513	0.237	0.123
	SD	0.022	0.20	0.022	0.01	0.34	0.27	0.058	0.008	0.008

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.8. 2002 Fatty Acid Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
			%	%	%	%	%	%	%	%	%
2002	CA	COT102	0.185	5.75	0.130	0.553	3.75	12.48	0.058	0.065	0.043
		Coker 312	0.165	5.42	0.128	0.523	3.68	11.68	0.063	0.058	0.040
		SD	0.01	0.50	0.009	0.039	0.26	1.18	0.004	0.007	0.004
	MS	COT102	0.150	4.69	0.118	0.443	3.00	10.68	0.068	0.053	0.038
		Coker 312	0.155	4.96	0.120	0.463	2.91	11.08	0.068	0.058	0.040
		SD	0.009	0.28	0.004	0.024	0.12	0.54	0.006	0.007	0.004

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.9. 2002 Fatty Acid Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
		%	%	%	%	%	%	%	%	%
2002	COT102	0.168	5.22	0.124	0.498	3.37	11.58	0.063	0.059	0.040
	Coker 312	0.160	5.19	0.124	0.493	3.30	11.38	0.065	0.058	0.040
	SD	0.010	0.41	0.007	0.032	0.20	0.92	0.005	0.007	0.004

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.10. 2001 Amino Acid Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Asp	Thre	Ser	Glu	Pro	Gly	Ala	Cys	Val
		%	%	%	%	%	%	%	%	%
2001	COT102	423	400	2340	787	1057	4597	880	963	953
	Coker 312	407	403	2270	770	1023	4450	850	940	950
	SD	27	36	85	25	29	159	31	25	29

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.11. 2002 Amino Acid Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	Asp	Thre	Ser	Glu	Pro	Gly	Ala	Cys	Val
			mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g
2002	CA	COT102	2525	785	1113	5740	1085	1155	1068	443	1313
		Coker 312	2605	805	1170	5925	1078	1168	1085	470	1325
		SD	83	17	29	201	32	30	29	35	35
	MS	COT102	2480	773	1098	5780	1025	1128	1063	468	1288
		Coker 312	2488	745	1190	5883	1083	1155	1095	463	1323
		SD	51	20	51	157	36	38	39	14	42

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.12. 2002 Amino Acid Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Asp	Thre	Ser	Glu	Pro	Gly	Ala	Cys	Val
		mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g
2002	COT102	2503	779	1105*	5760	1055	1141	1065	455	1300
	Coker 312	2546	775	1180*	5904	1080	1161	1090	466	1324
	SD	69	18	42	181	34	34	34	27	39

Means do not differ significantly from the control ($p \leq 0.05$) except where noted with a *.

Table 5.13. 2001 Amino Acid Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
		%	%	%	%	%	%	%	%	%
2001	COT102	1013	733	1330	540	1197	657	1003	2630	310
	Coker 312	987	710	1297	520	1153	643	990	2523	313
	SD	39	23	43	19	41	18	27	89	11

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.14. 2002 Amino Acid Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
			mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g
			Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
2002	CA	COT102	388	920	1608	718	1465	780	1223*	3148	263
		Coker 312	390	925	1633	735	1503	795	1270*	3275	270
		SD	24	22	33	20	41	22	9	141	12
	MS	COT102	413	905	1600	703	1450	760	1205	3098	268
		Coker 312	405	925	1640	735	1498	780	1250	3228	275
		SD	12	33	64	30	51	30	39	114	4

Means do not differ significantly from the control ($p \leq 0.05$) except where noted with a *.

Table 5.15. 2002 Amino Acid Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
		mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g
2002	COT102	400	913	1604	710	1458	770	1214*	3123	265
	Coker 312	398	925	1636	735	1500	788	1260*	3251	273
	SD	19	28	51	26	47	26	28	128	9

Means do not differ significantly from the control ($p \leq 0.05$) except where noted with a *.

B. Toxicants

Cottonseed samples collected from the same plots described above were analyzed for the presence of natural toxicants. Analyses were completed by Covance Laboratories, Inc. (Madison, WI) for total gossypol and cyclopropenoid fatty acids.

Total Gossypol

Gossypol is a naturally occurring toxin in cotton plants and is believed to provide them some degree of protection from insect damage. Because gossypol is also known to be toxic to livestock, it is a limiting factor in the use of whole cottonseed and cottonseed meal as feed sources (Adams, 1977). Total gossypol levels were measured in cottonseed from all locations. No significant differences were measured in total gossypol levels of event COT102 as compared to the non-transgenic parental Coker 312 line (Tables 5.16, 5.17, and 5.18).

Cyclopropenoid Fatty Acids

The cyclopropenoid fatty acids (CPFA) including sterculic, malvalic, and dihydrosterculic are unique fatty acids in cotton that are considered undesirable, anti-nutrients. The listed fatty acids were measured in 2002 and no statistical differences were noted (Tables 5.19 and 5.20).

References

Adams, R. and Geissman, T.J., (1977) Gossypol, pigment of cottonseed, Chem. Rev., 60, 555.

U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS). 2002. USDA/APHIS Petition 00-342-01P for Determination of Non-Regulated Status for Bollgard II Cotton Event 15985 Producing the Cry2Ab Insect Control Protein Derived from *Bacillus thuringiensis subsp. kurstaki.* Environmental assessment and finding of no significant impact.

Table 5.16. 2001 Total Gossypol Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Total Gossypol (% fresh wt. basis)
2001	COT102	0.877
	Coker 312	0.939
	SD	0.077

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.17. 2002 Total Gossypol Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	Total Gossypol (% fresh wt. basis)
2002	CA	COT102	0.864
		Coker 312	0.856
		SD	0.128
	MS	COT102	0.949
		Coker 312	1.025
		SD	0.071

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.18. 2002 Total Gossypol Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Total Gossypol (% fresh wt. basis)
2002	COT102	0.906
	Coker 312	0.940
	SD	0.104

Means do not differ significantly from the control ($p \leq 0.05$)

Table 5.19. 2002 Cyclopropene Fatty Acid Analysis of Cottonseed from COT102 and Coker 312 Within Location

Year	Location	Line	Sterculic	Malvalic	Dihydrosterculic
			%	%	%
2002	CA	COT102	0.248	0.320	0.100
		Coker 312	0.255	0.320	0.105
		SD	0.049	0.038	0.004
	MS	COT102	0.278	0.378	0.108
		Coker 312	0.260	0.408	0.113
		SD	0.089	0.037	0.019

Means do not differ significantly from the control ($p \leq 0.05$).

Table 5.20. 2002 Cyclopropene Fatty Analysis of Cottonseed from COT102 and Coker 312 Across Locations

Year	Line	Sterculic	Malvalic	Dihydrosterculic
		%	%	%
2002	COT102	0.263	0.349	0.104
	Coker 312	0.258	0.364	0.109
	SD	0.072	0.037	0.014

Means do not differ significantly from the control ($p \leq 0.05$)

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Chapter 6

QUANTIFICATION OF VIP3A AND APH4 PROTEINS IN EVENT COT102

A. Summary

To characterize the range of expression of transgenic proteins in cotton plants derived from event COT102, the concentrations of VIP3A protein (the active insecticidal principle) and APH4 protein (the selectable marker protein) were determined by ELISA for whole plants and designated plant tissues obtained from three separate field locations at six developmental stages. Isogenic, nontransgenic control plants were similarly collected for analysis. Pollen and nectar were collected from greenhouse-grown plants. For one or more plant developmental stages, quantifiable levels of VIP3A protein were present in leaves, squares, roots, bolls, seeds, pollen, and whole plants. VIP3A protein was not detectable in nectar or cotton fiber. Additionally, no protein of any kind was detectable in nectar or cotton fiber by standard protein assay.

For most tissues and sampling stages, VIP3A concentrations were generally comparable across all locations. Across all developmental stages and locations, mean VIP3A concentrations measured in whole-plant samples ranged from *ca.* 1 - 13 µg/g fresh wt. (1 – 73 µg/g dry wt.). Leaves had the highest mean VIP3A levels, which ranged from *ca.* 3 – 22 µg/g fresh wt. (5 – 118 µg/g dry wt.) across sampling stages. Mean VIP3A concentrations measured in squares, roots and bolls did not exceed *ca.* 4 µg/g fresh wt. (17 µg/g dry wt.), 2 µg/g fresh wt. (7 µg/g dry wt.) and 1 µg/g fresh wt. (9 µg/g dry wt.), respectively. For all test locations, mean VIP3A concentrations measured in seeds were *ca.* 3 µg/g on a fresh wt. and dry wt. basis. The VIP3A concentration measured in pollen was *ca.* 1 µg/g air-dried pollen. The values reported herein were not corrected for extraction efficiency, however, the estimated extraction efficiencies for the VIP3A quantitation method ranged from *ca.* 80 – 90% across the various cotton plant tissues analyzed.

Across all plant stages, estimates of the amount of VIP3A protein produced per acre of COT102-derived cotton plants ranged from a mean of *ca.* 3 g/acre (*ca.* 7 g/hectare) at the four-leaf stage to *ca.* 106 g/acre (*ca.* 261 g/hectare) at the peak bloom stage. By the pre-harvest stage, mean VIP3A levels had declined to *ca.* 19 g VIP3A/acre (46 g VIP3A/hectare). These estimates assumed a planting density of 60,000 plants/acre (148,200 plants/hectare).

APH4 was either not detectable in most COT102 plant tissues or the levels were too low to quantify. Pollen was the only tissue in which quantifiable levels, *ca.* 2.3 µg APH4/g air-dried pollen, were measured.

In addition, extraction efficiency experiments were performed to estimate the relative amount of VIP3A extracted from different tissues during routine procedures.

B. Materials and Methods

Source of field-grown plants for evaluation of VIP3A and APH4 levels. Using standard local agronomic procedures, plants representing one transgenic cotton line derived from event COT102 and an isogenic, nontransgenic control line (Coker 312) were field-grown concurrently in 2001 in Camilla, Georgia[1]; Maricopa, Arizona[2]; and Idalou, Texas (USA) (USDA Notification # 01-078-20n). In most cases, ten whole plants, including roots, from the transgenic COT102-derived line, plus two plants from the control Coker 312 line, were harvested at each of six developmental stages:

Four-Leaf stage, *ca.* 2 weeks post emergence
Squaring, *ca.* 4 weeks post emergence
First White Bloom, *ca.* 9 weeks post emergence
Peak Bloom, *ca.* 13 weeks post emergence
First Open Boll, *ca.* 15 weeks post emergence
Pre-harvest, *ca.* 22 weeks post emergence
(Sampling times varied depending on environmental conditions)

Harvested plants were shipped on ice overnight to the Syngenta Seeds Product Registration Laboratory, Research Triangle Park, NC. Upon receipt, five transgenic plants per 10-plant sample were separated into parts and five retained as whole-plant samples. One corresponding control plant was separated into parts and the other retained as a whole-plant sample. All retained samples of whole plants or plant parts were weighed and stored frozen at *ca.* –80°C until further processing of the tissues for extraction and analysis. Tissue samples from leaves, roots, squares, seeds, cotton fiber, bolls, and whole plants were processed and extracted as described below in **Plant tissue processing** and **Tissue extraction** and quantitatively analyzed for VIP3A and APH4 by ELISA. The specific tissues analyzed at each developmental stage are described in **Results**. In accordance with the study protocol, not all tissues collected were analyzed at all developmental stages, although all samples were retained.

Source of pollen and nectar for evaluation of VIP3A and APH4 levels. The samplings of field-grown plants did not yield pollen and nectar of sufficient quantity and quality for analysis. Therefore, it was necessary to sample the flowers of 15 - 25 greenhouse-grown plants to produce a single pooled sample of pollen and a single pooled sample of nectar for each genotype (COT102 transgenic and Coker 312 control).

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[1]For the first open boll sampling stage, polymerase chain reaction (PCR) evaluation of the plants received indicated that all were inadvertently nontransgenic. Therefore, no data on VIP3A and APH4 levels were obtained for this location at this developmental stage. An "X" has been placed in the data tables (Tables 6.1 – 6.4) to signify which data points were affected.

[2]For both the peak bloom and first open boll stages, PCR evaluation indicated that all plants sampled from this location at these developmental stages were inadvertently nontransgenic. An "X" has been placed in the data tables (Tables 6.1 – 6.4) to signify which data points were affected.

Source of tissue for supplemental analysis of VIP3A levels in young leaves. In the initial samplings of leaf tissue at six developmental stages (described above in **Source of field-grown plants for evaluation**), all plant leaves (young and older leaves) were combined to produce a single leaf tissue sample, per plant, for analysis. However, because cotton plants continuously produce new leaves, which might contain higher VIP3A levels than older leaves, a supplemental analysis of young leaves was conducted to provide data more representative of this specific plant tissue. One young, shiny leaf was collected from each of ten COT102 plants field grown in 2002 in two different locations, Louisburg, North Carolina and Victoria, Texas (USDA Notification # 02-072-17n and # 02-063-06n, respectively). The developmental stages sampled were squaring, first white bloom, peak bloom, and first open boll stage. Leaves from Texas were packaged with gel-packs of ice substitute ('blue ice') and shipped overnight to the Product Registration Laboratory, while those from North Carolina were hand delivered on ice within a day of collection to the Product Registration Laboratory. The ten leaves were pooled to make a single sample from each site. All leaves were stored at *ca.* –80°C until processed and analyzed as described below.

Source of data for estimations of grams of VIP3A protein per acre and per hectare. Using the VIP3A values measured for the whole-plant samples described above (see **Source of field-grown plants for evaluation**), estimates of the quantities of VIP3A protein that may be present in COT102 derived plants on a per-acre and a per-hectare basis were calculated. Assuming a density of 60,000 VIP3A cotton plants per acre, estimates were calculated, as follows, for plants at five developmental stages (four-leaf, first white bloom, peak bloom, first open boll, and pre-harvest):

$$\frac{\text{g VIP3A}}{\text{acre}} = \frac{\text{mean g VIP3A}}{\text{g dry wt.}} \times \frac{\text{mean g dry wt.}}{\text{plant}} \times \frac{\text{60,000 plants}}{\text{acre}}$$[1]

Sample calculation:

$$\frac{\text{37.12 g VIP3A}}{\text{acre}} = \frac{\text{0.00001774 g VIP3A}}{\text{g dry wt.}} \times \frac{\text{34.87 g dry wt.}}{\text{plant}} \times \frac{\text{60,000 plants}}{\text{acre}}$$

Similar calculations were also made using a value of 148,200 plants/hectare.[2]

Plant tissue processing. Whole plants and individual parts were reduced to a fine powder by processing, using either a coffee grinder, blender, Retsch Cutting Mill (Brinkmann Instruments, Westbury, NY), Grindomix™ grinder (Brinkmann Instruments), mortar with a pestle or mill, or a combination of these devices, in accordance with Syngenta Standard Operating Procedure (SOP) 2.10. All processing was done in the presence of either dry ice or liquid nitrogen. Samples were mixed well to ensure homogeneity. The entire plant tissue sample or a representative sub-sample was lyophilized and retained for analysis, allowing sufficient sample size for archival storage of

[1] Based on estimated average planting density for cotton in the United States.
[2] Based on estimated average planting density for cotton in the United States.

reserve plant tissue samples. The percent dry weight of each sample was determined in accordance with Syngenta SOP 2.11. Processed samples were stored at *ca.* –80°C until lyophilization.

Pollen from greenhouse-grown plants was collected, filtered through 100 µm sieves, and air-dried overnight. All pollen samples were stored at *ca.* –80°C. Pollen was extracted and analyzed as described in **Tissue extraction**. Nectar was collected and diluted (1:1) with extraction buffer to allow analysis.

Tissue extraction. With the exception of pollen and nectar, plant tissue and whole-plant samples were extracted in accordance with Syngenta SOP 2.50. For each sample analyzed, a 0.1 g aliquot of the powdered lyophilized material was weighed into a 15-ml polypropylene tube, suspended in 3 ml extraction buffer (100 mM sodium borate, 5 mM magnesium chloride, 0.05% Tween 20, 0.2% ascorbic acid, pH 7.8), and extracted using a Polytron® homogenizer (Brinkmann Instruments). Following centrifugation at 14,000 x g for 10 min at *ca.* 2° - 8°C, VIP3A and APH4 analyses by ELISA were performed on the supernatants. Total protein in the extracts was quantified using the Bio-Rad Reagent (Bio-Rad; Hercules, CA) in accordance with Syngenta SOP 2.53.

Pollen extracts were prepared by suspending air-dried pollen 1:30 (w/v) in extraction buffer. Following 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at *ca.* 15,000 psi, followed by centrifugation at 14,000 x g for 5 min at *ca.* 4°C (SOP 2.42). Nectar samples in extraction buffer were manually stirred. VIP3A and APH4 analyses by ELISA were performed on the pollen supernatants and mixed nectar samples. Total protein was quantitated as described above.

VIP3A quantification. The extracts prepared, as described above, were quantitatively analyzed for VIP3A by ELISA (Tijssen, 1985), in accordance with Syngenta SOP 2.38, using Protein A-purified polyclonal rabbit and immunoaffinity-purified polyclonal goat antibodies generated to VIP3A protein purified from recombinant *Escherichia coli* over-expressing the *vip3A(a)* gene. The lower limit of quantification of the double-sandwich ELISA was estimated based on the lowest concentration of pure reference protein lying on the linear portion of the standard curve, the maximum volume of a control extract that could be analyzed without background interference, and the corresponding weight of the sample that the aliquot represented (see Appendix 6A).

APH4 quantification. The extracts prepared as described above were quantitatively analyzed for APH4 by ELISA, in accordance with Syngenta SOP 2.49, using Protein A-purified polyclonal rabbit and immunoaffinity-purified sheep antibodies generated to APH4 protein purified from *E. coli* over-expressing the *aph4* gene. The lower limit of quantification of the double-sandwich ELISA was estimated based on the lowest concentration of pure reference protein lying on the linear portion of the standard curve, the maximum volume of a control extract that could be

analyzed without background interference, and the corresponding weight of the sample that the aliquot represented (see Appendix 6B).

VIP3A extraction efficiency. Extraction efficiency measurements were performed to estimate the relative amount of VIP3A extracted during routine procedures, compared to that which remained associated with the insoluble plant tissue pellet. Tissues extracted were leaves, roots, squares, seeds, and whole plants (Appendix 6C). Tissues were extracted as described in **Tissue extraction** and the insoluble material was collected and re-extracted. (For a complete description see Appendix 6C.) The levels of APH4 protein in COT102-derived plant tissues were too low to permit the estimation of extraction efficiencies.

C. Results

VIP3A protein levels in COT102 plant tissues at various cotton developmental stages. The lower limit of quantification (LOQ) for the VIP3A ELISA ranged from *ca.* 40 – 270 ng/g fresh weight, depending on the tissue and developmental stage (see Appendix 6A, Table A-1). On a dry weight basis, the LOQ was *ca.* 300 ng/g. VIP3A protein was detected in most, but not all, COT102 plant tissues analyzed (Tables 6.1, 6.2, 6.5, 6.6 and 6.7). For most tissues and sampling stages, VIP3A concentrations were generally comparable across all locations (where samples were available from more than one location). Leaves (Tables 6.1 and 6.2) had higher VIP3A levels than other individual tissues at all sampling stages (mean values measured ranged from *ca.* 3 – 22 μg VIP3A/g fresh wt. and *ca.* 5 – 118 μg VIP3A/g dry wt.), and levels generally declined at the later sampling times. The VIP3A levels in whole-plant samples typically paralleled those in leaves, although they tended to be somewhat lower across all sampling stages (means ranged from *ca.* 1 – 13 μg VIP3A/g fresh wt. and *ca.* 1 – 73 μg VIP3A/g dry wt). VIP3A concentrations measured in young leaves (*ca.* 1 – 19 μg VIP3A/g fresh wt.; *ca.* 5 – 66 μg VIP3A/g dry wt.; Table 6.7) were generally comparable, across sampling stages, to VIP3A concentrations in leaf samples (described above) that represented a composite of both young and older leaves. Mean VIP3A protein levels measured in roots (Tables 6.1 and 6.2) were uniformly low (*ca.* < 0.2 – 2 μg VIP3A/g fresh wt.; *ca.* < 0.4 – 7 μg VIP3A/g dry wt.) at all times sampled. Mean VIP3A levels in bolls were also low and declined from *ca.* 1 μg VIP3A/g fresh wt. (*ca.* 7 - 9 μg VIP3A/g dry wt.) at peak bloom to levels that were generally undetectable or too low to quantify at the pre-harvest stage. In squares, mean VIP3A concentrations at all stages sampled ranged from *ca.* 2 – 4 μg VIP3A/g fresh wt. (*ca.* 4 – 17 μg VIP3A/g dry wt.), except for the Georgia location at pre-harvest stage, for which the VIP3A levels were below the LOQ. Mean VIP3A levels in seeds were *ca.* 3 μg VIP3A/g fresh or dry wt. (Table 6.5). VIP3A was not detected in any samples of cotton fiber (Table 6.5). Moreover, no protein of any kind was detectable in fiber by the Bio-Rad protein determination method.

The pooled pollen sample contained *ca.* 1.1 μg VIP3A/g air-dried pollen (Table 6.6). VIP3A was not detectable in the pooled nectar sample, and no protein of any kind was detectable in nectar by the Bio-Rad method.

VIP3A extraction efficiency. The apparent extraction efficiency of VIP3A across the various tissues ranged from 78.0% in squares to 92.1% in leaves (Appendix 6C; Table C-1). The mean extraction efficiency across all tissues (including whole-plant samples) was found to be *ca.* 87%, indicating that the procedures used were well optimized for VIP3A. ELISA values provided in this report were not corrected for extraction efficiency.

APH4 protein levels in COT102 plant tissues at various cotton developmental stages. The LOQ for the APH4 ELISA ranged from *ca.* 25 – 137 ng/g fresh wt. across the tissues and stages analyzed (see Appendix 6B, Table B-1). This corresponded to a LOQ of *ca.* 150 ng/g dry weight. Quantifiable levels of APH4 were not found in any of the tissues sampled except pollen (Tables 6.3, 6.4, 6.5 and 6.6). APH4 quantitation results were generally consistent across sampling locations. Leaves and whole-plant samples had detectable but not quantifiable levels at all sampling times through the peak bloom stage, and APH4 was typically not detected at the later sampling stages (Tables 6.3 and 6.4). Bolls had detectable but not quantifiable APH4 levels at all stages analyzed. Roots and squares generally had detectable APH4 upon initial sampling, but APH4 was not detected in these tissues at the pre-harvest stage. APH4 was generally not detectable in seeds, except for some seed samples from Georgia in which the APH4 levels were below the LOQ (Table 5). APH4 protein was not detectable in cotton fiber (Table 6.5) or nectar (Table 6.6). Pollen contained *ca.* 2.3 μg APH4/g air-dried pollen.

Estimated total VIP3A protein per acre and per hectare in COT102 plants. Across all plant stages, estimates of the amount of VIP3A protein produced per acre ranged from a mean of *ca.* 3 g/acre (*ca.* 7 g/hectare) at the four-leaf stage to *ca.* 106 g/acre (*ca.* 261 g/hectare) at the peak bloom stage (Table 6.8; Figure 6.1). This stage also had the most variability between sites. This variability can be attributed to the large difference in the size of plants found at this stage; the plants from Georgia weighed about four times more than those from Texas. Environmental conditions, agronomic practices, and genotype adaptation for the different growing regions all likely contributed to this variation. By the pre-harvest stage, mean VIP3A levels had declined to *ca.* 19 g VIP3A/acre (46 g VIP3A/hectare).

References

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immunoassays. In, *Practice and theory of enzyme immunoassays*. (Laboratory techniques in biochemistry and molecular biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

Table 6.1. VIP3A Protein Levels on a Fresh-Weight Basis During Development of COT102 Plants

Tissue	Location	Mean µg VIP3A/g fresh wt.[a] ± Standard Deviation (N = 5 unless otherwise indicated; range)					
		4-Leaf	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-Harvest
Leaves	Georgia	15.08 ± 3.22 (9.39 – 15.08)	18.26 ± 4.92 (4[c]; 11.12 – 22.40)	9.86 ± 2.28 (7.66 – 13.31)	5.92 ± 2.84 (1.87 – 9.05)	X[b]	3.29 ± 3.31 (2[d]; 0.95 – 5.63)
	Texas	18.51 ± 2.45 (15.47 – 20.78)	21.51 ± 2.06 (19.01 – 23.75)	10.78 ± 1.22 (9.99 – 12.92)	4.66 ± 0.84 (3.73 – 5.46)	8.82 ± 1.49 (6.79 – 10.68)	7.24 ± 1.18 (5.41 – 8.50)
	Arizona	12.35 ± 6.26 (1.20 – 15.90)	12.87 ± 3.39 (7.04 – 15.70)	8.56 ± 3.48 (3.94 – 13.31)	X	X	3.65 ± 1.94 (4[d]; 1.08 – 5.17)
Roots	Georgia	1.27 ± 0.36 (0.82 – 1.78)	NA[e]	NA	1.18 ± 0.13 (1.01 – 1.35)	NA	1.21 ± 0.46 (0.53 – 1.78)
	Texas	1.57 ± 0.16 (1.31 – 1.73)	NA	NA	1.82 ± 0.69 (1.05 – 2.53)	NA	2.15 ± 0.32 (1.88 – 2.69)
	Arizona	< 1.33 (DNQ[f] – 1.86)	NA	NA	X	NA	< 0.17 (DNQ– 0.35)
Bolls	Georgia	---[g]	---	---	1.09 ± 0.10 (0.94 – 1.19)	X	nd[h]
	Texas	---	---	---	1.38 ± 0.82 (0.44 – 2.18)	0.45 ± 0.17 (0.33 – 0.74)	< 0.20 (DNQ)
	Arizona	---	---	---	X	X	< 0.36 (DNQ– 0.47)
Squares	Georgia	---	NA	3.72 ± 1.25 (2.01 – 5.45)	2.77 ± 0.12 (2.58 – 2.85)	X	< 0.22 (DNQ)
	Texas	---	NA	1.64 ± 0.43 (0.88 – 1.92)	2.64 ± 0.93 (1.42 – 3.54)	2.10 ± 0.34 (1.69 – 2.41)	4.00 ± 1.48 (1.62 – 5.64)
	Arizona	---	NA	3.11 ± 0.41 (2.68 – 3.70)	X	X	1.51 ± 0.31 (1.17 – 2.01)
Whole Plant	Georgia	13.22 ± 1.68 (11.70 – 15.13)	NA	4.53 ± 0.84 (3.63 – 5.91)	6.35 ± 0.35 (5.90 – 6.79)	X	0.72 ± 0.16 (0.47 – 0.86)
	Texas	12.37 ± 1.40 (10.53 – 14.30)	NA	5.46 ± 1.10 (4.24 – 6.86)	4.64 ± 0.75 (3.92 – 5.70)	5.28 ± 2.07 (1.87 – 7.36)	2.96 ± 1.04 (1.82 – 4.19)
	Arizona	10.75 ± 2.05 (7.72 – 12.65)	NA	5.16 ± 1.83 (2.39 – 7.49)	X	X	1.59 ± 0.35 (1.30 – 2.12)

[a] Values were determined by ELISA and were not corrected for extraction efficiency. All control tissues/plants had 0 ng VIP3A/g fresh wt.
[b] "X" indicates that PCR tests confirmed the plants sampled were inadvertently nontransgenic.
[c] Percent dry weight not determined for one sample, so conversion to fresh weight basis could not be calculated for that sample.
[d] Number of plants analyzed was <5 because leaves were not present on some plants.
[e] "NA" = not analyzed
[f] "DNQ" = sample had detectable, non-quantifiable amounts below the lower limit of quantification (LOQ) specified in Appendix 6A. Means were calculated by assuming VIP3A was present at the LOQ for such samples, and such means are preceded by "<" indicating that the mean is less than the quantity indicated.
[g] "---" = tissue not available at this stage
[h] "nd"=VIP3A was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

Table 6.2. VIP3A Protein Levels on a Dry-Weight Basis During Development of COT102 Plants

Tissue	Location	Mean µg VIP3A/g dry wt.[a] ± Standard Deviation (N = 5 unless otherwise indicated; range)					
		4-Leaf	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-Harvest
Leaves	Georgia	77.29 ± 19.45 (44.67 – 95.97)	118.22 ± 28.03 (68.67 – 135.60)	40.52 ± 9.53 (32.34 – 56.15)	30.05 ± 14.93 (10.40 – 46.88)	X[b]	4.58 ± 4.76 (2[c]; 1.21 – 7.95)
	Texas	96.26 ± 11.15 (81.42 – 108.81)	87.41 ± 9.40 (76.85 – 99.66)	50.42 ± 5.67 (46.47 – 60.10)	17.89 ± 2.60 (14.79 – 20.44)	35.31 ± 4.76 (28.19 – 40.14)	9.01 ± 1.56 (6.28 – 10.06)
	Arizona	64.61 ±32.82 (6.17 – 83.67)	58.50 ± 15.22 (32.59 – 71.70)	40.70 ± 15.00 (18.26 – 59.69)	X	X	6.17 ± 2.08 (4[c]; 3.38 – 8.41)
Roots	Georgia	6.30 ± 0.81 (5.08 – 7.28)	NA[d]	NA	4.37 ± 0.58 (3.76 – 5.19)	NA	3.39 ± 1.40 (1.32 – 5.12)
	Texas	7.03 ± 0.76 (6.16 – 7.84)	NA	NA	5.13 ± 1.95 (3.14 – 7.32)	NA	4.83 ± 0.58 (4.14 – 5.68)
	Arizona	< 5.09 (DNQ[e] – 7.45)	NA	NA	X	NA	< 0.43 (DNQ – 0.94)
Bolls	Georgia	---[f]	---	---	8.66 ± 1.09 (7.34 – 10.05)	X	nd[g]
	Texas	---	---	---	6.99 ± 4.04 (2.18 – 11.28)	2.12 ± 0.82 (1.66 – 3.56)	< 0.30 (DNQ)
	Arizona	---	---	---	X	X	< 0.91 (DNQ– 1.36)
Squares	Georgia	---	NA	16.57 ± 5.44 (8.81 – 23.69)	16.86 ± 1.36 (15.43 – 18.75)	X	< 0.30 (DNQ)
	Texas	---	NA	7.99 ± 2.02 (4.51 – 9.50)	11.42 ± 3.77 (6.84 – 15.55)	8.63 ± 1.42 (7.02 – 10.23)	4.75 ± 1.81 (1.77 – 6.43)
	Arizona	---	NA	16.69 ± 1.61 (14.93 – 19.06)	X	X	3.64 ± 1.91 (2.04 – 5.92)
Whole Plant	Georgia	72.82 ± 8.43 (62.76 – 81.81)	NA	17.74 ± 3.25 (13.49 – 22.63)	35.53 ± 2.81 (31.04 – 38.17)	X	1.29 ± 0.30 (0.82 – 1.55)
	Texas	65.36 ± 6.70 (57.84 – 76.07)	NA	26.88 ± 5.02 (21.51 – 32.99)	18.20 ± 2.53 (15.56 – 21.67)	19.96 ± 8.33 (6.54 – 28.54)	5.27 ± 1.98 (2.94 – 7.68)
	Arizona	54.97 ± 12.17 (35.42 – 65.90)	NA	26.76 ± 7.65 (14.96 – 36.34)	X	X	4.27 ± 0.94 (3.30 – 5.60)

[a] Values were determined by ELISA and were not corrected for extraction efficiency. All control tissues/plants had 0 ng VIP3A/g dry wt.
[b] "X" indicates that PCR tests confirmed the plants sampled were inadvertently nontransgenic.
[c] Number of plants analyzed was <5 because leaves were not present on some plants.
[d] "NA" = not analyzed
[e] "DNQ" = sample had detectable, non-quantifiable amounts below the lower limit of quantification (LOQ) specified in Appendix 6A. Means were calculated by assuming VIP3A was present at the LOQ for such samples, and such means are preceded by "<" indicating that the mean is less than the quantity indicated.
[f] "---" = tissue not available at this stage
[g] "nd" = VIP3A was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

Table 6.3. APH4 Protein Levels on a Fresh-Weight Basis During Development of COT102 Plants

Tissue	Location	Mean ng APH4/g fresh wt.[a] (N = 5 unless otherwise indicated; range)					
		4-Leaf	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-Harvest
Leaves	Georgia	< 29 [b]	< 32 (4 [c])	< 7 (nd - < 33)	< 20 (nd - < 33)	X [d]	nd (2 [e])
	Texas	< 29	< 32	< 33	< 20 (nd - < 33)	nd	nd
	Arizona	< 29	< 6 (nd - < 32)	< 33	X	X	nd (4 [e])
Roots	Georgia	< 20 (nd - < 25)	NA [f]	NA	nd [g]	NA	nd
	Texas	< 5 (nd - < 25)	NA	NA	nd	NA	nd
	Arizona	nd	NA	NA	X	NA	nd
Bolls	Georgia	--- [h]	---	---	< 25	X	< 20 (nd - < 100)
	Texas	---	---	---	< 25	< 30	< 60 (nd - < 100)
	Arizona	---	---	---	X	X	< 40 (nd - < 100)
Squares	Georgia	---	NA	< 32	< 30	X	nd
	Texas	---	NA	< 26 (nd - < 32)	nd	nd	nd
	Arizona	---	NA	< 7 (nd - < 32)	X	X	nd
Whole Plant	Georgia	< 29	NA	< 14 (nd - < 33)	< 32	X	nd
	Texas	< 29	NA	< 20 (nd - < 33)	< 26 (nd - < 32)	nd	< 60 (nd - < 75)
	Arizona	< 29	NA	< 20 (nd - < 33)	X	X	nd

[a] Values were determined by ELISA and were not corrected for extraction efficiency. All control tissues/plants had 0 ng APH4/g fresh wt.
[b] Samples had either detectable, non-quantifiable amounts below the lower limit of quantification (LOQ) specified in Appendix 6B or APH4 was nondetectable (nd). Means were calculated by assuming APH4 was present at either the LOQ for such samples or 0 if nd, and such means are preceded by "<" indicating that the mean is less than the quantity indicated.
[c] Percent dry weight not determined for one sample, so conversion to fresh weight basis could not be calculated for that sample.
[d] "X" indicates that PCR tests confirmed the plants sampled were inadvertently nontransgenic.
[e] Number of plants analyzed was <5 because leaves were not present on some plants.
[f] "NA" = not analyzed
[g] "nd"=APH4 was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.
[h] "---" = tissue not available at this stage

Table 6.4. APH4 Protein Levels on a Dry-Weight Basis During Development of COT102 Plants

Tissue	Location	Mean ng APH4/g dry wt.[a] (N = 5 unless otherwise indicated; range)					
		4-Leaf	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-Harvest
Leaves	Georgia	< 150[b]	< 150	< 120 (nd - < 150)	< 90 (nd - < 150)	X[c]	nd (2[d])
	Texas	< 150	< 150	< 150	< 90 (nd - < 150)	nd	nd
	Arizona	< 150	< 150	< 150	X	X	nd (4[d])
Roots	Georgia	< 120 (nd - < 150)	NA[e]	NA	nd[f]	NA	nd
	Texas	< 30 (nd - < 150)	NA	NA	nd	NA	nd
	Arizona	nd	NA	NA	X	NA	nd
Bolls	Georgia	---[g]	---	---	< 150	X	< 30 (nd - < 150)
	Texas	---	---	---	< 150	< 150	< 90 (nd - < 150)
	Arizona	---	---	---	X	X	< 60 (nd - < 150)
Squares	Georgia	---	NA	< 150	< 30	X	nd
	Texas	---	NA	< 120 (nd - < 150)	nd	nd	nd
	Arizona	---	NA	< 30 (nd - < 150)	X	nd	nd
Whole Plant	Georgia	< 150	NA	< 60 (nd - < 150)	< 150	X	nd
	Texas	< 150	NA	< 90 (nd - < 150)	< 90 (nd - < 150)	nd	< 120 (nd - < 150)
	Arizona	< 150	NA	< 90 (nd - < 150)	X	X	nd

[a] Values were determined by ELISA and were not corrected for extraction efficiency. All control tissues/plants had 0 ng APH4/g dry wt.

