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Petition for Determination of Non-regulated Status for Ultra-Low Gossypol Cottonseed TAM66274

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR 340.

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CERTIFICATION

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

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SUMMARY

Texas A&M AgriLife Research is submitting a Petition for Determination of Non-regulated Status for TAM66274 cotton. Texas A&M AgriLife Research requests the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) confirm that TAM66274 and any cotton lines derived from crosses between TAM66274 and conventional cotton or biotechnology-derived cotton granted non-regulated status by APHIS, no longer be considered regulated articles under 7 CFR Part 340.

Product Rationale and Description.

Although cottonseed is a rich source of relatively high quality protein and is used as a feed supplement for ruminant animals, it is not typically consumed by humans or monogastric animals due to the presence of the anti-nutrient gossypol. Historically, limitations on the use of cottonseed due to gossypol have led to efforts to reduce gossypol through processing and breeding. While these approaches to reducing gossypol in cottonseed and its products were not commercially viable, cottonseed products modified by mechanical or solvent extraction, or derived from glandless cotton varieties, are currently approved by the U.S. Food and Drug Administration (FDA) for use in human food provided the free gossypol content does not exceed 450 parts per million (ppm). Similarly, the Association of American Feed Control Officials (AAFCO) established standards for low gossypol cottonseed meal for use in monogastric animal feed provided the free gossypol content does not exceed 400 ppm.

Texas A&M University (TAMU) has developed a transgenic cotton plant (*Gossypium hirsutum* L. Merr. [*G. hirsutum*]), henceforth referred to as TAM66274, with ultra-low gossypol levels in the seed, while maintaining normal plant-protecting gossypol levels in the rest of the plant. The phenotype is attributable to RNA interference (RNAi)-mediated silencing of δ-cadinene synthase (*dCS*) genes that encode δ-cadinene synthase (dCS), a key enzyme involved in gossypol biosynthesis, using a seed specific promoter. Ultra-low gossypol cottonseed (ULGCS) is the latest and most promising step in a long-standing effort to reduce or eliminate gossypol in cottonseed and cottonseed products so this valuable source of protein may be used in approved food and feed applications. The technology promises to increase the value of cottonseed to farmers, with benefits to food and feed processors and end users in livestock and aquaculture industries and, ultimately, consumers.

TAM66274 was produced by *Agrobacterium tumefaciens* (*A. tumefaciens*)-mediated transformation of *G. hirsutum* cotton tissues from non-transgenic cultivar (cv.) Coker 312 using plasmid pART27-LCT66. The T-DNA region of plasmid pART27-LCT66 has two gene cassettes: a *dCS* RNAi cassette and a neomycin phosphotransferase II variant (*nptII* variant)

expression cassette. The *dCS* RNAi cassette is designed to silence the endogenous *dCS* genes in cottonseed. It is comprised of a highly seed-specific α-globulin B gene promoter (AGP) derived from cotton (*G. hirsutum*), a 604 base pair (bp) internal sequence (Trigger A) of the *dCS* gene from cotton (*G. hirsutum*), an intron from the pyruvate dehydrogenase kinase (*pdk*) gene from *Flaveria trinervia*, and a reverse complement of the Trigger A sequence (Trigger B), to make an intron-containing hairpin (ihp) RNA cassette. Expression of this cassette results in the formation of a double stranded RNA (dsRNA) transcript containing a fragment of the *dCS* genes in cotton. The dsRNA is recognized and processed by the cotton plant's RNAi machinery, ultimately resulting in suppression of expression of the endogenous *dCS* and its cognate protein. The *nptII* variant expression cassette serves as a plant selectable marker gene cassette. Expression of the *nptII* variant gene renders the transformed cells resistant to the antibiotic kanamycin, thus allowing the selection of the transformed cells in tissue culture.

Data and information presented in this petition to USDA APHIS demonstrate that TAM66274 is phenotypically, agronomically, ecologically and compositionally comparable to non-transgenic cv. Coker 312, except for the presence of the introduced *dCS* RNAi and *nptII* variant genes and the ULGCS trait expressed only in the cottonseed. TAM66274 is unlikely to increase plant pest risk or weediness potential compared to non-transgenic cv. Coker 312. These conclusions are based on the outcome of extensive data and evaluation, including the following:

- Cotton is a familiar crop that lacks characteristics commonly associated with weeds, and, with seed gossypol reduced to safe levels, has a history of safe use in food and feed products.
- Non-transgenic cotton variety Coker 312 is an appropriate comparator to TAM66274.
- Molecular characterization studies confirmed the integrity and stability of the T-DNA in the genome of TAM66274.
- Studies characterized and confirmed the safety of the products of expression of the *dCS* RNAi and *nptII* variant gene cassettes in TAM66274.
- With the exception of ultra-low levels of gossypol, TAM66274 cottonseed is compositionally equivalent to that of non-transgenic cv. Coker 312 and other conventional cotton varieties.
- TAM66274 is phenotypically, agronomically and ecologically equivalent to non-transgenic cv. Coker 312.
- TAM66274 will not adversely affect non-target organisms (NTOs) beneficial to agriculture.
- Deregulation of TAM66274 is not likely to impact cotton agronomic practices.
- TAM66274 is unlikely to present other risks to the environment relevant to plant pest risk.

Cotton is a familiar crop that lacks characteristics commonly associated with weeds, and, with seed gossypol reduced to safe levels, has a history of safe use in food and feed products.

Cotton has been cultivated for at least 7,000 years. Cotton fabrics woven by indigenous people were among the first products observed in the New World by Christopher Columbus. Cotton has been grown in what is now the United States since before colonial times, becoming a primary crop in the United States over the centuries.

Cotton is not considered to have weedy characteristics. Cotton is not listed as a Federal noxious weed species (7 CFR Part 360), nor does it possess attributes commonly associated with weeds. For example, commercial cotton varieties rarely display any dormancy characteristics. Cotton is a slow growing plant that does not compete well with weeds or native vegetation, and is not an invasive plant species. Cotton may grow as a volunteer only under favorable conditions, but volunteers are readily controlled by two primary methods: tillage or herbicide treatment. Although feral populations of cultivated variants of cotton exist in the U.S., these populations do not occur in cotton growing areas. Further, the ULGCS trait in TAM66274 would not be expected to confer a selective advantage or result in increased plant pest or weediness potential if crossing with feral populations were to occur. In the unlikely event that this should occur, progeny resulting from such a cross could easily be controlled through current agronomic practices used to control conventional cotton.

In the U.S., cotton is grown in 17 southern States, with major concentrations in the Texas High and Rolling Plains; the Mississippi, Arkansas and Louisiana Delta; Southern Georgia; and California's San Joaquin Valley. Upland cotton (*G. hirsutum*) is grown in all cotton-producing states, and accounts for over 95% of planted acreage. Pima cotton (*G. barbadense*) accounts for the remaining acreage and is grown only in California, Texas, Arizona and New Mexico. Cotton is cultivated primarily for fiber. However, cottonseed is processed into four major food and feed products: oil, meal, hulls and linters. Cottonseed oil is primarily used for human food applications. Linters are used to produce cellulose derivatives for both food and industrial applications. The hulls and meal are used for livestock feed, primarily cattle. Therefore, cottonseed is an economically important secondary product of cotton production that accounts for between 13-24% of crop value.

Non-transgenic cotton variety Coker 312 is an appropriate comparator to TAM66274.

Non-transgenic cotton cv. Coker 312 was transformed with plasmid pART27-LCT66 to produce TAM66274. TAM66274 and non-transgenic cv. Coker 312 have similar genetic backgrounds with the exception of the *dCS* RNAi and *nptII* variant expression cassettes. Therefore, non-transgenic cv. Coker 312 is the near isogenic line to TAM66274 and the most appropriate comparator to assess the effects of the *dCS* RNAi and *nptII* variant expression cassettes on potential plant pest and weediness risks of TAM66274.

Molecular characterization studies confirmed the integrity and stability of the T-DNA in the genome of TAM66274.

The molecular characterization of TAM66274 showed that the T-DNA of plasmid pART27-LCT66 was integrated as a single insert and single copy in the genome of TAM66274. The integrity of the T-DNA was maintained from the transformation plasmid to the insert in the genome of TAM66274, except that the entire right border (RB) T-DNA repeat was not integrated in the plant genome, and only seven nucleotides from the left border (LB) T-DNA repeat were included in the T-DNA insert in the cotton genome. No genetic elements from the backbone DNA of plasmid pART27-LCT66 were integrated in the TAM66274 genome. Analysis of the genomic DNA flanking the T-DNA insert in TAM66274 showed that the T-DNA integration occurred in an intron of a putative *α-hydrolase* gene. However, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed that there was no impact on mRNA expression from this gene in TAM66274 compared to expression of the same gene in non-transgenic cv. Coker 312. Therefore, integration of the T-DNA insert in the intron of the *α-hydrolase* gene had no effect on expression of this protein in TAM66274. The stability of the T-DNA insert in the genome of TAM66274, determined by Southern blot analyses of three breeding generations and by trait inheritance studies over plant breeding generations, demonstrated that the inserted DNA is stably integrated in the plant genome and was stably inherited through multiple generations. Moreover, the transgene insert displayed the expected Mendelian inheritance pattern for single locus integration in the segregating generations, confirming that the transgene insert in TAM66274 is stably integrated at a single chromosomal locus.

Studies characterized and confirmed the safety of the products of expression of the *dCS* RNAi and *nptII* variant gene cassettes in TAM66274.

The efficacy of the *dCS* RNAi cassette in suppressing expression of the *dCS* transcripts in cottonseed only, not in other plant parts, was demonstrated and, thereby, explains reduction of gossypol levels only in the cottonseed. Also, the food and feed safety of *dCS* RNAi was demonstrated not only from reviews of safety of dsRNA and nucleic acids in general, but by specific safety of *dCS* RNAi. Bioinformatic analyses were conducted with the 604 bp *dCS* gene trigger sequence using a BLASTN search of the National Center for Biotechnology Information (NCBI) database. The sequence was queried against human, cow, pig, chicken, fish, shrimp, dog and cat expressed sequence tags (ESTs) and showed no homology in any 20 bp contiguous stretch to any known transcripts in this database. This analysis confirms the unlikelihood of adverse non-target effects of *dCS* RNAi on humans and animals that are likely to consume the TAM66274 cottonseed or products derived from TAM66274 cottonseed. Also, with reduced levels of the dCS enzyme in TAM66274 cottonseed, the potential for *2E,6E*-farnesyl diphosphate (FDP), the first intermediate in the committed step to gossypol biosynthesis, to be diverted to other plant metabolic pathways, was addressed. A review of the literature suggested that a major portion of the FDP pool is used for primary plant metabolites (e.g., phytosterols,

polyisoprenoids, quinones, and farnesylated proteins) and only a minute portion is channeled into the gossypol biosynthesis pathway in the cotton plant. Therefore, silencing of the *dCS* genes in TAM66274 is unlikely to make a significant difference to the partitioning of FDP into other biosynthetic pathways.

Expression levels of the NPTII variant protein were measured by enzyme-linked immunosorbent assay (ELISA) in leaf, root, pollen and seed tissues of TAM66274 and non-transgenic cv. Coker 312. Among all the tissues evaluated, NPTII variant expression was highest in leaves of TAM66274, was lower in the root, was lowest in the seed (41.1 ng/g dry weight [DW]), and was not detected in pollen of TAM66274 at the detectable level of 25 ng/g DW. NPTII variant represents no more than 0.0000041% of the seed of TAM66274. The protein was not detected (N.D.) in any tissues of the non-transgenic cv. Coker 312 plants. It was determined that the first 24 nucleotides of the *nptII* variant gene in TAM66274 had been replaced with 51 nucleotides from the nopaline synthase (*nos*) gene. The remaining 768 nucleotides matched exactly with the original *nptII* gene in other commercial cotton and corn crops (e.g., Genuity® DroughtGard™ corn (MON 87460), YieldGard® Rootworm corn (MON 863), Bollgard® cotton (MON 531)). Thus, the NPTII variant protein in TAM66274 is an in-frame translational fusion of a short section of the *nos* gene at the amino terminal end with the *nptII* variant gene. This *nptII* variant coding sequence in TAM66274 is the same *nptII* coding sequence used to generate ringspot virus resistant papaya ("Sunset" lines 55-1 and 63-1) This product has been in commercial production since 1998, so there is a 19-year history of safe use of the same NPTII variant produced in TAM66274 as is produced in ringspot virus resistant papaya. On the basis of extensive safety studies previously conducted on NPTII, widespread use of food and feed crops containing this protein, the history of safe use of the same NPTII expressed in ringspot virus resistant papaya as in TAM66274, as well as the absence of amino acid sequence homology of the NPTII variant in TAM66274 to sequences of known allergens and toxins, it is concluded that the NPTII variant expressed in TAM66274 has the same food, feed and environmental safety characteristics as NPTII expressed in other commercial crops.

With the exception of ultra-low levels of gossypol, TAM66274 cottonseed is compositionally equivalent to that of non-transgenic cv. Coker 312 and other conventional cotton varieties.

Composition of cottonseed of TAM66274 and control non-transgenic cv. Coker 312 was measured from plants grown in replicated field trials at three locations in the U.S. during the summer of 2014, and at five locations in the U.S. during the summer of 2015. The components analyzed included proximates, fiber (total dietary, crude, acid and neutral detergent fibers), fatty acids, amino acids, minerals, alpha-tocopherol, and anti-nutrients (total and free gossypol, gossypol isomers, cyclopropenoid fatty acids and phytic acid). Further, cottonseed harvested from the five field trials in 2015 was analyzed for mycotoxins. Cottonseed compositional analyses showed that the intended ultra-low gossypol cottonseed trait was expressed in TAM66274, with mean levels of total gossypol in cottonseed of TAM66274 harvested from

2014 and 2015 field trials of 370 and 300 ppm on a DW basis, respectively, compared to levels of 10,300 ppm and 10,000 ppm in cottonseed of non-transgenic cv. Coker 312 harvested from the same field trials. The compositional analyses also showed that other than the intended reduction in seed gossypol levels, TAM66274 is compositionally equivalent to non-transgenic cv. Coker 312. Further, results of mycotoxin analyses of the cottonseed harvested from TAM66274 and non-transgenic cv. Coker 312 grown in the 2015 field trials showed no difference in mycotoxin levels in cottonseed of TAM66274 compared to non-transgenic cv. Coker 312. Therefore, the ULGCS trait does not confer any different susceptibility of TAM66274 cottonseed to mycotoxins compared to non-transgenic cv. Coker 312.

In summary, these compositional analyses demonstrated that introduction of plasmid pART27-LCT66 into the genome of non-transgenic cv. Coker 312 to produce TAM66274 achieved the intended effect of significantly reducing total seed gossypol levels compared to non-transgenic cv. Coker 312. The mean total gossypol levels in cottonseed of TAM66274 are also well below established safety standards for cottonseed products used in human food (450 ppm) and for monogastric animal feed (400 ppm). Therefore, TAM66274 cottonseed is appropriate for use in approved human food and monogastric animal feed applications. Further, introduction of plasmid pART27-LCT66 into the Coker 312 genome did not impact the nutritional composition of cottonseed produced by TAM66274. Results of these analyses demonstrate that other than the intended reduction in cottonseed gossypol levels, cottonseed from TAM66274 is compositionally equivalent to, and as nutritious as, cottonseed from non-transgenic cv. Coker 312, as well as other conventional cotton varieties. The results demonstrate that cottonseed from TAM66274 is appropriate for conventional food and feed uses of cottonseed and processed cottonseed products (e.g., cottonseed oil for human food; hulls and meal for livestock feed) and for currently approved uses of low gossypol cottonseed products for food and feed.

TAM66274 is phenotypically, agronomically and ecologically equivalent to non-transgenic cv. Coker 312.

The phenotypic, agronomic and ecological characteristics of TAM66274 were evaluated relative to non-transgenic cv. Coker 312 in field trials in 2014 and 2015. These were the same field sites from which cottonseed was harvested for compositional analyses (three sites in 2014 and five sites in 2015), and the sites were representative of areas of commercial cotton production. Phenotypic, agronomic and ecological characteristics encompassed six general categories: 1) seed germination, dormancy, and stand count; 2) vegetative growth; 3) reproductive development; 4) fiber quality; 5) plant mapping and 6) plant susceptibility to diseases and insect pests, as well as to rodents. Forty characteristics were measured at six in-season time points and at harvest. In addition, germination of seeds harvested from the eight field trials was evaluated in separate laboratory studies.

No statistically significant or biologically meaningful differences were detected in seed germination (both in laboratory and field studies), stand count, vegetative growth, or plant susceptibility to disease and insect pests or rodents for TAM66274 relative to non-transgenic cv. Coker 312 in either 2014 or 2015 field studies. Further, there were no statistically significant differences for the majority of the reproductive development, fiber quality and plant mapping parameters in both field trial seasons. Overall, statistical differences were detected in only 11.9% of all comparisons at individual locations over two years (i.e., 40 of 336 agronomic and germination comparisons). In the few instances where statistically significant differences were observed between the treatments, these differences were inconsistent between the two field trial seasons and, therefore, were not considered agronomically meaningful. The only parameter that was consistently statistically different between the treatments over the two field trial seasons was fiber length. Although fiber length of TAM66274 was slightly shorter than non-transgenic cv. Coker 312, it was within commercially acceptable limits and does not pose a risk of increased weediness or plant pest characteristics.

Results of these studies showed the lack of biologically meaningful differences in phenotypic, agronomic and ecological characteristics between TAM66274 and non-transgenic cv. Coker 312. These data demonstrated that TAM66274 is phenotypically, agronomically and ecologically equivalent to non-transgenic cv. Coker 312 and, therefore, is likely comparable to other conventional cotton varieties. Overall, the results demonstrate that the cultivation of TAM66274 poses no greater weediness risk or plant pest risk than does the cultivation of non-transgenic cv. Coker 312 and, therefore, is unlikely to pose greater weediness risk or plant pest risk than other conventional cotton varieties.

TAM66274 will not adversely affect non-target organisms (NTO's) beneficial to agriculture.

TAM66274 is genetically engineered for improved product quality and, therefore, has neither target nor non-target species. The *dCS* RNAi construct in TAM66274 interferes with expression of δ-cadinene synthase genes that encode a key enzyme in gossypol biosynthesis in cottonseed, while leaving gossypol levels unchanged in other plant tissues. Field evaluations of TAM66274 demonstrated that the ULGCS trait did not alter interactions with insects and diseases compared to non-transgenic cv. Coker 312. Further, evaluation of phenotypic and agronomic characteristics of TAM66274 under widely different environmental conditions in 2014 and 2015 field studies showed that TAM66274 responded to both abiotic and biotic stressors in the same manner as non-transgenic cv. Coker 312.

The genetic material inserted into TAM66274 is not toxic and does not produce any substance that would be considered toxic. The trigger sequences for RNAi-mediated suppression are highly specific to δ-cadinene synthase genes in cotton and share no significant homology to genes in other plant or animal species, nor do they encode a protein toxin or allergen. Additionally,

TAM66274 contains the *nptII* gene, which is widely distributed in nature and has previously been evaluated for human and environmental safety. Similarly, USDA APHIS has identified no human or environmental safety issues for use of *nptII* in genetically engineered plants and plant products.

Based on the phenotypic, agronomic and ecological equivalence of TAM66274 to non-transgenic cv. Coker 312, the specificity of the *dCS* RNAi-mediated suppression, and the absence of demonstrable human or environmental harm from exposure to the NPTII protein, no effects on NTOs beneficial to agriculture are likely from unconfined environmental release of TAM66274.

Deregulation of TAM66274 is not likely to impact cotton agronomic practices.

Field and laboratory studies confirm that, except for the ultra-low cottonseed gossypol levels, TAM66274 is phenotypically and agronomically comparable to non-transgenic cv. Coker 312. No significant impact is expected on general production practices, insect pest management, management of diseases and other pests, or weed management. Therefore, unconfined environmental release of TAM66274 is unlikely to significantly impact U.S. cotton agronomic practices, except for implementation of an identity preservation system to capture the increased value of the cottonseed.

TAM66274 is unlikely to present other risks to the environment relevant to plant pest risk.

The introduction of the ULGCS trait into TAM66274 does not alter the weediness characteristics of cotton. Agronomic properties of TAM66274 related to weediness, such as germination, emergence, seedling vigor, and response to environmental conditions have been shown to be substantially equivalent to non-transgenic cv. Coker 312.

The potential for TAM66274 to hybridize with cultivated, wild or feral cotton and persist in the environment is low due to the predominance of self-pollination, geographic isolation, and other reproductive barriers. If such crosses did occur, the ULGCS trait is unlikely to confer a selective advantage to or enhance the persistence of resulting progeny. Accordingly, the environmental consequences of gene flow from TAM66274 to sexually compatible species are considered to be negligible.

Furthermore, the ULGCS trait does not increase the insect pest or disease susceptibility of TAM66274 relative to non-transgenic cv. Coker 312 and is unlikely to have indirect plant pest effects on other agricultural products that are grown or stored in proximity to TAM66274.

Additionally, the transfer of genetic material from TAM66274 to sexually incompatible organisms is remote. If such a transfer were to occur, the ULGCS trait is unlikely to present a weediness or plant pest risk based on safety data presented in this petition.

The genetic material inserted into TAM66274 is not toxic and does not produce any substance that would be considered toxic. Based on the specificity of the *dCS* RNAi-mediated suppression of δ-cadinene synthase genes in TAM66274 and the absence of demonstrable human or environmental harm from exposure to the NPTII variant protein, no effects on NTOs beneficial to agriculture are likely from unconfined environmental release of TAM66274.

Finally, TAM66274 cottonseed is compositionally equivalent to non-transgenic cv. Coker 312, except for the intended reduction of the anti-nutrient, gossypol, in cottonseed. As a byproduct of one of the most widely cultivated crops globally, cottonseed is a readily available source of high quality protein that could be used in human food and feed for monogastric animals if not for the presence of gossypol. The availability of TAM66274 will enable approved uses of cottonseed for animal and human nutrition, which can increase the value of cottonseed to growers, livestock and aquaculture producers, and food processors.

Conclusion

Based on the data presented in this petition, Texas A&M AgriLife Research submits that TAM66274 is unlikely to pose a plant pest risk to U.S. agriculture or the natural environment. Therefore, Texas A&M AgriLife Research requests a determination from USDA APHIS that TAM66274 and any progeny derived from crosses between TAM66274 and conventional *Gossypium* cotton species or deregulated biotechnology-derived cotton be granted non-regulated status under 7 CFR Part 340.