[b] Samples had either detectable, non-quantifiable amounts below the lower limit of quantification (LOQ) specified in Appendix 6B or APH4 was nondetectable (nd). Means were calculated by assuming APH4 was present at either the LOQ for such samples or 0 if nd, and such means are preceded by "<" indicating that the mean is less than the quantity indicated.

[c] "X" indicates that PCR tests confirmed the plants sampled were inadvertently nontransgenic.

[d] Number of plants analyzed was <5 because leaves were not present on some plants.

[e] "NA" = not analyzed

[f] "nd"=APH4 was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

[g] "---" = tissue not available at this stage

Table 6.5. VIP3A and APH4 Protein Levels in Seeds and Cotton Fiber from the Pre-harvest Stage During the Development of Cotton Plants Derived from Event COT102

Tissue	VIP3A Levels[1]		APH4 Levels[1]	
	Mean µg VIP3A/g fresh weight ± S. D. (range)	Mean µg VIP3A/g dry weight ± S. D. (range)	Mean ng APH4/g fresh weight (range)	Mean ng APH4/g dry weight (range)
	N = 5			
Seeds				
Georgia	2.88 ± 0.28 (2.52 – 3.28)	3.23 ± 0.31 (2.86 – 3.65)	< 55[2] (nd[3] - <137)	< 60 (nd - < 150)
Texas	2.70 ± 0.27 (2.41 – 3.05)	2.99 ± 0.29 (2.65 – 3.35)	nd	nd
Arizona	2.51 ± 0.25 (2.14 – 2.82)	2.72 ± 0.28 (2.33 – 3.08)	nd	nd
Cotton Fiber[4]				
Georgia		nd		nd
Texas		nd		nd
Arizona		nd		nd

[1] Values were determined by ELISA and were not corrected for extraction efficiency. Values for all control plants corresponded to 0 ng VIP3A or APH4/g fresh or dry weight.

[2] Where traces of APH4 were found, but could not be quantitated, the value is indicated as less than (<) the lower limit of quantification for that tissue.

[3] "nd" = VIP3A or APH4 was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

[4] Only dry weight values for cotton fiber were calculated.

Table 6.6. VIP3A and APH4 Protein Levels in Nectar and Pollen Collected From Greenhouse-Grown Cotton Plants Derived from Event COT102

Tissue	µg VIP3A/g sample[1]	µg APH4/g sample[1]
Pollen[2]	1.09	2.25
Nectar[3]	nd[4]	nd

[1] Values were determined by ELISA and were not corrected for extraction efficiency. Values for all control plants corresponded to 0 ng VIP3A or APH4/g sample. Each plant represented a composite of pollen or nectar collected from 15 – 25 plants.
[2] Pollen values are reported on a g air-dried pollen basis.
[3] Nectar values are on a g nectar (as collected) basis.
[4] "nd" = VIP3A or APH4 was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

Table 6.7. VIP3A Protein Levels in Young Leaves of Event COT102-Derived Cotton Plants Grown at Two Locations and Sampled at Four Developmental Stages

Location	Stage	µg VIP3A/g fresh wt.[1]	µg VIP3A/g dry wt.[1]
North Carolina	Squaring	13.88	44.85
	First White Bloom	18.87	65.61
	Peak Bloom	12.33	45.20
	First Open Boll	3.90	15.37
Texas	Squaring	6.86	27.19
	First White Bloom	5.54	22.52
	Peak Bloom	1.55	7.11
	First Open Boll	1.33	5.04

[1] Values were determined by ELISA and were not corrected for extraction efficiency. Each sample represented a composite of 10 leaves from different plants.

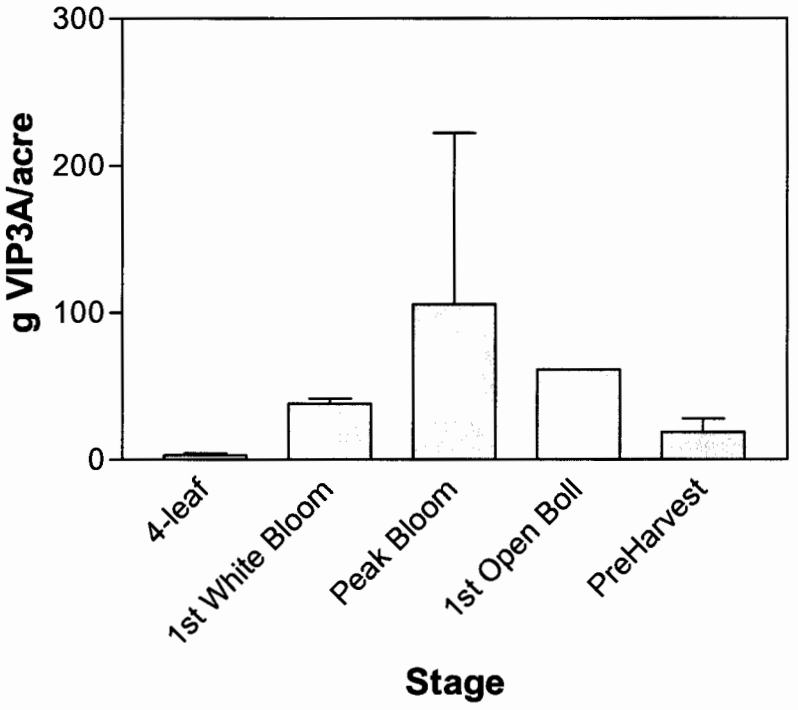
Table 6.8. VIP3A g/Acre and g/Hectare Estimates for COT102 Cotton Plants

Stage	Site	Mean Plant Dry Wt. (g)	g VIP3A acre[1]	g VIP3A hectare[1]
4 - Leaf	Georgia	1.02	4.46	11.01
	Texas	0.77	3.02	7.46
	Arizona	0.48	1.58	3.91
	Mean ± Standard Deviation		3.02 ± 1.44	7.46 ± 3.55
1st White Bloom	Georgia	34.87	37.12	91.68
	Texas	21.72	35.03	86.52
	Arizona	26.24	42.13	104.06
	Mean ± Standard Deviation		38.09 ± 3.65	94.09 ± 9.02
Peak Bloom	Georgia	88.30	188.24	464.95
	Texas	21.40	23.37	57.72
	Mean ± Standard Deviation		105.80 ± 116.56	261.33 ± 287.95
1st Open Boll	Texas	51.23	61.35	151.54
Pre Harvest	Georgia	123.12	9.53	23.54
	Texas	60.70	19.19	47.41
	Arizona	108.16	27.71	68.45
	Mean ± Standard Deviation		18.81 ± 9.10	46.46 ± 22.47

[1] All estimates were derived from mean ELISA values for whole plants presented in Table 6.2. Values were not corrected for extraction efficiency.

Figure 6.1. Estimated mean VIP3A protein levels per acre during the growing season in COT102 Plants.

Graphic representation of data presented in Table 6.8. Plants were obtained and analyzed as described in Materials and Methods.



APPENDIX 6A: VIP3A ELISA PROCEDURE

INTRODUCTION

Extracts were quantitatively analyzed for VIP3A protein by enzyme-linked immunosorbent assays (ELISA; Tijssen, 1985) using Protein A-purified polyclonal rabbit and immunoaffinity-purified goat antibodies generated to VIP3A protein purified from recombinant *E. coli* over-expressing the *vip3A(a)* gene. The ELISA method described here was adapted from procedures initially optimized for leaf tissue. The procedures, conditions employed, and sample calculations are described in detail and are restated from Syngenta SOP 2.38.

MATERIALS AND METHODS

ELISA Buffers

Borate Buffered Saline (BBS)

100 mM Boric acid
25 mM Sodium borate
75 mM Sodium chloride
pH 8.4-8.5

Blocking Buffer

10 mM Sodium phosphate, pH 7.4
140 mM Sodium chloride
1% Bovine serum albumin (BSA)
0.02% Sodium azide

Diluent

10 mM Sodium phosphate, pH 7.4
140 mM Sodium chloride
0.05% Tween-20
1% Bovine serum albumin
0.02% Sodium azide

Wash Buffer

10 mM Tris-HCl, pH 8.0
0.05% Tween-20
0.02% Sodium azide

Phosphatase Substrate Buffer

10 mM Diethanolamine, pH 9.8
5 mM Magnesium chloride

VIP3A ELISA Procedure

Vinyl 96-well plates (Costar, Cambridge, MA) were pre-washed for two hours with 95% ethanol and dried overnight. The plates were then coated with immunoaffinity-purified goat anti-VIP3A polyclonal antibodies specific for VIP3A protein purified from a

recombinant *E. coli* over-expression system. After incubation overnight at 2° - 8°C, the plates were washed three times with wash buffer and blocked for at least 45 min at room temperature with blocking buffer. Plates were washed three times and triplicate samples of each tissue extract (appropriate dilutions prepared in diluent) were applied (total volume was 50 µl per well). Following incubation at 2° - 8°C for 1.5 hr and for 0.5 hr at room temperature, the plates were washed three times and coated with Protein A-purified rabbit anti-VIP3A polyclonal antibodies. The plates were incubated for 1 hr at 35° - 39°C and then washed three times prior to coating with donkey anti-goat alkaline phosphatase conjugated antibody (1 µg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After incubation for 1 hr at 35° - 39°C, the plates were washed three times and phosphatase substrate (0.6 mg p-nitrophenyl phosphate/ml phosphatase substrate buffer) was added. Color was allowed to develop for 30 min at room temperature and the reaction stopped by the addition of 3N NaOH. Absorbance at 405 nm was measured using a Ceres 900C multi-well plate reader. The results were analyzed using the KC3 Curve fitting software program (Bio-Tek® Instruments, Inc.; Winooski, VT, USA) as described in Syngenta SOP 3.13. The four parameters algorithm was used to generate a curve.

Temperature and incubation times were adhered to, as the ELISA was not designed to go to equilibrium. Standard curves, prepared from purified VIP3A, were run on each plate. Data points were considered acceptable if the mean "Delta" OD value obtained lay within the linear range of the standards (between 10 and 60 ng/ml) in close approximation of the midpoint. Only analyses in which the coefficient of variance was less than 10% were accepted. If, due to technical error, one of the three aliquots did not yield a reliable absorbance value, then the mean absorbance of the remaining duplicate aliquots of the sample extract was used. VIP3A was considered to be undetectable if the mean absorbance obtained for the triplicate ELISA samples did not exceed that of the non-transgenic control. The lower limit of quantification (LOQ), below which VIP3A is not quantifiable but may be detectable, was estimated for various plant tissues at different developmental stages (Table A-1). Because the extracts were prepared on the same dry weight-to-volume ratio, the LOQ on a dry weight basis was identical for all samples. However, because the moisture content varied widely from tissue to tissue and throughout the growing season, the LOQ on a fresh weight basis varied widely.

Table A-1: Approximate Lower Limits of Quantification of VIP3A in Cotton Tissues Derived From Event COT102

Tissue	µg VIP3A/ g dry wt.	µg VIP3A/g fresh wt.					
	All stages analyzed	4 - Leaf	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-harvest
Leaves	0.3	0.06	0.04	0.07	0.07	0.07	0.21
Roots	0.3	0.07	NA[1]	NA	0.08	NA	0.12
Squares	0.3	---[2]	NA	0.06	0.06	0.07	0.22
Bolls	0.3	---	---	---	0.05	0.06	0.20
Seeds	0.3	---	---	---	---	---	0.27
Fiber	0.3	---	---	---	---	---	ND[3]
Whole plants	0.3	0.06	NA	0.06	0.06	0.08	0.15

[1] NA = Tissue not analyzed

[2] "---" = Tissue not available at this stage.

[3] ND = Dry weight not determined, assumed to contain zero moisture.

Lower limit of quantification =
lower limit of standard curve
(dry weight basis) g dry wt./ml extract at max. conc. usable in ELISA

Calculation for all samples:
10 ng VIP3A/ml = 300 ng or 0.3 µg VIP3A/g dry wt.
0.1 g dry wt./3 ml extract

To estimate the LOQ on a fresh weight basis, the LOQ on a dry weight basis is multiplied by the mean ratio of dry weight/fresh weight for that tissue (SOP 2.11).

Sample calculation for whole plants at four-leaf stage:

0.3 µg VIP3A/g dry wt. x 0.19 g dry wt./g fresh wt. = 0.06 µg VIP3A/g fresh wt.

VIP3A was determined to be stable for at least five days at *ca.* 4°C in extracts prepared as described in **Tissue extraction**, in the main text of this report (SOP 2.10, SOP 2.50). Range finder ELISAs were performed to allow dilutions of extracts to be prepared such that the mean absorbance of three replicates closely coincided with the midpoint of the linear portion of the standard curve. Generally, five plants were sampled for each time point, with one extract prepared from each sample.

VIP3A calculations:

$$\frac{\text{ng VIP3A}}{\text{ml extract}} = \frac{\text{ng VIP3A}^{1}}{\text{ml}} \times \frac{50\ \mu\text{l/well}}{\mu\text{l extract/well}}$$

$$\frac{\mu\text{g VIP3A}}{\text{g dry wt.}} = \frac{\text{ng VIP3A}}{\text{ml extract}} \times \frac{\text{ml extract}}{\text{g dry wt.}} \times \frac{1}{1000}$$

$$\frac{\mu\text{g VIP3A}}{\text{g fr. wt.}} = \frac{\mu\text{g VIP3A}}{\text{g dry wt.}} \times \frac{\text{g dry wt.}}{\text{g fr. wt.}}$$

REFERENCES

Standard Operating Procedures

SOP 2.10 Maize Tissue Preparation for Extraction
SOP 2.11 Dry Weight Determination
SOP 2.38 Quantitative Analysis for VIP3A by ELISA
SOP 2.50 VIP3A Extraction from Cotton
SOP 3.13 Ceres 900C™ Multi-well Plate Reader

Literature Citations

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immunoassays. In, *Practice and theory of enzyme immunoassays*. (Laboratory techniques in biochemistry and molecular biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

[1]ELISA value

APPENDIX 6B: APH4 ELISA PROCEDURE

INTRODUCTION

Extracts were quantitatively analyzed for APH4 protein by enzyme-linked immunosorbent assays (ELISA; Tijssen, 1985) using immunoaffinity-purified sheep and Protein A-purified polyclonal rabbit antibodies generated to APH4 protein purified from *E. coli* over-expressing the *aph4* gene. The ELISA method described here was adapted from procedures initially optimized for leaf tissue. The procedure, conditions employed, and sample calculations detailed are also described in Syngenta SOP 2.49.

MATERIALS AND METHODS

ELISA Buffers

Borate Buffered Saline (BBS)

100 mM Boric acid
25 mM Sodium borate
75 mM Sodium chloride
pH 8.4 - 8.5

Blocking Buffer

10 mM Sodium phosphate, pH 7.4
140 mM NaCl
1% Bovine serum albumin (BSA)
0.02% Sodium azide

Diluent

10 mM Sodium phosphate, pH 7.4
140 mM Sodium chloride
0.05% Tween-20
1% BSA
0.02% Sodium azide

Wash Buffer

10 mM Tris-HCl, pH 8.0
0.05% Tween-20
0.02% Sodium azide

Phosphatase Substrate Buffer

10 mM Diethanolamine, pH 9.8
5 mM Magnesium chloride

APH4 ELISA Procedure

Vinyl 96-well plates (Costar, Cambridge, MA) were pre-washed for two hours with 95% ethanol and dried overnight. The plates were then coated with donkey anti-sheep antibody. Following incubation overnight at 2° - 8°C, the plates were washed three times with wash buffer and blocked for at least 45 min at room temperature with blocking buffer. Plates were then washed at least three times and coated with immunoaffinity

purified sheep anti-APH4 antibodies specific for APH4 protein purified from a recombinant *E. coli* over-expression system. After washing the plates five times, triplicate samples of each tissue extract (appropriate dilutions prepared in diluent) were applied (total volume was 50 µl per well). Following incubation at room temperature for 1.5 hr, the plates were washed five times and coated with Protein A- purified rabbit anti-APH4 antibodies. The plates were incubated for 1 hr at 35° - 39°C and then washed five times prior to coating with donkey anti-rabbit alkaline phosphatase conjugated antibody (Kirkegaard and Perry Laboratories; Gaithersburg, MD). Following incubation at 35° - 39°C, plates were washed five times and phosphatase substrate (0.6 mg p-nitrophenyl phosphate/ml phosphatase substrate buffer) added. Color was allowed to develop for 30 min at room temperature. Absorbance at 405 nm was measured using a Ceres 900C multi-well plate reader. The results were analyzed using the KC3 Curve fitting software program (Bio-Tek® Instruments, Inc.; Winooski, VT) as described in SOP 3.13. The four parameters algorithm was used to generate a curve.

Temperature and incubation times were adhered to, as the ELISA was not designed to go to equilibrium. Standard curves, prepared from purified APH4, were run on each plate. Data points were considered acceptable if the mean "Delta" OD value obtained lay within the linear range of the standards (10 and 80 ng/ml) in close approximation of the midpoint. Only analyses in which the coefficient of variance was less than 10% were accepted. If, due to technical error, one of the three aliquots did not yield a reliable absorbance value, then the mean absorbance of the remaining duplicate aliquots of the sample extract was used. APH4 was considered to be undetectable if the mean absorbance obtained for the triplicate ELISA samples did not exceed that of the nontransgenic control. The lower limit of quantification (LOQ), below which APH4 is not quantifiable but may be detectable, was estimated for various plant tissues at different developmental stages (Tables B-1). Because the extracts were prepared on the same dry weight to volume ratio, the LOQ on a dry weight basis was identical for all samples. However, because the moisture content varied widely from tissue to tissue and throughout the growing season, the LOQ on a fresh weight basis varied widely.

Table B-1. Approximate Lower Limits of Quantification of APH4 in Cotton Tissues Derived From Event COT102

Tissue	ng APH4A/ g dry wt.	ng APH4/g fresh wt.					
	All stages analyzed	4-Leaf	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-harvest
Leaves	150	29	32	33	33	36	108
Roots	150	25	NA[1]	NA	47	NA	59
Squares	150	---[2]	NA	32	30	35	108
Bolls	150	---	---	---	25	30	100
Seeds	150	---	---	---	---	---	137
Fiber	150	---	---	---	---	---	ND[3]
Whole plants	150	29	NA	33	32	39	75

[1] NA = Tissue not analyzed

[2] "---" = Tissue not available at this stage.

[3] ND = Dry weight not determined, assumed to contain zero moisture.

$$\text{Lower limit of quantification (dry weight basis)} = \frac{\text{lower limit of standard curve}}{\text{g dry wt./ml extract at max. conc. usable in ELISA}}$$

Sample calculation for all samples:

$$\frac{5 \text{ ng APH4/ml}}{0.1 \text{ g dry wt./3 ml extract}} = 150 \text{ ng APH4/g dry wt.}$$

To estimate the lower limit of quantification on a fresh weight basis, the limit on a dry weight basis is multiplied by the mean ratio of dry weight/fresh weight for that tissue (SOP 2.11).

Sample Calculation for whole plants at four-leaf stage:

150 ng APH4/g dry wt. x 0.19 g dry wt./g fresh wt. = 29 ng APH4/g fresh wt.

Extracts were prepared as described in **Tissue extraction** in the main text of this report (SOP 2.10, SOP 2.50). Generally, five plants were sampled for each time point, with one extract prepared from each sample.

APH4 calculations:

$$\frac{\text{ng APH4}}{\text{ml extract}} \quad \underset{\text{ml}}{\_\_\_\_\_} = \frac{\text{ng APH4}^{1}}{\text{µl extract/well}} \times \frac{\text{50 µl/well}}{\_\_\_\_\_}$$

$$\frac{\text{ng APH4}}{\text{g dry wt.}} = \frac{\text{ng APH4}}{\text{ml extract}} \times \frac{\text{ml extract}}{\text{g dry wt.}}$$

$$\frac{\text{ng APH4}}{\text{g fr. wt.}} = \frac{\text{ng APH4}}{\text{g dry wt.}} \times \frac{\text{g dry wt.}}{\text{g fr. wt.}}$$

REFERENCES

Standard Operating Procedures

SOP 2.10 Maize Tissue Preparation for Extraction
SOP 2.11 Dry Weight Determination
SOP 2.49 Quantitative Analysis for APH4 by ELISA
SOP 2.50 VIP3A Extraction from Cotton
SOP 3.13 Ceres 900C™ Multi-well Plate Reader

Literature Citations

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immunoassays. In, *Practice and theory of enzyme immunoassays*. (Laboratory techniques in biochemistry and molecular biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

[1]ELISA value

APPENDIX 6C: EFFICIENCY OF VIP3A PROTEIN EXTRACTION

INTRODUCTION

Extraction efficiency measurements were performed to estimate the relative amount of VIP3A extracted during routine procedures compared with that which remains associated with the insoluble plant tissue pellet. Tissues extracted were leaves, roots, squares, seeds, and whole plants.

MATERIALS AND METHODS

Source of plant material. Tissues were obtained from various stage COT102 samples, as indicated in Table C-1.

Tissue extraction. All tissues (except pollen and nectar) were extracted in accordance with SOP 2.50. Lyophilized, powdered tissue aliquots of 0.1 g were suspended in 3 ml extraction buffer (0.1 M Tris, 0.2 M sodium borate, 5 mM magnesium chloride, 0.2% ascorbic acid, 0.05% Tween-20) and extracted using a Polytron® homogenizer (Brinkmann Instruments, Westbury, NY). Following centrifugation at 14,000x g for 10 min, the supernatant was retained for analysis. The pellet was suspended in an additional 3 ml of extraction buffer, re-extracted, and centrifuged as before. Total protein in the first and second extracts was determined using the Bio-Rad Protein Reagent (Bio-Rad; Hercules, CA, USA) in accordance with SOP 2.53. VIP3A protein levels were measured in the first and second extracts by enzyme-linked immunosorbent assays (ELISA, SOP 2.38) as described in Appendix 6A.

Pollen extracts were prepared from dried pollen suspended 1:30 in extraction buffer. After 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at *ca.* 15,000 psi, and centrifuged at 14,000x g for 5 min at *ca.* 4°C (SOP 2.42). The resulting pellet was suspended in 3 ml fresh extraction buffer and French pressed as before. Total protein in the extracts was determined as above.

VIP3A quantification. The first and second extracts from each sample were quantitatively analyzed for VIP3A protein by ELISA as described in Appendix 6A. Extraction efficiency was calculated as follows:

$$\% \text{ efficiency of first extraction} = 100 \times \frac{\text{ng VIP3A in } 1^{st} \text{ extract}}{\text{ng VIP3A in } 1^{st} \text{ extract} + 2^{nd} \text{ extract}}$$

$$\text{Sample calculation: } 100 \times \frac{2728.30 \text{ ng VIP3A}}{2728.30 + 233.15 \text{ ng VIP3A}} = 92.1\% \text{ efficiency}$$

RESULTS

Extraction efficiency ranged from 78.0% in squares to 92.1% for leaves (Table C-1). This indicates that extraction efficiencies were quite comparable and that the procedure followed was well optimized for VIP3A extraction from these cotton tissues.

Table C-1. Efficiency of VIP3A Extraction from COT102 Plant Tissues as Determined by ELISA

Tissue (Stage)	ng VIP3A			Percent Extraction Efficiency
	First Extraction	Second Extraction	Total VIP3A Extracted	
Leaves (squaring)	2728.30	233.15	2961.45	92.1
Roots (peak bloom)	237.70	30.75	268.45	88.5
Squares (1^{st} white bloom)	660.73	186.49	847.22	78.0
Seeds (pre-harvest)	81.04	16.16	97.20	83.4
Whole plant (4- leaf)	2293.60	209.34	2502.94	91.6

REFERENCES

Standard Operating Procedures

SOP 2.38 Quantitative Analysis for VIP3A by ELISA
SOP 2.42 Maize Pollen Isolation and Extraction For VIP3A Protein
SOP 2.50 VIP3A Extraction from Cotton
SOP 2.53 Bio-Rad Microtiter Protein Determination

Chapter 7

ENVIRONMENTAL SAFETY

VIP3A is one of a new class of insecticidal proteins identified by Syngenta that are produced during vegetative growth of various *Bacillus* species. These proteins have been given the acronym VIP, for vegetative insecticidal protein. One of these insecticidal proteins, VIP3A, isolated from *Bacillus thuringiensis* strain AB88, has been found to have activity against larvae of the tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*), pink bollworm (*Pectinophora gossypiella*), and other secondary lepidopteran cotton pests. The cotton line Coker 312 was transformed via *Agrobacterium* transformation procedures with a synthetic *vip3A(a)* gene encoding VIP3A protein, and the selectable marker gene *aph4* encoding the enzyme APH4. The transformation event that gave rise to the transgenic line being tested, designated event "COT102," was transformed with plasmid pCOT1 (see Section 3B. Donor Genes and Regulatory Sequences).

A. Potential for Exposure to Transgenic Proteins

The potential for environmental exposure to transgenic proteins expressed in event COT102 cotton is expected to be relatively low because of the minute amount of transgenic protein present in the plants as compared to the total plant protein, and the fact that species that feed upon cotton tissues will typically be pests. Further, the established target-specificity of VIP3A supports the lack of toxicity to non-lepidopteran species. Any transgenic proteins, including the marker gene product, ingested by non-lepidopterans are expected to be digested as conventional dietary proteins. Handling of transgenic plant materials by humans will not result in any measurable exposure, as the transgenic proteins are contained within the plant tissues. The human and mammalian safety of VIP3A and event COT102 are addressed in Chapter 8, 'Food and Feed Safety'.

B. Expression Levels of Transgenic Proteins

Section 6 describes in detail the quantification of VIP3A and APH4 proteins in cotton tissues and whole plants derived from transformation event COT102 and characterizes the range of expression of transgenic proteins in cotton plants derived from event COT102.

For most tissues and sampling stages, VIP3A concentrations were generally comparable across all locations. Across all developmental stages and locations, mean VIP3A concentrations measured in whole-plant samples ranged from *ca.* 1 - 13 µg/g fresh wt. (1 – 73 µg/g dry wt.). Leaves had the highest mean VIP3A levels, which ranged from *ca.* 3 – 22 µg/g fresh wt. (5 – 118 µg/g dry wt.) across sampling stages. VIP3A concentrations in leaves were highest in the early growth stages (four-leaf and squaring stage), and then declined as the plants matured. Similarly, VIP3A concentrations in whole plants were highest for young plants (four-leaf stage), and declined substantially as the plants matured. Mean VIP3A concentrations measured in squares, roots and bolls did not exceed *ca.* 4 µg/g fresh wt. (17 µg/g dry wt.), 2 µg/g fresh wt. (7 µg/g dry wt.) and 1 µg/g fresh wt. (9 µg/g dry wt.),

respectively. For all test locations, mean VIP3A concentrations measured in seeds were *ca.* 3 µg on a fresh wt. and dry wt. basis. The VIP3A concentration measured in pollen was *ca.* 1 µg/g air-dried pollen. The values reported were not corrected for extraction efficiency, however, the estimated extraction efficiencies for the VIP3A quantitation method ranged from *ca.* 80 – 90% across the various cotton plant tissues analyzed.

Across all plant stages, estimates of the amount of VIP3A protein produced per acre of COT102-derived cotton plants ranged from a mean of *ca.* 3 g/acre (*ca.* 7 g/hectare) at the four-leaf stage to *ca.* 106 g/acre (*ca.* 261 g/hectare) at the peak bloom stage. By the pre-harvest stage, mean VIP3A levels had declined to *ca.* 19 g VIP3A/acre (46 g VIP3A/hectare). These estimates assumed a planting density of 60,000 plants/acre (148,200 plants/hectare).

APH4 was either not detectable in most COT102 plant tissues or the levels were too low to quantify. Pollen was the only tissue in which quantifiable levels, *ca.* 2.3 µg APH4/g air-dried pollen, were measured.

C. Protein Equivalence Study

VIP3A protein produced in cotton plants derived from the transgenic cotton event COT102 was characterized for its biochemical and functional similarity to VIP3A expressed in recombinant *Escherichia coli* and VIP3A protein produced in event "Pacha"-derived transgenic maize (corn) plants (Privalle, 2002b). The details of that study are summarized here.