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ABBREVIATIONS AND TERMS

~	Approximately
4-MU	4-methylumbelliferone
aa	Amino acid
aadA	Adenylyl transferase
AAFCO	Association of American Feed Control Officials
ACN	Acetonitrile
ACT	Adenylate cyclase toxin
AD	Arbitrary degenerate primers
ADF	Acid detergent fiber
ADON	Acetyldeoxynivalenol
AGP	α-Globulin B gene promoter plus 5' untranslated region
ALS	Acetolactate synthase
AOAC	American Organization of Analytical Chemists
AOCS	American Oil Chemists Society
AOSA	Association of Official Seed Analysts
ATP	Adenosine triphosphate
BLASTN	Basic local alignment search tool - nucleotide
BLASTP	Basic local alignment search tool - protein
BLOSUM	Blocks substitution matrix
bp	Base pair
BSA	Bovine serum albumin
ºC	Degrees centigrade
cDNA	Complementary DNA
CDS	Coding sequence
CF	Crude fiber
CFR	Code of Federal Regulations
CPFA	Cyclopropenoid fatty acids
CPG	FDA compliance policy guide
Cq	Quantitation cycle
CTAB	Cetyltrimethyl ammonium bromide
cv.	Cultivar
CVM	Center for Veterinary Medicine
CWVI	Cool-warm vigor index
DAP	Days after planting
dCS	δ-Cadinene synthase genes
dCS	δ-Cadinene synthase enzyme
dCTP	Deoxycytidine triphosphate
DDBJ	DNA Data Bank of Japan

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate, a generic term referring to the four deoxyribonucleotides: dATP (deoxyadenosine triphosphate), dCTP, dGTP (deoxyguanosine triphosphate) and dTTP (deoxythymidine triphosphate)
DON	Deoxynivalenol
dpa	Days post anthesis
dsRNA	Double stranded RNA
DW	Dry weight
*Eco*RI	Restriction enzyme isolated from *E. coli*
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ESTs	Expressed sequence tags
EMBL	European Molecular Biology Laboratory
FAO/WHO	Food and Agriculture Organization/World Health Organization
FASTA	FAST- All - Algorithm used to find local high scoring alignments (protein or nucleotides)
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FDP	*2E,6E*-Farnesyl diphosphate
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMOC	Fluorenylmethyl chloroformate
FTP	File transfer protocol
FW	Fresh weight
g	Unit of gravitational force
G	Gossypol
GC	Gas chromatography
gDNA	Genomic DNA
Genbank	A public genetic database maintained by the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD
GFP	Green fluorescent protein
gfp	Green fluorescent protein gene
Gh histone 3A	Histone 3A gene from *Gossypium hirsutum*
GLP	Good laboratory practices
GM	Genetically modified
GUS	β-glucuronidase enzyme
gusA	β-glucuronidase gene from *Escherichia coli*
H1-H4	Heliocides 1-4
HE-TAIL PCR	High efficiency-thermal asymmetric interlaced polymerase chain reaction
HF buffer	High fidelity PCR buffer

HGQ	Hemigossypolone
HNR	Height to node ratio
HPLC	High pressure liquid chromatography
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
IgE	Immunoglobulin E
ihp	Intron-containing hairpin
ILSI	International Life Sciences Institute
IPGB	Institute for Plant Genomics and Biotechnology
JAOAC	Journal of the AOAC
Kb	Kilobase
Kcal	Kilocalories
kg	Kilogram
*Kpn*I	Restriction enzyme isolated from *Klebsiella pneumoniae*
L	Liter
LB	Left border
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
LSD	Least significant difference
mcs	Multiple cloning site
µg	Microgram
µl	Microliter
mic	Micronaire
mg	Milligram
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MUG	4-methyl umbelliferyl glucuronide
N	Normal
N.A.	Not applicable
ng	Nanogram
NCBI	National Center for Biotechnology Information
N.D.	Not detected
NDF	Neutral detergent fiber
NIH	National Institutes of Health
nm	nanometers
nos	Nopaline synthase gene from *Agrobacterium tumefaciens*
nptII	Neomycin phosphotransferase II gene from *Escherichia coli* Tn5
NPTII	Neomycin phosphotransferase II protein
N.R.	Not reported

NTOs	Non-target organisms
ocs	Octopine synthase gene from *Agrobacterium tumefaciens*
OD	Optical density
OECD	Organization for Economic Cooperation and Development
OPA	o-phthalaldehyde
ORF	Open reading frame
pART27-LCT66	Plasmid vector used to develop TAM66274
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pdk	pyruvate orthophosphate dikinase gene from *Flaveria trinervia*
PEB1	Protein extraction buffer 1
PIR	Protein Information Resource
ppb	Parts per billion
ppm	Parts per million
PRF	Protein Research Foundation
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
QTLAD	Arbitrary degenerate primers used in TAIL PCR and HE-TAIL PCR
RB	Right border
RBC	Randomized complete block
RefSeq	An open access, annotated and curated reference sequence database with a collection of publicly available nucleotide sequences (DNA, RNA) and their protein products.
REML	Residual maximum likelihood mixed-designed statistical model
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
rpm	Revolutions per minute
RQ	Relative quantification
SE	Standard error
S.E.M.	Standard error of the means
siRNA	Small interfering RNA
SSC	Saline sodium citrate
SDS	Sodium dodecyl sulfate
SwissProt	Swiss Protein Sequence Data Bank
T0, T1, T2, …	Generations of transformed plant
Taq	*Thermus aquaticus*, a thermophilic bacterium
T-DNA	Transfer deoxyribonucleic acid

TAM66274	A genetically modified cotton line, and the subject of this application, which produces ultra-low gossypol cottonseed
TAMU	Texas A&M University
TBE	Tris-borate-EDTA
TDF	Total dietary fiber
tex	A unit weight in grams of 1,000 meters of fiber
Tm	Melting temperature
TMB	Tetramethylbenzidene
TPA	Third party annotation
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
UHM	Upper half mean length
ULGCS	Ultra-low gossypol cottonseed
UniProt	Universal Protein Resource
U.S.C.	United States Code
USDA AMS	U.S. Department of Agriculture Agricultural Marketing Service
USDA APHIS	U.S. Department of Agriculture Animal and Plant Health Inspection Service
USDA ERS	U.S. Department of Agriculture Economic Research Service
USDA FS	U.S. Department of Agriculture Forest Service
USDA NASS	U.S. Department of Agriculture National Agricultural Statistics Service
UTR	Untranslated region
UV	Ultraviolet
WGS	Whole genome shotgun
ZEA	Zearalenone

Definitions of International System of Units (SI) base units and derived units may be found in NIST (2015).

1. RATIONALE FOR THE DEVELOPMENT OF TAM66274 COTTON

1.1 Introduction

Although cottonseed is a rich source of relatively high quality protein and is used as a feed supplement for ruminant animals, due to the presence of anti-nutrient gossypol, it is not typically consumed by humans or monogastric animals, which are more sensitive to gossypol toxicity (OECD, 2008, 2009; Risco and Chase, 1997). Texas A&M University (TAMU) has developed a transgenic cotton plant, henceforth referred to as TAM66274, with ultra-low gossypol levels in the seed, while maintaining normal plant-protecting gossypol levels in the rest of the plant. The phenotype is attributable to RNA interference (RNAi) mediated silencing of δ-cadinene synthase (*dCS*) genes that encode δ-cadinene synthase (dCS), a key enzyme (CAD1: EC 4.2.3.13, referred to as dCS in this petition) involved in gossypol biosynthesis, using a seed-specific promoter. Ultra-low gossypol cottonseed (ULGCS) is the latest and most promising step in a long-standing effort to reduce or eliminate gossypol in cottonseed so this valuable source of protein may be used in approved food and feed applications. The technology promises to increase the value of cottonseed to farmers, with benefits to processors and end users in livestock and aquaculture industries and ultimately consumers.

1.2 Gossypol Biosynthesis and Toxicity

Gossypol is a terpenoid produced in pigment glands of plants belonging to the genus *Gossypium* of the family Malvaceae (Adams et al., 1960). Gossypol and related terpenoids are present throughout the cotton plant in the glands of foliage, floral organs, bolls, roots and seeds (Stanford and Viehoever, 1918; Wang et al., 2009). Constitutive presence of these compounds protects the plant from both insects and pathogens (Hedin et al., 1992; Stipanovic et al., 1999) and they are also induced in response to microbial infections as well as insect herbivory (Bell et al., 1975; Bezemer et al., 2004; El-Sebae et al., 1981; Liu et al., 1999; McAuslane and Alborn, 1998; Opitz et al., 2008; Townsend et al., 2005). These terpenoids are derived from (+)-δ-cadinene. dCS catalyzes the conversion of FDP to δ-cadinene, the first committed step in the biosynthesis of terpenoids, including gossypol (Benedict et al., 1995; Chen et al., 1995; Davis et al., 1996). Gossypol is the predominant terpenoid present in the glands of the seed kernel and flower petals, whereas glands in other parts of the plant contain gossypol and additional protective terpenoids derived from the same biosynthetic pathway.

The terpenoid profile in cotton plants, including total gossypol content in the seed, varies across species and is influenced by weather conditions. Among cotton species, *G. barbadense* (Pima cotton) has higher average seed gossypol concentrations (1.41%) than *G. hirsutum* (upland cotton) (1.32%) (Percy et al., 1996). Pons Jr. et al. (1953) reported that the amount of gossypol in cottonseed kernels is negatively correlated with temperature and positively correlated with rainfall. Most commercial cottonseed contains 0.52% - 1.01% gossypol (Calhoun et al., 2004). Gossypol usually occurs as a mixture of two enantiomers, (−) and (+) gossypol (Hron et al.,

1999). The (−) gossypol is the more toxic form compared to the (+) gossypol (Bailey et al., 2000; Stipanovic et al., 2006). The proportion of these two enantiomers is genetically determined and varies among *Gossypium* species (Stipanovic et al., 2005).

Gossypol causes heart and liver damage in monogastric animals including humans (Gadelha et al., 2014; Risco and Chase, 1997). Gossypol poisoning has been reported in several species, including pigs (Haschek et al., 1989), broiler chicks (Henry et al., 2001), dogs (West, 1940; Uzal et al., 2005), sheep (Morgan et al., 1988), and goats (East et al., 1994). Monogastric animals, such as pigs, birds, fish, and rodents, are more susceptible to gossypol toxicity than ruminants (EFSA, 2008; Kenar, 2006; Randel et al., 1992; Zhang et al., 2007). Signs of acute gossypol toxicity in most animals include impaired body weight gain, weakness, anemia, respiratory distress, anorexia, apathy, heart failure and death after several days (East et al., 1994; Haschek et al., 1989; Henry et al., 2001; Morgan et al., 1988). Adult ruminant animals are able to tolerate a limited amount of gossypol in their diets because gossypol is bound during ruminal fermentation and becomes unavailable for intestinal absorption. Therefore, cottonseed is currently used mainly as feed for ruminant animals as either whole seed or cottonseed meal after oil extraction. However, even adult cattle can suffer from gossypol toxicity above a certain amount of cottonseed intake (Smalley and Bicknell, 1982) and it is recommended that daily intake of whole cottonseed be limited to less than 2.7 kg/cow (Blasi and Drouillard, 2002). Young animals, without fully developed rumen, are more sensitive to gossypol compared to the adult ruminants (Holmberg et al., 1988).

1.3 Long-standing Efforts to Remove Gossypol from Cottonseed

Historically, limitations on the use of cottonseed due to the presence of gossypol have led to efforts to reduce gossypol through processing and breeding. Production of high quality protein products from conventional cottonseed, but low in free gossypol, has been demonstrated using several processes: air classification, liquid cyclone processing, solvent extraction and screw-press techniques (Kadan et al., 1979; Liadekis et al., 1993; Vix et al., 1971). While all processes resulted in cottonseed protein products with free gossypol levels below limits established by the FDA, the processing steps were cost prohibitive (Rathore et al., 2008a). For example, Gardner et al. (1973) reported that the liquid cyclone process was capable of producing edible cottonseed for food uses that contained 400 ppm or less free gossypol and more than 65% protein. After the FDA approved ground cottonseed kernels produced by the liquid cyclone process as a food additive in 1972, a processing plant to produce deglanded, high protein edible cottonseed flour began commercial production at Plains Cooperative Oil Mill, Lubbock, Texas, in 1973 (Gardner et al., 1973). However, this facility was unable to remain financially viable and ceased operation within a short period of time.

A gossypol-free mutant cotton strain was identified in the 1950s and was used to breed the trait into commercial varieties (McMichael, 1959, 1960). The so-called “glandless” cotton, which

lacked the glands where gossypol and other terpenoids are stored, was used to conduct nutritional studies from the 1960s through 1980s that confirmed that glandless cottonseed meal was suitable for consumption by monogastric animals and humans (Alford et al., 1977; Alford et al., 1996; Bressani, 1965; Graham et al., 1970; Lusas and Jividen, 1987; Srikantia and Sahgal, 1968; Thomas et al., 1979). In an effort to realize the benefits of glandless cotton, FDA approval was sought and granted, setting the maximum allowable level of free gossypol in roasted or baked glandless cottonseed kernels used as human food at 450 ppm (FDA, 1976). This decision followed FDA prior approval of modified cottonseed products for human consumption derived from glanded cottonseed varieties that are mechanically or chemically processed to reduce free gossypol to less than 450 ppm (FDA, 1960; FDA, 1972). In a complementary scheme for animal feed, AAFCO, with technical and scientific assistance from the FDA Center for Veterinary Medicine (CVM), establishes definitions for ingredients that can be safely and legally used in animal feed. AAFCO has adopted definitions for low gossypol cottonseed meal used in animal feed as either mechanically or solvent-extracted meal in which free gossypol does not exceed 400 ppm (AAFCO, 1968a; 1968b).

1.4 Importance of Gossypol in the Rest of the Cotton Plant

Gossypol and related terpenoids derived from the same basic biosynthetic pathway are present in pigmented glands located on the surface throughout the cotton plant (Adams et al., 1960) and play an important role in defending the plant against pests and some diseases (Bell and Stipanovic, 1978; Bottger et al., 1964). Many of these terpenoids are also induced in response to fungal or bacterial infection and serve as phytoalexins (Abraham et al., 1999; Bell et al., 1975; Bell and Stipanovic, 1978; Liu et al., 1999; Stipanovic et al., 1999; Zhang et al., 1993). The discovery of the glandless cotton mutant and subsequent introgression of this trait into commercial cultivars by breeders generated a great deal of excitement and provided hope for the utilization of glandless cottonseed as feed for monogastric animals and for human food (Lusas and Jividen, 1987). Unfortunately, due to the lack of the glands and, therefore, the protective terpenoids in the vegetative and floral parts of the plant, glandless cotton varieties suffered more severe pest damage from traditional and also non-traditional cotton pests and had lower yields under field conditions (Bottger et al., 1964; Jenkins et al., 1966; Lukefahr et al., 1966; Maxwell et al., 1965; Vaissayre and Hau, 1985). Thus, although the glandless cottonseed proved fit as a source of food and feed, it was not widely accepted by cotton growers. The glandless cotton experience underscored the importance of maintaining the protective terpenoids in the vegetative and floral parts of the plant.

1.5 Development of ULGCS Event TAM66274

Texas A&M University has developed transgenic cotton plants containing an RNAi cassette for silencing the members of *dCS* gene family in the seed, which results in the ULGCS trait. TAM66274 was developed using *A. tumefaciens*-mediated transformation to stably incorporate the *dCS*-RNAi cassette into the genome of the recipient cv. Coker 312 (referred to as non-

transgenic cv. Coker 312 in this petition). This RNAi cassette, under the control of a seed-specific, α-globulin B gene promoter (AGP) from cotton (Sunilkumar et al., 2002), results in the formation of dsRNA and subsequently small interfering RNAs (siRNA) that are responsible for silencing the *dCS* genes in seed tissue. By interfering with the expression of *dCS* genes during seed development, gossypol biosynthesis was disrupted to generate cottonseed with ultra-low gossypol levels (Palle et al., 2013; Rathore et al., 2008a; Rathore et al., 2012; Sunilkumar et al., 2006). This silencing, restricted to the seed tissue only, results in significant reduction (approximately 97%) in the level of gossypol in the cottonseed while maintaining gossypol and related terpenoids at normal levels in the rest of the plant.

1.6 Projected Benefits of TAM66274 Cotton

As a secondary product of one of the most widely cultivated crops globally, cottonseed is a readily available source of high quality protein that could be used in human food and feed for monogastric animals if not for the presence of gossypol. Grown by 20 million farmers in 80 different countries, enough cottonseed is produced globally to meet the daily protein requirements of a half a billion people per year (Rathore et al., 2008b; Rathore et al., 2015; Sunilkumar et al., 2006). Rising incomes and diversifying diets in developing countries will increase demand for feed in livestock and aquaculture production (Narrod et al., 2008; Tacon and Metian, 2009; World Bank Report, 2013). ULGCS promises to remove current constraints on the use of cottonseed to help meet the increasing demands of a growing global population.

In the short term, the ULGCS trait in TAM66274 will be made available as a stand-alone trait in public sector cotton varieties. Eventually, it is anticipated that the ULGCS trait will be commercialized in stacked varieties through private sector breeding programs. A newly created identity preserved market for ULGCS will prevent contamination of ULGCS by conventional cottonseed and protect the added value of ULGCS for use in food and feed. Farmers who grow ULGCS will receive higher prices for their cottonseed. Dedicated cottonseed mills will handle ULGCS with lower oil refining costs, due primarily to decreased energy use, and increased prices due to expanded use of products derived from ULGCS. Livestock and aquaculture producers will reduce the cost of feed as they replace more expensive alternatives. Food processors will substitute cottonseed for more expensive tree nuts. Cost reductions by food producers will be passed on to consumers in the form of lower prices.

1.7 Basis for the Request for Non-regulated Status under 7 CFR §340.6

USDA APHIS has responsibility under the Plant Protection Act (7 U.S.C. §7701-7772) to prevent the introduction or dissemination of plant pests into or within the United States. APHIS regulation 7 CFR §340.6 provides that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and, therefore, should no longer be regulated. If APHIS determines that the

regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Texas A&M AgriLife Research is submitting this request to APHIS for a determination of non-regulated status for the ULGCS event TAM66274, any progeny derived from crosses between TAM66274 and conventional cotton, and any progeny derived from crosses between TAM66274 with biotechnology-derived cotton that have previously been granted non-regulated status under 7 CFR Part 340.

1.8 Submissions to Other Regulatory Agencies

TAM66274 falls within the scope of the FDA's policy statement concerning foods derived from new plant varieties, including those produced through recombinant DNA techniques (FDA, 1992). Texas A&M AgriLife Research initiated food safety consultations with FDA in 2012 in accordance with FDA's policy statement and industry guidance. Texas A&M AgriLife Research has prepared a safety and nutritional assessment of food and feed derived from TAM66274 and expects to submit its findings to FDA in 2017. Texas A&M AgriLife Research is examining opportunities to deploy TAM66274 in other countries and may seek import clearances in U.S. export markets that have functioning regulatory processes in place.

2. THE BIOLOGY OF COTTON AND TAM66274 RECIPIENT COTTON CULTIVAR

2.1 Overview of Cotton Biology

The biology of cotton is described in detail in the consensus document for *Gossypium hirsutum* L. Merr. prepared by the Organisation for Economic Co-operation and Development (OECD, 2008), and is briefly summarized in this Section of the petition

2.1.1. Cotton as a crop in the United States.

Two species of cotton are grown commercially in the United States, *G. hirsutum*, known as upland cotton; and *G. barbadense*, known as Pima or Egyptian cotton. In the U.S., cotton is grown in 17 southern States, with major concentrations in the following areas: Texas High and Rolling Plains; the Mississippi, Arkansas and Louisiana Delta; Southern Georgia; and California's San Joaquin Valley. Upland cotton is grown in all cotton-producing states, and accounts for over 95% of planted acreage. *G. hirsutum* in the U.S. is classified into four major types: Delta, Plains, Eastern and Acala, referring to the major producing regions of the country. Pima cotton accounts for the remaining acreage and is grown only in California, Texas, Arizona and New Mexico. Cotton production in the U.S. is described in detail in Section 8 of this petition (Table 8-1 shows cotton acreage planted by state and type for 2016). The fiber harvested from upland cotton varieties is used primarily for cordage and non-woven products as well as for textiles. In addition, the linters of upland cotton, which are the short fibers removed before seed crushing, are a major source of industrial cellulose. The fiber harvested from Pima cotton is valued for length and quality and is used primarily for sewing threads and luxury fabrics.

Cotton is cultivated primarily for the fiber. However, cottonseed is an economically important secondary product of cotton production that accounts for between 13-24% of crop value, depending on relative prices of fiber and various seed products (USDA ERS, 2017b; Liu et al., 2012). For every 100 pounds of fiber, the cotton plant also produces 145 to 165 pounds of cottonseed. The ginning process separates fiber for textile use from the seed. The resulting cottonseed can either be further processed or be used directly as cattle feed (OECD, 2009). Cottonseed is processed into meal (45% by weight), hulls (27%), crude oil (16%), linters (8%) and waste (4%) (NCPA, 2017). The oil is the most valuable product of cottonseed, a major oilseed crop in the U.S. Cottonseed oil makes up approximately 5-6% of the total U.S. domestic fat and oil supply, ranking third behind soybean and corn oil for human consumption (OECD, 2009).

2.1.2. Taxonomy of cotton.

Cotton belongs to the genus *Gossypium* of the tribe Gossypieae of the family Malvaceae of the order Malvales (Fryxell, 1979; Wendel, 1989). The genus *Gossypium* is comprised of approximately 50 species that are widely distributed and occur predominately in tropical and subtropical regions around the world (Percival et al., 1999). Worldwide there are four species of

cotton of commercial importance. These include two diploid species *G. arboreum* L. and *G. herbaceum* L., which evolved in Africa and the Middle East, and the two allotetraploid species *G. barbadense* and *G. hirsutum*, described above, which evolved in the Americas (Percival et al., 1999; Supak et al., 1992). In addition to *G. barbadense* and *G. hirsutum*, there are two *Gossypium* species native to the U.S., *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seeman (Fryxell, 1979; Wendel, 1989). These two native species of cotton grow in Arizona and Hawaii, respectively (USDA NRCS, 2017). *G. thurberi* Todaro (*Thurberia thespesiodes* Gray) is a diploid species and is found in the mountainous regions of southern Arizona in the counties of Graham, Gila, Pinal, Maricopa, Cochise, Santa Cruz and Pima, and also in the Bradshaw Mountains of Yavapai County (Fryxell, 1979). *G. thurberi* is generally found at elevations of 2,500 to 5,000 feet and is isolated from areas of cotton production. Any gene exchange between this species and tetraploid cotton, if it were to occur, would result in triploid sterile plants. *G. tomentosum* is a tetraploid and is found on the larger islands of Hawaii, as well as on Nihau and Kahoolawe. The plants grow on arid, rocky, or clay plains not far from the sea. On the larger islands, it is found chiefly on the dry, leeward side. On Oahu, it is common near Koko Crater, and grows scattered between Honolulu and Markus Balley. On Molokai, it is common on the southwestern end; elsewhere, it is rare except near Kamalo.

In addition to the native species *G. thurberi* and *G. tomentosum* in the U.S., there are naturalized populations of *G. hirsutum* in Florida, Puerto Rico, and the Virgin Islands, and naturalized populations grow in some of the Hawaiian Islands (Fryxell, 1979; USDA NRCS, 2017; Wunderlin et al., 2017). Naturalized populations of *G. barbadense* grow in Puerto Rico, the Virgin Islands and most of the Hawaiian Islands, but it is no longer grown as an agricultural commodity in Hawaii.

2.1.3. The genetics of cotton.

There are three major lineages of the diploid *Gossypium* species: Australian (C, G, K genomes), the American continents (D genome), and Africa/Middle East (A, B, E, F genomes) (Percival et al., 1999). The tetraploid species (2n=4x=52) including *G. hirsutum*, *G. barbadense* and *G. tomentosum* are comprised of the A and D nuclear genomes (AADD) and contain only the A chloroplast genome, indicating the seed parent of the original hybridization was of African or Middle Eastern descent (Percival et al., 1999). Diploid species (2n=2x=26) are distributed among tropical and subtropical regions worldwide and, as described above, two of the diploid species, *G. herbaceum* and *G. arboreum*, are of regional agronomic importance outside of the U.S.

2.1.4. Pollination of cotton.

Cotton is considered predominately self-pollinating. Pollen grains are large, heavy and somewhat sticky, which makes dissemination by wind negligible (Jenkins, 1993; McGregor, 1976; OECD, 2008). However, in the presence of suitable insect pollinators, cotton is also cross-pollinating at generally low levels (McGregor, 1976; OECD, 2008; Van Deynze et al., 2005). The extent of

spontaneous (unaided) or natural outcrossing depends greatly on the species pool, preferences, and abundance of pollinators, which can vary according to region, location, season, time of day, and use of insecticides (OECD, 2008). Additionally, outcrossing will decrease with increasing spatial isolation between the source and recipient plant populations and physical barriers. Farm scale studies with upland cotton indicate that outcrossing declines sharply with distance from the pollen source, typically below 1% beyond 10 meters (Van Deynze et al., 2005).

2.1.5. Weediness of cotton.

Upland cotton is not considered to have weedy characteristics and USDA has previously determined that "cotton is not considered to be a serious, principal or common weed pest in the U.S." (USDA APHIS, 1995). Cotton is not considered to have weedy characteristics in the U.S. and does not possess characteristics commonly associated with weeds. It is not effective in invading and competing in established ecosystems, and does not compete well with native vegetation. Although cotton seeds can have a natural capability of two to three months of innate or induced dormancy, "hard" seeds are undesirable for crop production, and the trait has been minimized or completely eliminated in modern cultivars through domestication and selective breeding (OECD, 2008). Cotton does not persist where freezing conditions occur and, therefore, there are only a few regions in the U.S. where cotton is capable of overwintering. Hence, in the continental U.S., wild populations of *G. hirsutum* exist only in the southern tip of Florida. It is recognized that in some agricultural systems, cotton can volunteer in a subsequent rotational crop. However, volunteers are easily controlled through tillage or use of appropriate herbicides (Morgan et al., 2011a; Morgan et al., 2011b). Additionally, cotton is not listed as a Federal noxious weed species (7 CFR Part 360).

2.2 Characteristics of the Recipient Cotton Cultivar

The publically available non-transgenic cv. Coker 312 (*G. hirsutum* L.) was used as the recipient cultivar for the generation of TAM66274. Coker 312 was developed by the cotton division of Coker's Pedigreed Seed Company and is an older commercial variety of upland cotton generated from a cross of Coker 100 Staple and Deltapine 15 and selected through successive generations of line selection (Bowman et al., 2006; Smith et al., 1999).

3. DESCRIPTION OF THE GENETIC MODIFICATION

TAM66274, which exhibits ultra-low levels of the anti-nutrient gossypol in the cottonseed, was generated by *A. tumefaciens*-mediated transformation of cotton tissues from non-transgenic cv. Coker 312 using plasmid pART27-LCT66 (described below in Section 3.2). The RNAi construct interferes with expression of *dCS* genes that encode a key enzyme in gossypol biosynthesis in the cottonseed, while leaving gossypol levels unchanged in other plant tissues (Palle et al., 2013; Rathore et al., 2012; Sunilkumar et al., 2006). This section describes the intended technical effect in TAM66274, the transformation plasmid vector (pART27-LCT66), the transformation method and breeding development of TAM66274, the donor genes for RNAi-mediated suppression, and the regulatory elements used in the development of TAM66274.

3.1 Intended Technical Effects in TAM66274 Cotton

3.1.1. Biosynthesis of gossypol and related terpenoids.

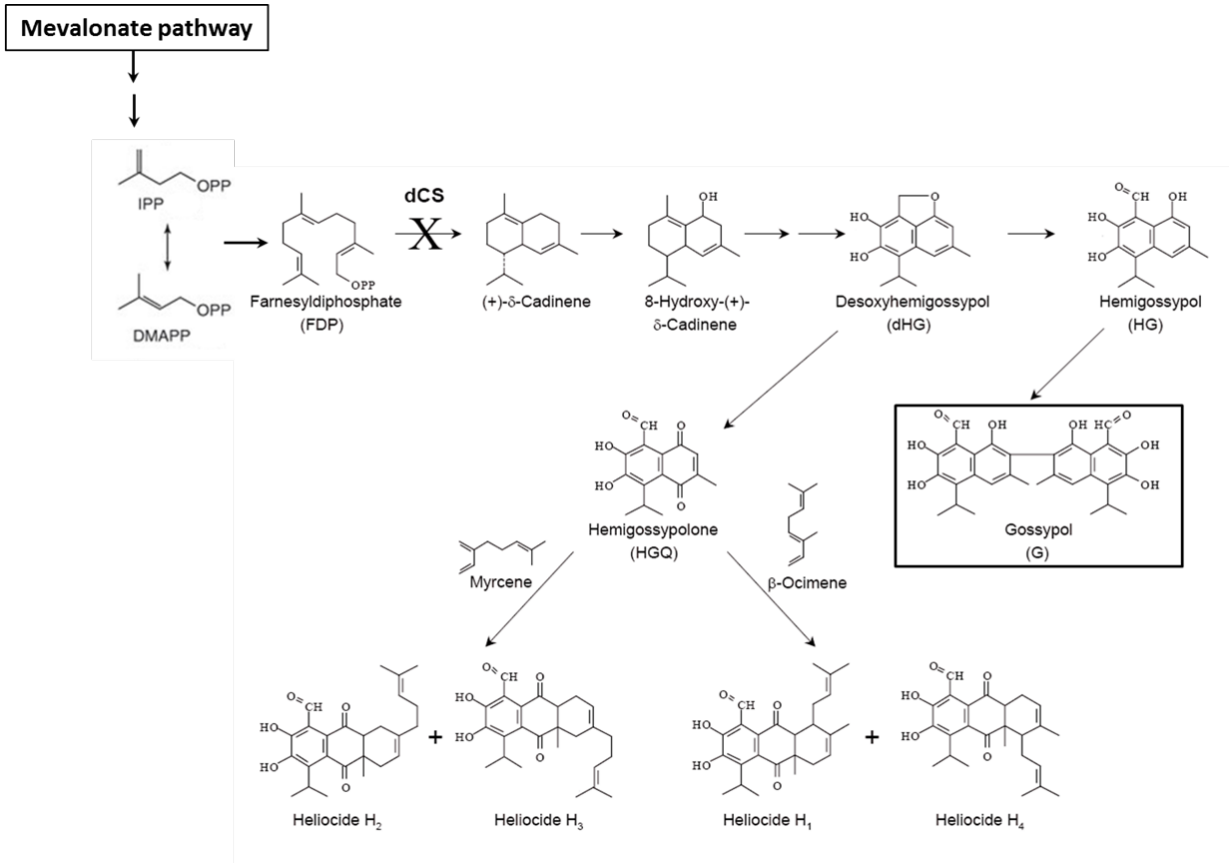
Gossypol is a terpenoid found in plants belonging to the genus *Gossypium* of the family Malvaceae. Gossypol and related terpenoids are present throughout the cotton plant in the glands of foliage, floral organs, bolls, roots and seeds. Constitutive presence of these compounds protects the plant from both insects and pathogens (Hedin et al., 1992; Stipanovic et al., 1999) and they are also induced in response to microbial infections as well as insect herbivory (Bezemer et al., 2004; McAuslane and Alborn, 1998; Opitz et al., 2008; Townsend et al., 2005). These terpenoids are derived from (+)-δ-cadinene. dCS catalyzes the first committed step involving the cyclization of FDP to (+)-δ-cadinene (Benedict et al., 2001; Chen et al., 1995; Stipanovic et al., 1999; Townsend et al., 2005). A proposed pathway for the biosynthesis of gossypol and related terpenoids from FDP in cotton is shown in Figure 3-1. Gossypol is the predominant terpenoid found in the cottonseed, with only traces of desoxyhemigossypol (dHG) and hemigossypol (Cai et al., 2010). Tissue-specific suppression of *dCS* expression by RNAi caused disruption of terpenoid biosynthesis in the seed resulting in the ULGCS trait, while retaining a full complement of gossypol and other protective terpenoids in the rest of the TAM66274 plant.

3.1.2. Plant selectable marker.

The selectable marker gene used to generate TAM66274 was the *nptII* variant gene. This gene was isolated from the *Escherichia coli* (*E. coli*) Tn5 transposon (Beck et al., 1982) and encodes the enzyme neomycin phosphotransferase type II variant (NPTII variant), which confers resistance to the antibiotics kanamycin and neomycin (Fraley et al., 1986). NPTII uses adenosine triphosphate (ATP) to phosphorylate kanamycin and neomycin, thereby inactivating the antibiotic and preventing it from killing the NPTII-producing cell. FDA has approved NPTII as an indirect food additive in genetically modified (GM) cotton, canola and tomatoes for human consumption (21 CFR §173.170) and in animal feed (21 CFR §573.130). The U.S Environmental Protection Agency (EPA) has granted an exemption from the requirement of a tolerance for

residues of NPTII in all food commodities when used as an inert ingredient in a plant-incorporated protectant (40 CFR §174.521).

Figure 3-1. Biosynthesis pathway for gossypol and related terpenoids in cotton (Modified from Cai et al., 2010).



3.2 Plasmid Vector pART27-LCT66

TAM66274 was generated through *A. tumefaciens*-mediated transformation of non-transgenic cv. Coker 312 with the binary vector pART27-LCT66. A circular map of pART27-LCT66 plasmid is shown in Figure 3-2. This plasmid is approximately 15.6 kilobase (kb). The backbone DNA sequences of pART27-LCT66 are based on the pART-27 binary vector (Gleave, 1992). The transfer DNA (T-DNA) region of pART27-LCT66 contains a *dCS* silencing cassette and a *nptII* variant expression cassette, which is delineated by the right border (RB) and left border (LB) repeat sequences (Figure 3-3).

3.2.1. Genetic elements of pART27-LCT66 T-DNA.

The 6.8 kb T-DNA region of plasmid pART27-LCT66 has two gene cassettes: a *dCS* RNAi cassette and a *nptII* variant expression cassette. The organization and orientation of these two cassettes are depicted in a linear map (Figure 3-3) and are described below. The location of each of the genetic elements on the T-DNA (base pair position) and its size are presented in Table 3-1.

Figure 3-2. Circular map of the binary vector pART27-LCT66.

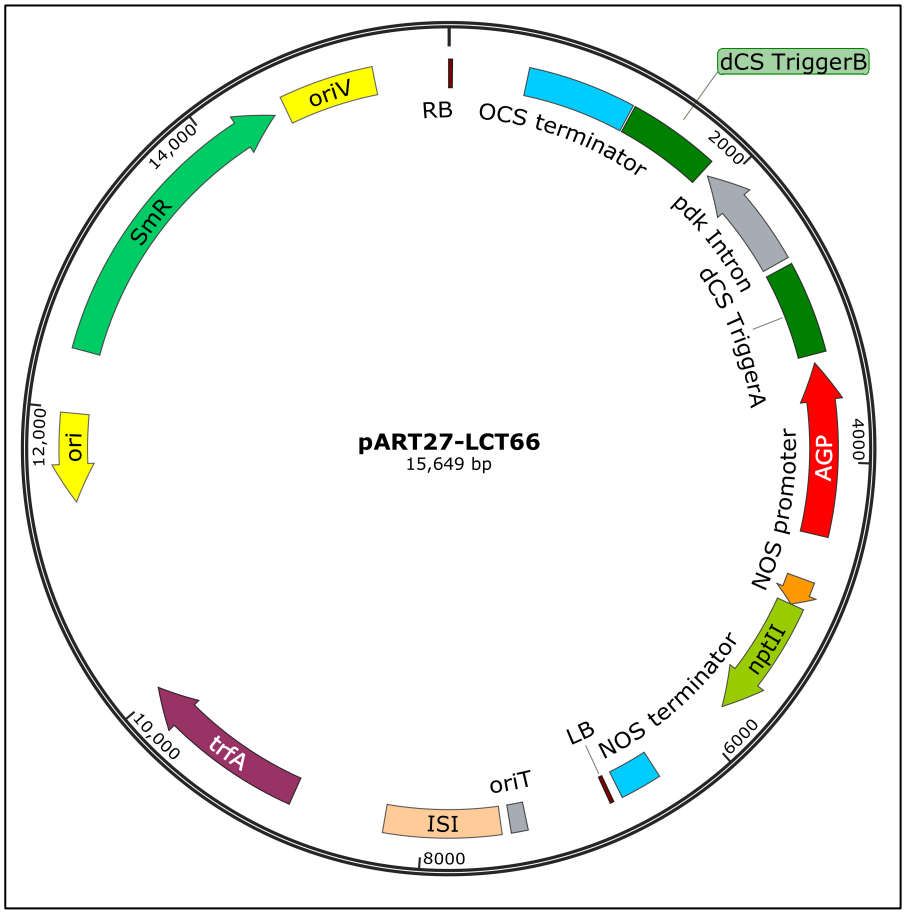
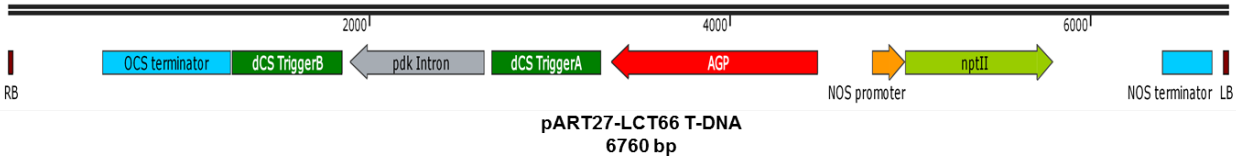


Figure 3-3. Linear map of the T-DNA region in the binary vector pART27-LCT66.



3.2.1.1. dCS RNAi cassette.

The *dCS* RNAi cassette is designed to silence the endogenous *dCS* genes in cottonseed. It is comprised of a highly seed-specific AGP derived from cotton (*G. hirsutum*) (Sunilkumar et al., 2002), a 604 bp internal sequence (Trigger A) of the *dCS* gene from cotton (*G. hirsutum*) (Sunilkumar et al., 2006), an intron from the *pdk* gene from *F. trinervia* (Wesley et al., 2001), a reverse complement of the Trigger A sequence (Trigger B) and the terminator sequence of the

octopine synthase (*ocs)* gene from *A. tumefaciens* (Wesley et al., 2001) (Figures 3-2 and 3-3, Table 3-1).

The putative AGP, comprised of 1108 bp of the promoter sequence and a 36 bp 5'-untranslated region (UTR) of the cotton α-globulin B gene, was isolated by genome walking (Sunilkumar et al., 2002; Rathore et al., 2009). The seed-specificity of the AGP, and its suitability for use in silencing *dCS* genes in cottonseed but not in other plant parts, was verified in studies before the development of TAM66274 (refer to Appendix A for details of these studies). In brief, the AGP region was fused to the β-glucuronidase (*gusA*) reporter gene in the binary vector pBI101.3 (Clontech) and the construct was used to determine promoter activity in transgenic cotton plants (Sunilkumar et al., 2002; Rathore et al., 2009). Quantitative, fluorometric β-glucuronidase (GUS) analysis revealed that AGP became active at 15 days post-anthesis (dpa), during the cotyledon expansion stage in the developing embryo. Its activity quickly increased thereafter and remained high until embryo maturation. In contrast, there was no detectable GUS activity in the stem, leaf, root, pollen, and floral bud tissues of the transgenic cotton plants. These analyses confirmed the seed-specificity of AGP in cotton. Moreover, AGP remained inactive in the foliage of cotton plants even under extreme water stress conditions (Appendix A). These results suggested that AGP-based seed-specificity of the ULGCS trait would be maintained under field conditions where plants are likely to experience water stress. This critical test was performed to determine the exclusivity of the AGP since some seed-specific promoters are known to exhibit leaky activity in the foliage under water stress. Indeed, in seven years of field trials at TAMU, there has been no significant change in the terpenoid profile in non-seed tissues of multiple ULGCS events under the control of AGP, including TAM66274, compared to non-transgenic cv. Coker 312 (Appendix A) and ULGCS plants retain terpenoid-based defenses under field conditions (Palle et al., 2013). This was verified in field studies with TAM66274 and non-transgenic cv. Coker 312, which detected no difference in susceptibility to insects or diseases between the two treatments (Section 7 of this petition).

dCS catalyzes the first committed step involving the cyclization of FDP to (+)-δ-cadinene. Chen et al., (1995; 1996) first cloned and functionally characterized a *CAD1* (referred to as *dCS* in this petition) from the A-genome diploid cotton *G. arboreum*. The dCS is encoded by a gene family that is divided into two subfamilies, *CAD1-A* and *CAD1-C (Cdn1-C1, Cdn1-C14, Cdn1-A,* and *Cdn1-C2*)[1] based on sequence similarities. The diploid genome of *G. arboreum* contains about six members of *CAD1-C* and a single copy of *CAD1-A* (Tan et al., 2000). Starting at 23 dpa, *dCS* transcripts increase dramatically in the developing embryo, closely followed by enzyme activity and gossypol accumulation (Martin et al., 2003; Meng et al., 1999). By probing a cDNA library, prepared from the mRNA obtained from developing *G. hirsutum* embryos, with the *G.*

[1] Members of the δ-cadinene synthase gene family are referred in various publications by alternative designations, which are considered synonyms in this petition.

arboreum-derived *cad*1-C1 gene (Genbank accession #AF174294), Sunilkumar et al. (2006) isolated a clone of the *dCS* gene. Sequencing confirmed that this clone belonged to the *CAD1-C* subfamily. A 604 bp internal fragment amplified from this cDNA clone was used as the trigger A sequence and a reverse complement of the Trigger A sequence (Trigger B) to make an ihp RNA construct using the pHANNIBAL/pART27 system. The expression of this cassette results in formation of a dsRNA transcript corresponding to a segment of the *dCS* genes in cotton. The dsRNA is recognized and processed by the cotton plant's RNAi machinery, ultimately resulting in suppression of dCS enzyme activity. The mode of action of the RNAi construct in TAM66274, which results in suppression of expression of *dCS* genes is discussed in more detail in Section 5 of this petition.

The selected trigger A sequence has 80.9–99.8% homology to several other published sequences of *dCS* genes from the diploid (*G. arboreum*) and tetraploid (*G. hirsutum*) cottons (Chen et al., 1995, Townsend et al., 2005, Sunilkumar et al., 2006). This sequence was intended to target all members of the *dCS* gene family, including *cad*1-A, because it bears several stretches (20–35 bp) of perfect homology to the selected sequence. AGP was used to control the expression of this ihp RNA sequence, thereby restricting the silencing of *dCS* gene(s) to the seed tissue only.

3.2.1.2. nptII variant expression cassette.

The *nptII* variant expression cassette serves as a plant selectable marker gene cassette. It consists of the promoter and 3'-UTR sequences from the nopaline synthase (*nos*) gene of *A. tumefaciens* and the *nptII* gene derived from *E. coli* Tn5 (An et al., 1988) (Figures 3-2 and 3-3). The *nptII* variant expression cassette in pART27 was derived from pGA643 (An et al., 1985; 1988). Expression of the *nptII* variant gene renders transformed cells resistant to the antibiotic (kanamycin), thus allowing selection of transformed cells in tissue culture.

3.2.1.3. T-DNA borders.

The T-DNA in pART27-LCT66 contains the RB and LB regions (Figure 3-2 and Table 3-1) that were derived from *A. tumefaciens* Ti-plasmid. Each border region contains a nick site that is the site of DNA exchange during transformation (Barker, et al., 1983; Depicker, et al., 1982; Zambryski, et al., 1982). The 25 bp repeat sequence of the borders delimits and acts as an end point of T-DNA transfer into the plant cells.

3.2.1.4. Genetic elements outside the T-DNA borders.

Genetic elements that exist outside of the T-DNA borders are those that are essential for the maintenance or selection of the pART27-LCT66 plasmid in bacterial cells, in steps prior to cotton transformation. The origin of replication *ori V* is required for maintenance of the plasmid in *A. tumefaciens* and is derived from the broad range host plasmid RK2 (Stalker et al., 1981). The origin of replication *ori* is required for maintenance of the plasmid in *E. coli* and is derived from plasmid vector pBR322 (Sutcliffe, 1979). *trfA*, which encodes a trans-acting replication

protein that binds to and activates *ori V*, is derived from *A. tumefaciens.* The selectable marker *smR* region, containing the coding sequence of adenylyl transferase (*aadA*) from transposon Tn*7*, confers resistance to spectinomycin and streptomycin (Fling, et al., 1985) both in *E. coli* and *A. tumefaciens*. Because these elements are outside the border regions, they are not expected to be transferred into the cotton genome. This was verified by molecular characterization of TAM66274, presented in Section 4 of this petition. Description of the genetic elements within and outside the T-DNA borders in pART27-LCT66 is provided in Table 3-1.

Table 3-1. Genetic elements in pART27-LCT66.

Genetic Element (GE)	Location in the Plasmid (bp)	GE Size (bp)	Description
T-DNA region (6760 bp)			
T-DNA Right Border repeat	1 - 25	25	Short direct repeat from *A. tumefaciens* containing the right border sequence required for transfer of the T-DNA into plant cells (Barker et al., 1983; Wang et al., 1984, 1987; Zambryski et al., 1982).
Right border overdrive element	26 - 177	152	Sequences flanking the right border repeat in the T-DNA (Gleave, 1992).
Intervening sequence	178 - 441	264	Sequences of *LacZ* promoter and 5' truncated *LacZ'* gene from pGEM-5Zf(-) vector (Promega, Madison, WI) used for cloning purposes.
mcs + ocs terminator	442 - 1230	789	Multiple cloning site sequences and 3' UTR terminator of octopine synthase gene derived from *A. tumefaciens* (Wesley et al., 2001).
Intervening sequence	1231 - 1243	13	Sequence used for DNA cloning.
dCS Trigger B	1244 - 1847	604	Trigger B of the δ-cadinene synthase gene from *G. hirsutum* (Sunilkumar et al., 2006).
Intervening sequence	1848 - 1891	44	Sequence used for DNA cloning.
pdk intron	1892 - 2635	744	Intron of the pyruvate orthophosphate dikinase gene from *F. trinervia* (Wesley et al., 2001).
Intervening sequence	2636 - 2679	44	Sequence used for DNA cloning.
dCS Trigger A	2680 - 3283	604	Trigger A of the δ-cadinene synthase gene from *G. hirsutum* (Sunilkumar et al., 2006).
Intervening sequence	3284 - 3339	56	Sequence used for DNA cloning.
AGP	3340 - 4485	1146	Promoter and 5' UTR derived from the α-globulin B gene of *G. hirsutum* (Sunilkumar et al., 2002).