Microbially-produced VIP3A protein has been used as the test substance in mammalian toxicity and other safety studies. Similarly, maize-produced VIP3A (as a VIP3A-enriched protein fraction) has been used in mammalian toxicity studies, environmental fate studies, and ecotoxicological studies. Therefore, it was necessary to demonstrate that these VIP3A protein preparations were substantially equivalent surrogates for cotton-expressed VIP3A protein, which cannot be purified for testing at comparable concentrations. [Additionally, natural toxicants (*e.g.*, gossypol) present in cotton may preclude the use of cotton-derived materials in toxicity tests.] VIP3A proteins from all three sources, *E. coli*, maize and cotton, were determined to have the predicted molecular weight of *ca.* 89,000 and cross-reacted immunologically with the same anti-VIP3A antibody. Through mass spectral analysis, it was possible to determine the amino acid sequence of peptides representing *ca.* 85% of the complete cotton-produced VIP3A protein. The resulting sequences corresponded identically to the predicted amino acid sequence of VIP3A and no evidence of any post-translational modification of the VIP3A protein was observed. The cotton-expressed VIP3A protein had the predicted N-terminal amino acids, beginning with asparagine-18. The 17 N-terminal amino acids not detected in the cotton-expressed VIP3A could represent *in planta* proteolysis or *in vitro* degradation. Comparisons of the biological activity of *E. coli*-expressed and cotton-expressed VIP3A protein in larval diet bioassays demonstrated very similar activities and the same rank order of VIP3A sensitivity among the four species tested. Based on the various functional and biochemical parameters evaluated, it can be concluded that VIP3A proteins from recombinant *E. coli*, Pacha-derived maize, and event COT102-derived cotton are substantially equivalent.

D. Potential Effects VIP3A Protein and VIP3A Cotton Event COT102 to Non-Target Organisms

To evaluate the environmental safety of the *Bacillus thuringiensis* (Bt) VIP3A insect control protein, standard single-species laboratory studies were conducted with representative avian, aquatic, insect, and soil invertebrate species (Table 7.1). These studies were conducted under conditions intended to maximize exposure to the VIP3A material used for testing to ensure an adequate margin of exposure above field-use conditions. Because the primary route of potential exposure of non-target organisms to VIP3A would be through dietary ingestion of plant tissues containing VIP3A protein, all of the non-target organism testing except that with daphnids utilized a dietary/oral exposure route. No adverse effects resulting from VIP3A exposure were observed among any of the non-target species tested.

The same data were submitted to the Environmental Protection Agency in December 2003 in support of a Section 3 Commercial Registration of the VIP3A protein as produced in event COT102.

In addition to the laboratory studies with VIP3A protein, two field studies have been completed, both of which were conducted with field corn (maize) expressing the VIP3A protein. One study was a caged semi-field study wherein hives of honeybees were exposed to VIP3A-expressing corn or isogenic control corn during pollen shed, and were additionally provisioned with pollen collected from the test plots. A second field study represented a comprehensive large plot census comparing non-target arthropod populations in VIP3A-expressing corn with populations in isogenic non-VIP3A corn and isogenic corn treated with conventional chemical insecticides for lepidopteran control.

The information summarized herein reinforces the characterization of VIP3A protein as a lepidopteran-specific toxin that will pose no risk of adverse effects to non-lepidopteran species if direct exposure were to occur. Additional extensive information supporting the species-specificity, mode of action, and non-target safety of the VIP3A protein have been provided in the Section 1. Included in that summary are references to additional studies in which VIP3A was screened for activity among pest species representing the orders Lepidoptera, Coleoptera, Diptera, Homoptera, and Thysanoptera.

Table 7.1. Summary of Non-Target Organism Studies

Non-Target Study	VIP3A Test Material	NOEC (= highest dose)	COT102 Margin of Exposure
Bobwhite Quail	VIP3A *E. coli* expressed protein	400 mg VIP3A/kg	~21X: leaves >1200X: seeds
Larval Honeybee	Maize inbred pollen (event Pacha)	2 mg pollen/larva (84 ppm VIP3A)	~ 77X conc. in pollen
Adult Honeybee; semi-field study	Hives caged w/ Pacha hybrids	Caged for 10 days, suppl. w/ VIP3A pollen cakes thru 21 days	>8X conc. in pollen
Adult Ladybeetle	Maize inbred pollen (event Pacha)	5% pollen in diet (pollen = 145 ppm VIP3A)	~ 133X conc. in pollen
Adult Lacewing	Maize inbred pollen (event Pacha)	15% pollen in diet (pollen = 145 ppm VIP3A)	~ 133X conc. in pollen
Daphnia magna	Maize inbred pollen (event Pacha)	120 mg pollen/L	N/A
Collembola	Maize leaf tissue (event Pacha)	50% of diet (43 ppm VIP3A in diet)	~6X whole plants >2100X soil EEC
Earthworm	Concentrated maize leaf protein (event Pacha)	1000 mg/kg soil (3.6 ppm VIP3A in soil)	~180X soil EEC
Bioactivity in 4 agricultural + 1 artificial soil	Concentrated maize leaf protein (event Pacha)	DT_{50}s = 1 – 5 days, after 3 – 12 day lag phase	N/A

1) Applicability of Surrogate VIP3A Protein Preparations to the Non-Target Safety Assessment of VIP3A as Expressed in COT102-Derived Cotton Plants

The majority of the non-target organism tests with VIP3A protein were conducted with test substances derived from VIP3A corn (maize), *e.g.,* corn pollen and lyophilized corn leaf tissue. These tests were initially conducted in the context of an ongoing environmental safety assessment of a VIP3A field corn product (derived from transformation event "Pacha") intended for control of lepidopteran pests in Brazil. However, the non-target studies conducted with VIP3A corn–derived test substances are also applicable to and valid for evaluating potential effects on non-target organisms associated with other crops expressing VIP3A, such as cotton. These studies are particularly applicable to VIP3A cotton because (1) VIP3A cotton expresses the same *vip3A(a)* gene as is expressed in VIP3A corn; (2) the test organisms used to assess the environmental safety of VIP3A corn represent core indicator species that have been employed in safety evaluations of other *Bacillus thuringiensis*-derived

plant-incorporated protectants (US EPA, 2001); (3) the non-target species in the cotton agro-ecosystem are comparable to those in corn; and (4) the use of cotton-derived plant materials as test substances may not be practical due to the potential presence of gossypol or other natural plant toxicants.

Additionally, although the various non-target toxicity studies were designed to expose the test organisms to VIP3A levels that were higher than the estimated environmental concentrations (EECs) for VIP3A corn, the EECs and highest plant tissue VIP3A concentrations for COT102 cotton plants are lower for all comparisons except leaves (Table 7.2, below). Moreover, despite the higher planting density of cotton (*e.g., ca.* 50,000 to 70,000 plants per acre) as compared to corn (*ca.* 25,000 to 27,000 plants per acre): (1) cotton pollen, in contrast to corn pollen, is not windborne and therefore has significantly lower potential for off-site dispersal to non-target species; (2) VIP3A protein is not detectable in nectar of event COT102 VIP3A plants, therefore, it is unlikely that organisms feeding upon nectar will consume biologically significant quantities of VIP3A protein; and (3) the practice of applying defoliants prior to harvesting cotton bolls substantially degrades or reduces the amount of plant tissue that may be potentially incorporated into soil through tillage practices.

Table 7.2. Highest Mean Plant Tissue Concentrations and Estimated Environmental Concentrations of VIP3A for Event COT102-Derived Cotton Plants as Compared to Event Pacha-Derived Field Corn Hybrids Used as a Source of VIP3A Test Materials

Description of Plant Tissue	Event COT102 VIP3A Cotton (plant stage)	Event Pacha VIP3A Field Corn Hybrids[a] (plant stage)
Leaves: Highest VIP3A conc. (μg VIP3A/g dry wt.; mean ± st. dev.)	83.72 ± 21.93[b] (4-leaf and squaring)	46.96 ± 4.37 (dough)
Seeds/Grain: Highest VIP3A conc. (μg VIP3A/g dry wt.; mean ± st. dev.)	2.98 ± 0.26[c]	26.39 ± 1.93 (dough)
Roots: Highest VIP3A conc. (μg VIP3A/g dry wt.; mean ± st. dev.)	6.14 ± 0.98[b] (4-leaf)	12.55 ± 4.22 (kernel maturity)
Pollen: VIP3A conc. (μg VIP3A/g air dried pollen)	1.09[d]	9.06 ± 0.21[g]
Nectar: VIP3A conc. (μg VIP3A/g)	0[d]	Not applicable
Estimated grams VIP3A/acre at peak VIP3A expression	105.80 ± 116.56[e] (peak bloom)	192.95 ± 47.16 (dough)
Estimated grams VIP3A/acre at latest stage analysed	18.81 ± 9.10[e] (pre-harvest)	133.60 ± 14.00 (senescence)
Estimated conc. of VIP3A in top 6" soil at latest stage analyzed (mg VIP3A/kg dry wt soil)[f]	0.02 (pre-harvest)	0.13 (senescence)

[a] Source: Privalle, 2002b
[b] Source: Table 6.2 of Chapter 6
[c] Source: Table 6.5 of Chapter 6
[d] Source: Table 6.6 of Chapter 6
[e] Source: Table 6.8 of Chapter 6
[f] Assumes entire plant is tilled into soil; 60,000 plants/acre for cotton; 26,500 plants/acre for field corn; see Part D. 2. for derivation of estimate for COT102 cotton.
[g] Pollen VIP3A concentrations for the inbred (homozygous transgenic) corn plants actually used as source of pollen test materials were substantially higher than for these hybrid (hemizygous transgenic) plants

As summarized in 7.C various biochemical and functional parameters have been used to compare VIP3A protein as produced in COT102-derived cotton with that produced in Pacha-derived corn as well as by recombinant *Escherichia coli* that over-expressed a *vip3A(a)* gene. These VIP3A preparations were shown to be substantially equivalent as measured by apparent molecular weight, immunoreactivity, absence of detectable post-translational glycosylation, mass spectral analysis of peptides, and the same rank-order of bioactivity against four species of VIP3A-sensitive lepidopteran larvae.

2) VIP3A Test Materials Used in Studies on Non-Target Organisms

Six primary types of test materials were used in the studies that evaluated the safety of VIP3A protein for non-target species. In general, the specific test material selected for a study was based on a consideration of the plant product most likely to result in exposure for the non-target organism being tested. Thus, pollen containing VIP3A was selected as the test substance for testing of daphnids, honeybees, ladybird beetles, and green lacewings; VIP3A-enriched leaf protein was tested on earthworms; and lyophilized leaf tissue was tested on collembola. Additionally, the avian toxicity study was conducted with VIP3A protein that had been produced in recombinant *Escherichia coli.*

Prior to use in any study, a sample of each test substance was bioassayed against a VIP3A-sensitive species (fall armyworm or black cutworm larvae) to confirm that the VIP3A protein displayed insecticidal activity, and the VIP3A concentration in the test substance was determined by ELISA. Western blot analysis was also conducted to verify the integrity (intactness) of the VIP3A protein. Following each study, the insecticidal activity of the test substance was again verified and VIP3A concentrations were re-analyzed by ELISA to confirm stability during shipping and storage at the testing laboratory. For most test material samples, VIP3A integrity was also re-assessed by western blot.

All studies using VIP3A corn-derived test materials also included non-transgenic corn controls. These consisted of the same type of test material (*i.e.,* pollen, concentrated leaf protein, or lyophilized leaves) collected or produced from isogenic, nontransgenic corn plants grown under the same environmental conditions as the transgenic corn. No VIP3A protein was detectable in these control substances. Neither the transgenic nor the control plants were treated with any insecticides, except for the greenhouse-grown plants from which some pollen was collected. The use of non-systemic insecticides was permitted on these plants up to two weeks prior to tasseling.

By including non-transgenic corn controls in the non-target organism studies, effects of the VIP3A protein could be distinguished from effects attributable to the presence of other

substances in the conventional, isogenic corn. Most studies also included negative control groups that were not exposed to any test or reference materials, but were otherwise maintained under the same conditions as the test organisms.

The specific test substances used, and the concentration of VIP3A protein in each, are described below:

1. **VIP3A protein produced in a microbial expression system (Test Substance VIP3A-0198).** VIP3A protein was produced in recombinant *E. coli* by over-expressing a *vip3A(a)* gene. The *vip3A(a)* gene was cloned into the inducible, over-expression pET-3d® vector (Novagen; Madison, WI) in *E. coli* strain BL21DE3pLysS. The VIP3A protein as encoded by the native *vip3A(a)* gene differs by a single amino acid as compared to the VIP3A protein encoded by the synthetic *vip3A(a)* gene in VIP3A cotton and VIP3A corn. The change at amino acid position 284 from lysine (in the native sequence) to glutamine (in VIP3A cotton and corn) is a conservative substitution in that both are polar amino acids and have a mol. wt. of 146. Following purification from *E. coli*, dialysis, and lyophilization, the resulting sample, designated Test Substance VIP3A-0198, was estimated to contain *ca.* 0.20g VIP3A/g as measured by ELISA. VIP3A represented *ca.* 31% of the total protein in this test substance, as estimated by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation.

   A comparable *E. coli*-produced VIP3A preparation (Test Substance VIP3A-0199) has been shown to be substantially equivalent to VIP3A produced in event COT102-derived cotton and event Pacha-derived corn, as measured by apparent molecular weight, immunoreactivity, absence of detectable glycosylation, mass spectral analysis of peptides, and the same rank-order of bioactivity against four species of VIP3A-sensitive lepidopteran larvae (Privalle, 2002c).

2. **VIP3A inbred corn (maize) pollen (Test Substance PHOPACHA-0199).** Pollen was collected from inbred field corn plants that were homozygous for the transgenes in VIP3A transformation event "Pacha". Pollen from inbred Pacha-derived corn plants contains higher concentrations of VIP3A protein than pollen from hybrid Pacha-derived plants because the hybrid plants (representing the plants that would potentially be grown commercially) are only hemizygous for the transgenes. The concentration of VIP3A in Test Substance PHOPACHA-0199 was *ca.* 83.8 μg/g pollen (83.8 ppm), as determined by ELISA.

3. **VIP3A inbred corn pollen (Test Substance PHOPACHA-0100).** A second lot of pollen collected from inbred Pacha-derived field corn plants that were homozygous for the transgenes was designated PHOPACHA-0100. The concentration of VIP3A in Test Substance PHOPACHA-0100 was *ca.* 144.8 μg/g pollen (144.8 ppm), as determined by ELISA.

4. **VIP3A/Cry1Ab 'stacked' hybrid corn pollen (Test Substance PHPACHABt11-0100).** This pollen sample was collected and tested as part of a program to evaluate experimental 'stacked' field corn hybrids expressing both VIP3A and Cry1Ab. The concentration of VIP3A protein in pollen sample PHPACHABt11-0100 was *ca.* 40.2

μg/g pollen, and the concentration of Cry1Ab was <90 ng/g pollen (the lower limit of quantification) by ELISA.

5. **VIP3A-enriched protein extracted from corn leaves (Test Substance LPPACHA-0199).** VIP3A-enriched corn leaf protein was prepared by extracting protein from leaves of event Pacha-derived hybrid field corn plants, concentrating the VIP3A protein by ammonium sulfate precipitation, dialyzing to remove salts, and lyophilizing the material to yield a fine protein powder. This material provided a source of concentrated VIP3A protein produced in transgenic corn; the resulting test substance, sample LPPACHA-0199, contained *ca.* 3.64 mg VIP3A/g.

6. **Lyophilized leaves of VIP3A hybrid corn plants (Test Substance LLPACHA-0100).** Leaves of event Pacha-derived field corn hybrids were collected from field-grown plants, powdered in liquid nitrogen, and lyophilized. The resulting material was designated Test Substance LLPACHA-0100 and contained *ca.* 86.7 μg VIP3A/g.

3) Laboratory Testing of VIP3A Protein on Non-Target Organisms

1. 14-Day Acute Oral Toxicity Study of VIP3A Protein in Bobwhite Quail (*Colinus virginianus*) *(Pedersen, 1999)*

An avian acute oral toxicity study on VIP3A protein was conducted according to US EPA Guideline No. 71-1. The test substance used was VIP3A-0198 (VIP3A protein produced in a microbial expression system), and contained *ca.* 0.20 g VIP3A/g as measured by ELISA. Five male and five female nine-week old bobwhite quail *(Colinus virginianus;* average body weight 158 g) were given a single oral dose of 2000 mg VIP3A-0198/kg body weight and observed for 14 days. The test material was administered in gelatin capsules. As a control group, five male and five female birds received gelatin capsules containing water. Throughout the study, all birds had continuous access to well water and a standard laboratory game bird diet, except that food was withheld for *ca.* 16 hours prior to dosing. No mortalities occurred during the study. No clinical signs of toxicity were observed in any birds during the study. No statistically significant changes in body weights were noted at any weighing interval (3, 7 or 14 days after dosing). Feed consumption values in the test and control groups were similar. Gross pathological examinations of all birds at study termination revealed no abnormalities. Tissues examined included the gastro-intestinal tract, liver, kidneys, heart, spleen, muscle, and subcutaneous fat. The results indicate that the LD_{50} of test substance VIP3A-0198 is greater than 2000 mg/kg body weight (corresponding to 400 mg VIP3A protein/kg body weight). The No Observable Effect Level (NOEL) in the study was 2000 mg test material/kg body weight (the highest dose tested).

2. In-Hive Larval Honeybee [*Apis mellifera* L. (Hymenoptera)] Development Study with VIP3A Corn Pollen (Maggi, 2002)

An in-hive honeybee study was conducted to determine whether ingestion of VIP3A inbred corn pollen had any measurable effects on larval honeybees (*Apis mellifera* L.) developing within honeycomb brood cells. Honeybees collect pollen from various plants and feed it to larval brood, either intact and/or in processed form (Winston, 1991). For cotton, it has been

suggested (Vaissière and Vinson, 1994) that honeybees do not extensively forage for cotton pollen because the pollen's spiny shape makes it difficult for honeybees to pack it in their pollen baskets. Pollen deposition on honeybees, and the ability of honeybees to pollinate cotton, is a consequence of foraging for nectar. [No VIP3A protein has been detected in nectar of COT102-derived cotton plants (Chapter 6)]. Nevertheless, honeybee larvae can be considered a surrogate species for other hymenopteran pollinators, such as bumblebees, which may have greater potential for dietary exposure to cotton pollen. Bumblebee larvae eat most of the pollen supply brought back to the nest; adult bees eat relatively little (Free and Butler, 1959). However, the extent to which cotton pollen, *per se*, may contribute to the total pollen diet of bumblebees and other bee species is uncertain.

The test pollen [test substance PHOPACHA-0199; VIP3A inbred corn (maize) pollen] used in the larval honeybee development study was estimated to contain *ca.* 83.8 μg of VIP3A protein/g pollen, as measured by ELISA. A single-exposure study was conducted in which *ca.* 2 mg of VIP3A corn pollen, moistened with a drop of 30% sucrose solution, was administered into the individual brood cells of three to five day-old honeybee larvae. Additional test groups included a negative control group in which larval bees received no treatment but were handled similarly, a reference pollen control group that received a 2 mg dose of non-transgenic isogenic inbred corn pollen and a drop of 30% sucrose solution per brood cell, and a positive control group that received 2 mg control inbred pollen plus 1000 ppm potassium arsenate in a drop of 30% sucrose solution. Each treatment group consisted of four replicates of 20 larvae each, for a total of 80 bees per treatment. The bee larvae were allowed to consume the pollen and were then returned to their source hives for capping of the brood cells by nurse bees. The hives were maintained under natural environmental conditions. Twelve days after treatment, the frames of capped brood cells were moved to a growth chamber where they were maintained under controlled conditions until emergence of adult bees from the brood cells. Emergence began 12 days after treatment and was complete by 19 days after treatment. Test endpoints included evaluating larval bee survival to capping, survival to emergence of adult honeybees, and the behavior and morphology of emerged adults.

Mean survival to capping and mean survival to adult emergence were 76.3% in the VIP3A corn pollen group and 77.5% in the control corn pollen group. Mean survival to capping and mean survival to adult emergence were 87.5% for the negative control group. (All mortalities in all study groups occurred prior to capping of the brood cells.) The differences among these three study groups were not statistically significant. Mean survival to capping and mean survival to adult emergence were 20% in the positive control group, which was statistically significantly lower than in the other three study groups. No behavioral or morphological abnormalities were noted among the emerged adult bees, and no differences in mean emergence times were observed. Based upon these results, the NOEL was 2 mg of VIP3A inbred corn pollen per honeybee larva.

3. 21-Day Chronic Toxicity Study of VIP3A Corn Pollen in Pink Spotted Lady Beetles [Coleomegilla maculata (Coleoptera)] (Teixeira, 2002a)

Coleomegilla maculata (pink spotted lady beetle or ladybird beetle) adults are generalist predators, but also feed directly on plant pollens. A study assessing the chronic dietary

toxicity of (1) pollen from VIP3A inbred corn plants and (2) pollen from hybrid corn plants expressing both VIP3A and Cry1Ab (another lepidopteran-active plant-incorporated protectant) to adult *C. maculata* was conducted according to US EPA OPPTS Guideline No. 885.4340. The adult lady beetles were 8 – 9 days old at study initiation. Test groups included lady beetles provided diet containing VIP3A inbred corn pollen (Test Substance PHOPACHA-0100; VIP3A inbred corn pollen) at 5% w/w, a group provided diet containing control inbred corn pollen at 5% w/w, a group provided diet containing pollen from hybrid corn plants expressing both VIP3A and Cry1Ab (Test Substance PHPACHABt11-0100; VIP3A/Cry1Ab 'stacked' hybrid corn pollen) at 5% w/w, a group provided diet containing control hybrid pollen at 5% w/w, a negative control group that received diet without any pollen added, and a positive control group provided diet containing 50 mg thiodicarb/kg diet (50 ppm). The VIP3A concentration in the inbred test pollen PHOPACHA-0100 was estimated at 144.8 µg VIP3A protein/g pollen, while the concentrations of VIP3A and Cry1Ab in test substance PHPACHABt11-0100 were *ca.* 40.2 µg/g and <90 ng/g, respectively, as measured by ELISA. To maximize stability of the VIP3A and Cry1Ab proteins in the lady beetle diet, diets for all test and control groups were replaced daily. Each treatment group consisted of three replicates of 25 beetles each, for a total of 75 beetles per treatment. All groups had continuous access to deionized water. Study duration was 21 days.

After 21 days of exposure to test or control diet, mean lady beetle survival was 93% in the group that received diets containing VIP3A inbred corn pollen, 92% in the group that received diets containing control inbred corn pollen, 85% in the group that received the VIP3A/Cry1Ab hybrid pollen, 99% in the group that received control hybrid pollen, and 97% in the negative control group. All lady beetles exposed to diet containing 50 ppm thiodicarb died within 7 days. Mean survival was not statistically significantly different among the groups of beetles provided diet containing VIP3A inbred pollen, control inbred pollen, VIP3A/Cry1Ab hybrid pollen, control hybrid pollen, or no pollen. No adverse effects were observed in lady beetles consuming diets containing 5% VIP3A inbred corn pollen, control inbred corn pollen, VIP3A/Cry1Ab hybrid corn pollen, or control hybrid corn pollen for 21 days. Therefore, the NOEC was 5% pollen in the diet (highest concentration tested), which corresponded to a VIP3A concentration of *ca.* 7.24 ppm in diets supplemented with test substance PHOPACHA-0100, and 2.01 ppm VIP3A and <4.5 ppb Cry1Ab in diets supplemented with test substance PHPACHABt11-0100.

4. 13-Day Toxicity Study of VIP3A Corn Pollen in Lacewings [Chrysoperla carnea (Neuroptera)] (Teixeira, 2002b)

Chrysoperla carnea adults feed directly on plant pollens, in addition to nectar and aphid honeydew. Unlike the larval stage, adult *C. carnea* are not generalist predators. However, other chrysopid species are predatory as adults (Principi and Canard, 1984), and thus *C. carnea* can be considered a surrogate indicator for these species. An advantage of testing *C. carnea* adults was, because pollens are a natural component of their diet, direct exposure to VIP3A pollen as the only source of pollen in the diet provided a worst-case exposure scenario. As discussed further in Parts D. 3. and D. 4., there is minimal potential for exposure of chrysopids to VIP3A *via* consumption of prey organisms that have consumed VIP3A in cotton plant material (*i.e.*, via a tri-trophic interaction). This is because the pests

that feed upon VIP3A cotton plants are expected to retain little if any VIP3A protein, in comparison to any VIP3A concentrations present in the plant tissues.

A study assessing the dietary toxicity of VIP3A inbred corn pollen to adult *C. carnea* (green lacewings) was conducted according to US EPA OPPTS Guideline No. 885.4340. Test groups included lacewings exposed to VIP3A inbred corn pollen (Test Substance PHOPACHA-0100; VIP3A inbred corn pollen) mixed into the diet at 15% w/w, lacewings exposed to control inbred corn pollen mixed into the diet at 15%, a negative control group that received diet containing 15% BEE-PRO® Pollen Substitute w/w, and a positive control group exposed to diet containing 100 mg thiodicarb/kg diet (100 ppm) (concentration increased to 500 ppm on day 10). The VIP3A concentration in the test pollen, as measured by ELISA, was *ca.* 144.8 µg VIP3A/g pollen. To maximize stability of the VIP3A protein in the diet, fresh diets were provided to all groups of lacewings daily. The adult lacewings were four days old at study initiation. Each treatment consisted of three replicates of 25 lacewings each, for a total of 75 lacewings per treatment. All groups had continuous access to deionized water. Eggs produced during the study were removed from the culture vessels regularly. The study duration was 13 days[1]. Study endpoints included survival, observations on sublethal effects, and observations on egg laying.

After 13 days of exposure to test or control diet, mean survival was 99% among the lacewings that received diets containing VIP3A inbred corn pollen, 97% among those that received diets containing control inbred corn pollen, and 99% in the negative control group. Mean survival was 45% in the positive control group. No statistically significant differences in mean survival were observed, except in comparison to the thiodicarb positive control group. Eggs were produced in all replicates for all treatments during the study and eggs were present in all replicates at test termination. Prior to termination, lacewings in all replicate vessels were actively feeding. Black or darkened abdomens were observed in lacewings exposed to the positive control substance (thiodicarb); no abnormalities were observed in lacewings in the other test or control groups.

No adverse effects were observed in adult lacewings consuming diets containing 15% VIP3A inbred corn pollen or control inbred corn pollen for 13 days. The LC_{50} for lacewings exposed to test or control pollen was >15% w/w in the diet, and the NOEC for the test or control pollen groups was 15% w/w in the diet (highest concentration tested); for the test group, these LC_{50} and NOEC values corresponded to a VIP3A concentration of *ca.* 21.7 ppm.

5. 14-Day Toxicity Study of VIP3A Corn Leaf Protein in Earthworms (Eisenia foetida) (Teixeira, 2002c)

A 14-day acute toxicity study examining the effects of VIP3A protein on earthworms was conducted according to OECD Guideline #207. Earthworms were placed in a defined artificial soil medium (with low organic matter content) to which VIP3A-enriched corn leaf protein (Test Substance LPPACHA-0199; VIP3A-enriched protein extracted from corn

[1] The study protocol originally specified that the lacewings would be exposed for 14 days. However, the study was terminated one day early due to an impending weather emergency, which forced the temporary closure of the testing facility on the day the study was scheduled to terminate. This did not compromise the validity of the study.

leaves) or control corn leaf protein had been added at a concentration of 1000 mg test or control substance/kg dry weight soil (1000 ppm dwt). The study design also included a negative control group, which was exposed to artificial soil only, without any added corn leaf protein. The test substance was estimated to contain *ca.* 3.64 mg VIP3A/g as measured by ELISA. Each test or control group consisted of four replicates containing ten worms per replicate, for a total of 40 worms/treatment group. Test endpoints included earthworm survival, body weights, and observations for sublethal effects.

After 14 days of exposure to test or control soil, earthworm survival was 100% in the VIP3A corn leaf protein group, the control corn leaf protein group, and the negative control group. No abnormal behavior or signs of toxicity were observed at the weekly observation times (days 7 and 14) in any test group. Earthworm weights were not statistically different among the three study groups before or after the 14-day exposure. A positive control reference test using chloroacetamide was also conducted on this lot of test worms; results were within the historical control range at the testing facility for chloracetamide, verifying the health and sensitivity of the test worms. The 14-day LC_{50} was >1000 mg corn leaf protein/kg dry weight soil (>3.6 ppm VIP3A in the test soil) and the NOEC was 1000 ppm (the highest concentration tested).

The results of a separate VIP3A soil bioactivity study support the conclusion that the earthworms in the VIP3A toxicity study were exposed to active VIP3A protein in the artificial soil substrate during a substantial portion of the 14-day exposure. As described in details in Part M of this Section, the persistence of VIP3A bioactivity was evaluated using the same VIP3A test substance and the same type of artificial soil as were used in the earthworm study. (Four live agricultural soils were also evaluated.) Two concentrations of the VIP3A test substance in soil were incubated for up to 29 days and aliquots of these soil mixtures were tested at weekly intervals for bioactivity against VIP3A-sensitive black cutworm *(Agrotis ipsilon)* larvae. The mixtures of VIP3A in artificial soil displayed initial lag phases of *ca.* 5 or 3 days, depending on the test concentration, during which there was no appreciable loss in bioactivity. Following this, the corresponding DT_{50}s (times to dissipation of 50% of the initial VIP3A bioactivity) were *ca.* 2 or 5 days for the same mixtures. The combined lag phases and DT_{50}s for each test concentration (5 + 2 days; 3 + 5 days) allow a conclusion that the earthworms were likely exposed to bioactive VIP3A protein for more than 7 – 8 days during the 14-day study. Measurements of larval mortality and weight following 7 days of soil incubation indicated that the VIP3A protein retained some of its initial activity in artificial soil, but this had largely dissipated after 14 days of incubation (see Tables 7.5 and 7.6).

6. 28-Day Survival and Reproduction Study of VIP3A Corn Leaf Tissue in *Folsomia candida* (springtails; Collembola: Isotomidae) *(Privalle, 2002e)*

A 28-day chronic *Folsomia candida* (Collembola: Isotomidae; springtail) survival and reproduction study was conducted to determine the potential effects of the lepidopteran-active VIP3A and Cry1Ab proteins on these organisms. The test material chosen for this study was lyophilized leaves, because the EPA had previously requested that collembola studies be conducted with lyophilized leaf material (US EPA, 2001). Forty juvenile collembola (four replicates of 10 collembola each) were exposed daily to freshly-prepared

diets containing equal parts of yeast (the standard *in vitro* diet) and powdered lyophilized leaves from transgenic VIP3A Event "Pacha"-derived or Cry1Ab Event "Bt11"-derived corn hybrids. Additional groups were similarly exposed to the corresponding corn leaf preparations from isogenic control hybrids, as well as from a transgenic hybrid that produced both VIP3A and Cry1Ab proteins, but was otherwise isogenic to the VIP3A hybrid. Yeast diet alone was provided to the negative control group, and yeast diet with thiodicarb (500 ppm) was provided to a positive control group. After 28 days of exposure, mean survival among the adult collembola that had matured from the initial juveniles ranged from *ca.* 78% – 83% among the corn leaf treatment groups and the negative control, and the differences among the groups were not statistically significant. Mean survival in the positive control group was *ca.* 3%, which was statistically lower than each of the other six treatment groups. The mean number of juvenile collembola recovered (resulting from eggs produced by the original 10 juveniles per replicate culture) in the corn leaf treatment groups ranged from *ca.* 344 to 533 per replicate and was, in most cases, statistically significantly higher than the mean number of juveniles produced in the negative control cultures (*ca.* 219 per replicate). A nutritional benefit of the corn leaf material may be responsible for these differences; it was evident that the collembola had consumed the leaf material, because the adults were observed to have green material internally. The differences between the VIP3A corn and Cry1Ab corn treatments and their respective corn controls were not statistically significant. Although the mean number of juveniles recovered from the combined VIP3A/Cry1Ab corn leaf treatment was statistically lower than in the corresponding control corn leaf cultures, this is likely the result of the relatively high number of juveniles produced in these control cultures as compared to all the other corn leaf treatments, and is not likely to reflect a biologically meaningful difference. The mean number of juveniles produced in the positive control cultures was significantly lower than in all other treatments (*ca.* 4 per replicate).

Reproduction was also analyzed as the number juveniles per surviving adult to normalize the numbers of offspring recovered in each replicate of a treatment for varying numbers of surviving adults among the replicates. The numbers of juveniles per adult survivor ranged from *ca.* 45 to 66 per replicate among all the corn leaf treatment groups and 29 per replicate in the negative control group. The differences between the transgenic corn leaf treatments and their respective control corn leaf treatments were not statistically significant. The numbers of juveniles per adult survivor were statistically lower in the negative control group as compared to the VIP3A corn group, the Cry1Ab corn group and the group representing the control for VIP3A and VIP3A/Cry1Ab corn leaves.

Dietary ingestion of corn leaf material containing VIP3A protein, Cry1Ab protein, or both proteins did not adversely affect survival or reproduction of collembola when compared to control corn leaf material or to a negative control group that did not receive any corn leaf material in the diet. The No-Observable Effect Concentrations (NOECs) of VIP3A and Cry1Ab in the diet were *ca.* 43.1 µg/g dry wt. (43.1 ppm) and 17.1 µg/g dry wt. (17.1 ppm), respectively, the highest concentrations tested in a diet consisting of 50% lyophilized corn leaves.

7. 48-Hour Static Renewal Toxicity Study of VIP3A Corn Pollen in Daphnia magna (Putt, 2002)

A 48-hour static-renewal test with VIP3A inbred corn pollen and isogenic control inbred corn pollen was conducted on *Daphnia magna* according to US EPA Guideline No. 72-2. The test pollen used (Test Substance PHOPACHA-0199; VIP3A inbred corn (maize) pollen) was estimated to contain *ca.* 83.8 μg of VIP3A protein/g pollen as measured by ELISA. Daphnids were less than 24 hours old at the time of study initiation, and were exposed to a single concentration of 120 mg test or control (isogenic) pollen/liter of water. This concentration was chosen as the maximum practical test concentration because, in previous studies, higher concentrations of control corn pollen were associated with unacceptable reductions in dissolved oxygen concentrations in the culture vessels. A negative control group, which was not exposed to any pollen in the test water, was also included in the study design. Each test or control group consisted of three replicate test vessels containing 10 daphnids each, for a total of 30 daphnids/treatment group. Daphnids were not fed during the exposure period. Daphnids were exposed for 48 hours, with complete renewal of the test or control suspensions after 24 hours. Each exposure vessel was aerated gently during the exposure period to maintain acceptable dissolved oxygen concentrations.

Survival was 100% in all replicates for the VIP3A inbred pollen, control inbred pollen, and negative control groups. All daphnids appeared normal throughout the study, and no immobilization or sublethal signs of toxicity were observed in any group. Dissolved oxygen concentrations and pH of the culture water were not different between the test and control pollen groups. After 48 hours of exposure, the EC_{50} based on immobilization was >120 mg pollen/liter for both the transgenic and control pollen groups, and the NOEC (No Observable Effect Concentration) was 120 mg VIP3A inbred corn pollen or control inbred corn pollen/liter (the highest concentration tested).

8. 30-Day Feeding Study in Channel Catfish (*Ictalurus punctatus*) with Fish Feed Prepared from VIP3A Corn Grain

Fish feed (Test Substance FFKPACHA-0100) was prepared from VIP3A corn grain, using methods that maximized the proportion of corn grain and the quantity of VIP3A protein in the fish diet and minimized the opportunity for degradation of the VIP3A protein during feed preparation. A commercial fish diet was formulated using standard feed components, however, the corn grain proportion of the diets (50% by weight) represented the maximum practical amount of corn grain in fish feed. This percentage of corn grain was approximately 25% higher than the proportion of corn grain that would typically be included in a commercial fish feed diet. During preparation, the feed was processed into pellets using a "cold pelleting" process. This served to minimize the potential for VIP3A degradation that might otherwise have occurred using typical feed pelleting and extrusion processes, which utilize significantly higher heat. The test feed was estimated by ELISA to contain *ca.* 7.1 μg VIP3A/g feed (7.1 ppm). Control fish feed was prepared in the same manner using corn grain from nontransgenic plants that were isogenic to the VIP3A corn. Test groups included a group of juvenile catfish receiving diet prepared with VIP3A corn grain and a group of catfish receiving diet prepared with control corn grain.

A 30-day feeding study was conducted following standard subchronic fish testing procedures. Three replicate test aquaria were established for each test and control group, with 10 fish per replicate (total of 30 fish per group). At study initiation, the mean individual wet weight of the fish in the VIP3A corn diet group and the control diet group was *ca.* 1.8 g/fish. Feed was

provided three times daily, at a daily feeding rate that represented *ca.* 6% of the total biomass in each aquarium. On test day 15, all fish were re-weighed and the amount of feed adjusted to the new total biomass. Throughout the feeding study, the measured pH, dissolved oxygen concentration, and temperature of the aquarium water were unaffected by the VIP3A corn fish feed or the control fish feed and remained within acceptable ranges for the survival of channel catfish. After 30 days, there were no statistically significant differences in mean wet weight increase for catfish exposed to the diet prepared from VIP3A corn grain compared to fish exposed to the diet prepared from isogenic control corn grain; the mean weight increase was *ca.* 1.5 g/fish in both groups. No abnormalities were noted among the catfish during the study except for one thin fish in the control corn diet group, which died on the last day of the study.

E. Estimated Exposure of Nontarget Organisms to VIP3A Protein

1. VIP3A Expression in Event COT102-Derived VIP3A Cotton Plants

Transgenic cotton line COT102 has been engineered for broad-spectrum lepidopteran insect resistance *via* expression of a gene encoding the VIP3A protein. Additionally, COT102-derived plants contain a gene encoding APH4, an enzyme that serves as a selectable marker.

The concentrations of VIP3A protein and APH4 protein were determined by ELISA for several plant tissues and whole plants at six developmental stages, for plants grown in multiple locations (Chapter 6).

For the primary plant tissues relevant to an assessment of potential non-target impacts, the VIP3A expression data are summarized in Table 7.3. For summary purposes, the data provided below represent the mean VIP3A concentrations measured across all locations for relevant plant stages, as well as the range of measured values across all locations, where applicable. The values summarized in Table 7.3 were used to calculate the "margin of exposure" estimates that appear elsewhere in this summary (see Part F, below). Data provided in Table 7.3 are expressed on a fresh-weight tissue basis. Chapter 6 also provides VIP3A concentrations on a dry-weight basis.

Table 7.3. VIP3A Concentrations (Ranges and Means) for Relevant Tissues of Event COT102-Derived Cotton Plants as Measured by ELISA[1]

Plant Tissue (Stage)	Approximate VIP3A Concentrations (µg VIP3A/g fresh wt.)
Whole plants (includes roots)	
Range across all locations and plant stages	0.47 – 15.13
Mean ± St. Dev. (peak bloom)	5.50 ± 1.21
Mean ± St. Dev. (pre-harvest)	1.76 ± 1.13
Leaves	
Range across all locations at four-leaf and squaring[2]	1.20 – 23.75
Mean ± St. Dev. (four-leaf and squaring)	16.43 ± 3.60
Mean ± St. Dev. (pre-harvest)	5.22 ± 1.94

Plant Tissue (Stage)	Approximate VIP3A Concentrations (μg VIP3A/g fresh wt.)
Seeds	
Range across all locations at pre-harvest stage	2.14 – 3.28
Mean ± St. Dev. (pre-harvest)	2.70 ± 0.20
Pollen (μg/g air-dried pollen)	1.09
Nectar	Not detectable[3]
Roots	
Range across all locations and plant stages	Below limit of quantification[4] – 2.69
Mean (all stages)	< 1.34

[1] Values are derived from Tables 6.1, 6.5 and 6.6 in Chapter 6, and are not corrected for extraction efficiency, which ranged from *ca.* 83.4 – 92.1% across the various cotton tissues described above. Due to the limited quantity of sample available, extraction efficiency was not established for pollen but it can be predicted as *ca.* 90% based on similar methodology for VIP3A corn pollen (Privalle, 2002b).

[2] Four-leaf and squaring stages had the highest VIP3A expression levels.

[3] Mean absorbance generated during ELISA did not exceed that of nectar from control plants.

[4] The lower limit of quantification by ELISA for root tissue was ca. 0.07 μg VIP3A/g fresh wt. (Table A-1 in Chapter 6).

2. Estimated Quantity of VIP3A Produced Per Acre and Estimated VIP3A Concentrations in Soil

Using the VIP3A values measured for the whole-plant samples (including root tissue), estimates of the quantities of VIP3A protein that may be present in COT102 derived plants on a per-acre and a per-hectare basis were calculated and are reported in the accompanying Section 6. Assuming a density of 60,000 VIP3A cotton plants per acre (EPA, 2001)(and 148,200 plants/hectare), estimates were calculated for COT102 plants at five developmental stages: four-leaf, first white bloom, peak bloom, first open boll, and pre-harvest (see Table 6.8 in Chapter 6). Using the mean measured VIP3A concentration in whole plants at the pre-harvest stage, the estimated VIP3A concentration in soil has been calculated (see below). These estimates are based on a six-inch (15-cm) soil depth, which is representative of a typical tilling depth. Therefore, after harvest, the whole cotton plants (including roots and bolls) are assumed to be chopped and tilled into the soil to a depth of six inches.

This estimation of the VIP3A concentration in soil incorporates several conservative assumptions, including the assumption that complete plants are tilled into the soil at harvest. The latter assumption likely results in a significant overestimation of VIP3A expression in soil because, in practice, defoliants are typically applied to cotton fields prior to harvest. Therefore, VIP3A in the leaves will likely degrade before being tilled into the soil. Additionally, for most COT102 plant tissues including leaves and whole plants, the lowest VIP3A expression levels were seen at the pre-harvest stage, the last stage sampled. This suggests that VIP3A concentrations in plant tissues likely continue to decline between the pre-harvest stage and the actual tilling of post-harvest plant residue into the soil.

The mean VIP3A concentration at pre-harvest stage is calculated as 42.46 g/hectare (18.81 g/acre) (see Table 6.8 in Chapter 6). After correcting for 91.6% extraction efficiency for whole-plant samples (see Table C-1 in Chapter 6), the corresponding value is 50.72 g

VIP3A/hectare (20.53 g VIP3A/acre). The top 15 cm (6 inches) of a 1-hectare field contains 1.5×10^9 cm^3 of soil; based on a density of 1.5 g/cm^3, this represents 2.25×10^9 g dry wt. soil (2.25×10^6 kg dry wt. soil). The estimated concentration of VIP3A in soil is calculated as follows:

$$\frac{50.72 \text{ g VIP3A}}{\text{ha}} \times \frac{1000 \text{ mg}}{\text{g}} \times \frac{\text{ha}}{2.25 \times 10^6 \text{ kg dry wt. soil}} = 0.02 \text{ mg VIP3A/kg dry wt.soil}$$

The estimated soil concentration of 0.02 mg VIP3A/kg dry wt. soil was used to evaluate potential exposure of non-target soil invertebrates to VIP3A protein. Although it is theoretically possible that VIP3A cotton plants might release VIP3A protein from roots into soil, thereby potentially exposing non-target organisms, such release would likely be inconsequential given that (1) VIP3A bioactivity substantially dissipates in a matter of days after introducing the protein into soil (Part M.) and (2) only sensitive organisms (*i.e.,* lepidopteran larvae) living in the soil zone adjacent to the roots might potentially be impacted, if sufficient VIP3A exposure were possible.

3. Estimated VIP3A Concentrations in Avian and Mammalian Wildlife Feed Items

In comparison to other types of agricultural habitats, conventional cotton is recognized as a relatively poor avian and wildlife habitat, primarily because cotton fields, including the field borders, are heavily managed for pest control. The most likely feed items for birds and small mammals that may feed in cotton are insects, soil invertebrates, and seeds. Some species may possibly ingest cotton leaves or seedlings, although observations suggest that these are not a preferred feed item, possibly because of the texture of cotton leaves and/or natural toxicant properties. Within a cotton field, insects and soil invertebrates that may have ingested VIP3A cotton plant material would only represent a secondary potential source of VIP3A exposure to foraging birds and small mammals. Two recent studies have indicated that pests feeding upon Cry1Ab Bt corn (events Bt11 and MON810) retain no detectable Cry1Ab protein (as in the case of aphids feeding upon corn phloem sap) or contain Cry1Ab concentrations that are *ca.* 9 – 143 times lower than those in the corn leaves consumed by the pests (as seen for various species of lepidopteran larvae)(Head *et al.*, 2001; Raps *et al.*, 2001). These studies support the assumption that indirect VIP3A exposure via by consumption of prey species feeding upon VIP3A cotton will be minimal. Therefore, the safety of VIP3A cotton will be evaluated by directly comparing concentrations of VIP3A in cotton leaves and seeds (Table 7.3) to results of avian and mammalian testing with purified protein. Estimates of avian and mammalian exposure to VIP3A protein are based on measured values of VIP3A in cotton tissues expressed on a fresh weight basis because, if these organisms ingest cotton tissue, they will ingest fresh tissue, not dried tissue.

The highest measured concentrations of VIP3A protein in leaves occurred in young, actively growing plants (measured at the four-leaf and squaring stages; Table 7.3). The highest VIP3A concentration measured in any individual sample of cotton leaves was 23.75 µg VIP3A/g fresh wt (23.75 ppm). The highest VIP3A concentration measured in cotton seeds was 3.28 µg VIP3A/g fresh wt. (ppm).

4. Estimated VIP3A Concentrations in Feed Items of Non-Target Beneficial Insects

Non-target beneficial insects may be directly exposed to VIP3A protein from COT102 plants through consumption of certain plant tissues, such as pollen. Insects feeding on cotton leaves will typically be considered pests. Nectar feeders will not be exposed to VIP3A protein, or will only consume a negligible quantity, because no VIP3A protein was detected in nectar (Table 7.3). To a minimal extent, indirect exposure may occur by ingestion of prey, such as lepidopteran larval pests, that have consumed plant tissue containing VIP3A. Detritivores may ingest VIP3A-containing plant tissue in their role of degrading plant tissue and recycling nutrients, although the potential VIP3A concentrations in decaying plant matter are expected to be significantly lower than those in fresh tissues. Non-target beneficial insects might also be exposed by contact to plant tissues expressing the VIP3A protein; however, the protein must be ingested to exert toxicity, so contact with these tissues does not represent a potential hazard to non-target insects or other invertebrates (*e.g.*, spiders).

Many foliar non-target beneficial insects consume a varied diet, which may include prey organisms along with pollen. The greatest potential exposure of these organisms is from ingestion of pollen containing VIP3A protein. As discussed above (Part E. 3.), two recent studies (Head *et al.*, 2001; Raps *et al.*, 2001) indicate that pest species feeding upon Bt corn plants retained little Cry1Ab protein (9 – 143 times lower concentrations than were present in the Bt plant tissue) or no Cry1Ab protein after feeding upon Bt corn plants expressing Cry1Ab. It can be inferred from these data that VIP3A concentrations will be uniformly low to zero in pest organisms that consume VIP3A cotton tissues.

The available evidence supports the conclusion that the only significant route of non-target exposure to VIP3A protein from VIP3A plants will be from direct consumption of plant tissue, and not via consumption of prey species that have fed upon VIP3A plants. Therefore, exposure of non-target beneficial insects to pollen containing VIP3A likely represents the highest potential exposure of these organisms to VIP3A protein. Measured concentrations of VIP3A in cotton pollen were 1.09 μg/g air-dried pollen (1.09 ppm)(Table 7.3).

Detritivores may be exposed to VIP3A protein in plant tissue that enters the litter zone of soil. Representative concentrations to which these organisms may be exposed range from concentrations in plant tissue alone (e.g., roots, decaying leaves) to concentrations in plant tissue mixed into soil (see above). Measured VIP3A concentrations in pre-harvest whole plant tissue averaged 1.76 μg/g fresh wt. (ppm) , while mean measured concentrations in pre-harvest leaves were 5.22 μg/g fresh wt. (ppm)(Table 7.3). Both of these values likely represent overestimates of the potential VIP3A concentrations that may be present in decaying plant matter. Root tissue of COT102 plants had uniformly low levels of VIP3A protein across all plant stages sampled; the mean concentration was < 1.34 μg/g fresh wt. (ppm)(Table 7.3).

5. Lack of Exposure to Aquatic Environments

The most likely exposure of aquatic organisms to VIP3A protein produced by VIP3A cotton would be through deposition of pollen on nearby water surfaces. However, cotton is not wind-pollinated, and the relatively heavy and sticky characteristics of cotton pollen preclude

significant dispersal by wind currents. Cotton is largely self-pollinated and, to some extent, insect pollinated, so the primary mechanism of cotton pollen dispersal would be *via* organisms (e.g., nectar feeders, pollinators) that visit cotton flowers. Therefore, it is unlikely that significant quantities of pollen from cotton plants expressing VIP3A protein would be deposited offsite on water bodies. Data concerning the offsite movement of cotton pollen expressing an introduced *nptII* gene (encoding neomycin phosphotransferase) confirm the low potential for cotton pollen movement (US EPA, 2001). By tracking the expression of the *nptII* gene in a buffer zone of commercial cotton, the resulting data indicate rapid and consistent reduction in pollen dissemination by insects with distance from a test plot of cotton expressing the introduced gene. By approximately 7 m from the test plot containing the transgenic cotton, outcrossing decreased from 5% to <1%, with low but detectable outcrossing out to a distance of 25 m (Umbeck *et al.*, 1991, as cited by US EPA, 2001). Based on the above information it can be concluded that there will be negligible to very low exposure of aquatic organisms to VIP3A protein. This conclusion is consistent with the EPA's assessment regarding the potential exposure of aquatic organisms to Bt cotton (US EPA, 2001).

F. Risk Assessment of VIP3A to Non-Target Organisms

1. Lack of Effects on Non-Target Organisms

No non-target organisms among the various representative organisms tested displayed adverse effects from exposure to VIP3A protein; in all cases, the no-observed effect concentration (NOEC) corresponded to the highest VIP3A concentration tested. Therefore, margins of exposure (MOEs) for different groups of non-target organisms were calculated as the ratio of the NOEC to the estimated exposure concentration (EEC). For birds and mammals, the VIP3A test quantities are expressed as doses (rather than concentrations), and the margins of exposure are calculated accordingly based on the NOEL (no observed effect level).

2. Margins of Exposure for Birds and Mammals

The margins of exposure (MOEs) for birds and small mammals that may ingest cotton plant material (leaves and/or seeds) containing VIP3A protein were calculated based on assumptions of feed ingestion rates for small birds and mammals. This approach is necessary because VIP3A content in cotton tissues is reported as a concentration (μg/g; ppm), whereas testing results are reported as a dose of VIP3A administered to the test animals (*i.e.*, by capsule or oral gavage). Therefore, estimates of daily feed ingestion rates, as a percentage of body weight, are needed to convert the concentration in plant tissue to a dose that birds and mammals would receive if they ingested a given quantity (defined by the feed ingestion rate) of plant tissue. Therefore:

μg/g VIP3A in plant tissue X g feed ingested/day (as a percent of animal body weight) = dose

- Assessments for birds, such as a small passerine, consuming primarily an invertebrate or herbivorous diet are based on a 10 g bird ingesting 80% of its body weight/day (US EPA, 1993) = 8 g/day.

- Assessments for birds, such as quail, consuming primarily a seed diet are based on a 180 g bird ingesting 10% of its body weight/day (US EPA, 1993) = 18 g/day.

- Assessments for mammals consuming primarily an herbivorous diet are based on a 35 g mammal, such as a vole, ingesting 40% of its body weight/day (US EPA, 1993) = 14 g/day.

- Assessments for mammals consuming a seed diet are based on a 20 g mammal, such as a mouse, ingesting 20% of its body weight/day (US EPA, 1993) = 4.0 g/day.

Because the same daily ingestion rate is used for birds and mammals consuming herbivorous diets and seeds, the daily dose (in mg/kg body weight) will be the same despite differences in body weight and the quantities of feed ingested.

Using the above assumptions, the estimated dose for birds consuming an herbivorous diet based on the maximum measured VIP3A concentration in leaves (23.75 µg/g; Table 7.3) is 19.0 mg VIP3A/kg body weight (bwt), and the estimated dose based on the mean measured VIP3A concentration in leaves of young plants (16.43 µg/g at four-leaf and squaring growth stages; Table 7.3) is 13.14 mg VIP3A/kg bwt. Compared to a maximum tested avian dose of 400 mg VIP3A/kg bwt (see Part D.3.1.), the MOEs are 21X and 30X for the maximum and mean doses that small birds may ingest by consuming leaves of young COT102 plants.

For a small mammal consuming primarily an herbivorous diet, based on the maximum measured VIP3A concentration in young leaves (23.75 µg/g), the estimated dose is 9.5 mg VIP3A/kg bwt. The estimated dose based on the mean measured VIP3A concentration in leaves of young COT102 plants (16.43 µg/g) is 6.57 mg VIP3A/kg bwt. Compared to the NOEL of 3675 mg VIP3A/kg bwt (corresponding to the highest dose tested) in an acute oral mouse study (Vlachos, 2002b), the MOEs for small mammals are 387X and 559X for doses derived from maximum and mean VIP3A concentrations, respectively, in leaf tissue of young COT102 plants.

For seed-eating birds the estimated dose based on maximum measured VIP3A concentrations in COT102 seeds (3.28 µg/g; Table 7.3) is 0.33 mg VIP3A/kg bwt, and the estimated dose based on mean measured concentrations in seeds (2.70 µg/g; Table 7.3) is 0.27 mg VIP3A/kg bwt. Compared to a maximum tested avian dose of 400 mg VIP3A/kg bwt (see Part D.3.1.), the MOEs are > 1200X and nearly 1500X, respectively.

For small seed-eating mammals, the estimated dose based on the maximum measured VIP3A concentration in COT102 seeds (3.28 µg/g) is 0.66 mg VIP3A/kg bwt, and the estimated dose based on the mean measured VIP3A concentrations in seeds (2.70 µg/g) is 0.54 mg VIP3A/kg bwt. Compared to the NOEL and maximum mammalian test dose of 3675 mg VIP3A/kg bwt, MOEs for small seed-eating mammals are > 5500X and > 6800X, respectively.

3. Margins of Exposure for Pollinators

As discussed above, cotton is primarily self-pollinated, although insect pollination does occur. Therefore, non-target pollinating insects such as bees are likely to be exposed to pollen containing VIP3A. However, as discussed in Part D.3.2., above, cotton pollen does not appear to be a preferred pollen for honeybee foragers because it does not fit well into the pollen basket of honeybees (Vaissière and Vinson, 1994). Other hymenopterans, such as bumblebees, however, may forage for cotton pollen as well as play a role in pollinating cotton. Pollinating insects may be exposed to VIP3A-containing pollen through both contact and, for some species, through ingestion. While the exposure may be greater through contact as bees are visiting flowers, VIP3A must be ingested by an insect to affect susceptible species. Pollinating insects will typically consume nectar produced by cotton flowers; however assays of COT102 nectar have not detected any VIP3A protein (Table 7.3).

As discussed in Part D.3.2., above, bumblebee larvae eat most of the pollen supply brought back to the nest; adult bees eat relatively little (Free and Butler, 1959). However, the extent to which cotton pollen, *per se*, may contribute to the total pollen diet of bumblebees and other bee species is uncertain. In testing, larval honeybees were directly exposed to 2 mg corn pollen containing 83.8 µg/g VIP3A through ingestion (see Part D.3.2., above). This concentration in corn pollen is 77-fold greater than the measured concentrations of VIP3A in cotton pollen (1.09 µg/g; Table 7.3). No adverse effects were noted on larval survival, adult emergence, or behavior or morphology of emerged adults.

4. Margins of Exposure for Foliar Beneficial Insects

A variety of predatory and omnivorous beneficial insects may be present in the foliage of cotton plants. Because VIP3A must be ingested to exert its effects on susceptible species, contact of these organisms with cotton tissues expressing VIP3A does not raise concerns. Similarly, as described in Part E.3., above, data from two recent studies on Cry1Ab Bt corn (Head *et al.*, 2001 and Raps *et al.,* 2001) indicate that lepidopteran larval pests that consumed Bt corn tissue retained uniformly low amounts of Cry1Ab protein as compared to the Bt plant tissue consumed, and that aphid pests retained no detectable Cry1Ab. These data suggest that beneficial predatory insects will not consume significant quantities of VIP3A protein via multi-trophic mechanisms. Moreover, Bt insecticidal proteins do not possess characteristics that are consistent with any potential bioaccumulation through the food chain; they are not preferentially sequestered in fatty tissue, nor are they resistant to metabolic degradation.

Two representative foliar beneficial insects, lady beetles and green lacewings, have been tested by incorporating corn pollen into artificial diets developed for these test species. Young adult lady beetles were exposed to diets containing pollen at 5% w/w (5 mg pollen/100 mg diet), while young adult lacewings were exposed to diets containing pollen at 15% w/w (15 mg pollen/100 mg diet). The VIP3A concentration in the tested pollen was 144.8 µg/g. Lady beetles were exposed to the test diets for 21 days, while lacewings were exposed for 13 days; fresh diet was presented to the insects daily during the studies. Based on the VIP3A content of the test pollen the lady beetles were exposed to a dietary concentration of 7.24 µg VIP3A/g diet (ppm), and the lacewings were exposed to a dietary

concentration of 21.72 µg VIP3A/g diet (ppm). No adverse effects were noted on either adult lady beetle survival or behavior, or on lacewing survival or behavior. The pollen used in testing contained approximately a 130-fold greater VIP3A concentration than concentrations measured in VIP3A cotton pollen (1.09 µg VIP3A/g; Table 7.3). Therefore, even if lady beetles or lacewings were to actually consume diets containing a greater proportion of VIP3A pollen than was used in the test diets, an adequate margin of exposure exists for COT102 plants based on the fact that a much higher VIP3A concentration was present in the corn test pollen than has been measured in cotton pollen from COT102 plants.

5. Margins of Exposure for Soil Invertebrates

Two species of beneficial soil invertebrates, earthworms and collembola, were tested using corn leaf material expressing VIP3A. As described in Part D.3.5., above, earthworms were tested by incorporating VIP3A-containing test material into soil at a concentration of 1000 mg/kg dry wt. soil; based on the VIP3A content of the tested leaf material of 0.36% w/w, this represents a VIP3A concentration of 3.6 mg/kg dry wt. soil (ppm). Earthworms were exposed for 14 days to soil containing VIP3A protein. No adverse effects were noted on earthworm survival or body weights. As discussed in Part E.2., above, the estimated concentration of VIP3A in the uppermost 6 inches (15 cm) of soil is 0.02 mg/kg dry wt. soil (ppm). The VIP3A concentration to which earthworms were exposed *via* soil was 180X higher than this conservatively estimated soil concentration.

As described in Part D.3.6., above, collembola were exposed to VIP3A in their diet during a 28-day study survival and reproduction study. A lyophilized preparation of VIP3A leaves material was mixed into the diet daily at 50% of the diet, for a final VIP3A concentration of 43.1 ppm on a dry wt. basis. No adverse effects on survival or reproduction of collembola were noted. As described above, measured VIP3A concentrations are highest in the leaves of young plants and are lowest late in the growing season. The VIP3A concentrations in pre-harvest stage (the latest plant stage for which data are available) COT102 whole plants can be used to conservatively estimate the potential VIP3A concentrations in plant litter to which collembola may be exposed, although decaying post-harvest plant residue will likely contain significantly lower VIP3A concentrations because cotton plants are typically defoliated shortly before harvest, and the remaining plant will undergo senescence before being shredded and tilled into the soil (see discussion in Part E.2.). Maximum and mean VIP3A concentrations in pre-harvest whole plants are 7.68 and 3.61 µg/g dry wt. (ppm), respectively (derived from Table 6.2 in Chapter 6). Collembola dietary test concentrations of VIP3A were *ca.* 6X and 12X higher than these VIP3A concentrations in whole pre-harvest COT102 plants. If the estimated soil concentration of VIP3A [0.02 mg VIP3A/kg dry wt. soil (ppm); see Part E. 2.)] is alternatively used as an EEC for collembola, the MOE based on the NOEC from the 28-day collembola study is > 2100X.

6. Margins of Exposure for Aquatic Organisms

As described in Part E.5., above, the potential exposure of fish and aquatic invertebrates to cotton pollen containing VIP3A protein is very low. Additionally, EPA waived the data requirements for aquatic species testing because of a lack of exposure for Cry1Ac cotton.

Only very limited amounts of pollen would be available for drift and exposure to aquatic invertebrates (US EPA, 2001).

As described in Part D.3.7., above, *Daphnia magna,* a freshwater indicator species was tested using corn pollen containing VIP3A protein; the NOEC represented the highest concentration tested, 120 mg pollen/liter. Based on the VIP3A concentration in the tested pollen, this represents a concentration of 10 μg VIP3A/liter. Based on the expectation that there will be negligible to very low exposure of daphnids or other aquatic species to VIP3A cotton pollen, the calculation of a MOE is not warranted. This conclusion also applies to fish and other aquatic species.

Although the channel catfish feeding described above (see Part D.3.8.) demonstrated no adverse effects on juvenile fish exposed to feed containing VIP3A protein for 30 days, the report of this study (Dionne, 2002) is not yet available. However, in view of the probable lack of environmental exposure of fish to VIP3A protein from COT102 plants, Syngenta Seeds is requesting that any requirement for submitting the report of this fish toxicity study be waived.

G. Conclusions of Laboratory Tests on Non-Target Species

In summary, VIP3A presents a negligible risk to non-target birds, wild mammals, aquatic organisms, and beneficial or non-target foliar and soil organisms. VIP3A concentrations in cotton-based avian and mammalian wildlife feed items are well below the highest VIP3A doses tested, which had no adverse effects on the tested species. VIP3A is expressed in low concentrations in cotton pollen, and the properties of cotton pollen (heavy, sticky, and not wind-borne) result in a low potential for dispersal of cotton pollen to water bodies. Testing with pollinators (honeybees) and representative omnivorous predatory beneficial insects (ladybeetles and lacewings) by incorporating pollen expressing high concentrations of VIP3A into their diet resulted in no adverse effects at concentrations greater than these organisms are likely to be exposed to from VIP3A in cotton. Similarly, earthworms showed no adverse effects when exposed to VIP3A protein in soil at concentrations greater than those expected from VIP3A cotton, and collembola showed no adverse effects when tested with dietary concentrations of VIP3A greater than those expected from ingesting VIP3A cotton plant material. Additionally, testing using high aquatic concentrations of VIP3A corn pollen expressing much higher concentrations of VIP3A than does COT102 cotton pollen resulted in no adverse effects on sensitive invertebrate indicator species. All the available evidence indicates that no non-lepidoteran species will be directly impacted by cotton plants expressing VIP3A protein.

H. Field Testing

In addition to the laboratory testing described above, field tests with corn expressing VIP3A have been conducted to evaluate potential effects on honeybees and other non-target terrestrial invertebrates.

1. Honeybee Semi-Field Study (Dively, 2002) (USDA Permit # 01-022-07R/M)

The impact of transgenic lepidopteran-resistant VIP3A field corn on honeybee colonies was assessed in a semi-field setting. Foraging behavior, bee mortality and fitness, and colony performance of hives placed in plots of a VIP3A hybrid were compared to hives in plots of an isogenic control hybrid. The study design consisted of four replicate plots of corn expressing VIP3A protein and four replicate plots of an isogenic control corn. Duplex nuclear hives were placed in each plot. Each plot was enclosed in a cage that was approximately 25 feet long by 25 feet wide, so that approximately 375 plants were enclosed in the cages. The caged areas were surrounded by additional VIP3A hybrid or isogenic hybrid corn. Exclusive access to corn pollen during anthesis and consumption of provisioned pollen cakes from additional pollen collected from the same plots exposed hives for three weeks to levels of corn pollen higher than those encountered in nature. The percentage of bees returning with pollen pellets was significantly different over time but not between hybrids. Pre- and post-exposure measurements of bees, food stores and brood development per hive showed that the overall performance of colonies to produce brood, manage stores of food, and recruit new bees was not affected by exposure to the VIP3A protein. Furthermore, survival of developing pupae and the consequent body size of newly-emerged bees were not affected by exposure to pollen from the transgenic hybrid. Although more dead bees were found around hives in transgenic plots, it was not possible to accurately measure mortality or express it as a percent of the total bees in the hive because of different initial populations of bees in the hives. The difference in the number of dead bees was attributed in part to the larger colonies that were placed in the transgenic plots.