Genetic Element (GE)	Location in the Plasmid (bp)	GE Size (bp)	Description
Intervening sequence	4486 - 4790	305	Sequences of multiple cloning sites from pHANNIBAL (Wesley et al., 2001), 3' truncated *LacZ*' gene and multiple cloning sites from pGEM-5Zf(-) vector (Promega, Madison, WI) used for cloning purposes.
nos promoter	4791 - 4974	184	Nopaline synthase promoter derived from *A. tumefaciens* (An et al., 1985, 1988; Fraley et al., 1986).
Partial *nos* gene	4975 - 5025	51	Partial sequence of the 5' end of the nopaline synthase gene coding sequence derived from *A. tumefaciens* (An et al., 1985, 1988).
nptII gene	5026 - 5796	771	Neomycin phosphotransferase II gene derived from *E. coli* Tn5, which confers resistance to kanamycin (An et al., 1985, 1988; Beck et al., 1982).
Intervening sequence	5797 - 5969	173	Partial sequence of the 5' end of the bleomycin resistance gene coding sequence derived from *E. coli* (An et al., 1985, 1988).
Intervening sequence	5970 - 6400	431	Partial sequence of the 3' end of the nopaline synthase gene coding sequence derived from *A. tumefaciens* (An et al., 1985, 1988).
nos terminator	6401 - 6675	275	Nopaline synthase terminator and poly(A) signal (An et al., 1985, 1988).
Intervening sequence	6676 - 6735	60	Sequence used for DNA cloning (Gleave, 1992).
T-DNA Left Border repeat	6736 - 6760	25	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983; Wang et al., 1984, 1987; Zambryski et al., 1982).
Plasmid backbone region (8889 bp)			
Plasmid backbone sequence	6761 - 7313	553	Plasmid backbone sequences (Gleave, 1992).
ori T	7314 - 7423	110	RK2 derived origin of transfer (oriT) necessary for conjugal transfer (Gleave, 1992).
Plasmid backbone sequence	7424 - 7482	59	Plasmid backbone sequences (Gleave, 1992).
IS*1*	7483 - 8250	768	IS1R prokaryotic mobile element (Gleave, 1992).
Plasmid backbone sequence	8251 - 8874	624	Plasmid backbone sequences (Gleave, 1992).
trfA	8875 - 10023	1149	Codes for trans-acting replication protein that binds to and activates oriV (Smith and Thomas, 1984; Gleave, 1992).
Plasmid backbone sequence	10024 - 11382	1359	Plasmid backbone sequences (Gleave, 1992).

Genetic Element (GE)	Location in the Plasmid (bp)	GE Size (bp)	Description
Ori	11383 - 11971	589	Origin of replication in colicin E1 plasmid (Tomizawa et al., 1977) required for the maintenance of the plasmid in *E. coli.*
Plasmid backbone sequence	11972 - 12389	418	Plasmid backbone sequences (Gleave, 1992).
smR	12390 - 14453	2064	Coding sequence for an aminoglycoside-modifying enzyme, 3"(9)-O-nucleotidyltransferase from the transposon Tn7 that confers resistance to spectinomycin and streptomycin (Fling et al., 1985).
Plasmid backbone sequence	14454 - 14536	83	Plasmid backbone sequences (Gleave, 1992)
ori V	14537 - 15157	621	Origin of replication from the plasmid RK2 for maintenance of plasmid in *A. tumefaciens* (Smith and Thomas, 1984; Stalker et al., 1981).
Plasmid backbone sequence	15158 - 15649	492	Plasmid backbone sequences derived from *A. tumefaciens* (Gleave, 1992).

3.3 Description of the Transformation System

The disarmed *A. tumefaciens* strain LBA4404 (Ooms et al., 1982) carrying binary vector pART27-LCT66 was used to transform cotyledon, cotyledonary-petiole and hypocotyl segments (explants) of 10-day-old cotton (*G. hirsutum* cv. Coker 312) seedlings. *A. tumefaciens*-mediated transformation was carried out using a procedure described by Sunilkumar and Rathore (2001) and Rathore et al. (2006, 2015). Briefly, cotton seeds were germinated on basal media, and the cotyledon, cotyledonary petiole and hypocotyl segments were excised from a 10-day-old seedling and infected with *A. tumefaciens* strain LBA4404 carrying the pART27-LCT66 vector. After co-cultivation for three days, these explants were placed on selection medium containing kanamycin (50 mg/L) and carbenicillin (400 mg/L) in order to inhibit the growth of untransformed plant cells and arrest the growth of *A. tumefaciens* cells, respectively. Somatic embryos developed from selected events were obtained after 6-8 rounds of culture on various media (P1, P7 and MSEm). These embryos were further cultured on EG3 and MS3 media for 2-3 months to promote shoot and root development. The detailed protocol along with the recipes for the various media used in the tissue culture experiments can be found in Rathore et al. (2006, 2015). The rooted plants (T0) with normal phenotypic characteristics were transferred to soil for growth and various analyses. Southern blot analysis was performed on the genomic DNA obtained from the leaves of transgenic plants to confirm the presence and determine the copy number of the transgene. A scheme showing the steps in the development of TAM66274 is presented in Figure 3-4. Additional breeding steps with TAM66274 to generate plants used in characterization studies including molecular characterization, gene expression, cottonseed composition and phenotypic, agronomic and ecological characteristics, which are presented in Sections 4, 5, 6 and 7, respectively, in this petition and are shown in Figure 3-5.

Figure 3-4. Schematic of the development of TAM66274.

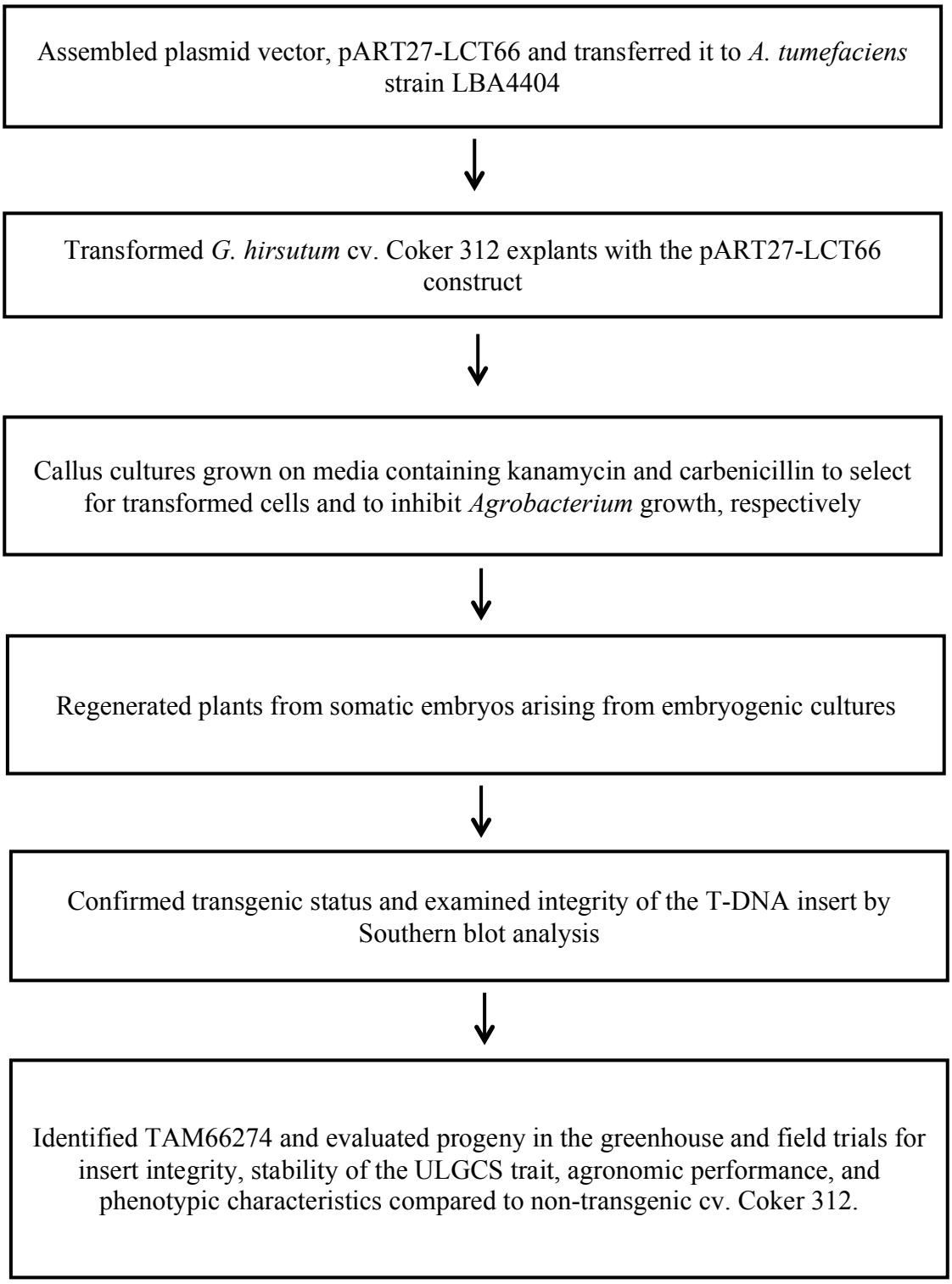
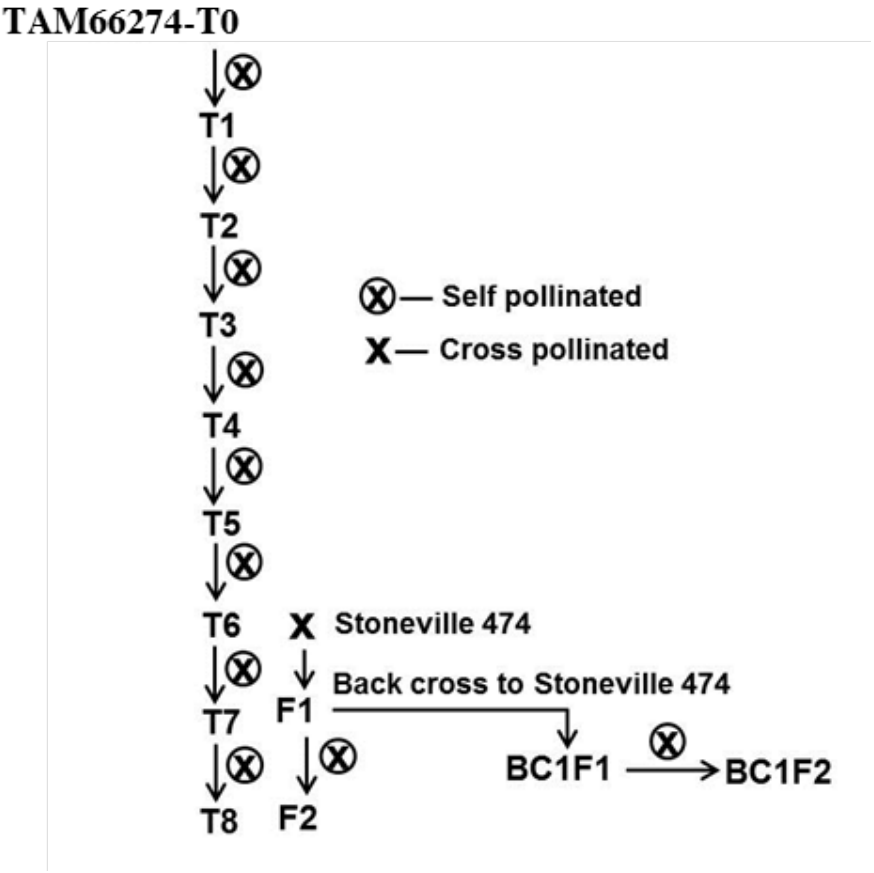


Figure 3-5. Propagation of TAM66274 used in characterization studies.



3.4 TAM66274 and Comparator Plants Used for Characterization Studies

Table 3-2 presents the breeding generations of TAM66274 used in the characterization studies of TAM66274 presented in this petition. The comparator plants were derived from non-transgenic cv. Coker 312, which was also used to produce TAM66274. Non-transgenic cv. Coker 312 was deemed the appropriate comparator as it is a near isogenic cultivar to TAM66274.

Table 3-2. Test and control materials used for characterization of TAM66274.

Analysis	Petition Section	TAM66274 Generation	Comparator
Molecular analysis	4	T1, T2, T3, T6	Coker 312
Segregation analysis (Single generation)	4	T2	None
Segregation analysis (Breeding generations)	4	ST474 x TAM66274 (F2), ST474 x TAM66274 (BC1F2)	None
Gene expression analysis	5	T6 for vegetative tissues, T7 for seed tissues	Coker 312
Composition analysis	6	T6 for 2014 studies, T7 for 2015 studies	Coker 312
Agronomic analysis	7	T5, T6	Coker 312
Germination and dormancy	7	T6 for 2014 studies, T7 for 2015 studies	Coker 312

4. MOLECULAR CHARACTERIZATION OF TAM66274

4.1 Overview of Molecular Characterization

This section contains a comprehensive molecular characterization of the genetic modification present in TAM66274. It provides information on the integration of the T-DNA into the genome of TAM66274, and additional information related to the stability, segregation, integrity and genomic organization of the introduced genetic material. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 30, 31, 32, and 33 (Codex, 2003a).

Molecular analyses were performed to characterize the integrated T-DNA in TAM66274 (T6 generation). Southern blot hybridizations were performed to investigate the presence or absence of the vector backbone sequences that are outside of the T-DNA borders, and to characterize the integration pattern of the T-DNA in TAM66274. Southern blot analysis was also used to demonstrate the stability of the inserted T-DNA in three different breeding generations. The sequence of genomic DNA directly adjacent to the T-DNA borders was determined by High-Efficiency Thermal Asymmetric InterLaced Polymerase Chain Reaction (HE-TAIL PCR) (Tan and Singh, 2011). The integrity and genomic organization of the T-DNA insert of TAM66274 were determined by overlap PCR amplification, followed by sequencing of the amplified products. Genotypic analysis of a segregating generation was performed by event-specific PCR. A high level correlation between the genotype and phenotype (visible color difference of the glands in ULGCS and non-transgenic cv. Coker 312 cottonseed kernels) was established by event-specific PCR. Subsequently, phenotypic analysis was used to further investigate the segregation of the ULGCS trait in breeding generations. Materials and methods used for molecular characterization of TAM66274 are described in detail in Appendix B.

Genomic DNA from non-transgenic cv. Coker 312 digested with the appropriate restriction enzyme was used as a negative control in Southern blot analyses to account for potential specific and non-specific endogenous hybridization signals. Non-transgenic cv. Coker 312 genomic DNA digested with the appropriate restriction enzyme and spiked with pART27-LCT66 plasmid DNA digested with the same enzyme at a level approximately equivalent to one copy of the transgene per cotton genome was used as a positive control for the Southern hybridizations. One kb DNA molecular weight markers served as size standards for agarose gel electrophoresis and Southern blot analysis. Probes for Southern blot hybridizations were generated by PCR using the transformation plasmid (pART27-LCT66) as the template.

Molecular characterization of TAM66274 showed that the T-DNA of plasmid pART27-LCT66 was integrated as a single insert and single copy in TAM66274, and that the integrity of the T-DNA was maintained from the transformation plasmid to the insert in TAM66274. HE-TAIL PCR analysis also showed that the RB T-DNA repeat was not integrated in the plant genome,

and only seven nucleotides from the LB T-DNA repeat were included in the T-DNA insert in TAM66274. No genetic elements from the backbone DNA of the plasmid were integrated in the TAM66274 genome. Analysis of the genomic DNA flanking the T-DNA insert in TAM66274 showed that the T-DNA integration occurred in an intron of a putative *α-hydrolase* gene. However, qRT-PCR analysis showed that there was no impact on mRNA expression from this gene in TAM66274 compared to non-transgenic cv. Coker 312. Therefore, integration of the T-DNA insert in the intron of the *α-hydrolase* gene had no effect on expression of its cognate protein in TAM66274. The stability of the T-DNA insert in TAM66274, determined by Southern blot analyses of three breeding generations and by trait inheritance studies over plant breeding generations, demonstrated that the inserted DNA is stably integrated in the plant genome and was stably inherited through multiple generations. Moreover, the transgene insert displayed the expected Mendelian inheritance pattern for single locus integration in the segregating generations, confirming that the transgene insert in TAM66274 is stably integrated at a single chromosomal locus.

4.2 Southern Blot Analyses of the T-DNA Insert in TAM66274

To interpret the results of Southern blot analyses, a linear map of the binary vector is provided showing the location of each specific probe (Figure 4-1B, Table 4-1) and the location of the restriction enzyme sites used for DNA digestion. Maps shown in Figures 4-1A, B, 4-3 and 4-5 serve as guides for the blots shown in Figures 4-2, 4-4A, B and 4-6A, B, respectively. In addition, comparisons of the expected and observed hybridization bands are presented in Tables 4-2 and 4-3 for each Southern blot. In the tables and figures corresponding to the Southern blots, the sizes of the hybridization bands are rounded to the nearest tenth and expressed in kb.

4.2.1. Absence of plasmid backbone sequences in TAM66274.

To examine the presence or absence of the plasmid backbone sequences in TAM66274, ten separate Southern blot analyses were performed. Ten overlapping probes (Probes 3-12, Table 4-1 and Figure 4-1B) spanning the *nos* terminator of the *nptII* variant expression cassette and the entire backbone region of plasmid pART27-LCT66 were generated and hybridized to *Eco*RI digested genomic DNA samples from TAM66274. Non-transgenic cv. Coker 312 genomic DNA spiked with *Eco*RI digested pART27-LCT66 plasmid DNA was used as a positive control and *Eco*RI digested non-transgenic cv. Coker 312 genomic DNA was used as a negative control for each Southern blot. As expected, and as shown in Figure 4-2A, Probe 3 hybridized to the *nptII* variant expression cassette, which resides within the T-DNA. Probe 3 spans the *nos* terminator of the *nptII* variant expression cassette and the vector backbone adjacent to the LB of the T-DNA. A single band (~3.6 kb size) was detected on the Southern blot (also see discussion on LB integration under Section 4.2.3). The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the *Eco*RI restriction site at bp 4509 within the T-DNA (Figure 4-1B) and a potential *Eco*RI site in the cotton genomic DNA flanking the 3' end of the T-DNA insert.

The Southern blot depicted in Figure 4-2A shows a difference in band intensity between the positive control and single hybridization band (~3.6 kb size) in the TAM66274 sample despite the expectation that each lane represents one copy of the transgene per cotton genome. This unintended difference is most likely due to a higher amount of plasmid loaded in the positive control lane (i.e., a pipetting error when the linearized plasmid was mixed with control DNA digest before loading). Additionally, after the digestion of the cotton genomic DNA, it is our practice to precipitate and re-dissolve genomic DNA in a smaller volume of buffer in order to fit all of the digested DNA into a well of the gel before electrophoresis. At times, the precipitated DNA may not be completely dissolved before loading the samples onto the gel, resulting in reduced intensity of the band of integrated DNA in Figure 4-2A.

Due to the presence of high amounts of polysaccharides and polyphenolics, isolation of genomic DNA from cotton leaves require the use of CTAB (cetyl trimethylammonium bromide) in combination with high concentrations of sodium chloride. Polysaccharides and polyphenolics have physicochemical properties similar to those of nucleic acids and are difficult to remove entirely from cotton genomic DNA preparations. Thus, the presence of co-purified contaminants and salts introduced by the purification procedure affects quantitation of the genomic DNA, which may have contributed to inaccurate DNA loading and the discrepancy in intensities of the bands seen in Southern blots in Figure 4-2A.

We have performed additional Southern blots (e.g., Figures 4-4, 4-6, 4-8) that do not show such differences indicating that the differences in band intensity seen in Figure 4-2A are likely due to laboratory technique issues limited to particular Southern blots.

Hybridization of *Eco*RI digested genomic DNA samples of TAM66274 with vector backbone Probes 4-12 did not show any hybridizing bands (Figures 4-2B-J), indicating the absence of the plasmid backbone sequences in TAM66274. All probes containing vector backbone DNA sequences (Probes 3-12, Figures 4-2A-J) hybridized with the positive control, *Eco*RI digest of the non-transgenic cv. Coker 312 genomic DNA spiked with *Eco*RI digested pART27-LCT66 plasmid DNA, and produced a hybridizing band of 13.8 kb. The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the *Eco*RI restriction sites at bp 4509 and bp 2668 in the plasmid (see Figure 4-1A, B). Further, no hybridizing bands were observed with any of the backbone probes for the *Eco*RI digests of genomic DNA from non-transgenic cv. Coker 312. Therefore, these Southern blots confirmed the absence of any plasmid backbone sequences in TAM66274 and also confirmed the presence of the *nos* terminator in the T-DNA insert in TAM66274.

Table 4-1. List of probes and their positions in transformation plasmid pART27-LCT66.

Probe	Position in pART27-LCT66 (Starting from RB T-DNA repeat)	Size (bp)	Description
3	6320 - 7219	900	*nos* terminator + vector backbone
4	7206 - 8192	987	Vector backbone
5	8174 - 9167	994	Vector backbone
6	9154 - 10131	978	Vector backbone
7	10120 - 11111	992	Vector backbone
8	11100 - 12077	978	Vector backbone
9	12061 - 13030	970	Vector backbone
10	13003 - 13976	974	Vector backbone
11	13936 - 14898	963	Vector backbone
12	14871 - 15649	779	Vector backbone

Figure 4-1. Schematics of genetic elements in plasmid pART27-LCT66, and probes and restriction enzyme sites used in Southern blot analyses of TAM66274.

A. Circular diagram of the transformation plasmid pART27-LCT66 showing genetic elements and positions of *Eco*RI and *Kpn*I sites, the two enzymes used for Southern blot analyses.

B. Diagrammatic representation of the transformation plasmid pART27-LCT66 (linear diagram) and the probes (red colored bars) used for Southern blot analyses. Probes 3-12 represent overlapping sequences covering *nos* terminator of the *nptII* variant cassette and the entire backbone of the binary vector. LB and RB indicate the left and the right border of the T-DNA, respectively.