2. **Non-target Terrestrial Invertebrate Field Testing with VIP3A Corn (Dively et al., 2002)** (USDA Permit #'s 00-024-01R and 01-022-07R/M)

A large-scale two-year field study was conducted to determine the possible effects of a "stacked" transgenic lepidopteran-resistant field corn (maize) hybrid on the non-target invertebrate community. This corn hybrid produces two lepidopteran-active insecticidal proteins, VIP3A (from transformation event Pacha) and a truncated Cry1Ab (from an independent transformation event), which have been combined in the same genotype by conventional breeding. Use of a hybrid producing these two proteins, which are present season-long throughout the plant, is expected to provide a more conservative estimate of potential non-target effects than a hybrid producing either protein alone. The summary below describes the findings from the first year of the study, as a detailed summary of the second year results is not yet available, however, the findings from the second year of testing were generally consistent with the first year's results.

The field study was conducted in an agricultural region of Queen Anne's County, Maryland, under the direction of Dr. Galen Dively, University of Maryland Professor and Extension Specialist. The lepidopteran-resistant (LR) transgenic hybrid was compared to 1) a non-transgenic, isogenic hybrid without additional insecticide treatments (isogenic control; non-LR) and 2) a non-transgenic, isogenic hybrid treated with a foliar insecticide for lepidopteran control (non-LR sprayed). Within the interior of a 28-acre field, nine one-acre plots with each hybrid replicated three times, were arranged as a Latin square. Buffer zones between blocks and the remaining area of the field were planted in non-transgenic field corn. Plots were planted no-till on 1 June 2000 into old soybean stubble. Standard agronomic practices were used, including seeds treated with fungicidal protectants, starter and side-dress

applications of fertilizer, burn-down and residual herbicides at planting to control weeds, and rotary mowing after harvest to shred the crop refuse. No other agronomic practices were applied, except for the foliar insecticide treatments targeted to control lepidopteran pests. The non-LR sprayed hybrid received two broadcast applications of lambda-cyhalothrin, the first for black cutworm (*Agrotis ipsilon*) control at 14 days after planting, and the second at peak anthesis for control of second-generation larvae of European corn borer (*Ostrinia nubilalis*). Both treatments were applied at the rates and timing consistent with standard pest management practices for these pests.

The abundance and diversity of invertebrates representing both foliage-dwelling and soil surface fauna were monitored by visual inspections of plants, pitfall traps, sticky cards, and ear/litter extractions throughout the growing season and post-harvest. All sampling was conducted on a weekly or biweekly basis within a central area within each plot to avoid potential edge effects. The treatment and time effects on the frequency of occurrence and mean abundance of each taxon were tested by ANOVA. The Shannon diversity index and the mean number of taxa were used to compare community composition of the invertebrate fauna. The principal response curve method was used to distill the time-dependent, community-level effects of the hybrid treatments into a graphical form. A Monte-Carlo permutation method was used to determine if the time by treatment effects were statistically different from the control.

A total inventory of over 200,000 organisms representing 78 families in the corn system was enumerated. For the carabid beetles alone, 30 species in 15 genera were recorded. In terms of diversity and abundance, approximately fifty-two percent of the invertebrates were saprophytes, mainly dominated by soil-litter detritivores such as springtails (Collembola), broad mites (Parsonemidae), oribatid mites (Oribatida), psocids (Psocoptera), sowbugs (Isopoda), millipedes (Diplopoda), and various fly (Diptera) larvae. Included in this group was a diverse assemblage of seven families (Corylophidae, Phalacridae, Crytophagidae, Mycetophagidae, Lathridiidae, Oedemeridae, Pselaphidae) of beetles (Coleoptera) that feed primarily on fungal growth associated with degraded pollen and senescent plant tissue. Sap beetles (Nitidulidae) occupied many niche-places of the corn plant and surface litter, and their feeding behavior trophically bridged the detrital and herbivore guilds of the food web.

Forty-two percent of the invertebrates recorded were herbivorous insects feeding on various parts of the corn plant. The most dominant taxa were aphids (*Rhopalosiphum maidis*; Aphididae), thrips (Thripidae), leafhoppers (Cicadellidae), leaf miners (Agromyiidae), seed-feeding ground beetles (primarily *Amara* spp., Carabidae), and lepidopteran larvae. A smaller portion of the invertebrate community consisted of organisms in the higher trophic levels. Five percent were foliage- and ground-dwelling predators, primarily minute pirate bugs (Anthocoridae), ladybird beetles (primarily *Coleomegilla maculata*), lacewings (*Chrysopa*), predaceous mites (Mesostigmata), ants, spiders (primarily Lycosidae), ground beetles (Carabidae), rove beetles (Staphylinidae), and centipedes (Geophilomorpha). Many of these organisms are omnivorous and known to shift to pollen and extrafloral nectar sources when prey items are scarce. Also, many carabid and staphylinid beetles feed on seeds and fungal spores. Only about one percent of the invertebrates were parasitic.

This study clearly demonstrated that VIP3A and Cry1Ab protein expression in the stacked transgenic hybrid has a significant impact on target lepidopteran species. The incidence of seedling defoliation caused by an outbreak population of saltmarsh caterpillars (*Estigmene acrea*) was reduced by 81% as compared to the non-transgenic control hybrid. The percentage of ears damaged by corn earworm (*Helicoverpa zea*) averaged 89% and 2%, respectively, in the non-transgenic control and LR hybrids. Moderate levels of stalk injury by European corn borer were reduced by 98% in the LR hybrids. These effects mediated by the host plant also had a significant indirect impact on certain other non-lepidopteran herbivores. Sap beetle populations were significantly lower in the LR corn community as a response to less stalk and ear injury by lepidopteran species. These beetles are attracted to the injury and frass produced by corn borers feeding on tassels and stalks, and by corn earworms feeding on developing kernels. It was also predicted that dipterans would experience similar changes in abundance because they were presumed to be attracted to injured plant parts and by-products of caterpillar feeding. However, population densities of dipteran families were virtually the same in the transgenic and isogenic control plots. Apparently, flies were able to use food resources of the corn plant and ground litter in niches that were independent of plant injury.

Aside from the anticipated effects on lepidopteran target pest species and the apparent indirect effects on sap beetles, the stacked LR hybrid had no significant negative effects on the invertebrate communities enumerated by the various sampling methods. There was no evidence of any cascading effects on upper trophic levels, such as predators, associated with the lower densities of sap beetles and lepidopteran larvae. The diversity and abundance of minute pirate bugs, ladybird beetles, lacewings, damselbugs (Nabidae), and other foliage-inhabiting predators did not significantly change over time in the LR corn community relative to the non-LR isogenic control. Pitfall captures of surface-dwelling invertebrates, primarily springtails, spiders, ants, ground beetles, centipedes, rove beetles, slugs (Agrolimacidae), sowbugs, and crickets (Gryllidae), were not significantly affected by the LR hybrid. Numbers of parasitic hymenopterans and flies captured on sticky cards followed closely the densities of these same taxa in the non-LR control plots. After the crop was harvested, the invertebrate communities found in the surface litter in the LR and sprayed non-LR plots were generally similar to the control community. The only deviation from the control community occurred during the last two sampling dates. On those dates, densities of spiders, psocids, springtails, and fungivorous beetles were significantly higher in the LR corn compared to the non-LR control. This response may have been related to the plants because plant senescence was slightly delayed and the foliage stayed green longer in the LR hybrid.

As expected, the pyrethroid insecticide applications to the non-LR sprayed plots had a significant negative impact on both pest species and many non-target invertebrate taxa. The sprayed plots had 67% less stalk damage and 18% less ear damage caused by lepidopteran pests compared to the non-LR control. Most changes in community diversity and abundance were predictable based on previous reports of the non-target effects of broad-spectrum insecticides. Sowbug populations were drastically reduced after both insecticide spray applications and showed no signs of recovery throughout the study. Densities of many key predators, including spiders, ladybird beetles, minute pirate bugs, rove beetles, and certain genera of carabid beetles significantly declined for three to four weeks after the second insecticide spray was applied. Most of these predators showed trends toward population recovery later in the crop season. Fungivorous insects, mainly minute fungus beetles

(Corylophidae), shining mold beetles (Phalacridae), and psocids, and herbivores such as leafhoppers and flea beetles (Alticinae) were particularly sensitive to the pyrethroid insecticide. Spider mites, aphids, and sap beetles were initially reduced in numbers for several weeks but then their populations rebounded to higher levels later in the crop season. Two groups, springtails and parasitic wasps, increased significantly after the second insecticide treatment. High recruitment rates of adult parasitoids emerging from host stages (*e.g.*, eggs, pupae, mummies) that were not affected by the insecticide, and negative insecticidal effects on predators of springtails are possible explanations.

Results of this first-year study lend strong support to the findings of other published reports that there are no unexpected multitrophic effects from transgenic lepidopteran-resistant crops on non-target organisms (see summary by ABSTC-NTO, 2002; MRID #45652001). Some changes in certain taxa did occur but were indirectly linked to plant-mediated factors and the absence of feeding injury by target pest species. The communities of non-target invertebrates inhabiting the foliage and soil surface in the insecticide-sprayed plots displayed statistically significant changes in species abundance, diversity, and trophic interactions that resulted in resurgence in certain pest species; a number of these non-target groups showed evidence of recovery towards the end of the growing season.

I. Potential for Exposure of Non-Target Lepidopteran Larvae to VIP3A Protein from VIP3A Cotton Plants

Non-target butterfly and moth larvae do not feed directly on cotton plants. (Lepidopteran species that feed directly on cotton are, by definition, pest species.) The opportunities for exposure of non-target lepidopteran larvae *via* VIP3A pollen deposition on their host plants will be quite limited, given (1) cotton pollen is relatively heavy, clumpy and moisture-laden, and cotton is not wind-pollinated but is largely self-pollinated, factors that greatly limit the potential for off-site movement through drift; (2) the very small amounts of pollen that might theoretically be deposited on host plants by pollinating insects; and (3) the relatively few host plants that occur within or sufficiently close to cotton fields. Additionally, not all non-target lepidopteran species are expected to be sensitive to VIP3A protein. For example, in a controlled, no-choice laboratory study, first-instar *Danaus plexippus* (monarch butterfly), the stage most sensitive to specific Bt Cry proteins (Hellmich *et al.,* 2001), showed no mortality or growth delay when exposed to 1000 ng VIP3A/cm^2 diet surface (standard Monarch Watch laboratory diet) for 96 hours (Syngenta Seeds, unpublished data). This VIP3A test concentration was higher than the 96-hour LC_{90}s (the concentrations causing 90% mortality) for first-instars of the VIP3A-sensitive pest species *Spodoptera frugiperda* (LC_{90} = 341.1 ng VIP3A/cm^2; 95% confidence interval = 264.4 – 555.8), *Agrotis ipsilon* (LC_{90} = 180.3 ng VIP3A/cm^2; 95% confidence interval = 142.8 – 265.6), and *Helicoverpa zea* (LC_{90} = 872.0 ng VIP3A/cm^2; 95% confidence interval = 426.1 – 52,417.1) that had been exposed to the same test material (Privalle, 2002f).

J. Endangered Species Considerations

1. Endangered Lepidopterans

Endangered or threatened moths and butterflies may conceivably be sensitive to VIP3A protein, given that the protein is selectively toxic to certain lepidopteran species. All current federally listed (US Fish and Wildlife Service, 2002) endangered or threatened moth and butterfly species are shown below in Table 7.4. While it is not possible, due to their status, to directly test listed endangered species for sensitivity to VIP3A protein, it can be concluded that larvae of these species will not be exposed to VIP3A expressed in cotton. In general, these species occur on very limited acreage and are endangered or threatened because of habitat destruction and/or reduced availability of the single or few species of host plants that will support larval survival for a specific species. None of the listed species feeds directly on cotton plants and their potential for exposure to cotton pollen is negligible. Cotton is primarily self-pollinated, although some insect pollination occurs. There is minimal potential for cotton pollen to become windborne or drift, although small amounts of cotton pollen may be dispersed by pollinators As described in the section concerning potential exposure of aquatic organisms, there is a very low potential for off-site movement of cotton pollen, and therefore exposure of endangered lepidopterans.

In its recent reassessment of the environmental safety of *Bt* cotton (expressing a *Bt* Cry1Ac lepidopteran-active toxin), the EPA concluded that, although three endangered or threatened lepidopteran species occur in cotton-growing counties of California (Quino Checkerspot butterfly and Kern Primrose Sphinx moth) and North Carolina (St. Francis' Satyr butterfly), the larvae of these species do not feed on cotton and will not be exposed to the Bt protein because their habitats do not overlap with cotton fields and the amounts of cotton pollen (if any) that might be deposited on their host plants would be negligible and have no impact (US EPA, 2001). These conclusions for Cry1Ac Bt cotton will also be applicable to VIP3A cotton.

In addition, no VIP3A cotton will be grown in proximity to areas of Hawaii or Florida where gene outcrossing to wild or weedy relatives of cotton might be possible. Therefore, such plants will not potentially serve as a source of VIP3A-containing pollen that might be consumed by endangered or threatened species in those areas.

Table 7.4. Lepidopteran insect species federally listed as endangered or threatened (US Fish and Wildlife Service, 2002)

Status[1]	Species Name
T	Butterfly, bay checkerspot (*Euphydryas editha bayensis*)
E	Butterfly, Behren's silverspot (*Speyeria zerene behrensii*)
E	Butterfly, callippe silverspot (*Speyeria callippe callippe*)
E	Butterfly, El Segundo blue (*Euphilotes battoides allyni*)
E	Butterfly, Fender's blue (*Icaricia icarioides fenderi*)
E	Butterfly, Karner blue (*Lycaeides melissa samuelis*)
E	Butterfly, Lange's metalmark (*Apodemia mormo langei*)
E	Butterfly, lotis blue (*Lycaeides argyrognomon lotis*)
E	Butterfly, mission blue (*Icaricia icarioides missionensis*)
E	Butterfly, Mitchell's satyr (*Neonympha mitchellii mitchellii*)
E	Butterfly, Myrtle's silverspot (*Speyeria zerene myrtleae*)
T	Butterfly, Oregon silverspot (*Speyeria zerene hippolyta*)
E	Butterfly, Palos Verdes blue (*Glaucopsyche lygdamus palosverdesensis*)
E	Butterfly, Quino checkerspot (*Euphydryas editha quino*)
E	Butterfly, Saint Francis' satyr (*Neonympha mitchellii francisci*)
E	Butterfly, San Bruno elfin (*Callophrys mossii bayensis*)
E	Butterfly, Schaus swallowtail (*Heraclides aristodemus ponceanus*)
E	Butterfly, Smith's blue (*Euphilotes enoptes smithi*)
E	Butterfly, Uncompahgre fritillary (*Boloria acrocnema*)
E	Moth, Blackburn's sphinx (*Manduca blackburni*)
T	Moth, Kern primrose sphinx (*Euproserpinus euterpe*)
E	Skipper, Carson wandering (*Pseudocopaeodes eunus obscurus*)
E	Skipper, Laguna Mountains (*Pyrgus ruralis lagunae*)
T	Skipper, Pawnee montane (*Hesperia leonardus montana*)

[1] T =Threatened; E = Endangered

2. Endangered Non-Lepidopteran Insects

In addition to the endangered or threatened lepidopteran species discussed above, several species of beetles (Coleoptera), one dragonfly (Odonata) species, one fly (Diptera) species, one naucorid (Hemiptera) species and one grasshopper (Orthoptera) species are federally listed as endangered or threatened (US Fish and Wildlife Service, 2002). Due to the demonstrated lack of VIP3A toxicity to non-lepidopteran species, it is not expected that these other insect species would be sensitive to the VIP3A protein, even if opportunities existed for exposure. This conclusion is supported by the laboratory studies demonstrating lack of VIP3A toxicity in non-target species representing several invertebrate Orders.

K. Gene Flow and Weediness

EPA recently concluded its environmental reassessment of the registered *B.t.* plant-incorporated protectants and summarized its findings in the "Biopesticides Registration

Action Document" for these products (US EPA, 2001). In its reassessment of Bt cotton, EPA reviewed the potential for gene capture and expression of the Cry1Ac endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories, and concluded that the possibility for gene transfer exists only in limited geographic locations where wild or feral cotton relatives exist, *i.e.* in Florida, Hawaii, and the Caribbean. In addition, the USDA/APHIS has made this same determination under its statutory authority. These conclusions made with respect to commercial Bt cotton (producing a Cry1Ac endotoxin) are also applicable to VIP3A Bt cotton. Accordingly, the same geographical restrictions (*e.g.,* no commercial plantings in Hawaii and south Florida) that are currently in effect for Cry1Ac Bt cotton are expected to be applicable to VIP3A cotton.

L. Potential for Horizontal Gene Transfer

In its recent reassessment of the environmental safety of Bt plant-incorporated protectants, EPA conducted an extensive review of information relevant to the theoretical potential for horizontal gene transfer, *i.e.,* the possibility that genes from Bt crops, including marker genes conferring antibiotic resistance, might be transferred to soil organisms (US EPA, 2001). EPA concluded that the possibility of this occurring is very remote, and that the potential consequences of horizontal gene transfer, if it occurred, would pose no significant risk. This assessment is also relevant to event COT102-derived cotton plants, which express the *vip3A(a)* gene from *Bacillus thuringiensis* and the *aph4* marker gene isolated from *E. coli.* Both of these transgenes (or genes with high homology and functional equivalence to these genes) are known to occur naturally in soil microbes.

M. Environmental Degradation of VIP3A Protein in Soils

In the context of conducting an ongoing environmental assessment of a VIP3A corn product destined for use in Brazil, Syngenta has evaluated the loss of VIP3A biological activity after introducing the protein into samples of various live agricultural soils. A summary of that study is provided below, and a complete report, titled "Biological Activity of VIP3A Maize (Corn) Leaf Protein (Sample LPPACHA-0199) in Various Soils," is being submitted concurrently with this Section 3 application (Privalle, 2002c).

Based on the measured concentrations of VIP3A in COT102-derived cotton plants (Chapter 6) and using highly conservative assumptions, an estimated soil concentration of 0.02 mg VIP3A/kg dry weight of soil (0.02 ppm) was calculated and used to evaluate potential exposure of non-target soil invertebrates to VIP3A protein (see accompanying summary report titled "Environmental Safety Assessment of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Event to Non-Target Organisms"; Vlachos and Habig, 2002). The levels of VIP3A protein tested in the described soil bioactivity study (58 and 14 μg VIP3A/g dry wt. equivalent soil, or 58 and 14 ppm) were in great excess (2900X and 700X, respectively) to the conservative estimate of expected VIP3A protein levels in soil. Therefore, the existing VIP3A soil bioactivity study adequately addresses any prospective concerns regarding the potential persistence or accumulation of VIP3A protein in agricultural soils.

Study Summary: Biological Activity of VIP3A Maize (Corn) Leaf Protein (Test Substance LPPACHA-0199) in Various Soils (Privalle, 2002d)

Four live soils (three from different agricultural regions of Brazil; one from Illinois, USA) and one artificial soil were used to assess whether VIP3A protein retained biological activity over time following incorporation into diverse, representative soil types. The source of VIP3A protein for this study was test substance LPPACHA-0199, a lyophilized material prepared by extracting protein from leaves of transgenic VIP3A maize (corn) plants and concentrating the VIP3A fraction. The soils used in this study represented four textures: clay, sandy clay loam, sandy loam, and silt loam. Test substance LPPACHA-0199 was tested at two concentrations, 16 mg/g and 4 mg/g dry wt. equivalent soil (corresponding to *ca.* 58 and 14 µg VIP3A/g dry wt. equivalent soil, respectively). The soil mixtures were sampled over a 29-day period and tested for bioactivity against a target lepidopteran larval pest of VIP3A maize, the black cutworm *(Agrotis ipsilon).* The loss of bioactivity in the soil samples, as defined by a decrease in insect mortality, was used to estimate the DT_{50} (time to dissipation of 50% of the initial bioactivity) for each soil and test concentration. Following an initial short lag phase for all soil types at both LPPACHA-0199 test concentrations, there was a rapid decline in VIP3A bioactivity. The initial lag phase may have represented an equilibration period, during which microbial communities adapt to the test conditions before exponential microbial growth and subsequent increased microbial activity. Lag phases for all the soils ranged from 3 - 12 days for the 16 mg/g test concentration and from 5 - 11 days for the 4 mg/g test concentration. Following the initial lag phases, the estimated DT_{50}s ranged from 1 - 5 days at the 16 mg/g test concentration and from 2 - 4 days at the 4 mg/g test concentration. Although the characteristics of the various soils tested were diverse (*e.g.,* with regard to pH, clay content, cationic exchange capacity, and organic carbon content), there were no substantial differences in the rates at which bioactivity declined among the soil types. All had negligible effect on the rate of dissipation of bioactivity. These results indicate that any VIP3A protein residues that may be incorporated into agricultural soils from VIP3A plants (*e.g., via* post-harvest tillage) will likely not persist or accumulate, but will degrade rapidly.

Table 7.5. Characteristics of four live soils and one artificial soil used in soil activity study

Soil Source	Soil Texture	pH	Clay (% dry wt.)	Organic Carbon (% dry wt.)	Cationic Exchange Capacity (mmol/z/100g dry wt.)
Cascavel	clay	6.0	51	3.3	23.78
Mateo	sandy clay loam	4.5	28	1.9	11.27
Uberlândia	clay	4.6	59	2.4	13.77
Illinois	silt loam	5.9	27	2.9	22.66
Artificial	sandy loam	7.4	19	2.4	7.48

Table 7.6. Estimated lag phases and DT_{50}s for loss of VIP3A activity in various soils

Soil Source	16 mg LPPACHA-0199/g soil		4 mg LPPACHA-0199/g soil	
	Lag Phase (days)	DT_{50} (days)	Lag Phase (days)	DT_{50} (days)
Cascavel	12	4	11	3
Mateo	6	2	7	3
Uberlândia	7	1	7	3
Illinois	6	3	6	4
Artificial	3	5	5	2

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Chapter 8

FOOD AND FEED SAFETY

A. Presence of VIP3A-Like Proteins in Formulated Microbial Bt Products

Syngenta Seeds examined several commercial lepidopteran-active formulations of Bt-based microbial insecticides for the presence of VIP3A protein (Syngenta Seeds, unpublished data). The products examined were all US EPA-registered formulations exempt from food and feed tolerance requirements. ELISAs were conducted using protein A-purified polyclonal rabbit and immunoaffinity-purified goat antibodies specific for VIP3A protein. All eight Bt products evaluated contained quantifiable (*ca.* 0.4 - 32 μg/g sample) material that cross-reacted with the VIP3A antibody. In some formulations, sufficient immunoreactive material was present to visualize by SDS-PAGE on an 8% polyacrylamide gel followed by western blot analysis using polyclonal goat anti-VIP3A antibody. The products Dipel®, Javelin® and Condor® insecticides, for example, were observed to contain immunoreactive proteins of comparable molecular weight (*ca.* 89,000) as VIP3A protein.

In a preliminary screening program, Baretto *et al.* (1999) determined that the supernatants of two Bt strains with high activity against *Spodoptera frugiperda* (fall armyworm) larvae had heat-labile proteins of comparable molecular weight as VIP3A. Recently, Donovan *et al.* (2001) demonstrated that VIP3A protein is partially responsible for the insecticidal activity of Bt subsp. *kurstaki* strain HD1 toward *Agrotis ipsilon* (black cutworm) and *Spodoptera exigua* (beet armyworm). Strain HD1 is used in registered microbial insecticide products. The investigators modified the wild-type HD1 strain to replace the *vip3A* gene with a *vip3A* allele containing a 'knock-out' deletion mutation. Compared with the wild-type HD1 strain, the strain lacking a functional *vip3A* gene was one-fourth as toxic to *A. ipsilon* larvae and less than one-tenth as toxic to *S. exigua* larvae. When streptomycin was included in the *S. exigua* diet to inhibit the germination of spores or the growth of Bt after ingestion by the insect, the toxicity of the modified HD1 strain was *ca.* half that of the wild-type HD1 strain. Addition of HD1 spores increased the toxicity of purified Cry1 protein more than 600-fold against *S. exigua*, whereas addition of spores from the *vip3A*-deleted HD1 strain increased toxicity of Cry1 protein *ca.* 10-fold. These results strongly suggest that an important component of Bt insecticidal activity against *S. exigua* is the synthesis of VIP3A protein by Bt cells after ingestion of spores and crystal proteins by insect larvae. It is possible that VIP3A, or related proteins, contribute to the lepidopteran toxicity and pathogenicity of many Bt strains and to what has been described as the insecticidal "spore effect" that is not attributable to δ-endotoxins (Donovan *et al.*, 2001).

Since VIP3A (or a very similar protein, based on size and/or immunoreactivity) appears to be present in registered biological insecticide products used on food crops, including fresh market produce, it is conceivable that small quantities of VIP3A protein are present in the food supply. Additionally, because Bt, the native source of VIP3A protein, is found on plants and in soils,

trace amounts of VIP3A protein may be present on raw agricultural commodities that have not been treated with microbial insecticides.

B. VIP3A Protein and Potential Mammalian Exposure

The potential for human exposure to VIP3A protein from COT102-derived cotton plants is minimal. The VIP3A protein is contained within plant cells, therefore dermal exposure is unlikely. The presence of gossypol, a natural toxin, in cotton plants and cottonseed somewhat limits use of the crop and some of its byproducts in food and feed. The primary food uses of cotton are refined cottonseed oil and cottonseed "linters." Refined cottonseed oil is highly processed using heat, solvent, and alkali treatments. Its food uses are primarily as salad or cooking oil, and smaller amounts are used as shortening and in margarine (National Cottonseed Products Association, 1999). Linters consist of essentially 100% pure cellulose fibers and are subjected to heat and solvent extraction. They are used in foods as a source of fiber in baked goods, salad dressings, snack foods, and processed meats. Linter fiber is also used to improve the viscosity of dressings and is commonly used to bind solids in pharmaceutical preparations such as tablets. As described in Appendix B "Analysis of Processed COT102 Cottonseed Products for Yield and Presence of Gossypol and VIP3A Protein", refined cottonseed oil produced from COT102-derived cotton plants had no detectable VIP3A protein as measured by ELISA. Similarly, as described in Chapter 6 "Quantification of VIP3A and APH4 Proteins in Cotton Tissues and Whole Plants Derived from Transformation Event 'COT102' ", cotton fiber from COT102-derived plants had no detectable VIP3A protein. Moreover, refined cottonseed oil and cotton fiber contain little to no protein of any kind. Therefore, the potential for human dietary exposure to VIP3A protein *via* COT102-derived plants is negligible.

The primary animal feed uses of cotton are cottonseed or seed by-products including cottonseed meal, seed hulls and "gin trash." As described in Chapter 6, the quantity of VIP3A protein measured by ELISA in COT102-derived whole cottonseed was approximately 3 micrograms per gram of seed (3 ppm on a fresh weight and dry weight basis). Cottonseed meal is used as a primary protein source in some rations for mature ruminants such as beef cattle, dairy cattle, and sheep. As described in Appendix B "Analysis of Processed COT102 Cottonseed Products for Yield and Presence of Gossypol and VIP3A Protein", the concentration of VIP3A protein in COT102-derived cottonseed meal prior to toasting measured approximately 3 micrograms per gram, essentially the same concentration as was measured in cottonseed samples. However, following a standard toasting procedure that included a steam heat treatment of 110°C for 40 minutes, the VIP3A concentration measured in the toasted cottonseed meal was reduced to less than one-tenth (approximately 0.2 micrograms/gram sample; 0.2 ppm) of the concentration prior to toasting. Livestock consuming rations containing COT102-derived cottonseed may receive low exposure to VIP3A protein, however, the dietary concentration would be expected to be significantly less than 1 ppm, accounting for dilution by other components in the diet ration.

As described below (see Part C), the lack of observed toxicity to rodents acutely exposed to high oral doses of VIP3A indicates that any residues of VIP3A that may be present in cotton by-products used in animal feeds will not pose a safety concern. Any VIP3A protein consumed will

not accumulate in animal tissues but will be digested and metabolized as conventional dietary protein.

C. APH4 Protein and Potential Mammalian Exposure

Syngenta Seeds is unaware of prior dietary exposure to the APH4 protein, either *via* food or feed. No human dietary exposure to APH4 protein is expected to result from the commercial use of COT102-derived cotton plants, as the cotton products that enter the food supply (primarily cottonseed oil and cottonseed linters; see Part B.1., above) are essentially devoid of protein of any kind. Moreover, APH4 protein was not detectable by ELISA in cotton fiber and in most samples of whole cottonseed (Chapter 6). In the relatively few cottonseed samples in which APH4 protein was detectable, the quantity was too low to quantify (< 150 ng/g dry wt.; < 150 ppb). Although whole cottonseed and its byproducts are used in some livestock feed rations, the potential for exposure to APH4 *via* animal feed containing these products is expected to be extremely low (*i.e.,* significantly less than 50 ppb, accounting for dilution by other components in the diet ration), if any exposure occurs at all.

As described below (see Part I), the lack of observed toxicity to rodents acutely exposed to a high oral dose of APH4 indicates that any APH4 residues that may be present in cotton by-products used in animal feeds will not pose a safety concern. APH4 protein consumed will not accumulate in animal tissues but will be digested and metabolized as conventional dietary protein.

D. Dietary Exposure to Nucleic Acids

The nucleic acids (DNA and the RNA encoded by it) present in COT102-derived cotton plants as a result of transformation will not present a dietary safety concern. Based on the ubiquitous occurrence and known safety of nucleic acids in the food supply, a tolerance exemption under the Federal Food Drug and Cosmetic Act regulations has been established for residues of nucleic acids that are part of plant-incorporated protectants or associated inert ingredients (US EPA, 2001a).

E. Results of Mammalian Toxicity Testing of VIP3A Protein

Four separate acute oral rodent toxicity studies were conducted with VIP3A protein preparations. No treatment-related adverse effects were observed in any of the studies, and the VIP3A protein can be considered to be non-toxic. Three of these studies were conducted with microbially produced VIP3A protein and one was conducted with VIP3A-enriched leaf protein material extracted from VIP3A corn plants. Two of the studies conducted with microbially produced material (Kuhn, 1997 and Glaza, 2000) utilized VIP3A preparations that had minor amino acid sequence differences from that encoded by the *vip3A(a)* gene in event COT102 cotton, due to sequence differences in the genes over-expressed in the recombinant *Escherichia coli* strains. The third study with microbially produced material (Glaza, 2002a) utilized a gene that encoded the identical VIP3A amino acid sequence encoded by the *vip3A(a)* gene in event COT102 cotton.

The studies with *E. coli*-produced VIP3A protein were conducted with maximum doses that ranged from 1616 – 3675 mg VIP3A/kg body weight. In the study with plant-derived VIP3A material (Glaza, 2002b), it was only possible to achieve a relatively lower dose of 18 mg VIP3A/kg body weight, due to practical limitations. These limitations included extracting significant quantities of active protein from VIP3A plants and acutely dosing large volumes of plant protein to mice. Nevertheless, the study with plant-derived VIP3A protein has been provided as supplemental information.

Because toxicity was not observed at the highest VIP3A tested, it can be concluded that the No Observed Effect Level (NOEL) was 3675 mg VIP3A/kg body weight. The LD_{50} can be estimated as >3675 mg VIP3A/kg body weight.