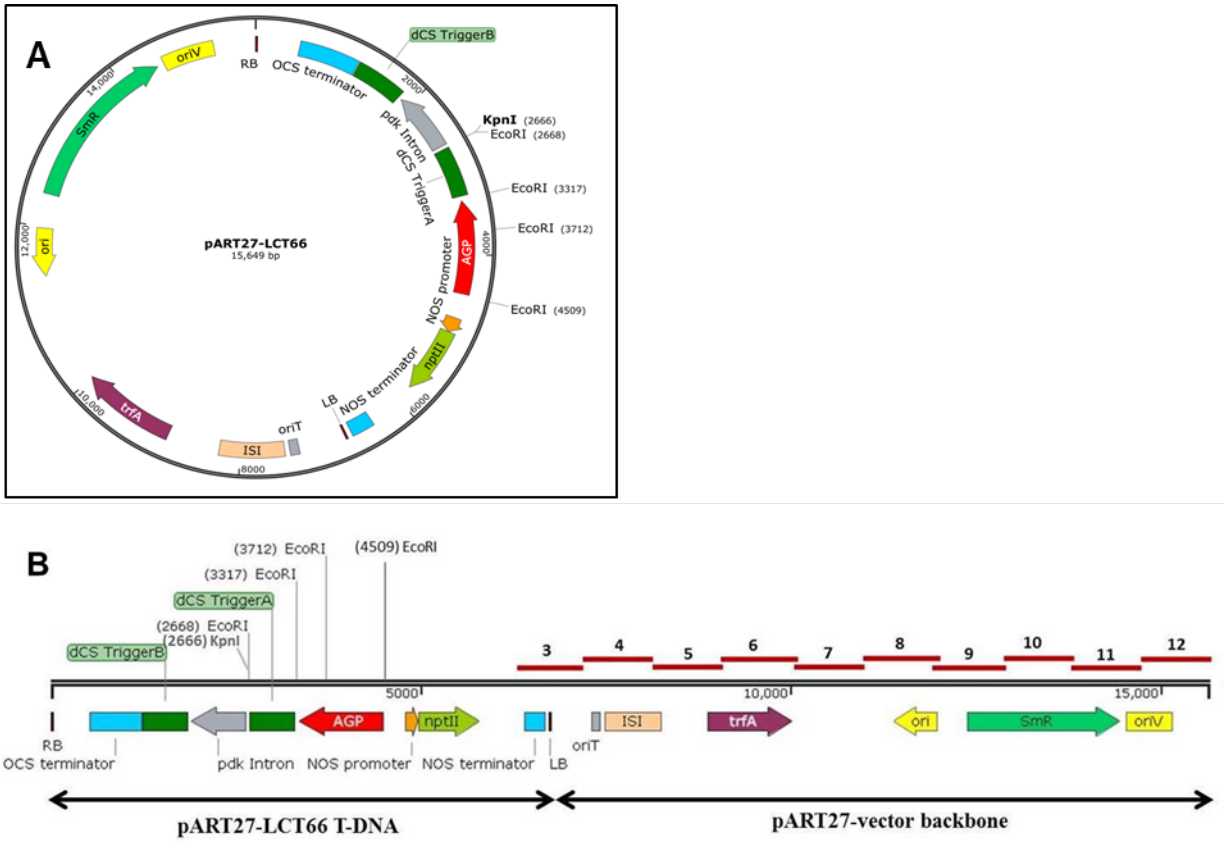


Figure 4-2 (A-D). Plasmid backbone analysis in TAM66274 - Southern blot analyses.
Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI digested pART27-LCT66 plasmid DNA at approximately one copy equivalent per cotton genome. Negative control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membranes were hybridized separately with individual radiolabeled probes (Probes 3-6, shown in Figure 4-1B).

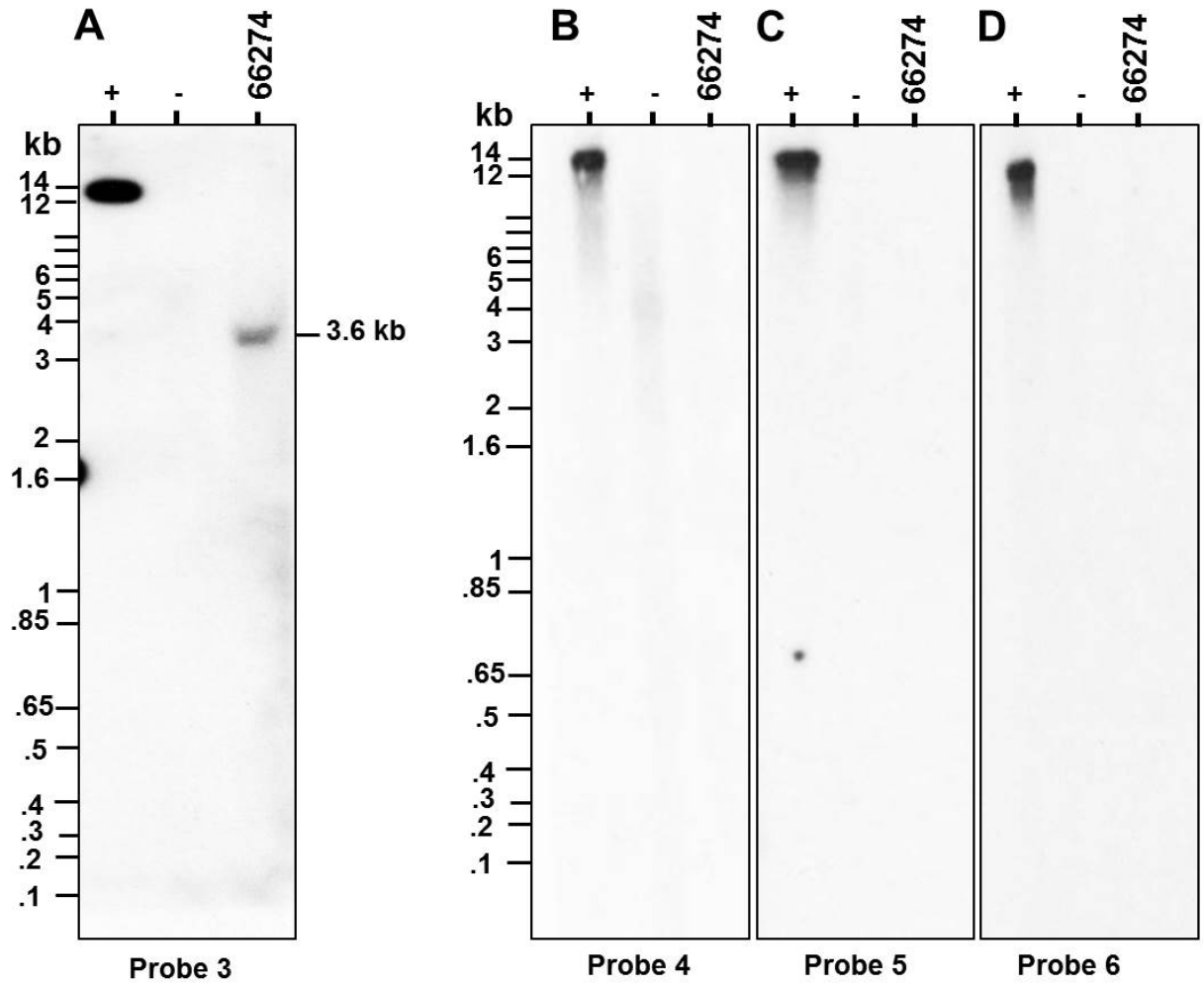


Figure 4-2 (E-G), continued. Plasmid backbone analysis in TAM66274 - Southern blot analyses.
Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI digested pART27-LCT66 plasmid DNA at approximately one copy equivalent per cotton genome. Negative control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membranes were hybridized separately with individual radiolabeled probes (Probes 7-9, shown in Figure 4-1B).

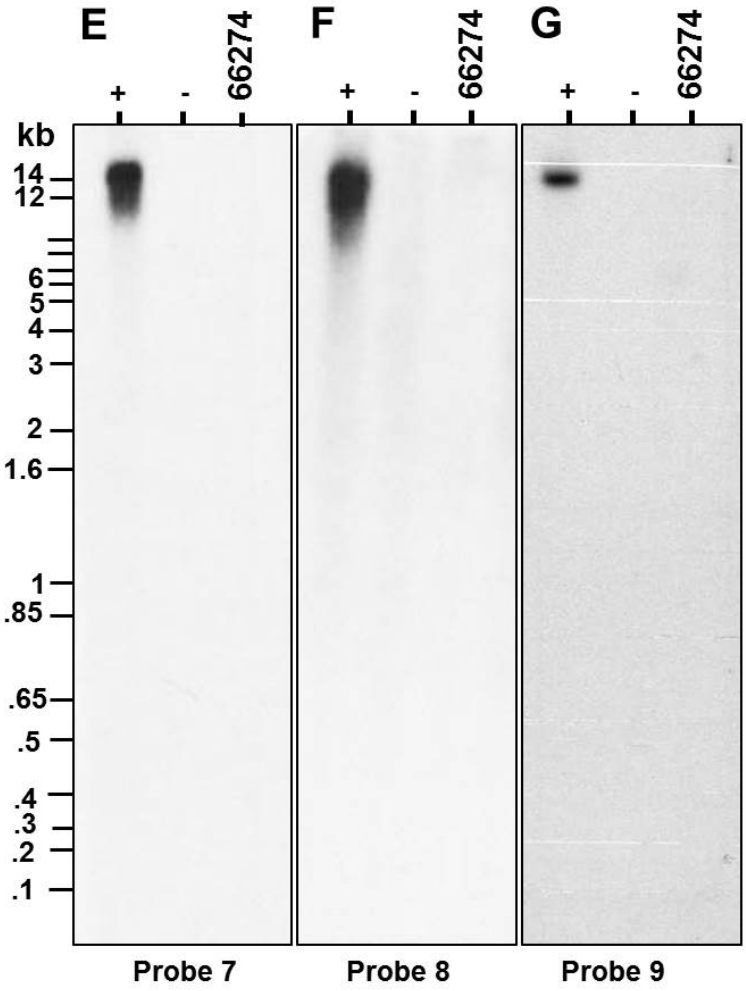
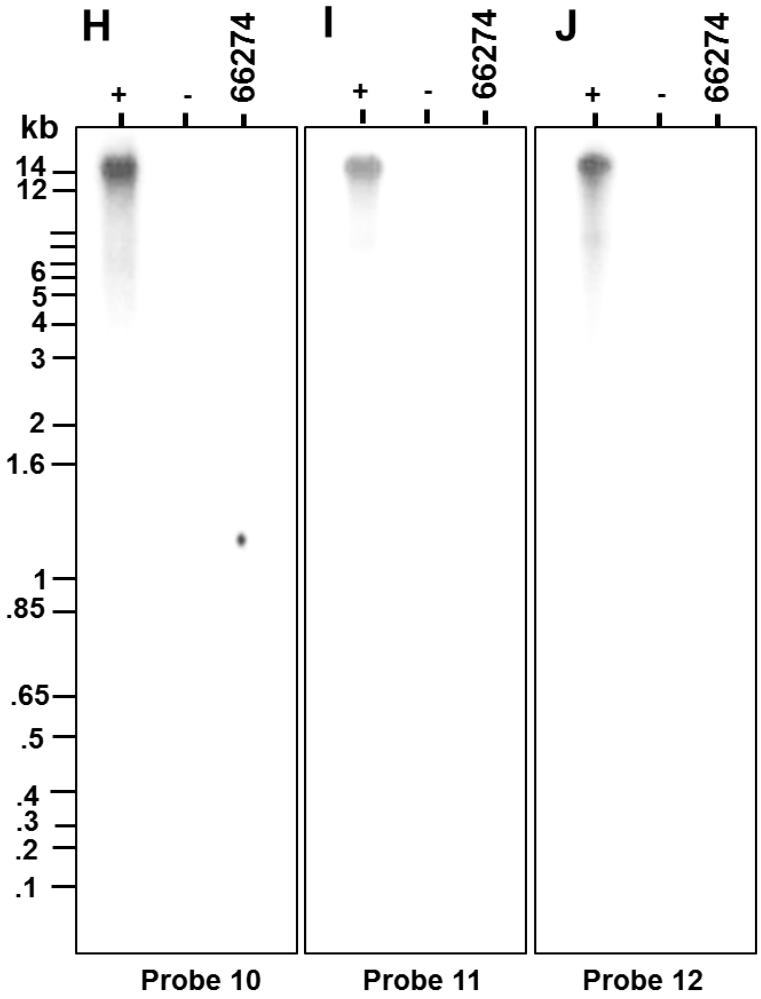


Figure 4-2 (H-J), continued. Plasmid backbone analysis in TAM66274 - Southern blot analyses.
Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI digested pART27-LCT66 plasmid DNA at approximately one copy equivalent per cotton genome. Negative control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membranes were hybridized separately with individual radiolabeled probes (Probes 10-12, shown in Figure 4-1B).



4.2.2. RB integration in TAM66274.

The RB integration pattern of T-DNA in TAM66274 was investigated by Southern blot analysis. Genomic DNA from TAM66274 was digested with the restriction enzyme *Eco*RI, followed by hybridization with Probe 13, which corresponds to the *ocs* terminator genetic element (Figure 4-3, Table 4-2). With this restriction enzyme and probe combination, any hybridizing band >2668 bp is considered a transgene integration in the cotton genome. As observed in the Southern blot (Figure 4-4A, Table 4-2), a single hybridization band of ~3.4 kb size was observed in TAM66274. The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the *Eco*RI restriction site at bp 2668 within the T-DNA (Figure 4-3) and a potential *Eco*RI site upstream in the cotton genomic DNA flanking the 5' end of the T-DNA insert. This Southern blot thus confirmed integration of the *ocs* terminator region at the 5' end of the T-DNA in TAM66274. Probe 13 hybridized with the positive control, *Eco*RI digest of the non-transgenic cv. Coker 312 genomic DNA spiked with *Eco*RI digested pART27-LCT66 plasmid DNA, and produced a hybridizing band of 13.8 kb (Figure 4-4A). This is the same DNA fragment that hybridized with Probes 3-12 containing vector backbone DNA (Figure 4-2 [A-J]), and the origin of this DNA fragment is described above (Section 4.2.1). No hybridizing bands were observed in the negative control, the *Eco*RI digest of genomic DNA from non-transgenic cv. Coker 312.

The RB integration pattern of T-DNA in TAM66274 was further confirmed by an additional Southern blot analysis. Genomic DNA from TAM66274 was digested with the restriction enzyme *Kpn*I, followed by hybridization with Probe 13 (Figure 4-3). With this restriction enzyme and probe combination, any hybridizing band >2666 bp is considered a transgene integration in the cotton genome (Table 4-2). As observed in the Southern blot (Figure 4-4B), a single hybridization band of ~4.3 kb size was observed in TAM66274. The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the *Kpn*I restriction site at bp 2666 within the T-DNA (Figure 4-3) and a potential *Kpn*I site upstream in the cotton genomic DNA flanking the 5' end of the T-DNA insert. Thus, the results from this Southern blot are consistent with those from the *Eco*RI/Southern blot (Figure 4-4A) showing that the RB region of the T-DNA integrated at a single locus in TAM66274. Probe 13 also hybridized with the positive control, *Kpn*I digest of non-transgenic cv. Coker 312 genomic DNA spiked with *Kpn*I digested pART27-LCT66 plasmid DNA, and produced a hybridizing band of 16.0 kb (Figure 4-4B). The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the single *Kpn*I restriction site at bp 2666 in the plasmid (see Figure 4-1A, B) and, therefore, is the complete linearized plasmid pART27-LCT66. Further, no hybridizing bands were observed with Probe 13 for the *Kpn*I digest of genomic DNA from non-transgenic cv. Coker 312.

Figure 4-3. RB T-DNA integration analysis.
Diagrammatic representation of the location of the Probe 13 within the T-DNA region of pART27-LCT66. Probe 13 was used for the determination of RB integration.

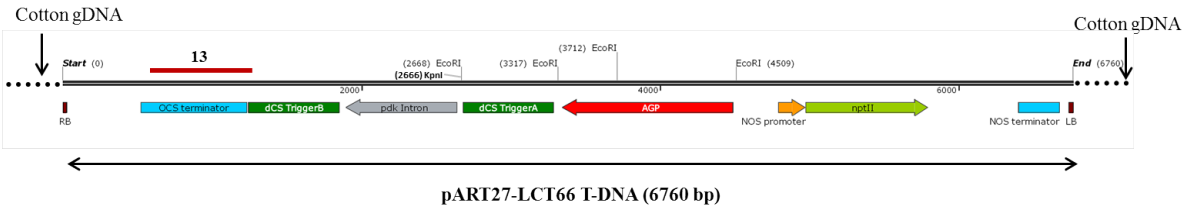
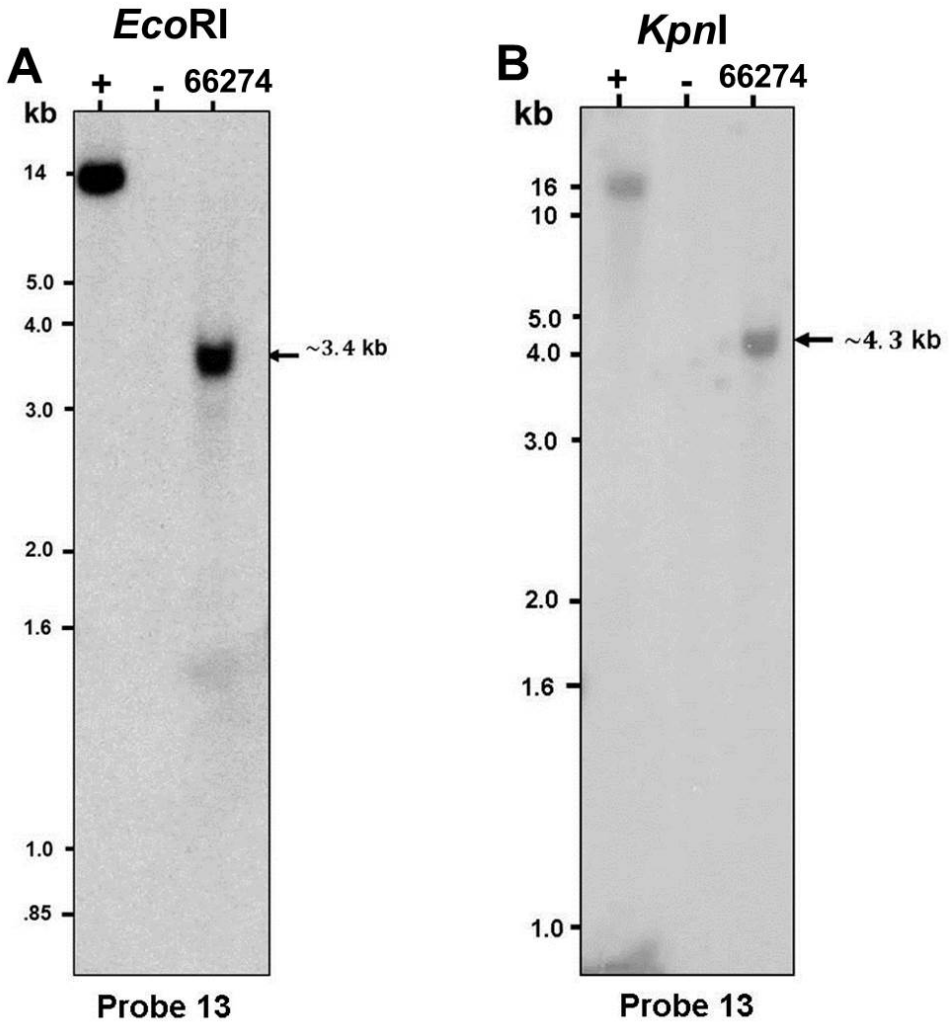


Table 4-2. RB T-DNA integration analysis.
Size(s) of the expected and observed hybridization bands in TAM66274.

RB T-DNA integration - TAM66274					
Restriction enzyme and position	**Probe**	**Probe position and size (bp)**	**Expected band(s) (kb)**	**Observed band(s) (kb)**	**Expected band for the positive control plasmid (kb)**
*Eco*RI (2668)	13	523-1230 (708)	>2.7	~3.4	14.0
*Kpn*I (2666)	13	523-1230 (708)	>2.7	~4.3	16.0

Figure 4-4. RB T-DNA integration - Southern blot analyses.
Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI or *Kpn*I digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI or *Kpn*I digested pART27-LCT66 plasmid DNA at approximately one copy equivalent per cotton genome. Negative control included approximately 12 µg of *Eco*RI or *Kpn*I digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membrane was hybridized with radiolabeled *ocs* terminator sequence (Probe 13).



4.2.3. LB integration in TAM66274.

The LB integration pattern of T-DNA in TAM66274 was investigated by Southern blot analysis. Genomic DNA from TAM66274 was digested with *Eco*RI restriction enzyme, followed by hybridization with Probe 1 that corresponds to the *nos* promoter and part of the *nptII* variant coding sequence (Figure 4-5). With this restriction enzyme and probe combination, any hybridizing band >2252 bp is considered an integration in the cotton genome. A single hybridization band of ~3.6 kb size was observed in TAM66274 (Figure 4-6A and Table 4-3). The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the *Eco*RI restriction site at bp 4509 within the T-DNA (Figure 4-5) and a potential *Eco*RI site downstream in the cotton genomic DNA flanking the 3' end of the T-DNA insert. This is the same DNA fragment produced by *Eco*RI restriction digest of TAM66274 that hybridized with Probe 3 (Table 4-1; Figure 4-2A) as described above (Section 4.2.1). Collectively, these results confirmed integration of the *nos* promoter, the *nptII* variant coding sequence, and the *nos* terminator adjacent to the LB region of the T-DNA in TAM66274. The results shown in Figures 4-6A suggest a single copy integration of the T-DNA in TAM66274. Probe 1 hybridized with the positive control, *Eco*RI digest of the non-transgenic cv. Coker 312 DNA spiked with *Eco*RI digested pART27-LCT66 plasmid DNA, and produced a hybridizing band of 13.8 kb (Figure 4-6A). This is the same DNA fragment that hybridized with Probes 3-12 containing vector backbone DNA (Figure 4-2 [A-J]), and the origin of this DNA fragment is described above (Section 4.2.1). No hybridizing bands were observed with the *Eco*RI digest of genomic DNA from non-transgenic cv. Coker 312.

The LB integration pattern of T-DNA in TAM66274 was further confirmed by an additional Southern blot analysis. Genomic DNA from TAM66274 was digested with the restriction enzyme *Kpn*I, followed by hybridization with Probe 1 (Figure 4-5). With this restriction enzyme and probe combination, any hybridizing band >4095 bp is considered an integration in the genome (Table 4-3). A single hybridization band of ~10 kb size was observed in TAM66274 (Figure 4-6B). The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the *Kpn*I restriction site at bp 2666 within the T-DNA (Figure 4-5) and a potential *Kpn*I site in the cotton genomic DNA flanking the 3' end of the T-DNA insert. Thus, the results from this Southern blot are consistent with those from the *Eco*RI/Southern blot (Figure 4-6A) showing the LB region of the T-DNA integration at a single locus in TAM66274. Probe 1 also hybridized with the positive control, *Kpn*I digest of non-transgenic cv. Coker 312 DNA spiked with *Kpn*I digested pART27-LCT66 plasmid DNA, and produced a hybridizing band of 16.0 kb (Figure 4-6B). The size of this hybridizing band is consistent with the production of a single DNA fragment defined by the single *Kpn*I restriction site at bp 2666 in the plasmid (see Figure 4-1A, B) and, therefore, is the complete linearized plasmid pART27-LCT66. Further, no hybridizing bands were observed with Probe 1 for the *Kpn*I digest of genomic DNA from non-transgenic cv. Coker 312.