F. Equivalence of VIP3A Proteins in Test Substances Used and VIP3A Produced in COT102-Derived Cotton Plants

A "bridging" study has been completed that demonstrates the substantial equivalence of VIP3A contained in test substances used in VIP3A safety studies and VIP3A protein as produced in COT102-derived cotton plants. VIP3A proteins from three sources, recombinant *E. coli*, VIP3A corn (event Pacha) and VIP3A cotton (event COT102), were determined to have the predicted molecular weight of *ca.* 89,000 and cross-reacted immunologically with the same anti-VIP3A antibody. Through mass spectral analysis, it was possible to determine the amino acid sequence of peptides representing *ca.* 85% of the complete cotton-produced VIP3A protein. The resulting sequences corresponded identically to the predicted amino acid sequence of VIP3A and no evidence of any post-translational modification of the VIP3A protein was observed. The cotton-expressed VIP3A protein had the predicted N-terminal amino acids, beginning with asparagine-18. The 17 N-terminal amino acids not detected in the cotton-expressed VIP3A could represent *in planta* proteolysis or *in vitro* degradation. Comparisons of the biological activity of *E. coli*-expressed and cotton-expressed VIP3A protein in lepidopteran larval diet bioassays demonstrated very similar activities and the same rank order of VIP3A sensitivity among the four species tested. Based on the various functional and biochemical parameters evaluated, it can be concluded that VIP3A proteins from recombinant *E. coli*, Pacha-derived corn and event COT102-derived cotton are substantially equivalent. Using similar methods to compare the VIP3A proteins biochemically and functionally, a previous bridging study (Privalle, 2002e) established the substantial equivalence of VIP3A produced in the same *E. coli* preparation to that produced in VIP3A corn (event Pacha).

G. Results of in vitro Digestibility Testing of VIP3A Protein

The susceptibility of VIP3A protein to proteolytic degradation was tested in simulated mammalian gastric fluid (SGF) containing pepsin. VIP3A from two sources, recombinant *E. coli* and leaves of transgenic corn plants (event Pacha), was evaluated. VIP3A from these sources has been shown to be substantially equivalent to that produced in COT102 VIP3A cotton (Privalle, 2002a). VIP3A from both sources was susceptible to pepsin degradation. No intact VIP3A (*ca.* 89,000 molecular weight) was detected upon immediate sampling of the digestion

reaction mixtures, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blot analysis or staining with Coomassie blue. Using *E. coli*-produced VIP3A (at a significantly higher concentration than was feasible for corn-produced VIP3A), two lower molecular weight (*ca.* 9,000 and 6,000) VIP3A polypeptides were still detectable as minor bands after two minutes in SGF. A progressive decline in intensity of these bands during the 60-minute incubation in SGF indicated that they represented transient VIP3A degradation products that were susceptible to pepsin digestion. These data support a conclusion that VIP3A expressed in transgenic plants will be readily digested as conventional dietary protein under typical mammalian gastric conditions.

The observation that some food allergens exhibit proteolytic stability has led to the common belief that proteins that are resistant to gastric digestion are more likely to become food allergens. However, some researchers (reviewed by Fu *et al.,* 2002) have questioned the validity of digestion stability as a criterion for protein allergenicity assessment. A recent comparison of 23 allergenic and 16 putative non-allergenic proteins belonging to each of four protein groups (storage proteins, plant lectins, contractile proteins and enzymes) found similar ranges of digestive stability among allergenic and non-allergenic proteins of similar cellular function (Fu, 2002; Fu *et al.*, 2002). For example, three out of the 23 known allergens were not digested after two hours in SGF, whereas four out of the 16 non-allergens were not digested after two hours in SGF. The ratio of pepsin to protein in the assays appeared to influence whether some proteins appeared to be digestible or stable. Additionally, food allergens with high allergenicity were not necessarily more resistant to SGF digestion than proteins with low allergenicity, as defined by the percentage of allergic patients having IgE to the protein. There was a similar lack of correlation for results using simulated intestinal fluid.

Similarly, Veiths *et al.* (1999) showed a lack of correlation between digestibility and allergenicity among peanut proteins and hazelnut allergens. Among the proteins studied by Kenna and Evans (2000), 13 out of 17 food allergens were partially or completely stable for at least 60 minutes, while 10 out of 24 nonallergenic proteins showed similar stability in SGF. Additional ongoing research on known allergens and non-allergens, as well as standardization of digestibility testing protocols[1] and data interpretation, should help clarify the utility of gastric digestibility as a predictive criterion for potential food allergenicity.

H. Evidence that the Mode of Action of VIP3A Protein is Not Relevant to Mammals

1. Mode of Action of VIP3A Protein

As VIP3A is one of a novel class of insecticidal proteins, information relating to its mode of action is being actively generated by Syngenta scientists. The investigative strategy has been to conduct similar studies to those previously done with the Bt Cry1A δ-endotoxins. Cry1 toxins, the most studied of Bt endotoxins, are solubilized in the alkaline pH of the

[1] through an ongoing effort sponsored by the Protein Allergenicity Technical Committee of the International Life Sciences Institute (ILSI)

lepidopteran midgut and activated by midgut proteases. In sensitive larvae, the activated toxin then binds to specific receptor(s) located on the epithelial cell brush border membranes. After binding, the toxin is integrated into the midgut membrane to form pores, which result in ion imbalances and cause insect death.

VIP3A toxin is also proteolytically activated to a toxin core in the lepidopteran larval midgut and forms pores (ion channels) in the gut membranes of sensitive species, a mechanism that appears to correlate with its toxicity. However, VIP3A has been shown to have significantly different receptor binding properties and pore forming properties than does Cry1Ab protein, indicating that VIP3A has a different target and specific mode of action than the δ-endotoxins in the Cry1 family (Lee *et al.,* 2002).

2. Symptomatology and histological effects of VIP3A protein in sensitive larvae

The general symptomatology displayed by sensitive lepidopteran larvae following ingestion of VIP3A protein resembles that caused by Bt δ-endotoxins, *i.e.,* cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death (Yu *et al.*, 1997). Histopathological examination of intoxicated larvae reveals that VIP3A specifically impacts the midgut epithelium, which is also the target tissue of δ-endotoxins. Following exposure of *Agrotis ipsilon* (black cutworm) larvae to 100 – 200 ng VIP3A/cm^2 diet cube for 24 hours, Yu *et al.* (1997) observed morphological changes in columnar and goblet cells. By 48 hours, the midgut lumen was filled with cellular debris, and by 72 hours, desquamation of the epithelial layer was complete and the larvae were dead. Similar histopathology was observed for *Spodoptera frugiperda* (fall armyworm), but not for *Ostrinia nubilalis* (European corn borer), which is relatively insensitive to VIP3A.

In similar studies, second-instar *A. ipsilon* and *O. nubilalis* were exposed to 200 ng VIP3A/cm^2 diet surface, and second-instar *S. frugiperda* were exposed to 150 ng VIP3A/cm^2 diet surface (Syngenta Seeds, unpublished data). Control insects were provided diet without VIP3A protein. Larval tissue sections were examined by Dr. Marcia Loeb, an insect physiologist at the USDA Agricultural Research Service (Beltsville, MD). After six hours of VIP3A treatment, the *A. ipsilon* and *S. frugiperda* larvae exhibited significant morphological changes in areas of the midgut. Many cell blebs were present in the midgut lumen, and the columnar cells, goblet cells, stem cells, peritrophic membrane and basal membrane were abnormal or degenerate. Similar changes were not observed among the untreated larvae or the *O. nubilalis* larvae.

3. Histopathological examination and VIP3A immunolocalization studies in gastrointestinal tissues of VIP3A-treated mice

In contrast to the profound changes in gut morphology observed in VIP3A-susceptibile insects (see Part H. 2., above), no detectable microscopic changes in morphology of gastrointestinal tissues were observed in mice treated with high oral doses of VIP3A protein (*ca.* 1616, 2700 or 3675 mg VIP3A/kg body weight as reported in Kuhn, 1997, Glaza, 2000

and Glaza, 2002a), respectively. The tissues were examined by veterinary pathologists and included the glandular and nonglandular stomach, duodenum, jejunum, ileum, cecum, colon, and rectum.

Despite the absence of any detectable changes in mouse gut tissue morphology, a more detailed study was undertaken to assess whether specific binding of VIP3A protein could be detected in mammalian gastrointestinal tissues. An immunohistochemical evaluation was conducted on gastrointestinal tissue sections that had been preserved from the studies in which the mice were acutely exposed to 2700 or 3675 mg VIP3A/kg body weight (Glaza, 2000 and Glaza, 2002a, respectively). This evaluation was conducted on all test and control mice sacrificed 6 hours after oral gavage exposure to *ca.* 3675 mg VIP3A/kg body weight, and 24 hours or 14 days after exposure to *ca.* 2700 mg VIP3A/kg body weight. Three mice/sex were examined for each of the 6- and 24-hour sacrifice groups, and five mice/sex were examined in the 14-day sacrifice group. Using standard immunohistochemical methods, sections of stomach (glandular and nonglandular), duodenum, jejunum and ileum were probed with rabbit anti-VIP3A antibody and further processed to visualize any bound antibody. The immunostained mouse tissue sections were counter-stained with Mayer's hematoxylin and evaluated by a veterinary pathologist. Negative control slides were also included for each VIP3A-treated and control animal; these consisted of slides exposed to the same staining procedure, but without anti-VIP3A antibody.

There was no antibody staining visible microscopically within or on the cytoplasmic membrane of the gastrointestinal cells of any of the VIP3A-treated mice. The mucosa appeared to be intact. The morphology of the mucosa, including the nonglandular stomach keratin and underlying epithelial cells, appeared normal, and there were no apparent morphological differences between the VIP3A-treated and control animals. It can be concluded that VIP3A does not bind to the gastrointestinal tract of mice and that the insecticidal mode of action of VIP3A is not relevant to mammals. The presence of lightly stained immunoreactive material within the gut lumen within 24 hours of treatment was not unexpected and is considered to reflect the very large dose of VIP3A protein delivered in a carboxymethylcellulose (CMC) suspension and the high sensitivity of the anti-VIP3A antibody to trace amounts of VIP3A peptides remaining. CMC is not susceptible to breakdown by pepsin or pancreatin (Massatsch and Steudel, 1941). Moreover, CMC has been shown to form complexes with some proteins as well as to bind to pepsin and inhibit its activity (Valaris and Harper, 1973). Additionally, CMC has bioadhesive properties and has been shown to bind to the gastrointestinal mucosa of rodents (Mathiowitz *et al.,* 1997).

I. Other Physico-Chemical Properties of VIP3A Protein

1. Lack of amino acid sequence homology with toxins or other known proteins

An extensive bioinformatics search was performed to determine whether the amino acid sequence of the VIP3A protein shows homology with proteins known to be toxins. VIP3A showed no significant homology with any non-VIP3A proteins in the public GenBank

database, including other proteins from Bt and proteins identified as toxins. Therefore, VIP3A represents a novel protein with no significant amino acid homology to other proteins for which amino acid sequences are publicly available.

2. Effects of heat and pH on VIP3A protein

Unlike the thermostabile non-proteinaceous β-exotoxin secreted by some Bt strains, VIP3A protein is thermolabile (Estruch *et al.,* 1996; Barreto *et al.,* 1999). Syngenta Seeds evaluated the stability of VIP3A protein under a range of heat and pH conditions, as described in Appendix A "Effects of Heat and pH on the Stability of VIP3A Protein". Instability of the protein was measured as the loss of bioactivity against VIP3A-sensitive fall armyworm (FAW; *Spodoptera frugiperda*) larvae. Although incubation of VIP3A at ambient temperature or at 37°C for 30 minutes had no apparent effect on its bioactivity, VIP3A protein was inactivated by heating at 55°C for 30 minutes. VIP3A bioactivity was not apparently affected by incubation in buffer for 30 minutes at pH's ranging from 4.0 to 9.5. In a separate study (Privalle, 2002b), exposure of purified VIP3A protein to *ca.* pH 1.0 – 1.2, in the absence of any protease, immediately caused some degradation of the intact protein, although immunoreactive VIP3A peptides were still present after 60 minutes.

As described above (Part B), when cottonseed meal prepared from COT102 VIP3A cotton was subjected to a standard toasting procedure that included a steam heat treatment of 110°C for 40 minutes, the VIP3A concentration measured in the toasted cottonseed meal was reduced to less than one-tenth (approximately 0.2 micrograms/gram sample; 0.2 ppm) of the concentration prior to toasting. The above information supports the conclusion that VIP3A protein is susceptible to inactivation and degradation by conventional processing methods.

J. Evidence That VIP3A is Unlikely to Become a Food Allergen

While virtually all allergens are proteins, only a few of the many proteins found in foods are allergenic. Although the probability that any specific novel protein will become a food allergen is, therefore, small, the potential allergenicity of the VIP3A protein was evaluated using an extensive weight-of-evidence approach. The methods employed generally followed a decision tree for allergenicity assessments, as recommended and described by several international organizations[1] and other experts (Metcalfe *et al.*, 1996; FAO/WHO, 2001; Taylor, 2002).

Four general concerns regarding potential allergenicity arise in the context of producing novel proteins in food plants:

- Is the novel protein derived from a source known to produce allergenic proteins and, therefore, might individuals previously sensitized to one or more of these allergens be

[1] The International Food Biotechnology Council (IFBC), the Allergy and Immunology Institute of the ILSI, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO)

inadvertently exposed *via* food from the modified crop?

- Is the novel protein sufficiently similar to known protein allergens such that it might elicit an allergic cross-reaction in sensitized individuals?

- Does the novel protein have particular physico-chemical characteristics that would make it more likely to sensitize some individuals, if sufficient dietary exposure occurred?

- Would the novel protein be present in sufficiently high concentrations in food to promote sensitization in the minority of individuals who might be predisposed to sensitization?

The following discussion presents specific test results and information regarding VIP3A protein to address each of these concerns. Based on the weight of evidence from diverse sources, it can be concluded that VIP3A is very unlikely to represent a potential allergen in food.

1. VIP3A will not be present in food items produced from VIP3A cotton

Many allergenic proteins, especially those in commonly allergenic foods, are abundant in the offending food, and are present at concentrations typically ranging between 1% and 80% of total protein (Metcalfe *et al.*, 1996). Food products derived from COT102 VIP3A cotton (refined cottonseed oil and cellulose linters) are not expected to contain any VIP3A protein, therefore no exposure is expected. Even for proteins with potentially allergenic properties, in the absence of measurable exposure, there is essentially no opportunity for allergic sensitization to occur.

2. VIP3A protein is not derived from a known source of oral allergens

As described in Chapter 1, the VIP3A protein was initially isolated from Bt strain AB88, and VIP3A or VIP3A-like proteins are present in many Bt strains and in some commercial Bt microbial insecticide products. Bacteria have no history of allergenicity (Taylor and Hefle, 2001; FAO/WHO, 2001). Additionally, despite decades of widespread use of Bt insecticides on food crops, there have been no reports of oral allergies to these preparations, and the US EPA has stated that laboratory animal studies submitted to the Agency have not indicated any potential for allergic reactions to Bt or its components (US EPA, 2001b).

3. VIP3A does not have amino acid sequence homology to known allergens

An extensive bioinformatics search was performed to determine whether the amino acid sequence of the VIP3A protein shows homology with proteins known or suspected to be allergens. Three different similarity searches were performed comparing the VIP3A protein to the entries in the Syngenta Biotechology Incorporated (SBI) Allergen Database. This database was compiled from entries identified as allergens or putative allergens in public protein databases and was supplemented with additional amino acid sequences identified from the scientific literature. First, the entire VIP3A protein sequence was compared to the

allergen sequences using the FASTA search algorithm (Pearson and Lipman, 1988). Second, contiguous VIP3A peptides of 80 amino acids, overlapping by 10 amino acids, were compared to the allergen sequences using the FASTA search algorithm. Third, the VIP3A protein sequence was screened for matches of eight or more contiguous amino acids using a program (developed by Syngenta) that compares every possible peptide of eight[1] contiguous amino acids between VIP3A and the allergen sequences. The results of these analyses revealed no significant similarity of the VIP3A protein to known or putative allergens for which amino acid sequences were available.

4. VIP3A protein is unstable to heat and food processing

Many food allergens are stable to heat and processing. However, the VIP3A protein has been demonstrated to lose activity upon moderate heating, and is substantially degraded or eliminated by standard cottonseed processing methods (see Part B).

Additionally, the VIP3A protein is unlikely to be stabilized by disulfide bonds. The amino acid sequence of the VIP3A protein includes only three cysteine residues. Because a disulfide bond within a protein molecule requires the presence of two cysteine residues, the VIP3A protein can theoretically have, at most, only one disulfide bridge. Such stabilizing bonds appear to contribute to the allergenicity of some food proteins, as evidenced by the mitigating effect of the reducing agent thioredoxin on wheat and milk allergenicity (Buchanan *et al.,* 1997; del Val *et al.*, 1999). The low likelihood of disulfide bonds within the VIP3A molecule, in addition to its lability to heat and processing, suggests that the VIP3A molecule does not have features that contribute to high stability and, presumably, higher allergenic potential.

5. VIP3A protein is susceptible to gastric digestion

As described in Part G, above, VIP3A is susceptible to digestion by pepsin under simulated gastric conditions. (As also discussed above, the utility of digestibility assays as a predictors of potential allergenicity has recently been called into question.)

6. VIP3A protein is not apparently glycosylated

Using mass spectral analysis of VIP3A peptides, VIP3A protein from COT102-derived plants showed no evidence of glycosylation or other post-translational modifications.

7. VIP3A did not elicit a sensitization response in an experimental atopic dog model of human allergy

[1] Eight contiguous amino acids is believed to generally represent the minimum size of allergenic linear epitopes (Metcalfe et al, 1996).

The VIP3A protein was evaluated for allergenic potential in an atopic dog testing model. This model is a promising system for predicting the potential for a novel transgenic protein to be a food allergen in humans (Ermel *et al.,* 1997; Buchanan, 2001; Teuber *et al.*, 2002). The dog is one of the few species other than humans that develops allergies naturally upon normal environmental exposure to a broad spectrum of allergens (reviewed by Teuber *et al.*, 2002). The atopic dog model has been used previously to investigate allergic responses to known food allergens, and a correlation between the sensitivity of these dogs towards known human food allergens, as measured by gastrointestinal inflammation and skin sensitivity towards these same allergens, has been established (Buchanan *et al*, 1997; Ermel *et al*, 1997; Teuber *et al.*, 2002). Moreover, based on measurements of the mean amount of allergen eliciting a skin response in dogs, the hierarchy of reactivity by skin testing appears to be similar to the clinical experience in humans (*e.g.,* peanut > tree nuts > wheat > soy > barley>)(Buchanan *et al.*, 1997; del Val *et al.*, 1999; Buchanan, 2001; Teuber *et al.*, 2002).

An inbred colony of high IgE-producing dogs, developed and maintained at the Animal Resources Service, School of Veterinary Medicine, University of California, Davis, was employed to further assess the allergenic potential of VIP3A protein (Buchanan, 2001).[1] These dogs were selected based on a genetic predisposition to allergies and a previous history of high sensitivity to pollens and foods. The dogs used for the VIP3A evaluation were inbred for seven generations from the original colony of high IgE producers. Following methods established at this University of California laboratory (Ermel *et al*, 1997; Buchanan *et al.*, 1997; Teuber *et al.*, 2002), a colony of 18 one-day old atopic, spaniel/basenji-type dogs were sensitized by subcutaneous injection of commercial protein extracts of foods known to induce a range of allergic responses: peanuts (very strong allergen), cow's milk (moderately strong allergen) and soy (moderately weak allergen). In the same manner, the dogs were concurrently immunized with an extract of VIP3A corn leaves containing VIP3A protein. Appropriate negative controls (including conventional, non-transgenic corn leaf extract) as well as a positive standard (ragweed pollen) were also tested. Hypersensitivity responses of the dogs to the various substances were assessed 9, 18 and 23 months after initial immunization by intradermal administration of the same food or pollen extracts, VIP3A corn leaf extract, control corn leaf extract, and/or purified VIP3A protein produced in recombinant *E. coli*. The relative sizes of the resulting dermal wheals were read blindly by the same experienced observer at each time point.

At the 9-month challenge test, peanut and soy extracts induced strong responses, and by the 18-month evaluation all the known allergens had produced a response. The VIP3A corn leaf extract showed essentially no response throughout the trial period, and at no time was the response significantly different than the control corn leaf extract. At 23 months, the relatively pure VIP3A preparation (produced in *E. coli*) elicited no response at concentrations up to 380 times higher than the VIP3A concentration in the minimum amount of VIP3A corn leaf extract eliciting a wheal. At the 23 month time-point, the relative skin test response of

[1] To maintain confidentiality at the time the study data were presented publicly, the VIP3A protein in the test preparation was referred to by the investigator as the "protein of interest."

VIP3A leaf extract was *ca.* 1/5000th the intensity of the response to peanut allergen, 1/900th the response to ragweed, 1/700th the response to milk, and 1/50th the response to soybean extracts. A comparison of skin test responses between the VIP3A corn leaf extract and the control corn leaf extract indicated no significant difference in the hypersensitivity response to the two preparations. The data derived from this animal model provide compelling evidence that the VIP3A protein has no demonstrable allergenic potential.

8. Summary of characteristics indicating that VIP3A will not be allergenic

Food products derived from cotton (refined cottonseed oil and cellulose "linters" fiber) are highly processed and are essentially devoid of any proteins. Moreover, no VIP3A protein was detected in refined cottonseed oil or cotton fiber produced from event COT102-derived VIP3A cotton plants. Therefore, no human dietary exposure to VIP3A protein is expected to occur *via* VIP3A cotton. Even if dietary exposure to VIP3A protein were to occur, data derived from bioinformatic analyses as well as direct *in vitro* and *in vivo* testing collectively indicate that the VIP3A protein is unlikely to have allergenic potential. The amino acid sequence of VIP3A is not homologous to that of any known or putative allergens described in public databases. The VIP3A protein is not derived from a known source of allergens and does not display characteristics commonly associated with allergens, including glycosylation or stability to heat and food processing. Additionally, VIP3A is susceptible to gastric digestion by pepsin and did not provoke an allergic response in an experimental atopic dog model of human food allergy.

K. Results of Mammalian Toxicity Testing of APH4 Protein

1. APH4 Test Substance Employed in the Mammalian Toxicology Study

Because it is not possible to extract sufficient APH4 protein from COT102 transformed plants for toxicology studies, APH4 protein was produced in recombinant *E. coli* by over-expressing the same *aph4* gene that was introduced into VIP3A cotton event COT102. The *aph4* gene was cloned into the inducible, over-expression pET-3a® vector (Novagen, Madison, WI) in *E. coli* BL21DE3pLysS. The APH4 protein, as encoded in this vector, was identical in amino acid sequence to that encoded by the plant transformation vector, pCOT1, except for an additional 11 amino acids from the T7 Tag™ and three amino acids from the vector polylinker. Following purification from *E. coli*, dialysis and lyophilization, the resulting sample, designated Test Substance APH4-0102, was estimated by ELISA to contain *ca.* 42.6% APH4 protein by weight. The test material material was confirmed to be enzymatically active.

It has not been possible to confirm the equivalence of APH4 protein in Test Substance APH4-0102 with that produced in event COT102 cotton plants, because it has not been possible to extract sufficient APH4 protein from the plants for these analyses. In most tissues of COT102 plants, APH4 has not been detected or the levels have been too low to quantify by ELISA (< 150 ng/g dry wt; < 150 ppb). However, given that the *aph4* gene cloned into *E.*

coli for over-expression was the same gene that was introduced into VIP3A cotton event, it can be predicted that the resulting APH4 proteins are equivalent.

2. Acute Oral Mouse Toxicity Study with APH4 Protein

An acute mouse oral toxicity study was conducted at the Syngenta Central Toxicology Laboratory (Alderley Park, Macclesfield, Cheshire, UK) according to US EPA Test Guideline OPPTS 870.1100 (Johnson, 2002). Test substance APH4-0102 (see above description of test substance) was administered to 5 male and 5 female mice [strain Alderley Park albino mouse (AP_fCD-1); 8 - 9 weeks old] *via* a gavage dose of 1828 mg/kg body weight. The test substance contained *ca.* 42.6% APH4 protein by weight. Therefore, the mice received *ca.* 779 mg APH4/kg body weight. A negative control group (5 mice/sex) concurrently received the dosing vehicle alone, a suspension of 1% methylcellulose, at the same dosing volume as used for the test material mixture. Food was provided *ad libitum*, except during the *ca.* one hour prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study. Observations for mortality and clinical/behavioral signs of toxicity were made at least twice on the day of dosing, and at least once daily thereafter for 14 days. Detailed clinical observations were made for each animal at each observation time. Body weights were recorded daily and food consumption was recorded weekly. Surviving animals were euthanized 14 days post dosing and subjected to gross necropsy. Organ weights (brain, liver with gall bladder, kidneys and spleen) were recorded and principal tissues were processed for microscopic examination.

No mortalities occurred during the study, and no clinical signs of toxicity were observed in either the test or control groups. There were no treatment-related effects on body weight, food consumption, or organ weights, nor were any treatment-related effects observed following macroscopic or microscopic examination. APH4-0102 is not acutely toxic to mice. There is no evidence of toxicity of the test substance at 1828 mg APH4-0102/kg body weight, representing *ca.* 779 mg APH4 protein/kg body weight. The estimated LD_{50} value for pure APH4 protein in male and female mice is >779 mg/kg body weight, the single dose tested.

L. Lack of Homology of APH4 Protein with Known Toxins or Allergens

An extensive bioinformatics search was performed to determine whether the amino acid sequence of the APH4 protein shows homology with proteins known to be toxins. APH4 showed no significant homology with any proteins identified as toxins in the public GenBank database.

M. Results of in vitro Digestibility Testing of APH4 Protein

The susceptibility of hygromycin phosphotransferase (APH4) protein to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin and simulated

mammalian intestinal fluid (SIF) containing pancreatin. APH4, produced in recombinant *Escherichia coli*, was rapidly degraded in both SGF and SIF. No intact APH4 (*ca.* 42,000 molecular weight) was detected upon immediate sampling of the reaction mixtures, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie blue staining or western blot analysis. These data support a conclusion that APH4 expressed in transgenic plants will be readily digested as conventional dietary protein under typical mammalian gastric conditions. Furthermore, in the unlikely event that APH4 protein survives the gastric environment, it will be degraded rapidly in the intestines.

N. Evidence That APH4 is Unlikely to Become a Food Allergen

As described in Part B, above, derivatives of cottonseed (*e.g.,* refined cotton seed oil) and fiber (*e.g.*, linters, which are essentially 100% cellulose) are used in some food products. However, APH4 was not detected in most of the samples of COT102-derived cotton seed analyzed or any of the cotton fiber samples analyzed (see Chapter 6 of this Petition). In the few cottonseed samples in which APH4 was detectable, the quantities were below the limit of quantification (<137 ng APH4/g fresh wt; <150 ng APH4/g dry wt). It is expected that any trace quantities of APH4 in cottonseed will be eliminated by standard seed processing methods. As demonstrated by the analysis of cottonseed products for VIP3A protein (Artim, 2002c), no VIP3A was detected in refined cottonseed oil from COT102-derived plants, despite the presence of *ca.* 3 μg VIP3A/g seed (fresh or dry wt.). Additionally, no protein of any kind was detected in the same sample of refined cottonseed oil. It can be concluded that APH4, as produced in COT102-derived cotton plants, does not pose a risk of becoming allergenic *via* food, because there will be no exposure *via* food. Moreover, the APH4 protein shows no amino acid sequence homology to known allergens (MRID No. 45766502; Vlachos, 2002); is not derived from a source known to produce allergens (MRID No. 45766501; Artim, 2002a); and is not targeted to a cellular pathway for glycosylation in the plant. Additionally, APH4 is rapidly degraded upon exposure to simulated gastric and intestinal fluids (see Part J, above).

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APPENDIX 8A

Effects of Heat and pH on the Stability of VIP3A Protein

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Syngenta Seeds, Inc.
Research Triangle Park, North Carolina USA

Syngenta Seeds Biotechnology Report No. SSB-007-02

INTRODUCTION

The stability of VIP3A protein was evaluated under a range of heat and pH conditions. Loss of bioactivity against VIP3A-sensitive fall armyworm (FAW; *Spodoptera frugiperda*) larvae was used to indicate instability of the protein after exposure to the various treatments.

MATERIALS AND METHODS

Test Substance. The source of VIP3A protein for this study was test substance VIP3A-0199, produced by over-expression of VIP3A in recombinant *Escherichia coli*. Prior characterization of this test substance determined that it contained *ca.* 54% VIP3A protein by weight and retained bioactivity against FAW larvae. The preparation and characterization of this test substance are described in detail in Novartis Seeds Biotechnology Report No. NSB-004-99 (Novartis Seeds, 1999). A separate study has established that VIP3A as contained in VIP3A-0199 is substantially equivalent to VIP3A as produced in transgenic maize plants (Syngenta Seeds, 2002). Since its preparation, test substance VIP3A-0199 has been stored desiccated at *ca.* –20°C.

Stability Experiments. For the heat stability analysis, a 1 mg/ml solution of VIP3A-0199 was prepared in standard buffer (50 mM Tris-HCl, 2 mM EDTA, pH 9.5) and incubated at ambient temperature or at 37, 55, or 95°C for 30 minutes. For the pH stability analysis, 1 mg/ml solutions of VIP3A-0199 were prepared in buffer titrated to pH 4.0, 5.1, 7.5, or 9.5 and incubated at ambient temperature for 30 minutes. The treated samples of VIP3A-0199 were assayed for insecticidal activity against first-instar FAW using a meridic diet surface bioassay procedure (Standard Operating Procedure 2.33). For each treatment, 100 µl of VIP3A-0199 solution was applied to each of two 50 x 9 mm petri dishes (#08-757-19, Gelman Sciences; Ann Arbor, MI, USA), and 10 larvae were placed in each dish. Similarly, positive control cultures received 100 µl of a 1 mg/ml solution of untreated VIP3A-0199. Negative control larvae were exposed to diet only or to diet treated with buffer at the various test pHs. The pH 9.5 buffer control treatment also served as a negative control for the heat stability treatments, which were

conducted at the same pH. All cultures were incubated at *ca.* 28°C, 50% relative humidity in the dark, and mortality was recorded after *ca.* 48 and 72 hours.

RESULTS

The results shown in Table A-1 indicate that VIP3A protein was inactivated by heating at 55°C for 30 minutes. There were no significant differences in larval mortality at 48 or 72 hours between the treatments at 55 or 95°C as compared to the diet-only control or the standard buffer control. Incubation at ambient temperature or at 37°C for 30 minutes had no apparent effect on VIP3A bioactivity; larval mortality was similar to the untreated VIP3A positive control. Similarly, VIP3A bioactivity was not apparently affected by incubation in buffer for 30 minutes at pH's ranging from 4.0 to 9.5, as significant larval mortality was evident in these treatments. (Analyses completed on 7 Jun 02; Notebook #312, pp. 23 – 29.)

REFERENCES

Product Registration Laboratory Standard Operating Procedures

SOP 2.33 Diet Surface Bioassay of Lepidopteran Insects

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Table A-1. Effect of Temperature and pH on Bioactivity of VIP3A as Measured by Toxicity to First Instar Fall Armyworm

Treatment	48 Hours			72 Hours		
	# Dead/10 larvae		%	# Dead/10 larvae		%
	Rep 1	Rep 2	Mortality	Rep 1	Rep 2	Mortality
Diet control	0	0	0	0	0	0
Temperature						
Buffer control (pH 9.5 buffer)	0	1	5	0	1	5
VIP3A positive control (4°C)	7	5	60	10	10	100
VIP3A - ambient temp.	7	5	60	10	9	95
VIP3A - 37°C	8	7	75	10	9	95
VIP3A - 55°C	0	0	0	2	0	10
VIP3A - 95°C	0	0	0	0	0	0
pH						
pH 4.0 buffer	0	0	0	0	0	0
VIP3A at pH 4.0	4	7	55	8	10	90
pH 5.1 buffer	0	0	0	0	0	0
VIP3A at pH 5.1	6	5	55	9	9	90
pH 7.5 buffer	1	0	5	1	0	5
VIP3A at pH 7.5	5	5	50	9	10	95
pH 9.5 buffer	0	1	5	0	1	5
VIP3A at pH 9.5	5	6	55	10	10	100

APPENDIX 8B

Product Registration Laboratory
Syngenta Seeds, Inc.
Research Triangle Park, North Carolina USA

Analysis of Processed COT102 Cottonseed Products for Yield and Presence of Gossypol and VIP3A Protein

Syngenta Seeds Biotechnology Report No.SSB-017-02

SUMMARY

Processing of cottonseed to produce defatted toasted meal and refined oil was performed on cottonseed from the transgenic cotton line, event COT102, and a non-transgenic control, Coker 312. Comparisons of yield for fractions produced during the processing showed no significant differences between COT102 and Coker 312. The products were analyzed for VIP3A protein by enzyme linked immunosorbent assay (ELISA). A quantifiable level of VIP3A protein was found in the defatted toasted meal but was not detectable in the refined oil. Gossypol levels were similar for both the transgenic and control cottonseed products.