Figure 4-5. LB T-DNA integration analysis.
Diagrammatic representation of the location of the Probe 1 within the T-DNA region of the construct pART27-LCT66. The probe was used for the determination of LB integration.

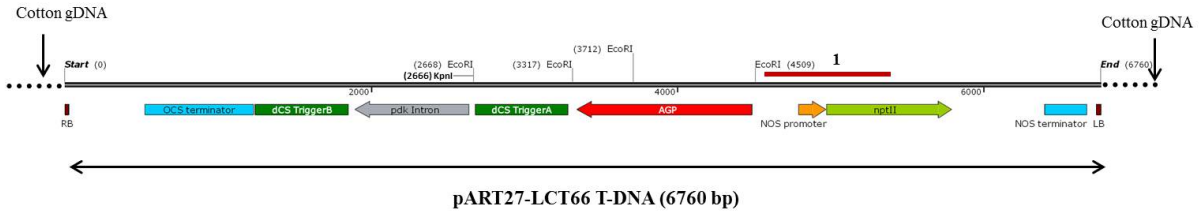
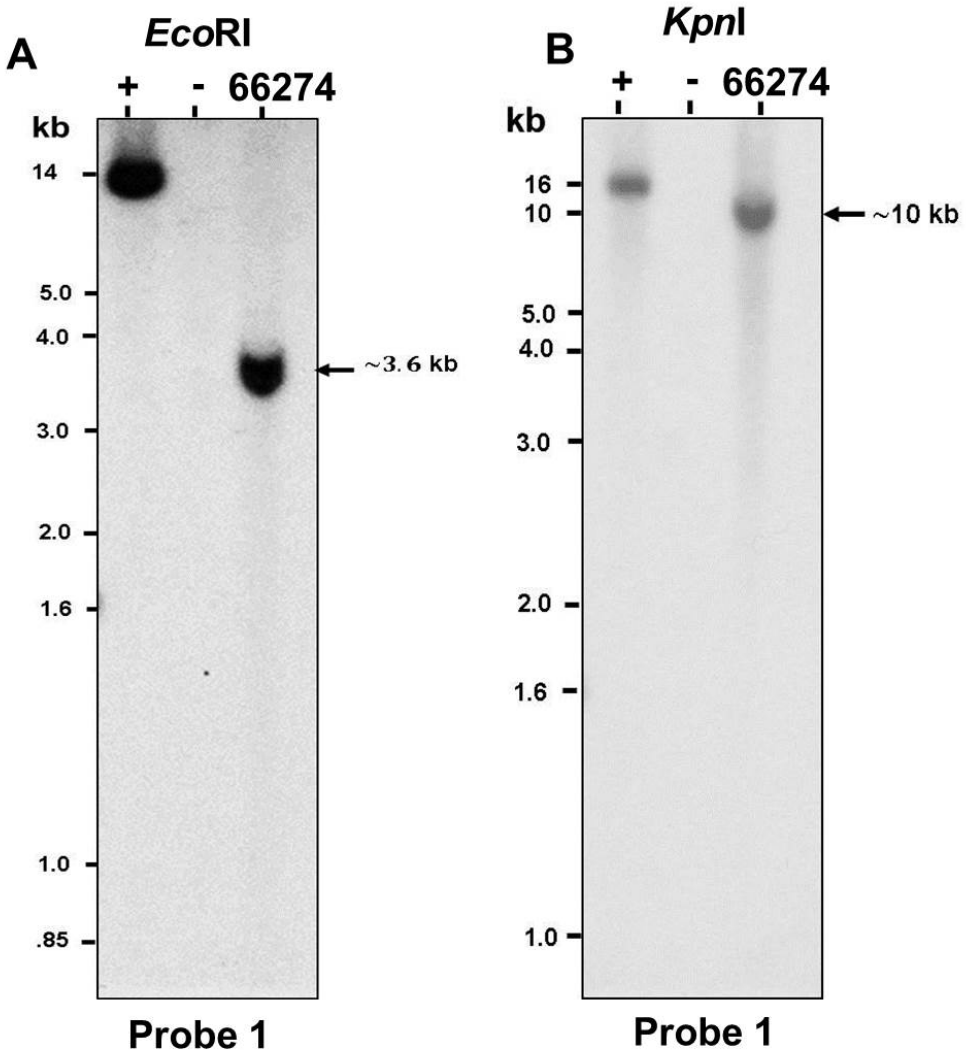


Table 4-3. LB T-DNA integration analysis.
Size(s) of the expected and observed hybridization bands in TAM66274.

LB T-DNA integration - TAM66274					
Restriction enzyme and position	**Probe**	**Probe position and size (bp)**	**Expected band (kb)**	**Observed band (kb)**	**Expected band for the positive control plasmid (kb)**
*Eco*RI (4509)	1	4528-5439 (912)	>2.3	~3.6	14.0
*Kpn*I (2666)	1	4528-5439 (912)	>4.1	~10	16.0

Figure 4-6. LB T-DNA integration - Southern blot analyses.
Approximately 12 µg of genomic DNA was digested with *Eco*RI **(A)** or *Kpn*I **(B)** and loaded per Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI or *Kpn*I digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI or *KpnI* digested pART27-LCT66 plasmid DNA at approximately one copy equivalent per cotton genome. Negative control included approximately 12 µg *Eco*RI or *Kpn*I digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membrane was hybridized with radiolabeled *nos* promoter + *nptII* variant sequence (Probe 1).



4.2.4. Integration of internal genetic elements of the T-DNA.

Results from the RB and LB integration Southern blot analyses using probes corresponding to the T-DNA borders showed single integrations of the *ocs* terminator region and the *nptII* variant cassette in TAM66274. In order to establish that the genetic elements between these two regions of the T-DNA, along with the RB and LB, are integrated in TAM66274 as a single T-DNA insert, a separate set of Southern blot analyses was conducted. These Southern blots were conducted following digestion of the genomic DNA with *Kpn*I since this enzyme cuts only once within the T-DNA. The *Kpn*I restriction site is present at position 2666 bp of the T-DNA, and it delineates the genetic elements adjacent to the RB of the T-DNA, which include the *ocs* terminator, the *dCS* Trigger B and the *pdk* intron, from genetic elements adjacent to the LB of the T-DNA, which include the *dCS* Trigger A, AGP and the *nptII* variant gene cassette. Three different probes (14, 15 and 16) were used to hybridize with individual Southern blots each carrying *Kpn*I digested DNA from TAM66274 (Figure 4-7 and Table 4-4).

Probe 14 corresponds to the *dCS* trigger coding sequences and, as expected, showed multiple hybridization bands in the lane containing the genomic DNA from non-transgenic cv. Coker 312 (a dominant band at ~16 kb and less intense lower molecular weight bands at ~8.0 kb, ~3.9 kb and ~3.3 kb) (Figure 4-8A). The results from this Southern blot are consistent with the fact that the *dCS* coding sequence is a member of a multigene family found in the cotton genome, with other members exhibiting high levels of sequence homology. Therefore, hybridizing bands in the non-transgenic cv. Coker 312 lane, which are produced by *Kpn*I restriction digest of the cotton genomic DNA and hybridized with Probe 14, correspond to the multigene family members of the endogenous *dCS* gene(s). In the TAM66274 lane, in addition to the hybridizing bands described for non-transgenic cv. Coker 312, there are hybridizing bands of sizes ~4.3 kb and ~10 kb. The ~4.3 kb band is consistent with hybridization of Probe 14 with the *dCS* Trigger B coding sequence in a DNA fragment produced by the *Kpn*I restriction site at bp 2666 within the T-DNA (Figure 4-7) and a potential *Kpn*I site upstream in the cotton genomic DNA flanking the 5' end of the T-DNA insert. This ~4.3 kb hybridizing band is the same DNA fragment produced by *Kpn*I digest of TAM66274 DNA which hybridized with Probe 13 (corresponding to the *ocs* terminator), as shown in Figure 4-4B. This result supports the conclusion that the *ocs* terminator and *dCS* Trigger B are contiguous in the T-DNA as shown in Figure 4-7. The ~10.0 kb band is consistent with hybridization of Probe 14 with the *dCS* Trigger A gene in a DNA fragment produced by the *Kpn*I restriction site at bp 2666 within the T-DNA (Figure 4-7) and a potential *Kpn*I site downstream in the cotton genomic DNA flanking the 3' end of the T-DNA insert. The ~10.0 kb hybridizing band is the same DNA fragment produced by *Kpn*I digest of TAM66274 DNA which hybridized with Probe 1 (corresponding to the *nos* promoter and part of the *nptII* variant gene), as shown in Figure 4-6B. This result supports the conclusion that the *dCS* Trigger A gene and the *nptII* variant gene cassette are on the same *Kpn*I digest fragment of the T-DNA as shown in Figure 4-7. The positive control (*Kpn*I digested non-transgenic cv. Coker 312 genomic DNA spiked with *Kpn*I digested pART27-LCT66 plasmid DNA) with Probe 14 showed the same

hybridizing bands as the *Kpn*I digested non-transgenic cv. Coker 312 genomic DNA negative control (Figure 4-8A). This was expected since *Kpn*I digest of the plasmid pART27-LCT66 produces a single hybridizing band of 16.0 kb, which corresponds to a DNA fragment defined by the single *Kpn*I restriction site at bp 2666 in the plasmid (see Figure 4-1A, B) and, therefore, is the complete linearized plasmid pART27-LCT66. This hybridizing band co-migrated with the ~16.0 kb band produced by *Kpn*I digest of non-transgenic cv. Coker 312 DNA.

Probe 15 corresponds to the *pdk* intron, which is located adjacent to the *dCS* Trigger B coding sequence in plasmid pART27-LCT66 (Figure 4-1A, B), and is at the RB end of the T-DNA delineated by the *Kpn*I restriction site at bp 2666 (Figure 4-7). Therefore, *Kpn*I restriction digest of TAM66274 DNA hybridized with Probe 15 was expected to yield the same ~4.3 kb band as observed with the same *Kpn*I digest and hybridization with Probes 13 and 14, as described above. This was indeed the case (Figure 4-8B), which supports the conclusion that the *pdk* intron is on the same *Kpn*I digested fragment of the T-DNA as the *ocs* terminator and *dCS* Trigger B sequences as shown in Figure 4-7. Also, as expected, a single hybridizing band of 16.0 kb was observed in the positive control, *Kpn*I digested non-transgenic cv. Coker 312 genomic DNA spiked with *Kpn*I digested pART27-LCT66 plasmid DNA, corresponding to the linearized plasmid pART27-LCT66 hybridized with Probe 15, and no hybridizing bands were observed in the negative control, *Kpn*I digested non-transgenic cv. Coker 312 genomic DNA (Figure 4-8B).

Probe 16 corresponds to the AGP sequence, which is located adjacent to the *dCS* Trigger A coding sequence in plasmid pART27-LCT66 (Figure 4-1A, B), and is at the LB end of the T-DNA delineated by the *Kpn*I restriction site at bp 2666 (Figure 4-7). Therefore, *Kpn*I restriction digest of TAM66274 DNA hybridized with Probe 16 was expected to yield the same 10.0 kb band as observed with the same *Kpn*I digest and hybridization with Probes 14 and 1, as described above (hybridizing with the *dCS* Trigger A coding sequence and the *nos* promoter and part of the *nptII* variant coding sequence, respectively). This was indeed the case (Figure 4-8C), which supports the conclusion that the AGP sequence is on the same *Kpn*I digested fragment of the T-DNA as the *dCS* Trigger A sequence and the *nptII* variant gene cassette, as shown in Figure 4-7. Several hybridizing bands were observed for the non-transgenic cv. Coker 312 *Kpn*I digest hybridized with Probe 16 (~16.0 kb, ~6.5 kb and ~5.5 kb) (Figure 4-8C). This was to be expected since the AGP sequence is derived from the cotton α–globulin B gene and the three bands correspond to homeologues in the A and D genomes of cotton. There is 82% sequence homology between the promoters from the two genomes. The positive control lane (*Kpn*I digest of the non-transgenic cv. Coker 312 genomic DNA spiked with *Kpn*I digested pART27-LCT66 plasmid DNA) with Probe 16 showed the same hybridizing bands as the negative control, *Kpn*I digested non-transgenic cv. Coker 312 genomic DNA (Figure 4-8C). This was expected since *Kpn*I digest of the plasmid pART27-LCT66 produces a single hybridizing band of 16.0 kb that corresponds to a DNA fragment defined by the single *Kpn*I restriction site at bp 2666 in the plasmid (see Figure 4-1A, B) and, therefore, is the complete linearized plasmid pART27-LCT66.

This hybridizing band co-migrated with the 16.0 kb band in the negative control, *Kpn*I digest of non-transgenic cv. Coker 312 genomic DNA.

The Southern blot in Figure 4-8C shows a slight difference in the intensity of the hybridization bands of 6.5 kb in the positive and negative control lanes compared to the TAM66274 sample lane. As noted above, Probe 16 hybridizes with several AGP sequences from homeologues in the A and D genomes of cotton resulting in three hybridizing bands common to both TAM66274 and non-transgenic cv. Coker 312 (~16.0 kb, ~6.5 kb and ~5.5 kb). In Figure 4-8C, the ~16.0 kb band is more intense in the positive control lane most likely because the expected plasmid control band (16.0 kb) runs on top of the endogenous band, which is observed in the negative control lane and in the TAM66274 sample. The ~5.5 kb band appears slightly darker in the TAM66274 sample compared to the positive and negative control most likely due to genomic DNA quantitation error as previously described above. Figure 4-8C also exhibits a diffuse band at ~6.5 kb in all three samples, but the TAM66274 sample appears slightly darker than the corresponding band in the positive and negative control. This is also most likely due to genomic DNA quantitation error as previously described, combined with its poor resolution at this size range in the agarose gel. These differences are an artifact of Southern blot technique, which does not invalidate the accuracy of the data or lead to any different conclusions about the depicted results.

Figure 4-7. Analysis of integration of internal genetic elements of the T-DNA.
Diagrammatic representation of the location of Probes 14, 15 and 16 within the T-DNA region of the construct pART27-LCT66. These probes were used to determine the intactness of the inserted T-DNA in the TAM66274 genome.

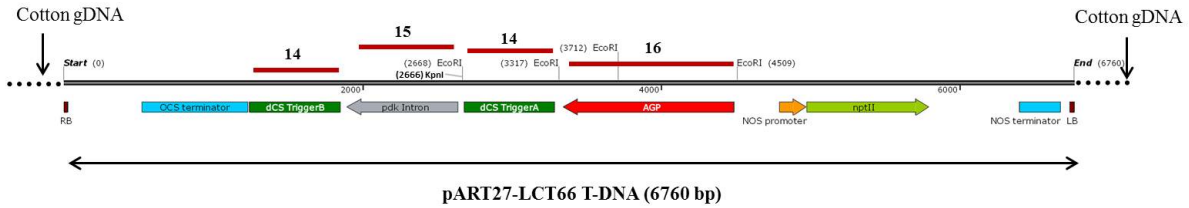
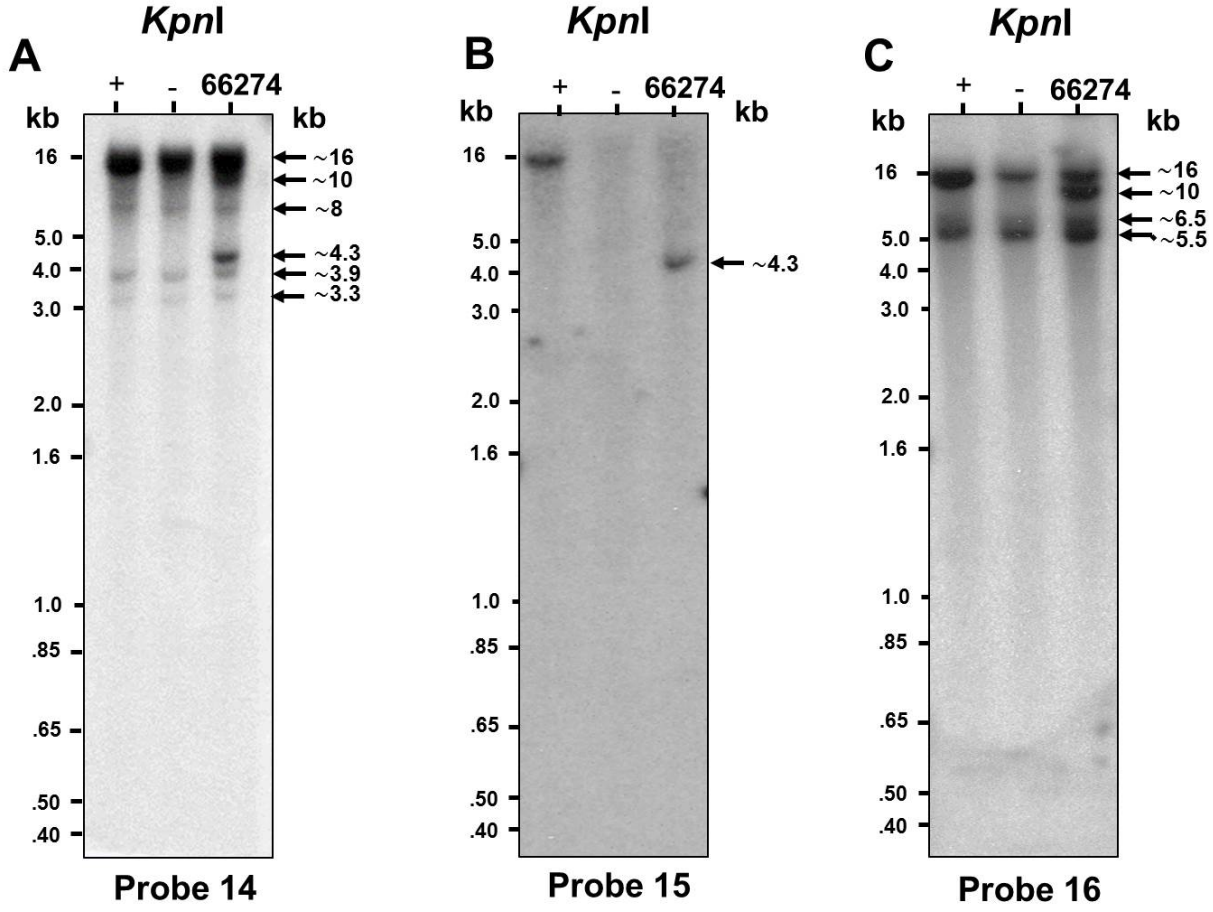


Table 4-4. Analysis of integration of internal genetic elements of the T-DNA.
Size(s) of the expected and observed hybridization bands in TAM66274.

Internal Elements integration - TAM66274					
Restriction enzyme and position	**Probe**	**Probe position and size (bp)**	**Expected band(s) (kb)**	**Observed band(s) (kb)**	**Expected band for the positive control plasmid (kb)**
*Kpn*I (2666)	14	1267-1847 (581)	>2.7, >4.1	~16, ~10, ~8, ~4.3, ~3.9, ~3.3	16.0
*Kpn*I (2666)	15	1950-2524 (575)	>2.7	~4.3	16.0
*Kpn*I (2666)	16	3340-4479 (1140)	>4.1	~16, ~10, ~6.5, ~5.5	16.0

Figure 4-8. Integration of internal genetic elements of the T-DNA - Southern blot analyses. Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Kpn*I digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Kpn*I digested pART27-LCT66 plasmid DNA at approximately one copy equivalent per cotton genome. Negative control included approximately 12 µg of *Kpn*I digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membrane was hybridized with radiolabeled **(A)** *dCS* trigger (Probe 14), **(B)** *pdk* intron (Probe 15) or **(C)** AGP (Probe 16).



The Southern blot analyses conducted following digestion of the genomic DNA with *Eco*RI and hybridized with Probes 1 and 13, and *Kpn*I digest and hybridized with Probes 1, 13, 14, 15 and 16 show clearly the contiguous nature of the genetic elements in the integrated T-DNA in TAM66274. Thus, the results presented in Figures 4-4, 4-6 and 4-8 together show a complete and single copy integration of the T-DNA in TAM66274.

4.3 Stability of the T-DNA Insert Across Generations

In order to assess the stability of the inserted T-DNA in TAM66274 across generations, two unique fingerprint Southern blot analyses were performed using DNA samples obtained from three generations (T1, T2 and T3). The first fingerprint was the *Eco*RI digest hybridized with Probe 13 which produced a border DNA fragment of the RB end of the T-DNA and cotton genomic DNA (Section 4.2.2, Figure 4-4A). The second was the *Eco*RI digest hybridized with Probe 1 which produced a border DNA fragment of the LB end of the T-DNA and cotton genomic DNA (Section 4.2.3, Figure 4-6A). The breeding generations used for this analysis are depicted in Section 3 of this petition in Figure 3-5. Probe 13 corresponding to the *ocs* terminator was used for hybridization in combination with an *Eco*RI digest. As observed earlier (Figure 4-4A), this probe and restriction digest combination produces a unique hybridizing band of 3.4 kb, which includes the *ocs* terminator at the RB end of the T-DNA and cotton genomic DNA which is contiguous with the T-DNA insert. Therefore, this probe and restriction digest provides a unique fingerprint of the T-DNA insert in the cotton genome, and can be used to determine stability of the insert across breeding generations. All three generations of TAM66274 showed a single and identical hybridization band (3.4 kb with the *ocs* terminator probe corresponding to the RB T-DNA region) (Figure 4-9). A similar Southern blot analysis was performed on DNA samples from three generations of TAM66274 using Probe 1 corresponding to the *nos* promoter and part of the *nptII* variant gene. As observed earlier (Figure 4-6A), this probe and *Eco*RI restriction digest combination produces a unique hybridizing band of 3.6 kb, which includes the *nptII* variant cassette at the LB end of the T-DNA and cotton genomic DNA which is contiguous with the T-DNA insert. Therefore, this probe and restriction digest provides a unique fingerprint of the T-DNA insert in the cotton genome, and can be used to determine stability of the insert across breeding generations. All three breeding generations of TAM66274 showed a single hybridization band of 3.6 kb (Figure 4-10).

The positive control, *Eco*RI digested non-transgenic cv. Coker 312 spiked with *Eco*RI digested pART27-LCT66 plasmid DNA, showed the same hybridizing band of 13.8 kb and probed with either Probe 1 or 13 (Figures 4-9 and 4-10). The origin of this band has been described in Sections 4.2.2 and 4.2.3 above. No hybridizing bands were observed in the negative control, *Eco*RI digested non-transgenic cv. Coker 312 genomic DNA lanes.