INTRODUCTION

The products created by the milling of cottonseed are numerous and diverse including cottonseed meal, refined oil, hulls, and linters. Cottonseed meal is used principally as feed for livestock and represents the material remaining after the extraction of cottonseed oil by solvent methods. Meal is considered sufficient as a sole source of protein for mature ruminants such as beef cattle, dairy cattle, and sheep. It is also used in formulated swine, poultry, and fish diets. Cottonseed oil can be extracted mechanically, chemically (solvent extraction), or by a combination of the two methods. Refined cottonseed oil is a highly valuable commodity for human food uses such as cooking, frying, baking, and as an ingredient in many processed foods. Cottonseed hulls are primarily sold into the cattle feed market as a fiber source (Smith, C.W., 1999). Cottonseed linters are produced by the mechanical removal of the short fiber fragments left on the cottonseed after ginning. Cottonseed linter fibers are nearly 100% cellulose and are used for various purposes. Products such as paper, diapers, mattress padding, and even currency are manufactured from linters. Linters are also used as a source of dietary fiber for baked goods, salad dressings, snack foods, and processed meats (Jones, L. A. and Kersey, J.H. 2002).

Gossypol is a naturally occurring toxin in cotton plants that protects them from insect damage. Because gossypol is known to be toxic to livestock, it is a limiting factor in the use of whole cottonseed and cottonseed meal as feed sources (Adams, R. 1977). Gossypol exists in two

forms, free and bound. The free form is toxic, while the bound form is considered nontoxic since it is not released in the animal rumen. In whole unprocessed cottonseed, almost all of the gossypol is in the free form. During processing, gossypol partitions into the meal and oil components. Although some of the gossypol in meal remains as the free form, much of it becomes bound to proteins and, therefore, detoxified. Gossypol in oil is eliminated during the refining process.

The described study was undertaken to evaluate and compare the processing of event COT102 with its non-transgenic counterpart, Coker 312, and determine levels of gossypol and VIP3A protein in the defatted meal and processed oil.

MATERIALS AND METHODS

Source of cottonseed. Cottonseed from event COT102 and Coker312 were obtained from field-grown plants produced in Leland, MS during the 2001 planting season. Approximately 3 lbs. of fuzzy cottonseed from each line were sent to the Food Protein Research and Development Center, Texas A&M University (College Station, TX, USA) for processing.

Cottonseed processing.
The processing of the transgenic and control cottonseed was performed, under the direction of Steven R. Gregory, in the Food Protein Research and Development Center, Texas A&M University, in accordance with the methods described in *Cottonseed Lab Standard Processing Procedure* (Appendix A). The primary products of this procedure were defatted meal and refined oil. These products were shipped to the Syngenta Seeds, Inc. Product Registration Laboratory (Research Triangle Park, NC, USA) and stored at room temperature upon arrival. The cottonseed meal samples were subsequently returned to Texas A&M for toasting upon realization that the toasting process was not originally performed. Toasting included a steam heat treatment of 230°F for 40 minutes. The toasted meal was returned to Syngenta Seeds, Inc. Product Registration Laboratory for reanalysis.

VIP3A protein extraction. Samples were extracted in accordance with SOP 2.41. An aliquot (0.1 g) of the meal or oil was weighed into a 15-ml polypropylene tube, suspended in 3 ml extraction buffer, and homogenized using a Polytron® homogenizer (Brinkmann Instruments). Following centrifugation for 10 min at 14,000 x g at *ca.* 2°-8°C, VIP3A analyses by ELISA were performed on the supernatants.

VIP3A quantitation. The sample extracts were quantitatively analyzed for VIP3A protein by enzyme-linked immunosorbent assay (ELISA; Tijssen, 1985), in accordance with SOP 2.38, using immunoaffinity-purified polyclonal goat and protein A-purified rabbit antibodies specific for VIP3A. The immunization schedule followed to generate these antibodies is described in Notebook #3124, p. 175 ff.

Gossypol Analysis. Gossypol analysis was carried out by Woodson-Tenent Laboratories, Inc, Memphis, TN, USA using the following standard practices: Total gossypol, AOCS 1987Ba8-78, Spectrophotometric method; Free gossypol, J. AOAC 1892 59(12), modified HPLC method.

Protein determination. Total protein for the cottonseed meal sample was determined by Woodson-Tenent Laboratories, Inc, Memphis, TN, USA using standard practices. Method for total protein determination by Kjeldahl procedure, AOAC method 988.05. Official Methods of the AOAC, 16th Edition, 1995.

RESULTS AND CONCLUSIONS

When comparing percent yield of the processed fractions, there were no significant differences with respect to hulls and lint, kernels, refined oil, and defatted meal (see Table 1). Yields were comparable for both event COT102 and Coker 312 and similar to ranges previously reported for processed cottonseed fractions from other cotton cultivars (Cherry, J.P., 1984).

There were no differences in the levels of total and free gossypol in the defatted (non-toasted) meal and refined oil from either event COT102 or Coker 312 (Table 2). The processing steps used to produce the refined cottonseed oil effectively removed any free gossypol. However, gossypol did fraction with the cottonseed meal during the solvent extraction phase, as is indicated by detectable and quantifiable levels of total and free gossypol in defatted (non-toasted) meal

As described in Table 2, VIP3A protein was present in the defatted (non-toasted) cottonseed meal from COT102 at a level of 2.75μg/g and was not detectable in the control when initially analyzed. After the toasting process was completed, VIP3A protein concentration dropped significantly to 0.23 μg/g in COT102 meal (*ca.* 10-fold decrease). The total protein content of COT102 and Coker 312 meal was 48.75% and 47.82%, respectively, which is consistent with commercial grade cottonseed meal. The industry standard is to produce meal with at least 41% protein (Smith, C.W., 1999). VIP3A protein was not detectable in the refined oil. Additionally, no total protein was detected in the oil using a standard Coomassie blue protein assay (Biorad, Pisquataway, NJ, USA).

RECORDS RETENTION: Raw data, the original copy of this report, and other relevant records are archived at Syngenta Biotechnology, Inc., 3054 Cornwallis Rd., Research Triangle Park, NC, USA 27709.

***CONTRIBUTING SCIENTISTS*:** Analytical work reported herein was conducted at Syngenta Seeds, Inc. Product Registration Laboratory, Research Triangle Park, NC by Kim Hill, B.S. and Xiaoxu Jiang, M.S.

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Product Registration Laboratory Standard Operating Procedures

SOP 2.38 Quantitative Analysis for VIP3A by ELISA
SOP 2.41 VIP3A Extraction from Maize Tissues and Silage

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Table B-1: Cottonseed Extraction Yield Data.

	Yield		% Yield	
Processed Fraction	COT102	**Coker 312**	COT102	**Coker 312**
Initial seed moisture (%)	5.90	6.00		
Fuzzy cottonseed to huller (g)	1279.0	1282.5		
Hulls and lint (g)	599.2	622.5	46.9%	48.5%
Loss in the huller (g)	8.6	8.8	0.7%	0.7%
Kernels to flaker (g)	671.2	651.2	52.5%	50.8%
Loss in flaker (g)	19.6	19.4	2.9%	3.0%
Flakes to cooker/extractor (g)	651.6	631.8		
Free fatty acid (crude oil) (%)			0.91%	1.4%
Refined oil (g)	155.5	133.0	12.2%*	10.4%*
Defatted meal (g)	441.0	467.1	34.5%*	36.4%*
Total protein in defatted meal (%)			48.75%	47.82%
* percent weight of fuzzy cottonseed				

Table B-2: VIP3A and Gossypol Levels in Processed Cottonseed Products

	VIP3A μg/g sample	Total Gossypol (% fresh wt.)	Free Gossypol (%fresh wt.)
COT102			
Non-toasted meal[1]	2.75 ± 0.12	1.45	0.829
Non-toasted meal[2]	2.57 ± 0.03	Nt[5]	Nt
Toasted meal[2]	0.23 ± 0.02	Nt	Nt
Refined oil[1]	Nd[3]	0.012	Na[4]
Coker 312			
Non-toasted meal[1]	Nd	1.44	0.714
Non-toasted meal[2]	Nd	Nt	Nt
Toasted meal[2]	Nd	Nt	Nt
Refined oil[1]	Nd	<0.010	Na[4]

Values were determined by ELISA and were not corrected for extraction efficiency. Values for all control samples corresponded to 0 ng VIP3A ug/g sample. "nd" = VIP3A was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

[1] ELISA analysis completed on May 9, 2002
[2] ELISA analysis completed on November 15, 2002.
[3] Nd = Not detectable <looks like there is an extra space above this line>
[4] Na = Not applicable for oil
[5] Nt = Not tested

Chapter 9

IMPACT ON AGRICULTURAL PRACTICES

A. Insect Resistance Management

COT102 offers a number of important benefits to growers and to society at large. The Bt-derived protein expressed in event COT102, VIP3A, is highly selective and provides excellent control of several economically important lepidopteran pests in cotton. The deployment of COT102 will provide to cotton producers an additional pest control tool to chose from that will lead to increased yields and greater profits.

A common concern with respect to transgenic crops that express Bt-derived insecticidal proteins is the selective pressure that these crops may place on the insect pests that feed on them. This concern focuses on the theoretical possibility that such selective pressure might result in the evolution of widespread resistance to Bt-derived insecticidal proteins, which, in turn, might lead to the loss of a valuable crop protection resource. Insect resistance management (IRM) practices are measures that are undertaken to reduce the potential for insects to develop resistance to a pesticide. Implementing appropriate IRM practices is of particular importance with Bt-derived insecticidal proteins because of their unique value as environmentally benign, highly selective pesticides that are extremely effective against a number of economically important pests.

EPA already mandates a rigorous IRM program for currently registered Bt cotton products. The centerpiece of this IRM program is a requirement that growers plant a "refuge" of non-Bt cotton whenever they plant cotton that contains a Bt-derived plant-incorporated protectant. This refuge must conform to specific requirements pertaining to size, placement, and refuge management. The effectiveness of the existing IRM program for Bt cotton is demonstrated by the fact that there have been no reported instances of insect resistance developing toward Bt cotton in the field since the first registration of Bt cotton varieties in 1995.

Conceptually, COT102 presents many of the same insect resistance issues as the currently registered Bt cotton products. However, COT102 differs from currently registered Bt cotton cultivars in ways that *reduce* the possibility of insect resistance developing, as compared to currently registered products. In particular, the VIP3A protein expressed by COT102 operates through a novel mode of action and a novel binding site that differ from currently registered Bt cotton plant-incorporated protectants. In addition, the available evidence indicates that, unlike some currently registered Bt cotton products, COT102 expresses VIP3A in a "high dose" for all target pests. Both of these features enhance the ability of COT102 to prevent the development of insect resistance.

As event COT102 may be less prone to insect resistance than currently used Bt cotton products, it should be subject to less stringent IRM requirements. Nevertheless, Syngenta is proposing that COT102 be subject to the same IRM program that applies to currently registered Bt cotton products. There should be no question that the current IRM program is more than adequate to protect against the development of insect resistance to VIP3A.

Moreover, by maintaining a single set of IRM requirements that apply to all registered Bt cotton products, grower confusion should be minimized, thereby enhancing overall compliance with the IRM requirements for Bt cotton.

B. Current Agronomic Practices and the Impact of VIP3A Cotton on Pest Management

Approximately 5.4 billion pounds of cotton are ulitized annually by the U.S. textile industry and approximately two to three billion additional pounds are grown for export markets worldwide (National Cotton Council. 2001a). The efficient production of this large quantity of high quality cotton fiber typically requires high levels of production inputs. Current cost estimates for major U.S. field crops indicate that cotton is second only to rice on a per acre basis (USDA-ERS 2001). A major production cost is chemical inputs, for which cotton costs are approximately $60 per acre (Carpenter, 2002). Cotton is exclusively grown in warm climates where insect pests are a season-long problem, and chemical pesticides are the predominant control measures. Twenty-five percent of all pesticides used globally are applied to cotton crops. In the U.S. conventional insecticides represent approximately 23-28% of the cost associated with cotton production (Table 9.1) between the years of 1999 and 2001.

Table 9.1. Chemical Production Cost Information

	Grower $ Spend			% Grower Spend		
Pesticide Type	1999	2000	2001	1999	2000	2001
Insecticide	$180.6M	$208.3M	$175m	26%	28%	23%
Fungicide	$13.9M	$14.9M	$15.2m	2%	2%	2%
Herbicide	$285M	$312.5M	$334.9m	41%	42%	44%
PGR[1]	$215.5M	$208.3M	$235.9m	31%	28%	31%
TOTAL	$695M	$744M	$761M	100%	100%	100%

[1] Plant Growth Regulators

In 1999 approximately 81 million pounds of pesticides were applied to upland cotton (USDA-NASS 2001d). The major chemical classes used (organophosphates, carbamates, synthetic pyrethroids) are relatively inexpensive and broad spectrum. New guidelines outlined in the Food Quality Protection Act of 1996 require a decrease in the use of many broad-spectrum pesticides, such as pyrethroids and organophosphates (Alabama Cooperative Extension System 2001). New classes of insecticides have been developed which are significantly more selective toward specific insect pests and are less disruptive to predatory insects and other non-target insect populations. Additionally, cotton farmers worldwide are adopting new biotechnology-derived products that provide an alternative to chemical pesticides. (Carpenter, 2002)

A significant and anticipated result of the adoption of Bollgard®, cotton which expresses an insecticidal protein throughout the plant, has been the major reduction in the use of conventional synthetic insecticide sprays for control of agronomically important lepidopteran species. Numerous studies have been conducted in the US, Australia, China, Mexico, and

Spain and have overwhelmingly reported a reduction in chemical use with the adoption of insect protected biotechnology derived products. Table 9.2 provides specific study references and reduction of insecticide spray by geographic region (USDA/APHIS. 2002).

Table 9.2. Reduction in Insecticide Application on Bollgard® Cotton Varieties Relative to Conventional Cotton

Location	Reduction in number of sprays per year	Reference
Australia	7.7	Anderson, 1999
Spain	5.6	Novillo *et al.*, 1998
Mississippi	5.5	Davis *et al.*, 1995
Arkansas	5.0	Bryant *et al.*,1997
Spain	4.4	Novillo *et al.*, 1999
South Carolina	4.0	ReJesus *et al.*, 1997
South Carolina	3.6	Roof and Durant, 1997
Arkansas	3.0	Bryant *et al.*, 1997
South Carolina	2.9	Roof and Durant, 1997
Georgia	2.5	Stark, 1997
North Carolina	2.5	Bacheler *et al.*, 1997
Southern and Southeastern US	2.4	Mullins and Mills, 1999
Mid-south and Southeastern US	2.2	Benedict and Altman, 2000
Georgia	2.0	Carlson *et al.*, 1998
Mexico	1.0	Obando-Rodriquex *et al.*, 1999
Average across studies	**3.6**	

Reduction in needed insecticide spays directly translates into cost savings for the grower. Additional savings are realized by a lower required investment in supplies, equipment, and labor (Benedict and Altman, 2000; Rejesus et al., 1997; Benedict, 1996; and Benedict et al, 1996). As a result, cotton producers are economically benefiting from biotechnology-based insect control methods and are adopting those products at a considerable rate. Data shown in Table 9.3 indicates that Bt cotton use has increased steadily from 1998 through 2001 and seems to have levelled off in 2002.

Table 9.3. Cotton Acres Grown by Seed Type (Doane, 2003)

Seed Type	1998		1999		2000		2001		2002	
	Acres grown	% Share	Acres grown	% Share	Acres grown	% Share	Acres grown	% Share	Acres grown	% Share
Bt[1]	964694	7.5	1488503	10.2	992283	6.4	385809	2.4	245578	1.7
Bt[1]+RR[2]	918375	7.1	17073134	11.7	2637382	17.0	4449885	27.5	3895584	27.1
BXN[3]	562384	4.4	997644	6.8	862773	5.6	391676	2.4	154547	1.1
Bt[1]+BXN[3]	287873	2.2	0	0	0	0	0	0	24466	0.2
Conv.	7759263	60.0	6300217	43.2	5135160	33.1	4304130	26.6	3786630	26.3
RR[2]	2440165	18.9	4107298	28.1	5904398	38.0	662524	41.1	6274183	43.6
Bt Total	**2170941**	**17.0**	**3195817**	**22.0**	**3629665**	**23.0**	**4835694**	**30.0**	**4165628**	**29.0**
Total	12932753	100	14600975	100	15531995	100	16194024	100	14380987	100

1 Bollgard® Bt cotton

2 Roundup-Ready® cotton

3 Oxynil herbicide tolerance, including bromoxynil and ioxynil.

Although direct side-by-side comparisons of VIP3A expressing cotton and Bollgard® and Bollgard II® varieties have not been conducted, many of the field trials to date with VIP3A cotton have been conducted by University investigators who have also tested the currently registered products and are able to make general comparisons regarding product performance. Insect efficacy data collected to date on VIP3A cotton is presented in Chapter 4 of this Petition and clearly indicates the product is highly efficacious in controlling both major and secondary Lepidopteran cotton insect pests including cotton bollworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*), soybean looper (*Pseudoplusia includens*), beet armyworm (*Spodoptera exigua*), pink bollworm (*Pectinophora gossypiella*), and cabbage looper (*Trichoplusia ni*). One can predict from the spectrum and degree of insect control that the same insecticide reduction, economic, and environmental benefits realized by the use of Bollgard® cotton and expected from the use of Bollgard II® cotton will be gained from the adoption of VIP3A expressing cotton. Studies specifically detailing the insecticide reduction benefits of VIP3A cotton are currently in progress at multiple locations throughout the U.S. cotton belt.

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Chapter 10

STATEMENT OF GROUNDS UNFAVORABLE

The results of all field studies and laboratory tests indicate that no unfavorable grounds are associated with transformation event COT102 expressing the insecticidal protein VIP3A and the marker protein APH4. Syngenta requests that cotton event COT102, and any progeny derived from crosses of event COT102 with conventional cotton varieties, and any progeny derived from crosses of event COT102 with transgenic cotton varieties that have also received a determination of non-regulated status, no longer be considered regulated under 7 CFR Part 340.

CBI-deleted
03-155-01p

Appendix to
CBI Deleted Petition for the Determination of Non-Regulated Status:
Lepidopteran Insect Protected VIP3A Cotton Transformation Event COT102
(USDA Petition # 03-155-01p)

Study Reports Supporting Regulatory Approval

Volume	Study Title	Author	Year	# Pages
1	Characteristics of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Plants Derived from Event COT102	Artim	2002	19
2	Acute Oral Toxicity Study with Test Substance VIP3A-0196 Protein in Mice	Kuhn	1997	32
3	Acute Oral Toxicity Study with Test Substance VIP3A-0199 Protein in Mice	Glaza	2000	142
4	Acute Oral Toxicity Study with Test Substance VIP3A-0100 Protein in Mice	Glaza	2002	109
5	Acute Oral Toxicity Study with Test Substance LPPACHA-0199 Protein in Mice	Glaza	2002	75
6	Acute Avian Oral Toxicity (LD_{50}) Study with VIP3A-0198 in Bobwhite Quail	Pedersen	1999	47
7	VIP3A Maize (Corn) Pollen:Acute Toxicity to Daphnids (*Daphnia magna*) Under Static-Renewal Conditions	Putt	2002	42
8	VIP3A Maize (Corn) Leaf Protein:Acute Toxicity to Earthworms (*Eisenia foetida*)	Teixeira	2002	50
9	Assessment of the Chronic Toxicity of VIP3A and VIP3A/Cry1Ab Maize Pollen to Pink-Spotted Lady Beetle (*Coleomegilla maculata*)	Teixeira	2002	79
10	Analysis of Processed COT102 Cottonseed Products For Yield and Presence of Gossypol and VIP3A Protein	Artim	2002	16
11	Summary of Mammalian Safety Data for the VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Event COT102; Supplement to MRID No. 45766502	Vlachos	2002	29
12	In Vitro Digestibility of VIP3A Protein Under Simulated Mammalian Gastric Conditions	Privalle	2002	14
13	In Vitro Digestibility of APH4 Protein Under Simulated Mammalian Gastric and Intestinal Conditions	Privalle	2002	13
14	APH4-0102: Acute Oral Toxicity of APH4 Protein in the Mouse	Johnson	2002	79
15	Environmental Safety Assessment of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Event COT102 to Non-Target Organisms	Vlachos/Habig	2002	37
16	VIP3A Inbred Maize (Corn) Pollen: Toxicity to Green Lacewing (*Chrysoperla carnea*)	Teixeira	2002	69

Volume	Study Title	Author	Year	# Pages
17	Evaluation of the Dietary Effect(s) of Transgenic VIP3A Maize (Corn) Pollen (Sample PHOPACHA-0199) on Honeybee Development	Maggi	2002	62
18	Impact of VIP3A AND CRY1Ab Transgenic Maize (Corn) Leaf Tissue (Samples LLPACHA-0100, LLBt11-0100, AND LLPACHABt11-0100) on 28-Day Survival and Reproduction of Collembola (*Folsomia candida)*	Privalle	2002	21
19	Biological Activity of VIP3A Maize (Corn) Leaf Protein (Sample LPPACHA-0199) in Various Soils	Privalle	2002	30
20	Characterization of VIP3A Protein Produced in COT102-Derived Cotton and Comparison with VIP3A Protein Expressed in Both Maize (Corn) Derived From Event PACHA and Recombinant *Escherichia coli*	Privalle	2002	44
21	Impact of Transgenic Lepidopteran-Resistant VIP3A Field Corn (Maize) on Honey Bee Colonies in a Semi-field Setting	Dively	2002	34
22	Characterization of VIP3A Protein Produced in Pacha-Derived Maize (Corn) and Comparison with VIP3A Protein Expressed in Recombinant *Escherichia coli*	Privalle	2002	28

03-155-01p

syngenta

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Re: Application for the Determination of Non-Regulated Status for Lepidopteran Insect Protected VIP3A Cotton Transformation Event COT102 (Petition Number 03-155-01p): Response to Deficiency Letter (received February 5, 2004)

Dear Margaret

Please find attached our response to the COT102 deficiency letter. I hope that we have been able to clearly address all of your questions. Please do not hesitate to contact me should you need additional details.

Very Kind Regards,

Lori Artim

Enclosure

cc: Jeff Stein, Director of Regulatory Affairs, Syngenta Seeds, Inc.

4/21/04
Kay

03-155-01p

Syngenta Response to USDA Deficiency Letter
(03-155-01p)

April 20, 2004

1. Please submit to APHIS-BRS any additional data or studies submitted to EPA, including those that utilize cotton or *E. coli* derived VIP3A and APH4 test substances for non-target and soil degradation studies.

   Syngenta Response:
   Syngenta does not anticipate completing additional studies regarding non-target organism toxicity of VIP3A protein. A complete ecological toxicity package was submitted to EPA in support of commercial registration of VIP3A expressing cotton event COT102. No such tests are required for the selectable marker protein APH4 as it is considered by EPA as an 'inert ingredient'.

2. Page 19. Provide update regarding consultations with FDA and EPA.

   Syngenta Response:
   Syngenta has received from EPA a Deficiency Letter (dated January 13, 2004) relating to the commercial Registration of VIP3A expressing cotton Event COT102. The letter includes additional questions relating to product characterization and mammalian toxicity, ecological toxicity, insect resistance management, and the public interest finding. The product characterization and mammalian toxicity issues raised include very minor clarifications to be made to submitted data. The ecological toxicity issues have been discussed with EPA at a meeting held on March 2, 2004 with scientific staff. Additionally, we have submitted a written response as requested by EPA. The feedback from that meeting indicated that EPA had not completely evaluated the non-target organism studies in the context of actual exposure and with regards to expected safety margins or with regards to the demonstrated equivalence between the COT102 VIP3A protein and VIP3A as contained in the test substances used to expose the non-target organisms in these studies. Syngenta is optimistic that no new studies will be required prior to registration, and that ALL of the submitted ecological toxicity studies will be deemed acceptable in support of this product. Syngenta plans to meet with EPA shortly to discuss IRM related issues. The public interest finding issues raised were related to benefits claims. Syngenta has since collected new information which will be presented to EPA regarding benefits of this product.

   Syngenta has been granted an Experimental Use Permit for planting in 2004 (EUP-67979-2) and the associated time limited tolerance exemptions for both VIP3A and APH4.

   The FDA has completed review of the COT102 Pre-Market Biotechnology Notice. We have addressed minor questions and clarifications with FDA scientists and are now awaiting final FDA's letter expressing non-objection.

4/21/04
Kay

3. Pages 23 - 25. Submit Syngenta unpublished data, documenting VIP3A insect sensitivity and insensitivity, as referenced in Tables 1.1, 1.2 and 1.3.

Syngenta Response:

A. Sensitive Lepidopteran species:

Anticarsia gemmatalis, Cochylis hospes, Diatraea grandiosella, Diatraea saccharalis, Homoeosoma electellum, Manduca sexta, Pectinophora gossypiella, Pseudoplusia includens, Scirpophaga incertulas, Spodoptera ornithogall, Trichoplusia ni

Syngenta scientists carried out laboratory insect bioassays using purified VIP3A protein as a test substance. Eight to ten different VIP3A concentrations were tested, ranging from 10 $\mu g/cm^2$ to 10 ng/cm^2. The toxin solution was applied to the surface of a typical insect diet and allowed to air dry. Typically, three to five replicates per test concentration were evaluated with ten larvae per replicate. Mortality was recorded at 72 and 120 hours after initial treatment. Assays were repeated three to five times to confirm results and account for variability.

Chilo suppressalis, Helicoverpa armigera, Spodoptera littoralis

Syngenta scientists carried out laboratory insect bioassays using purified VIP3A protein as a test substance. Seven different VIP3A concentrations were tested ranging from 10 $\mu g/cm^2$ to 10 ng/cm^2. The toxin solution was applied to the surface of a cotton leaf and allowed to air dry. Typically, three replicates per test concentration were evaluated with 20 first instar larvae per replicate. Mortality was recorded at 72 hours after initial treatment. Assays were repeated three to five times to confirm results and account for variability.

Cnaphalocrocis medinalis, Helicoverpa punctigera

Sensitivity was determined for these insects in field trials conducted with plants expressing VIP3A protein. In China, rice plants expressing VIP3A provided excellent control of *Cnaphalocrocis medinalis*. In Australia, cotton plants expressing VIP3A provided excellent control of *Helicoverpa punctigera.*

Ostrinia furnacalis

VIP3A protein was tested for activity to Asian corn borer as indicated in the patent WO97/46105 (Method of Controlling Insect Pests). Larvae were allowed to feed on maize leaves dipped in purified VIP3A protein at varying concentrations (100, 50, 25, 12.5, and 6.25 ppm) for 120 hours. The number of dead larvae was recorded. Results indicate significant activity of VIP3A protein on larval mortality and larval feeding.

Sesamia nonagrioides

Maize expressing VIP3A plants were tested for activity to *Sesamia* as indicated in the patent WO97/26339 (Method of Protecting Crop Plants Against Insect Pests). Larvae were allowed to feed on maize leaves for 72 to 120 hours. The number of live larvae and developmental instar was recorded. Results indicate significant activity of VIP3A protein on larval mortality and larval feeding.

B. Insensitive Lepidopteran species:

Danaus plexippus, Plodia interpunctella
Syngenta scientists carried out laboratory insect bioassays using purified VIP3A protein as a test substance. A single high concentration was typically tested (1-5 µg VIP3A/cm^2). The toxin solution was applied to the surface of a typical insect diet and allowed to air dry. Typically, two to five replicates per test concentration were evaluated with ten larvae per replicate. Mortality was recorded at 72 to 120 hours after initial treatment.

Hyphantria cunea
Bacillus culture supernatants of strain AB88 were tested (containing VIP3A protein). Same procedure was use as described in the following:

Warren, G.W. (1997) Vegetative insecticidal proteins. *In*: Advances in insect control: The role of transgenic plants. N.B. Carozzi and M.G. Koziel, eds. Gunpowder Square, London.

C. Insensitive Non-Lepidopteran species:

Diabrotica longicornis, Diabrotica undecimpunctata, Anthonomus grandis
Syngenta scientists carried out laboratory insect bioassays using purified VIP3A protein as a test substance. A single high concentration was typically tested (1-5 µg VIP3A/cm^2). The toxin solution was incorporated into semi-cooled insect diet, mixed well, and allowed to solidify. Typically, three to five replicates per dose were evaluated with ten larvae per replicate. Mortality was recorded at 72 and 120 hours after initial treatment. Assays were repeated three to five times to confirm results and account for variability.

Myzus persica, Frankliniella occidentalis
Syngenta scientists carried out laboratory insect bioassays specific for aphid and thrip biology using purified VIP3A protein as a test substance. The toxin solution was incorporated into a sucrose solution, which the insects were allowed to feed upon. Typically, three to five replicates per dose were evaluated with five to ten insects per replicate. Mortality was recorded at 96 to 120 hours after initial treatment. Assays were repeated three to five times to confirm results and account for variability.

Popillia japonica, Musca domestica
Bacillus culture supernatants of strain AB88 were tested (containing VIP3A protein). Same procedure was use as described in the following:

Warren, G.W. (1997) Vegetative insecticidal proteins. *In*: Advances in insect control: The role of transgenic plants. N.B. Carozzi and M.G. Koziel, eds. Gunpowder Square, London.

4. Page 26. The current URL address for the APHIS Cotton Biology Document is: http://www.aphis.usda.gov/brs/cotton.html

5. Pages 29 and 30. Indicate whether the Agrobacterium was cleared from the transformed tissue.

   Syngenta Response:
   After initial incubation with Agrobacterium, transformed tissue was transferred to, and grown for four months on selective media containing 500 mg/L of the broad-spectrum antibiotic cefotaxime.

6. Page 32. Provide a citation where ColE1 was described, isolated, and characterized (publicly available database citations are acceptable) and donor source for ColE1.

   Syngenta Response:
   Itoh, T., & Tomizawa, J. (1978). Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H and DNA polymerase I. Cold Spring Harbor Symposium on Quantitative Biology, 43, 409-418.

7. Page 35. The text indicates that the size of the restriction fragment of pCOT1 between sites SmaI and AscI is approximately 7.2 kb. However, in Figures 3.1, 3.2c, 3.3b, when all the constituent fragments between sites SmaI and AscI are added up (NOS Terminator of 255 bp + aph4 of 1026 bp, and so on) the total is 7,914. The band in Figure 3.2a appears to be approximately 7.9 kb.

   Syngenta Response:
   The manner in which we annotate promoters may have not been clearly indicated. The "intron" portion of the promoter is indicated on the pCOT1 map, but is a part of the total length indicated for the respective promoters. For example, the Actin 2 promoter is 1402 bp in length and 453 bp of this 1402 bp is an intron. If the length of these introns is added in addition to the length of the respective promoters you will achieve 7.9 kb. The correct size for the SmaI and AscI fragment in question is 7.2 kb, as indicated on the pCOT1 plasmid map. Enclosed is an update for table 3.2 to clarify the manner in which introns are annotated on the pCOT1 plasmid map. The distance separating the 7.0 kb and 8.0 kb standards on the southern is approximately 3 mm on figure 3.2a, and the difference between 7.9 kb and 7.2 kb would be difficult to ascertain at that level of resolution. However, the resulting hybridization band seen in Event COT102 does appear to be the same size as the predicted 7.2 kb band from pCOT1.