In Figure 4-9, there appears to be two hybridization bands in the T2 generation sample compared to one band in T1 and T3 generation samples. The additional faint hybridization band in the T2

generation sample is most likely non-specific since the band is not seen in Figure 4-10. In our experience, Southern blots occasionally show artifacts like this and we would only be concerned with this type of observation if we saw an extra band in T1 generation that disappeared in T2 and T3 generation, which is not the case in Figures 4-9 and 4-10. We consider these differences to be an artifact of Southern blot technique, which do not invalidate the accuracy of the data or lead to any different conclusions about the depicted results.

The hybridization band (~ 3.4 kb size) in the T2 generation sample in Figures 4-9 and 4-10 appears to migrate slower and is less intense than the hybridization bands of T1 and T3 generation samples, despite the expectation of that each lane was loaded with identical quantities of genomic DNA. It is well known that when running a Southern blot gel, genomic DNA does not always migrate at the same rate in all the lanes. The apparent differences in migration rate is likely due to batch-to-batch variation in the co-purified contaminants and salts introduced by the cotton genomic DNA purification procedure that affected the quantitation of genomic DNA in these samples. Occasionally, when the comb is removed from the gel, some of the wells narrow more than others and in these wells the DNA will appear to migrate slower than in other wells. These samples were derived from progenies of the same event and the slight differences are likely due to differences in technique or quantitation errors. However, we consider these differences to be an artifact of Southern blot technique, which do not invalidate the accuracy of the data or lead to any different conclusions about the depicted results. As previously explained, slight differences in hybridization band intensity T2 compared to T1 and T3) can arise due to difficulties in isolation and processing of DNA samples from cotton tissues.

Figure 4-9. Generational stability - Southern blot analysis with Probe 13.
Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI digested, pART27-LCT66 plasmid DNA at approximately one copy equivalent of the plasmid per cotton genome. Negative control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb. The membrane was probed with radiolabeled probe corresponding to the *ocs* terminator region (Probe 13).

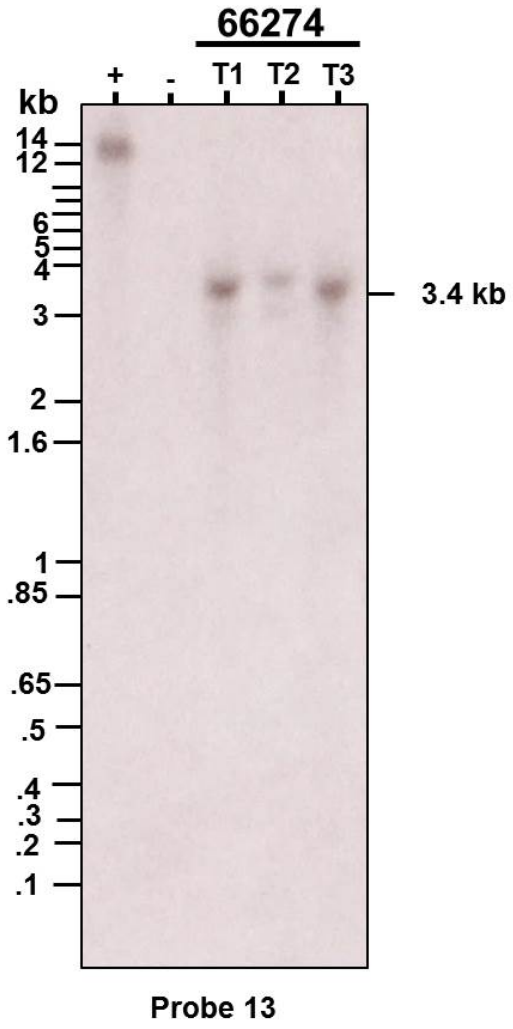
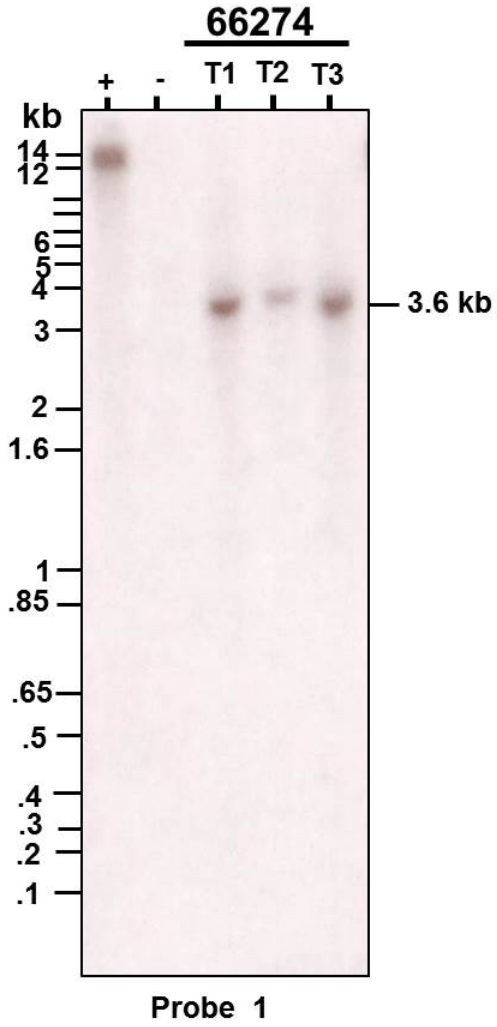


Figure 4-10. Generational stability – Southern blot analysis with Probe 1.
Lane marked (+) is positive control; (-) is negative control; and 66274 is TAM66274. Positive control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312 spiked with *Eco*RI digested, pART27-LCT66 plasmid DNA at approximately one copy equivalent of the plasmid per cotton genome. Negative control included approximately 12 µg of *Eco*RI digested genomic DNA from non-transgenic cv. Coker 312. Fragment sizes are shown in kb**.** The membrane was probed with a radiolabeled probe generated from the *nos* promoter + *nptII* variant region of the cassette (Probe 1).



4.4 Southern Blot Analysis Conclusions

The Southern blot analyses conducted following digestion of TAM66274 genomic DNA with *Eco*RI and hybridized with Probe 1 (corresponding to components of the *nptII* variant gene cassette at the LB region of the T-DNA) and Probe 13 (corresponding to the *ocs* terminator at the RB region of the T-DNA), showed single inserts of the T-DNA border regions in TAM66274. Further, *Kpn*I digest of TAM66274 and hybridized with Probes 1, 13, 14 (*dCS* trigger sequences), Probe 15 (the *pdk* intron) and Probe 16 (AGP) showed hybridizing bands that were consistent with a single copy of the T-DNA genetic elements integrated in TAM66274. The hybridizing bands also showed the contiguous nature of genetic elements of the T-DNA in TAM66274, confirming that the integrity of the T-DNA was maintained in TAM66274. Thus, the results presented in Figures 4-4, 4-6 and 4-8 together confirm a complete and single copy integration of the T-DNA in TAM66274. Further, no hybridizing bands were observed with any of the backbone probes for the *Eco*RI digests of genomic DNA from TAM66274. Therefore, these Southern blots confirmed the absence of any plasmid backbone sequences in TAM66274

In addition, Southern blot analysis of TAM66274 using the *ocs* terminator probe adjacent to the RB, and a probe including parts of the *nptII* variant cassette adjacent to the LB of the T-DNA, separately showed the same hybridizing bands for the T1, T2 and T3 generations of TAM66274 (Figures 4-9 and 4-10), and demonstrate stability of the transgene integration in the cotton genome over three breeding generations.

4.5 Characterization of the T-DNA Integration Site

4.5.1. Identification and analysis of genomic DNA flanking sequences.

Genomic DNA sequences flanking the inserted T-DNA LB and RB were determined by HE-TAIL PCR (Tan and Singh, 2011). The purpose of the flanking sequence analysis was: a) to determine if there is a correlation between the number of LB and RB T-DNA flanking sequences determined and the results of Southern blot analyses, b) to predict the presence of potential, newly created coding sequences (open reading frames or ORFs) in the 5' flanking genomic/T-DNA junction region and in the 3' flanking genomic/T-DNA junction region, c) to determine if any of the newly created ORFs have sequence similarity to any known allergens or toxins, and d) to determine if such flanking sequences provide information regarding disruption of important plant endogenous gene(s).

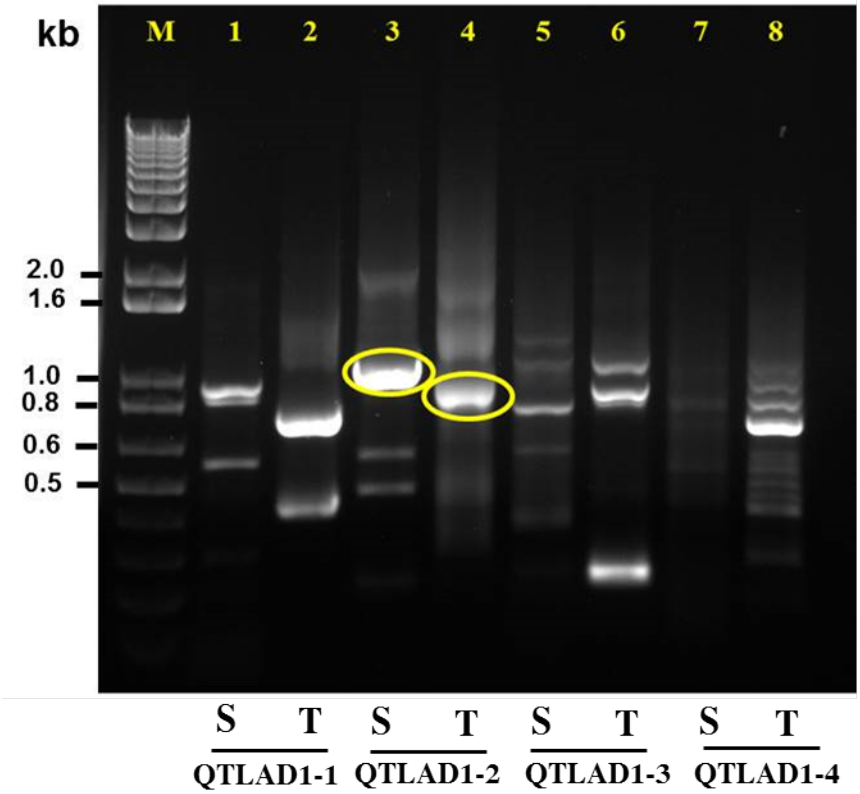
HE-TAIL PCR is an improved version of the original TAIL-PCR method (Liu and Chen, 2007) that enables preferential amplification of unknown genomic sequences using specially-designed primers. Although effective for the isolation of unknown genomic sequences flanking the T-DNA borders from several species, the original method is limited by the high frequency amplification of undesired smaller non-target sequences from large genomes such as cotton. This limitation is overcome in HE-TAIL PCR by the inclusion of arbitrary degenerate (AD) primers that have an additional, unique 15-mer sequence corresponding to the green fluorescent protein

(GFP) gene at their 5'ends (designated QTLAD1-1 to -4). The GFP sequence is used as a primer in the subsequent (primary, secondary and tertiary) PCR reactions following the pre-amplification step in order to favor amplification of target-specific PCR products. Amplicons of the secondary and tertiary PCR reactions that show an expected size shift on a gel image are considered target sequences and can be isolated from the gel for sequencing. Thus, HE-TAIL PCR was chosen as a method of choice to analyze the T-DNA flanking sequences from TAM66274. A description of the method and the PCR conditions used for the HE-TAIL PCR are given in Appendix B.

4.5.1.1. LB T-DNA flanking sequence analysis.

For LB flanking sequence analysis, amplicons from the tertiary reactions that showed ~150 bp shift (distance between New *nos*-2 and New *nos*-3 primers; Appendix B, Figure B-2) in comparison to the secondary reactions were considered target-specific. As shown in Figure 4-11, both the QTLAD1-1 and QTLAD1-2 primers produced amplicons with a similar and expected size shift [~1.0 kb in the secondary reaction (lanes 1 and 3); ~0.850 kb in the tertiary reaction (lanes 2 and 4)]. QTLAD1-3 and QTLAD1-4 primers did not produce any clear and specific amplicons with the expected size shift. A representative pair of amplicons from QTLAD1-2 secondary and tertiary reactions (circled in yellow in Figure 4-11) were extracted from the gel and sequenced. Results of the sequence analysis revealed that the terminal 18 bp of the LB repeat were not integrated in the TAM66274 genome, while the remainder of the LB region was integrated in the TAM66274 genome and was identical to the sequence in the pART27-LCT66 transformation plasmid. A 1152 bp TAM66274 genomic sequence flanking the LB T-DNA was obtained from this analysis and is presented in Appendix B (Figure B-4).

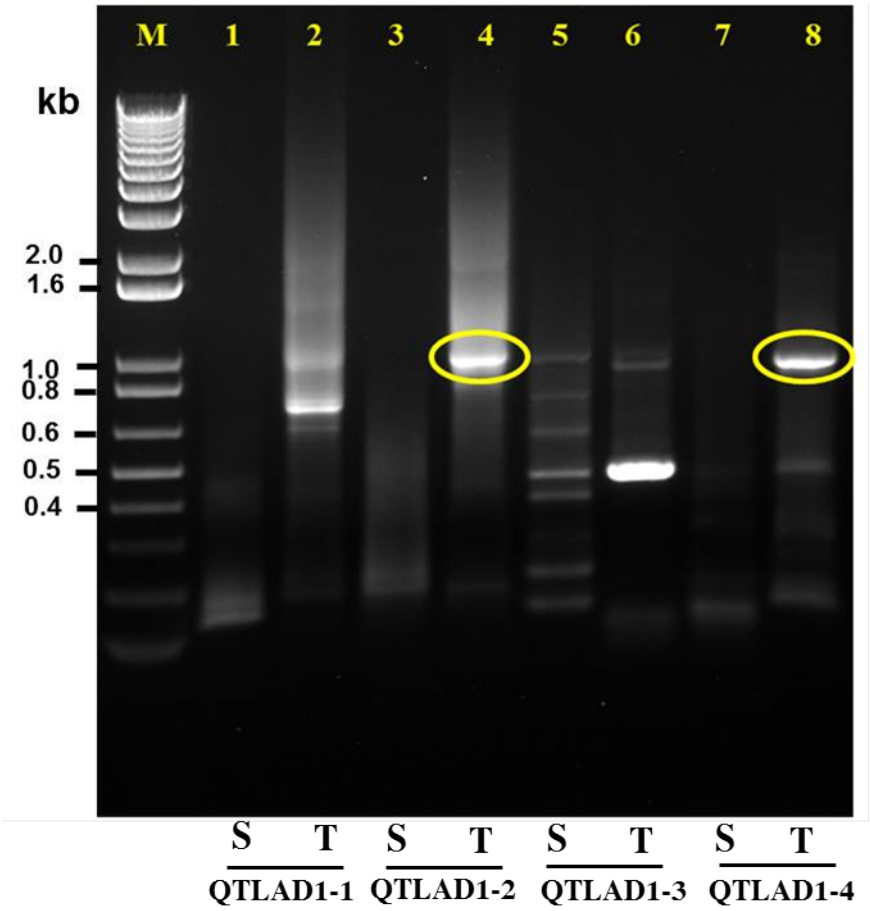
Figure 4-11. LB T-DNA flanking sequence analysis of TAM66274 using HE-TAIL PCR. Size shift in PCR bands from secondary to tertiary reactions with QTLAD1-1 and QTLAD1-2 degenerate primers can be clearly seen in the gel. A representative pair of amplicons (bands with yellow circles) was sequenced. S: Secondary PCR; T: Tertiary PCR; QTLAD1(1-4): Degenerate primers; M: 1 kb plus DNA ladder.



4.5.1.2. RB T-DNA flanking sequence analysis.

For RB flanking sequence analysis, amplicons from the tertiary reactions that showed ~129 bp (distance between New RB-2 and New RB-3 primers; Appendix B, Figure B-3) shift in comparison to the secondary reactions were considered target-specific. As shown in Figure 4-12, no prominent amplicons were obtained from any of the secondary reactions. This could be due to the presence of a very small amount of the target-specific product not detectable by ethidium bromide staining of the gel. The prominent tertiary amplicons of ~1 kb from the QTLAD1-2 primer (lane 4) and QTLAD1-4 primer (lane 8) were extracted and sequenced. Results of the sequence analysis revealed that the terminal 28 bp of the RB (25 bp RB repeat plus 3 bp RB overdrive element) were not integrated in the TAM66274 genome, while the remainder of the RB region was integrated in the genome and was identical to the sequence in the pART27-LCT66 transformation plasmid. A 1035 bp TAM66274 genomic sequence flanking the RB T-DNA was obtained from this analysis and is presented in Appendix B (Figure B-4).

Figure 4-12. RB T-DNA flanking sequence analysis of TAM66274 using HE-TAIL PCR. No prominent amplicons were obtained from any of the Secondary PCRs. However, prominent amplicons were obtained in some of the Tertiary PCRs. The bands with yellow circles were sequenced. S: Secondary PCR; T: Tertiary PCR; QTLAD1(1-4): Degenerate primers. M: 1 kb plus DNA ladder.



4.5.2. ORF analysis of the genomic DNA sequences flanking the LB and RB T-DNA.

ORF analyses were conducted on the cotton genomic DNA sequences flanking the RB and LB of the TAM66274 T-DNA, as well as for the T-DNA insert. Results of these searches are presented in Section 5 of this petition. Briefly, 33 putative translated ORFs were identified within the T-DNA and no putative ORFs in the genomic flanking sequences. The translated amino acid sequences encoded by the putative ORFs were screened for sequence similarity to known or putative allergens or toxins that would present any safety concerns. Results showed that the putative translated ORFs in TAM66274 exhibit no significant homology to allergens or protein toxins.

4.6 Organization and Integrity of the Inserted Genetic Elements in TAM66274

Southern blot analysis using probes adjacent to the RB, LB, and internal to the T-DNA suggested the presence of a complete and single copy of the T-DNA in TAM66274. The cotton genomic DNA sequences flanking the RB and LB were determined by HE-TAIL PCR followed by DNA sequencing of the amplified products (see Section 4.5.1. and Appendix B). Results of the flanking sequence analysis identified that 28 bp of the RB terminal section (the entire 25 bp RB T-DNA repeat plus three bp of the RB overdrive) and 18 bp of the LB T-DNA repeat were not integrated in TAM66274. The remainder of the T-DNA border regions inserted in TAM66274 is identical to the pART27-LCT66 plasmid**.**

Further, the organization and integrity of the genetic elements within the inserted T-DNA were confirmed by overlap PCR followed by DNA sequence analysis of the amplified products. Eight different pairs of primers were designed to amplify products to overlapping regions of the entire length of the insert (Figure 4-13B). Samples for the overlap PCR reactions included: non-transgenic cv. Coker 312 genomic DNA (negative control), TAM66274 genomic DNA, pART27-LCT66 plasmid DNA (positive control), and no template DNA (PCR control). The pART27-LCT66 plasmid DNA was not included in the PCR reactions where one of the primers was designed from the cotton genomic DNA sequence flanking the RB or LB of the T-DNA (Figure 4-13, Product A and H). The sequence of primers and the PCR conditions used are described in detail in Appendix B. Amplified products (Figure 4-13A, Products A-H) were electrophoresed on 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

As expected, there were no PCR products generated from the non-transgenic cv. Coker 312 genomic DNA or the no template DNA PCR controls (Figure 4-13A). The size of each of the amplified products matched the expected size of genetic elements in the T-DNA of pART27-LCT66. The amplified PCR products were subjected to DNA sequence analysis, and the results are presented in Appendix B (Table B-7). The results showed that the organization and sequence of each of the genetic elements in the T-DNA in TAM66274 are identical to those in pART27-LCT66. All of the genetic elements in the T-DNA that were integrated into TAM66274 are listed in Table 4-5 and depicted in Figure 4-14.

In conclusion, Southern blot analyses using probes adjacent to the RB, LB, and internal to the T-DNA suggested the presence of a complete and single copy of the T-DNA in TAM66274. Results from the overlap PCR analysis confirmed those obtained from Southern blot analyses. The integrity and organization of the T-DNA in TAM66274 was also shown to be identical to the T-DNA of the pART27-LCT66 plasmid, confirming that a single copy of T-DNA was inserted in TAM66274 as intended.

Figure 4-13. Overlap PCR analysis across the insert in TAM66274.
A. Agarose gel photograph of PCR products. PCR reactions were performed with eight pairs of primers on non-transgenic cv. Coker 312 and TAM66274 genomic DNA to generate overlapping PCR fragments. 1 kb plus DNA ladder is shown on both sides of the agarose gel photograph.
B. Diagrammatic representation of the T-DNA insertion in TAM66274. The expected size of each PCR product is shown.

Lane designations are as follows:

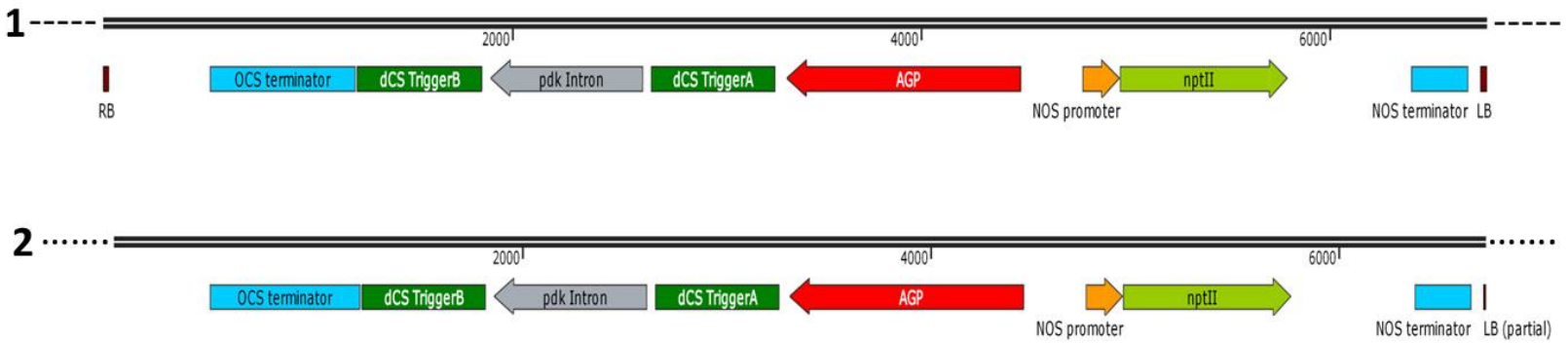
Lane	Sample	Lane	Sample
1	1 kb plus DNA ladder	18	1 kb plus DNA ladder
2	Coker 312 genomic DNA	19	Coker 312 genomic DNA
3	TAM66274 genomic DNA	20	TAM66274 genomic DNA
4	No template DNA control	21	pART27-LCT66 plasmid DNA
5	Coker 312 genomic DNA	22	No template DNA control
6	TAM66274 genomic DNA	23	Coker 312 genomic DNA
7	pART27-LCT66 plasmid DNA	24	TAM66274 genomic DNA
8	No template DNA control	25	pART27-LCT66 plasmid DNA
9	Coker 312 genomic DNA	26	No template DNA control
10	TAM66274 genomic DNA	27	Coker 312 genomic DNA
11	pART27-LCT66 plasmid DNA	28	TAM66274 genomic DNA
12	No template DNA control	29	pART27-LCT66 plasmid DNA
13	Coker 312 genomic DNA	30	No template DNA control
14	TAM66274 genomic DNA	31	Coker 312 genomic DNA
15	pART27-LCT66 plasmid DNA	32	TAM66274 genomic DNA
16	No template DNA control	33	No template DNA control
17	1 kb plus DNA ladder	34	1 kb plus DNA ladder

Table 4-5. Description of genetic elements in T-DNA insert (6714 bp) in TAM66274.