Table 1 (3.2. from original Petition) Summary of DNA sequences in pCOT1. (See figure 3.1)

Genetic Element	Base pair range (11801 bp plasmid)	Introduced during transformation
Left border region	11732-11801 bp and 1-60 bp	Partial Transfer
Left border	1-25 bp	Partial Transfer
Intervening Sequence	61-107 bp	Yes
nos terminator	108-362 bp	Yes
Synthetic Linker	363-394 bp	Yes
aph4 gene	395-1420 bp	Yes
Synthetic Linker	1421-1451 bp	Yes
ubiquitin-3 promoter, which contains: ubiquitin 3 intron	1452- 3172 bp 1452-1826 bp	Yes
Synthetic Linker	3173-3222 bp	Yes
actin-2 promoter, which contains: actin-2 leader exon actin-2 leader intron	3223-4630 bp 4124-4174 bp 4178-4630 bp	Yes
Synthetic Linker	4631-4639 bp	Yes
vip3A(a) gene	4640-7009 bp	Yes
Synthetic Linker	7010-7033 bp	Yes
nos terminator	7034-7288 bp	Yes
Intervening Sequence	7289-7449 bp	Yes
Right border region	7450-7615 bp	Partial Transfer
Right border	7494-7518 bp	Partial Transfer
Vector Backbone	7519-7630 bp	No
ColE1	7631-8437 bp	No
Vector Backbone	8438-9114 bp	No
VS1ori	9115-9519 bp	No
Vector Backbone	9520-9561 bp	No
RepA	9562-10635 bp	No
Vector Backbone	10636-10663 bp	No
Spectinomycin	10664-11452 bp	No
Vector Backbone	11453-11731 bp	No

8. Page 39. Plasmid numbering of restriction sites should be consistent in all diagrams. pCOT1 SmaI site is labeled 170 (Figure 3.2c) and 93 (Figure 3.1), and AscI is labeled 7393 (Figure 3.2c) and 7316 (Figure 3.1).

   Syngenta Response:
   Figures 3.2c (linear map of pCOT1 with the *vip3A(a)* probe) and Figure 3.4b (linear map of pCOT1 with *aph4* probe) were inconsistent compared to the other figures in Chapter 3

regarding position of restriction enzyme sites. These maps have been updated to reflect consistent numbering. See new figures attached at the end of this document.

9. Page 61. Cite method used for amino acid sequencing.

Syngenta Response:
The amino acid sequence for VIP3A and APH4 reported on page 61 is deduced from the nucleotide sequence. The VIP3A protein expressed in event COT102 has also been isolated and subjected to mass spectroscopic analysis. After a multi-step purification process from cotton leaves, in-gel digestions using various proteases generated peptides that were analyzed by tandem mass spectrometry. Mascot™ software (version 1.8, Matrix Sciences Ltd., London, UK) was used to match observed and predicted peptide masses. Peptides representing *ca.* 85% (673/789) of the complete VIP3A amino acid sequence were identified by mass spectral analysis of cotton-produced VIP3A protein and confirm the expected amino acid sequence.

10. Pages 62. APHIS records indicate that the 01-109-01n site was not planted.

Syngenta Response:
The site covered under that Notification was not planted. A termination report was submitted indicating that information.

11. Pages 62 and 63. Field test sites referenced in Table 4.1 (60 releases in 13 states) appears to be inconsistent with the information on pages 2 and 74 (51 releases in 12 states). Were all releases evaluated for insect efficacy and agronomic performance?

Syngenta Response:
Agronomic performance and insect efficacy data were formally generated and reported on a subset of the planted field trials noted in Table 4.1. Agronomic performance data reported in this Petition was generated at five locations in 2001 and 15 locations in 2002. Insect efficacy data reported was generated at 18 locations in 2002.

12. Pages 66 - 70. It is unclear what parameter was evaluated to determined Cumulative Percent Damage for Tables 4.4, 4.5, 4.6, 4.7 and 4.8.

Syngenta Response:
Calculations for cumulative season-long damage or infestation were completed as follows: For each individual site, the total number of squares (terminals, bolls, etc.) sampled throughout the season were determined. That total number was then divided by the number of damaged tissues recorded. Example: one location sampled twenty-five squares per assessment date at four times during the season, for a total of one-hundred squares sampled. If twelve squares were damaged, then 12% cumulative damage squares was reported.

13. Page 68. In Table 4.6, the relative difference in "Cumulative Percent Infestation" in Winnsboro, LA, of 2.8 and 10.6 appears to indicate an observation of significant difference, however, both are followed by the same letter.

    Syngenta Response:
    The value of 2.8 should have been followed by a 'b' to indicate a significant difference from the 16.6 value. We apologize for the error.

14. Page 70. In Table 4.8, the Cumulative Percent Damage lacks a letter designation for the Student-Newman-Keuls test.

    Syngenta Response:
    All of the COT102 Cumulative Percent Damage values should have been followed by a 'b' to indicate significant difference from the Coker 312 values. We apologize for the omission.

15. Page 71. In Table 4.9, the level of infestation for the Corpus Christi, TX location at 1.5% appear to be too low to evaluate at 100% reduction.

    Syngenta Response:
    Because of the very low insect pressure realized at the location, Syngenta agrees that the % reduction value reported is unreliable. We would suggest the value be removed from that table for that location.

16. Page 71. Table 4.10, the Newport, AR site infestation levels for the soybean looper, do not support a 60% reduction, if the statistical analysis of the data indicate that the levels of infestation are not significantly different.

    Syngenta Response:
    Same comment as #15. Low insect pressure did not allow for statistical separation, thus inferences on % reduction may not be valid.

17. Page 74. As an indication of the reproductive potential of the plant, where changes observed in the number of seeds produced per boll?

    Syngenta Response:
    Syngenta collected reproductive potential data from a single location (Leland, MS) planted in 2003. The seeds per boll were counted from 25 bolls collected from each genotype. The data were subjected to a simple analysis of variance. As part of this analysis, an F-test was used to test for evidence of a difference between the two genotypes. This test resulted in a probability level of 36.1%. In view of the fact that this probability level is far in excess of the customary 5% probability level, we conclude that the data provide no evidence to suggest that COT102 and Coker 312 differ with respect to the number of seeds per boll.

Table 2: Number of seeds per boll.

Genotype	# seeds per boll
Coker 312	27.8
COT102	29.0
Standard Deviation	1.2
F-Test Probability	36.1%

18. Pages 81 - 86. Figures 4.4 through 4.11 are either inaccurately referenced in the text or not referenced in the text.

Syngenta Response:
We agree with the errors cited with regards to the Table references. We would be willing to correct those pages and resubmit if APHIS suggested.

19. Page 87. Provide evidence for the statement that "non-target insect species were generally present at higher populations in the event COT102 and Coker 312 plots as compared to the sprayed treatments". Which field trials sites? What was evaluated? What are the results?

Syngenta Response:
It is Syngenta's standard protocol that insect efficacy trials are controlled for non-Lepidopteran species to clearly assess damage caused only by target pests. Plant bugs and sucking pests are controlled with the use of chemical treatments (ex. neonicotinoids and organophosphates). Field cooperators make general observations regarding the presence of non-target species in sprayed and unsprayed plots. Obviously, when chemical pesticides are applied, fewer non-target organisms are present in treated plots. No specific non-target insect counts were provided.

Syngenta has also conducted a non-target organism field survey study with Peter Ellsworth of the University of Arizona evaluating the presence of non-target species in large field plots of event COT102 plants. The final report from that study is not yet available.

20. Page 91. In Table 5.4 verify that the standard deviation for sodium (Na) is 212.

Syngenta Response:
The standard deviation of 212 ppm as reported for Na in Table 5.4 is correct. The statistical analysis we have conducted takes account of differences across location (samples taken from three locations in 2001), and produces an estimate of standard deviation that excludes the effect of location. This is standard practice and is entirely appropriate in this case.

21. Page 128. Explain how applying defoliants will reduce the amount plant tissue that is incorporated into the ground? Cite studies, or present data.

Syngenta Response:
Common agronomic practice includes the application of a defoliant prior to harvest. The defoliant causes the green leaves of the vegetative cotton plant to drop to the ground. Those leaves dry and decompose on the surface. The harvest machinery often further crushes decomposing leaves. Once cotton fiber has been harvested from the field, remaining stalks are typically mowed and left to over-winter. Tilling usually only occurs the following spring prior to planting. By the time tilling occurs, there is little to no leaf material present in the field thereby reducing the total amount of plant material incorporated through tillage.

22. Page 131. Reference the study guideline followed to generate the larval honeybee study.

Syngenta Response:
EPA Pesticide Assessment Guidelines, Series 885 – Microbial Pesticide Test Guidelines Group D – Nontarget Organism and Environmental Expression Test Guidelines, OPPTS Number: 885.4380, Honeybee Testing, Tier 1

23. Page 134. Provide evidence for the statement that "pests that feed upon VIP3A cotton plants are expected to retain little if any VIP3A protein".

Syngenta Response:
The indicated statement was made to provide evidence that the selection of lacewing adults as the test organism was more appropriate than lacewing larvae due to more likely direct exposure to adults.

In the Syngenta study [VIP3A Inbred Maize (Corn) Pollen: Toxicity to Green Lacewing (*Chrysoperla carnea*) (MRID No. 45835808)], adults were fed diets including maize pollen for 13 days. The concentration of VIP3A protein was 20X the concentration in COT102 pollen. Lacewing adults are known to consume nectar and pollen. The green lacewing larvae are predators, primarily of aphids. Adults were chosen for the study because they could be fed maize pollen directly in the laboratory test, and may ingest pollen in the field, whereas the larvae will not be directly exposed to active VIP3A protein in cotton fields. Dietary exposure, if any, to lacewing larvae would only occur at negligible amounts via tri-trophic mechanisms.

Many foliar non-target beneficial insects consume a varied diet, which may include prey organisms along with pollen. The greatest potential exposure of these organisms is from ingestion of pollen containing VIP3A protein. Two recent studies (Head *et al.*, 2001; Raps *et al.*, 2001) indicate that pest species feeding upon Bt corn plants retained little Cry1Ab protein (9 – 143 times lower concentrations than were present in the Bt plant tissue) or no Cry1Ab protein after feeding upon Bt corn plants expressing Cry1Ab. It can be inferred from these data that VIP3A concentrations will be uniformly low to zero in pest organisms that consume VIP3A cotton tissues.

Head, G., C.R. Brown, M.E. Groth and J.J. Duan (2001) Cry1Ab protein levels in phytophagous insects feeding on transgenic corn: implications for secondary exposure risk assessment. *Entomologia Experimentalis et Applicata* 99: 37-45.

Raps, A., J. Kehr, P. Gugerli, W.J. Moar, F. Bigler and A. Hilbeck (2001) Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the nontarget herbivore *Rhopalosiphum padi* (Homoptera: Aphidae) for the presence of Cry1Ab. *Molec. Ecology* 10: 525-533.

24. Page 135-136. Clearly explain what is being measured in this test. The text refers to number of juveniles per adult survivor and number of juveniles resulting from eggs produced by the original 10 juveniles. How is juvenile production a measure of sensitivity or insensitivity to a compound (an indicator of survival or reproduction)?

Syngenta Response:
The described Collembola study [Impact of VIP3A AND CRY1Ab Transgenic Maize (Corn) Leaf Tissue (Samples LLPACHA-0100, LLBt11-0100, AND LLPACHABt11-0100) on 28-Day Survival and Reproduction of Collembola (*Folsomia candida)* (MRID No. 45835810)] was designed to measure **survival** (mean survival of adults that had matured from the original juveniles), and **reproduction** (mean number of second generation juveniles produced from eggs of adults that had matured from the original juveniles).

The original juvenile Collembolla were exposed to VIP3A containing leaf material and matured normally into adults. Those adults also being exposed to VIP3A containing leaf material produced eggs and subsequent offspring (juveniles). The life cycle was completed on the test material and proved no significant effects when compared to appropriate control materials.

25. Page 136. Provide additional information regarding test substances. Which lines were used and what levels if any, VIP3A and/or Cry1Ab were produced? Define the genetic background relative to the test of the "control hybrid", "transgenic hybrid", "negative control cultures", "control corn leaf cultures" and "corn cultures". If the "negative control" is not the untransformed control, why not?

Syngenta Response:

Sources of test and reference substances.

Test Substance LLPACHA-0100. Hybrid VIP3A maize (field corn) plants of genotype 894 x CG00526-Pacha were field-grown in Illinois. Leaves were obtained from six-week old plants and shipped overnight on wet ice to the Syngenta Seeds, Inc. Product Registration Laboratory (Research Triangle Park, NC). The leaves were powdered using a Retsch cutting mill (Brinkmann, Inc.; Westbury, NY, USA) and dried by lyophilization.

The powdered lyophilized material was stored at room temperature under ambient conditions.

Test Substance LLPACHABt11-0100. Hybrid VIP3A/Cry1Ab maize (field corn) plants of genotype 894-Bt11 x CG00526-Pacha were field-grown in Illinois. Leaves were obtained from six-week old plants and shipped overnight on wet ice to the Syngenta Seeds, Inc. Product Registration Laboratory. The leaves were powdered, lyophilized, and stored as described for LLPACHA-0100.

Reference Substance LLPACHA-0100C. Hybrid maize (field corn) plants of genotype 894 x CG00526 were field-grown in Illinois. Leaves were obtained from six-week old plants and shipped overnight on wet ice to the Syngenta Seeds, Inc. Product Registration Laboratory. The leaves were powdered, lyophilized, and stored as described for LLPACHA-0100.

Test Substance LLBt11-0100. Leaves were harvested from four-week old, greenhouse-grown, hybrid Cry1Ab maize plants of the Attribute® Insect Protected sweet corn variety. The leaves were powdered, lyophilized, and stored as described for LLPACHA-0100.

Reference Substance LLBt11-0100C. Leaves were harvested from four-week old, greenhouse-grown, nontransgenic "Bonus" hybrid maize (sweet corn) plants that were isogenic to the Attribute® genotype. The leaves were powdered, lyophilized, and stored as described for LLPACHA-0100.

The term "culture" used in the test refers to the culture of Collembola on the powdered lyophilized leaf material mixed with the *in vitro* yeast diet.

See Table 3 (attached) indicating characterization of the test and reference substance used in this study.

26. Page 137. Define the relevant genetic background of the control corn grain (KKKPACHA-0100) for the Channel Catfish feeding study.

Syngenta Response:

Control corn grain (KPACHA-0100C) used for the preparation of the control fish food (FFKPACHA-0100C) was isogenic to the transgenic corn grain (i.e., of the same genetic background), and grown concurrently under the same environmental conditions.

27. Page 162. Provide evidence for substrate specificity of APH4. Does the cotton plant produce potential APH4 substrates that could result in the production toxic substances?

Syngenta Response:

APH4 catalyzes the phosphorylation of the 4-hydroxyl group on the hyosamine moiety of hygromycin B, thereby inactivating it. The enzyme has a narrow range of substrates, in that it phosphorylates hygromycin B, hygromycin B_2 and the closely-related antibiotics destomycin A and destomycin B, but does not phosphorylate other aminocyclitol or aminoglycoside antibiotics including neomycin, streptomycin, gentamicin, kanamycin, spectinomycin, tobramycin, and amikacin (Rao *et al.*, 1983). Hygromycin B is not used

in human clinical therapy, but is principally used as an antihelminthic agent in swine and poultry feeds.

Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, H.A. Kirst and J.W. Paschal (1983) Genetic and enzymatic basis of hygromycin B resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 24 (5): 689-695.

28. Page 162. Provide information on dermal exposure safety in humans. Is the APH4 protein contained within plant cells?

Syngenta Response:

No human dietary exposure to APH4 protein is expected to result from the commercial use of COT102-derived cotton plants, as the cotton products that enter the food supply (primarily cottonseed oil and cottonseed linters) are essentially devoid of protein of any kind. Moreover, APH4 protein was not detectable by ELISA in cotton fiber and in most samples of whole cottonseed. In the relatively few cottonseed samples in which APH4 protein was detectable, the quantity was too low to quantify (< 150 ng/g dry wt.; < 150 ppb).

The APH4 protein is contained within plant cells, therefore dermal exposure is unlikely.

29. Page 172. Provide search criteria and all databases searched for the presence of sequences homologous to allergens for APH4 and VIP3A.

Syngenta Response:

VIP3A: To determine whether the VIP3A protein had any significant amino acid homology with allergenic proteins, the VIP3A protein sequence (789 amino acids; GenBank accession No. L48811), as encoded by the *vip3A(a)* gene in transgenic maize, was systematically compared to the Syngenta Biotechnology, Inc. (SBI) Allergen Database. This database contains the amino acid sequences of known and putative protein allergens, and was initially compiled from entries in the following database sources:

1. All entries identified as allergens or putative allergens in the publicly available GenPept, PIR or SWISS-PROT protein databases. Allergen sequence entries from these public databases were identified using the program Lookup from the GCG Wisconsin Package version 10.1 as part of the SeqWeb Bioinformatics package (Accerlys, Inc., 2001).

2. Entries in the SWISS-PROT Allergen database (SWISS-PROT, 2001);

3. Entries in the List of Allergens database (International Union of Immunological Societies, 2001);

4. Entries in the FFARP Protein Allergen database (Food Allergy Research and Resource Program, 2001); and

5. Additional entries identified in the scientific literature as putative allergens, but which are not found in the public databases.

The SBI Allergen Database is updated on a semi-annual basis by searching the latest updates of the publicly available databases (listed as 1 – 4, above), and identifying any additional new allergens or putative allergens in these databases as well as in the scientific literature. A list of updated sequence entries is compiled, redundant sequences are removed, and the remaining unique set of updated sequences is added to the SBI Allergen Database. At the time the bioinformatic analysis of VIP3A was conducted, the latest update to the SBI Allergen Database was February 2002, at which time a total of 1523 entries had been compiled from among the sources listed. Some of these entries are redundant, *i.e.* they appear more than once because they were present in more than one of the source databases at the time the initial SBI database was compiled.

Three different similarity searches were performed comparing the VIP3A protein to the entries in the SBI Allergen Database. First, the entire VIP3A protein sequence was compared to the allergen sequences using the FASTA search algorithm[1] (Pearson and Lipman, 1988). Second, contiguous VIP3A peptides of 80 amino acids, overlapping by 10 amino acids, were compared to the allergen sequences using the FASTA search algorithm. Third, the VIP3A protein sequence was screened for matches of eight or more contiguous amino acids using a program (developed by Syngenta), which compares every possible peptide of eight contiguous amino acids between VIP3A and the allergen sequences. The results of these analyses revealed (1) no significant similarity with allergen sequences when comparing the entire sequence of the VIP3A protein, (2) no significant sequence alignments between the 80 amino acid-VIP3A peptides and any allergen sequences, where significance was defined as identity of greater than 35%, and (3) no alignments of eight or more contiguous identical amino acids between any peptides of VIP3A and the allergen sequences. Thus, VIP3A protein shows no significant homology to known or putative protein allergens.

APH4: To determine whether the APH4 protein had any significant amino acid homology with allergenic proteins, the APH4 protein sequence (341 amino acids; GenBank accession No. CAA85741), as encoded by the *aph4* gene in transgenic cotton, was also systematically compared to the Syngenta Biotechnology, Inc. (SBI) Allergen Database (described above).

Three different similarity searches were performed comparing the APH4 protein to the entries in the SBI Allergen Database. First, the entire APH4 protein sequence was compared to the allergen sequences using the FASTA search algorithm (Pearson and Lipman, 1988). Second, contiguous APH4 peptides of 80 amino acids, overlapping by 10 amino acids, were compared to the allergen sequences using the FASTA search

[1] In all the FASTA searches, the scoring matrix was blosum 62, the gap extension penalty was 2 and the gap creation penalty was 12.

algorithm[1]. Third, the APH4 protein sequence was screened for matches of eight or more contiguous amino acids using a program (developed by Syngenta), which compares every possible peptide of eight contiguous amino acids between APH4 and the allergen sequences. The results of these analyses revealed (1) no significant similarity with allergen sequences when comparing the entire sequence of the APH4 protein, (2) no significant sequence alignments between the 80 amino acid-APH4 peptides and any allergen sequences, where significance was defined as identity of greater than 35%, and (3) no alignments of eight or more contiguous identical amino acids between any peptides of APH4 and the allergen sequences. Thus, APH4 protein shows no significant homology to known or putative protein allergens.

Figure 3.2c Linear map of pCOT1 with the *vip3A(a)* probe

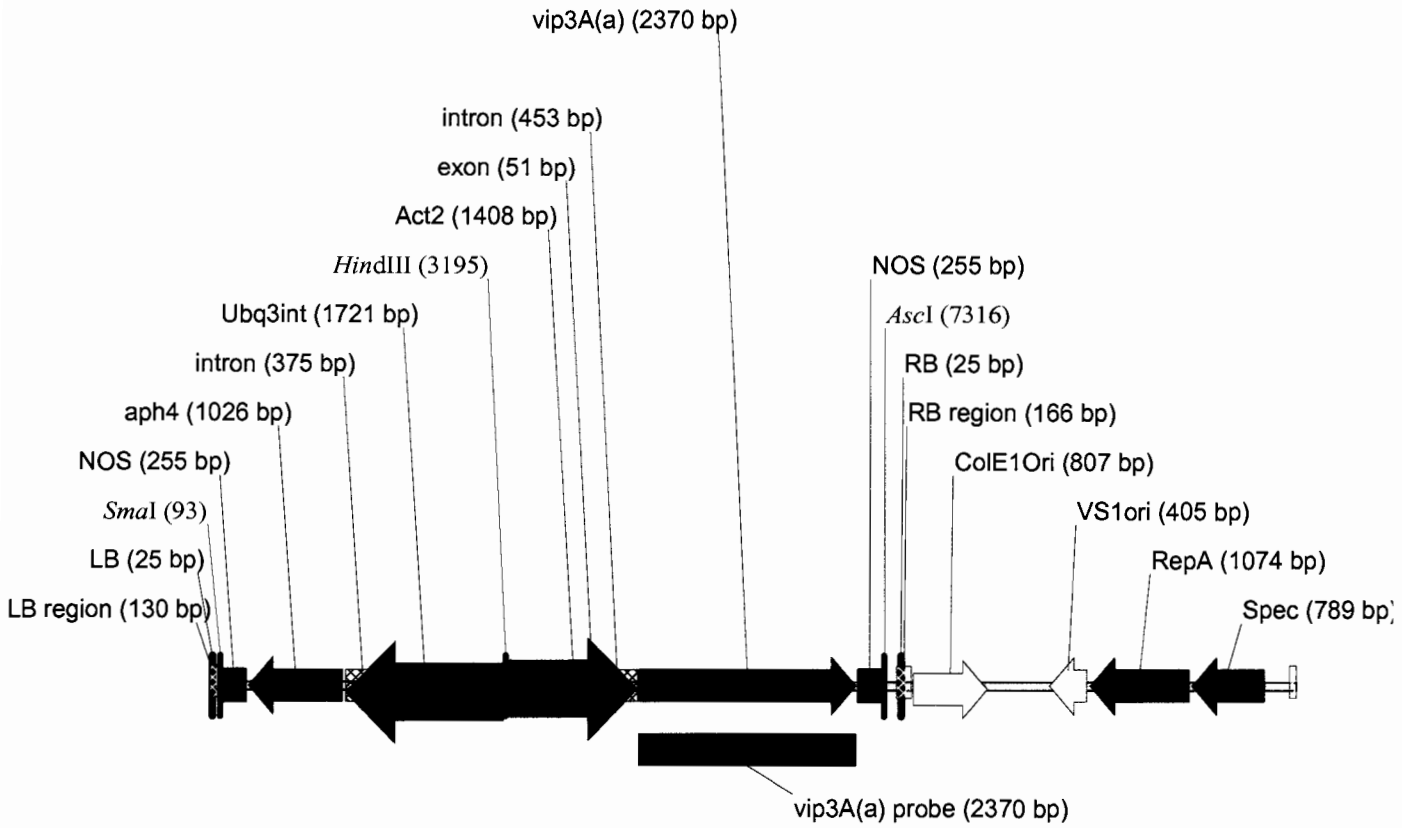

Figure 3.4b. Linear map of pCOT1 with *aph4* probe

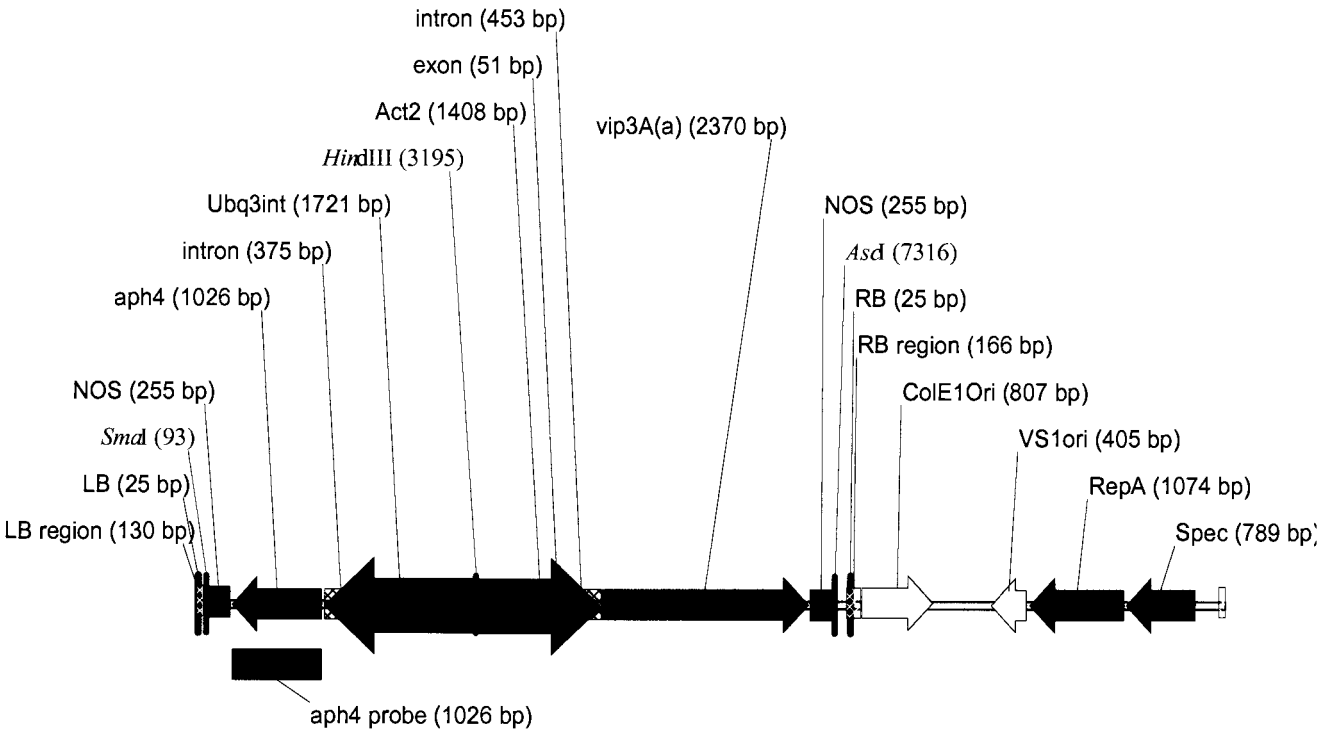

Table 3. Characterization of Test and Reference Substances

Test/Reference Substance	µg VIP3A/g		µg Cry1Ab/g		Bioactivity (% Mortality)			Estimated Conc. In Collembolan Diet	
	1[a]	2[a]	1	2	FAW[b]	ECB[c] 1	ECB[c] 2	VIP3A (µg/g)	Cry1Ab (µg/g)
LLPACHA-0100C	nd[d]	nd	NT[e]	NT	10	10.0	3.3	0.0	0.0
LLPACHA-0100	86.1	76.8	NT	NT	50	10.0	0.0	12.3	0.0
LLPACHABt11-0100	51.6	55.2	28.8	32.7	25	60.0	40.0	7.4	4.1
LLBt11-0100C	NT	NT	nd	nd	0	6.7	6.7	0.0	0.0
LLBt11-0100	NT	NT	34.2	20.6	0	13.3[f]	3.3[f]	0.0	4.9

[a] Analyses were performed prior to initiation (1) of the in-life phase of the 28 day study and after the in-life phase of the study (2).

[b] Sample homogenates were assayed in duplicate cultures, using 10 larvae per culture. Mortality was assessed after 72 hours and reported as the mean. Data are not shown for the initial (pre-test) FAW bioassay, because the sample extract applied to the larval diet surface did not contain sufficient VIP3A protein to observe bioactivity. Data shown are for the reanalysis conducted after the in-life phase of the study, for which a homogenate (rather than an extract) of the sample was applied to the larval diet surface. Mean mortality was 5% in control cultures in which the diet surface was treated only with 0.1% Triton-X 100 solution, the dispersing agent used in applying the sample homogenate to the test cultures.

[c] Sample extracts were assayed in triplicate cultures, using 10 larvae per culture. Effects were assessed after 120 hours.

[d] nd = not detectable

[e] NT = not tested

[f] Mortality did not exceed mortality in controls. (The buffer control had 13.3 and 6.7% mortality initially and upon reanalysis, respectively). However, absence of feeding and smaller larvae were noted for these samples in comparison with the controls.

Addendum to Syngenta Response to USDA Deficiency Letter
(03-155-01p)

May 26, 2004

Pages 66 - 70. It is unclear what parameter was evaluated to determined Cumulative Percent Damage for Tables 4.4, 4.5, 4.6, 4.7 and 4.8.

Syngenta Response:

Damage was assessed on the following tissues and reported in the cited tables: terminal leaves (Table 4.4), squares (Table 4.5), flowers (Table 4.6), the apical portion of immature bolls (Table 4.7), and developed green bolls (Table 4.8). Damage was determined in all tissues to consist of significant injury to each of the listed tissues and not merely superficial markings. Specifically, terminal leaves were considered damaged when significant feeding had occurred to the undeveloped leaves at the plant meristem. Squares were assessed to have significant damage when insects fed to the point of penetrating the square and moved into the undeveloped petal lying under the square. Flowers were visually assessed to have significant damage when clear feeding had occurred to the flower petals, stamen, and pollen. That damage would severely limit the likelihood of a flower normally progressing to the boll stage. The apical portion of immature bolls was considered damaged when insects had punctured through the carpel wall of the boll. Similarly, immature green bolls also were assessed as damaged when insects had fed through the carpel wall and entered the boll. Boll infestation and damage would result in a malformation of that boll which would affect yield. Often early boll damage caused an abortion of the boll formation and a cease in development.

Pages 81 - 86. Figures 4.4 through 4.11 are either inaccurately referenced in the text or not referenced in the text.

Syngenta Response:

Figure 4.4 is a graphical representation of the data described in Chapter 4 (Agronomic Performance), Section C (Morphological and Agronomic Characteristics), Subsection #2 (Trial Protocol Method #2 – Results), Part IV. Plant Height to Node Ratio (HNR) (page 81). Figure 4.4 should have been referenced in the text of that section.

Figure 4.5 is a graphical representation of the data described in Chapter 4 (Agronomic Performance), Section C (Morphological and Agronomic Characteristics), Subsection #2 (Trial Protocol Method #2 – Results), Part V. Total Number of Fruiting Branches (page 82). Figure 4.5 should have been referenced in the text of that section. Figure 4.4 is incorrectly mentioned in the text.

Figure 4.6 is a graphical representation of the data described in Chapter 4 (Agronomic Performance), Section C (Morphological and Agronomic Characteristics), Subsection #2 (Trial

Protocol Method #2 – Results), Part VI. Box Mapping Data and Node to 95% Yield Accumulation (page 83). Figure 4.6 should have been referenced in the text of that section. Figure 4.7 is incorrectly mentioned in the text.

Figures 4.7, 4.8, 4.9, 4.10, and 4.11 are graphical representations of the data described in Chapter 4 (Agronomic Performance), Section C (Morphological and Agronomic Characteristics), Subsection #2 (Trial Protocol Method #2 – Results), Part VIII. High Volume Instrument (HVI) Fiber Quality Analysis (page 84). Each of those figures refers to a particular measurement and should have been referenced in that section.

Addendum to Syngenta Response to USDA Deficiency Letter

(03-155-01p)

November 4, 2004

Item 27: Page 162. Provide evidence for substrate specificity of APH4. does the cotton plant produce potential APH4 substrates that could result in the production of toxic substances?

Syngenta Response:

Syngenta has not carried out specific studies to assess whether APH4 can phosphorylate substances within the cotton plant. However, given the relatively narrow substrate specificity of APH4, even among closely-related antibiotics of the same class, it is very unlikely that APH4 will phosphorylate any natural substances within the cotton plant. Moreover, it is even less likely that any such theoretically phosphorylated substances would lead to the production of new toxins within the cotton plant, or that these toxins would be present in sufficient concentrations in the relevant plant parts to represent a toxic hazard to organisms consuming the plant tissue or products thereof. It is worth noting that the natural toxicity of cotton (e.g., from gossypol) already limits its use for food and feed purposes.