Genetic Element (GE)	Location in T-DNA insert in TAM66274	GE Size (bp)	Description
Right border-flanking sequence		1035	Cotton genomic DNA flanking the right border of the transgene insert in TAM66274.
Transgene insert start			
Right border overdrive element	29 - 177	149	Sequences flanking the right border repeat in the T-DNA (Gleave, 1992).
Intervening sequence	178 - 441	264	Sequences of *LacZ* promoter and 5' truncated *LacZ'* gene from pGEM-5Zf(-) vector (Promega, Madison, WI) used for cloning purpose.
mcs + *ocs* terminator	442 - 1230	789	Multiple cloning site sequences + 3' UTR terminator of octopine synthase gene derived from *A. tumefaciens* (Wesley et al., 2001).
Intervening sequence	1231 - 1243	13	Sequence used for DNA cloning.
dCS Trigger B	1244 - 1847	604	Trigger B of the δ-cadinene synthase gene from *G. hirsutum* (Sunilkumar et al., 2006).
Intervening sequence	1848 - 1891	44	Sequence used for DNA cloning.
pdk intron	1892 - 2635	744	Intron of the pyruvate orthophosphate di kinase gene from *F. trinervia* (Wesley et al., 2001).
Intervening sequence	2636 - 2679	44	Sequence used for DNA cloning.
dCS Trigger A	2680 - 3283	604	Trigger A of the δ-cadinene synthase gene from *G. hirsutum* (Sunilkumar et al., 2006).
Intervening sequence	3284 - 3339	56	Sequence used for DNA cloning.
AGP	3340 - 4485	1146	Promoter and 5' UTR derived from the α-globulin B gene of *G. hirsutum* (Sunilkumar et al., 2002).
Intervening sequence	4486 - 4790	305	Sequences of multiple cloning sites from pHANNIBAL (Wesley et al., 2001), 3' truncated *LacZ*' gene and multiple cloning sites from pGEM-5Zf(-) vector (Promega, Madison, WI) used for cloning purposes.
nos promoter	4791 - 4974	184	Nopaline synthase promoter derived from *A. tumefaciens* (An et al., 1985, 1988; Fraley et al., 1986).
Partial *nos* gene	4975 - 5025	51	Partial sequence of the 5' end of the nopaline synthase gene coding sequence derived from *A. tumefaciens* (An et al., 1985, 1988).
nptII gene	5026 - 5796	771	Neomycin phosphotransferase II gene derived from *E. coli* Tn5, which confers resistance to kanamycin (An et al., 1985, 1988; Beck et al., 1982).

Genetic Element (GE)	Location in T-DNA insert in TAM66274	GE Size (bp)	Description
Intervening sequence	5797 - 5969	173	Partial sequence of the 5' end of the bleomycin resistance gene coding sequence derived from *E. coli* (An et al., 1985, 1988).
Intervening sequence	5970 - 6400	431	Partial sequence of the 3' end of the nopaline synthase gene coding sequence derived from *A. tumefaciens* (An et al., 1985, 1988).
nos terminator	6401 - 6675	275	Nopaline synthase terminator and poly(A) signal (An et al., 1985, 1988).
Intervening sequence	6676 - 6735	60	Sequence used for DNA cloning (Gleave, 1992).
Partial T-DNA left border repeat	6736 - 6742	7	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983; Wang et al., 1984, 1987; Zambryski et al., 1982).
Transgene insert end			
Left border-flanking sequence		1152	Cotton genomic DNA flanking the left border of the transgene insert in TAM66274.

Figure 4-14. Schematic representation of the T-DNA within pART27-LCT66 and the portion of the T-DNA that was integrated into TAM66274.



1 - pART27-LCT66 T-DNA

2 - T-DNA insert in TAM66274

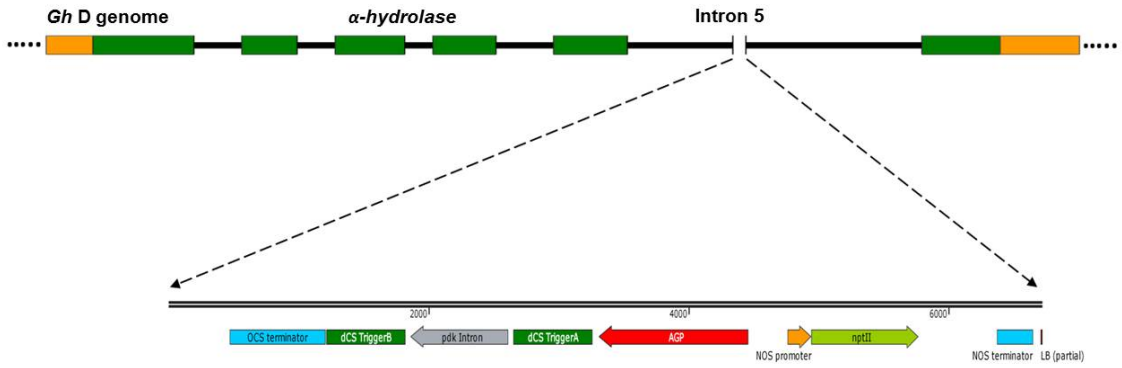
---------Vector backbone sequence in pART27-LCT66

…… Genomic DNA in TAM66274

4.7 Analysis of the T-DNA Insertion Site in the Genome of TAM66274

The T-DNA genomic flanking sequences were used to conduct a BLAST search of the *G. hirsutum* genomic sequence (Li et al., 2015; https://www.cottongen.org/), which indicated that the T-DNA insertion in TAM66274 had occurred within the last intron of an α-*hydrolase* gene. This was further confirmed by searching the Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST&method=Org_Graimondii) for the diploid cotton, *G. raimondii*. Primers were designed based on the RB and LB T-DNA flanking sequence and were used to conduct PCR on non-transgenic cv. Coker 312 genomic DNA. The amplicon generated was sequenced and the results confirmed that during the T-DNA insertion in TAM66274, 44 bp of the cotton genomic DNA was deleted (Appendix B; Figure B-4). The BLAST search also showed that the T-DNA in TAM66274 was inserted within the last intron of an α-*hydrolase* gene, at 364 bases from the 5' end of the intron-exon junction (Figure 4-15). According to genomic sequence data for *G. hirsutum* in the CottonGen database, this gene is located on Chromosome D7.

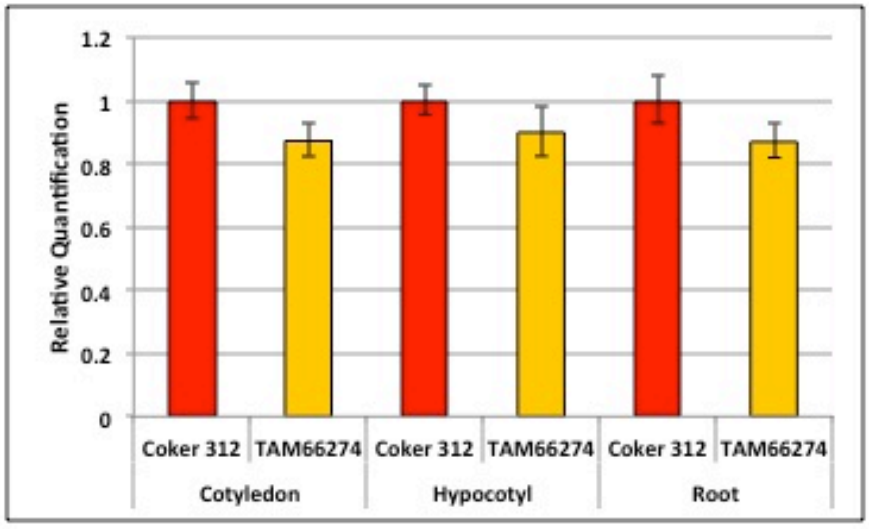
Figure 4-15. Diagrammatic representation of the T-DNA insertion site in TAM66274.
Insertion of the transgene caused a deletion of 44 bases in the intron of the *α-hydrolase* gene in TAM66274.



Since the integration of the T-DNA in TAM66274 occurred in an intron of a putative *α-hydrolase* gene, it is unlikely to affect its expression. Nevertheless, qRT-PCR analysis was performed to determine the effect of T-DNA integration on the mRNA expression of the putative *α-hydrolase* gene. Primers designed from the 3' UTR region of the putative *α-hydrolase* gene were such that these will amplify only the desired gene and not its close homologs/other gene family members. The qRT-PCR analysis was performed on the cotyledons, roots and hypocotyl of three-day-old seedlings of TAM66274 and non-transgenic cv. Coker 312 (Figure 4-16). No major differences were observed in the levels of mRNA expression of the putative *α-hydrolase* gene between TAM66274 and non-transgenic cv. Coker 312 (Figure 4-16). The results of qRT-PCR analysis comparing the *α-hydrolase* gene expression in three types of tissues in TAM66274

and non-transgenic cv. Coker 312 were not subjected to statistical analysis. Microsoft® Excel® was used to tabulate the raw data and graphically present the results in Figure 4-16. The error bars shown in Figure 4-16 represent the minimum and maximum values of two biological replicates and three technical replicates of TAM66274 and non-transgenic cv. Coker 312. When the results of replicated analysis for both genotypes were found to be this similar, it was not deemed necessary to do statistical analysis to have sufficient confidence that *α-hydrolase* gene expression in TAM66274 tissues was not adversely affected. This conclusion is corroborated by the absence of demonstrable differences in phenotypic, agronomic, and ecological characteristics of TAM66274 compared to non-transgenic cv. Coker 312, except for the intended reduction in seed gossypol. Materials and methods for the study are presented in Appendix B.

Figure 4-16. qRT-PCR results for the putative *α-hydrolase* gene in various tissues in TAM66274 and non-transgenic cv. Coker 312.
The relative quantification (RQ) values for *α-hydrolase* expression in non-transgenic cv. Coker 312 and TAM66274 are presented in the graph. Two biological replicates and three technical replicates were used for the qRT-PCR analyses of the seedling tissues. The top and bottom of the error bars represent RQ max and RQ min values, respectively. *Gh Histone 3A* was used as an internal control to normalize the expression of *α-hydrolase* gene.



Furthermore, phenotypic, agronomic and seed composition evaluations of TAM66274 and non-transgenic cv. Coker 312 confirmed that integration of the T-DNA in the intron of the *α-hydrolase* gene had no effect on plant metabolism or growth and development. Each of the measured phenotypic, agronomic and composition parameters provides an assessment of the cumulative result of numerous biochemical pathways in the plant. Results of these evaluations showed that TAM66274 is phenotypically, agronomically and morphologically equivalent to non-transgenic cv. Coker 312 (refer to Section 7 in this petition). In addition, it was shown that levels of nutrients and anti-nutrients in TAM66274 cottonseed are comparable to levels in non-transgenic cv. Coker 312 and other conventional cotton varieties, except for the intended

reduction of gossypol (refer to Section 6 in this petition). Therefore, other than the intended reduction in gossypol levels, TAM66274 cottonseed is compositionally equivalent to that of non-transgenic cv. Coker 312, as well as other commercial cotton varieties. These data confirm that integration of the T-DNA in the intron of the *α-hydrolase* gene had no effect on plant metabolism or growth and development. No secondary effects were anticipated and none have been observed or identified.

4.8 Trait Segregation Analysis

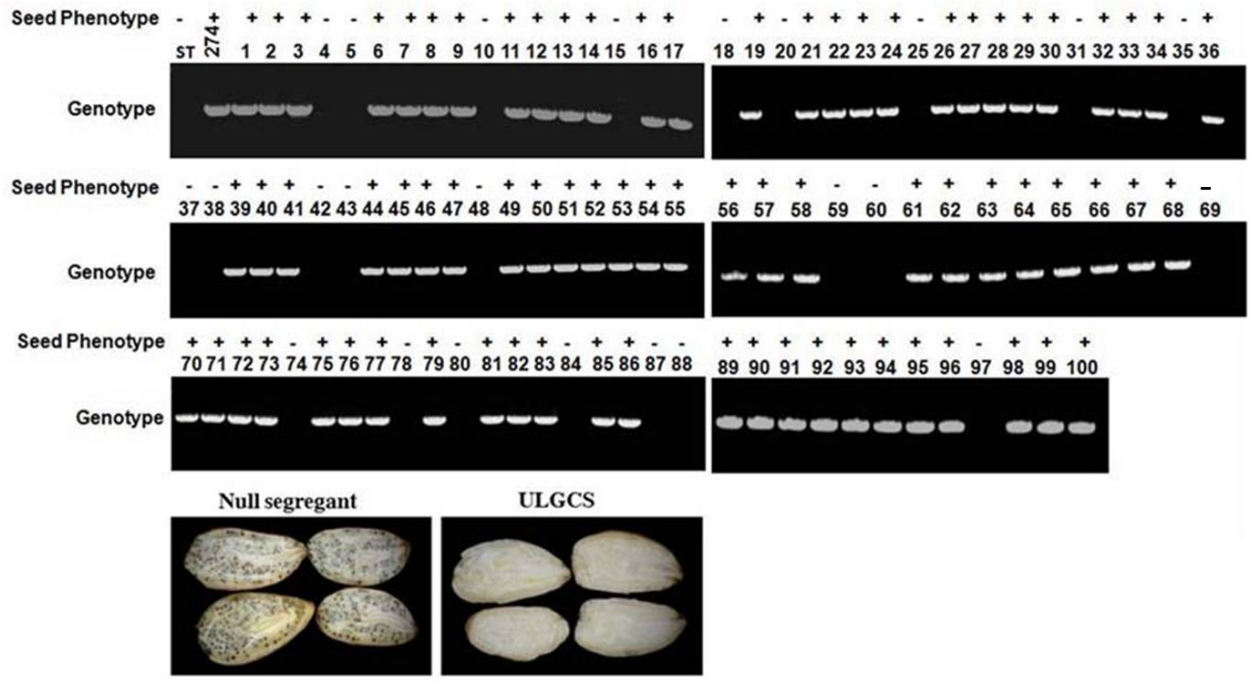
4.8.1. Phenotypic and genotypic analysis of segregating generations.

As indicated earlier, the T-DNA insert resides at a single locus in TAM66274, is stably integrated in the TAM66274 genome across breeding generations, and is expected to be inherited in a Mendelian fashion. Chi-square goodness-of-fit analysis of trait inheritance data within a segregating generation was conducted to confirm the Mendelian inheritance of the T-DNA insert.

Since the ULGCS trait confers a clearly visible phenotype (very light colored glands within the seed kernel compared to dark colored glands in non-ULGCS cottonseeds), a pilot study was conducted to examine if a high-level correlation exists between this phenotype and the presence of the transgene. For the segregation analyses, use was made of segregating progeny from crosses between TAM66274 and Stoneville 474. Phenotypic data based on the gland coloration in the seed was compared with results obtained from event-specific PCR analyses on individual cottonseeds within a BC_1F_2 generation of Stoneville 474/TAM66274. A total of 100 seeds from the BC_1F_2 generation (Stoneville 474/TAM66274) were first examined for the ULGCS phenotype. After removing the seed coat, one side of the seed kernel was scraped with a razor blade and visually examined to assess the intensity of the gland color. Out of the 100 seeds that were examined in this manner, 76 showed very light colored glands indicating their ULGCS status. The remaining 24 seeds had the usual dark-colored, distinct glands normally seen in the seeds of non-ULGCS, glanded cotton. Following this phenotypic evaluation, genomic DNA was extracted from each of these 100 seeds and subjected to event-specific PCR that was developed for TAM66274 (refer to Appendix C for method details). Each of the 76 seeds that showed the ULGCS phenotype also proved to be positive for the presence of the transgene by event-specific PCR, while those seeds that had the normal, non-ULGCS phenotype (dark glands) were negative in the PCR reactions (Figure 4-17, Table 4-6). These results also confirm TAMU observations during more than 10 years of working with a number of different ULGCS events, where a high-level correlation was observed between the phenotype and the presence of the transgene, which further validated the robustness of phenotypic observation as an effective means to identify the presence of the ULGCS trait.

Statistical analysis using the Chi-square goodness-of-fit test indicated that the ratio of 76 positive to 24 null segregants did not significantly differ from the expected Mendelian 3:1 segregation pattern for a single independent transgene locus in TAM66274 (Table 4-6).

Figure 4-17. Correlation between the phenotype and genotype for the ULGCS trait.
Event-specific PCR was performed on the genomic DNA samples obtained from 100 individual seeds of Stoneville 474/TAM66274-BC_1F_2 population. Prior to DNA extraction, each seed was given a + or – score based on the presence or absence of the ULGCS phenotype (very light colored glands, as shown), respectively. DNA from the Stoneville 474 seed was used as a negative control and DNA from TAM66274 seed served as a positive control.



4.8.2. Segregation analysis of breeding generations.

Based on the robustness of the phenotypic observation method, additional segregation analysis was conducted on 100 seeds each from two more segregating progenies, TAM66274-T_2 and Stoneville 474/TAM66274-F_2. Results from these analyses are shown in Table 4-6. Statistical analyses using a Chi-square goodness-of-fit test indicated that the ratio of 71 positive to 29 null segregants in the T_2 generation, and 78 positive to 22 null segregants in the F_2 generation did not significantly differ from the expected Mendelian 3:1 segregation pattern for a single independent locus integration (Table 4-6). Therefore, trait inheritance studies across breeding generations confirmed the expected trait segregation ratios, further confirming that the ULGCS trait in TAM66274 is conferred by a single functional T-DNA insert containing the RNAi cassette which interferes with expression of *dCS*, and that the DNA insert is inherited in typical Mendelian fashion and is stably integrated in the plant genome across multiple breeding generations.

Table 4-6. Segregation analysis of the progeny of TAM66274.

Generation	Test Method	Observed		Expected		Expected ratio	Chi-square	P-value[1]
		ULGCS	Null	ULGCS	Null			
TAM66274 (T_2)	Phenotype	71	29	75	25	3:1	0.85	0.356
ST474/TAM66274 (F_2)	Phenotype	78	22	75	25	3:1	0.48	0.488
ST474/TAM66274 (BC_1F_2)	Phenotype	76	24	75	25	3:1	0.05	0.823

[1] Based on a Chi-Square goodness of fit test

4.9 Summary of the Molecular Characterization

The T-DNA insert in TAM66274 was characterized using a combination of Southern blots, overlap PCR amplification, and HE-TAIL PCR analyses. Southern blots used a combination of *Eco*RI restriction digests, *Kpn*I restriction digests, and probes to the *ocs* terminator adjacent to the RB of the T-DNA and probes to parts of the *nptII* variant gene cassette adjacent to the LB of the T-DNA. Results showed single inserts of the *ocs* terminator and *nptII* variant gene cassette in TAM66274. Additional Southern blot analyses conducted following digestion of genomic DNA with *Kpn*I and hybridization with probes corresponding to *dCS* trigger sequence, *pdk* intron and AGP (internal genetic elements within the T-DNA) further confirmed the integrity of the T-DNA insert and its integration as a single copy in TAM66274. Overlap PCR amplification and sequence analyses of the amplicons provided additional proof that the integrity of the T-DNA from the *ocs* terminator to the *nptII* variant gene cassette was maintained from the transformation plasmid to the T-DNA insert in TAM66274, therefore showing that the single inserts of the *ocs* terminator and *nptII* variant gene cassette are connected in a single T-DNA insert in TAM66274. HE-TAIL PCR analysis also showed that the entire RB repeat was not integrated in the plant genome, and only seven nucleotides from the LB repeat were included in the T-DNA insert in TAM66274. No genetic elements from the backbone of the pART27-LCT66 plasmid were integrated in TAM66274 as shown by Southern blots using *Eco*RI restriction digest and overlapping probes to the entire backbone of the plasmid. Analysis of cotton genomic DNA flanking the T-DNA insert in TAM66274 showed that the T-DNA integration occurred in an intron of a putative *α-hydrolase* gene. However, qRT-PCR analysis showed that there was no impact on mRNA expression from this gene in TAM66274 compared to non-transgenic cv. Coker 312. Also, companion studies on phenotypic, agronomic, morphological and cottonseed composition showed that TAM66274 and non-transgenic cv. Coker 312 are comparable with respect to these parameters, and confirm that integration of the T-DNA insert in the intron of the *α-hydrolase* gene had no effect on plant metabolism or growth and development.

The stability of the T-DNA insert in TAM66274 was determined by Southern blot analyses of three generations (T1, T2 and T3), and by trait inheritance studies over plant breeding

generations. Southern blot analyses demonstrated that the inserted DNA is stably integrated in TAM66274 and was stably inherited through multiple generations. Moreover, the transgene insert displayed the expected Mendelian inheritance pattern for single locus integration in the segregating generations, confirming that the transgene insert in TAM66274 is stably integrated at a single chromosomal locus.

5. CHARACTERIZATION AND SAFETY OF THE NPTII VARIANT PROTEIN, PUTATIVE T-DNA ORFs AND IMPACTS OF dsRNA PRODUCTION IN TAM66274

In Section 4 of this petition, it was shown that the T-DNA insert in the genome of TAM66274 contains two gene cassettes. First, the RNAi cassette which silences *dCS* genes that encode dCS, a key enzyme involved in gossypol biosynthesis, and results in ultra-low gossypol levels in TAM66274 cottonseed. The second gene cassette introduced in TAM66274 contains the selectable marker *nptII* variant gene, which was used to select transformed cotton cells and therefore used to generate TAM66274.

The *dCS* RNAi cassette in TAM66274 was designed to specifically silence the endogenous *dCS* genes in cottonseed, but not have any effect on *dCS* genes in other parts of the cotton plant. The *dCS* RNAi cassette contains a 604 bp long internal sequence (Trigger A) of the *dCS* gene from cotton and a reverse complement of the Trigger A sequence (Trigger B). The expression of this cassette in TAM66274 results in the formation of a dsRNA transcript containing a fragment of the *dCS* genes in cotton, and it is recognition and processing of this dsRNA by the cotton plant's RNAi machinery that results in suppression of expression of the dCS protein in cottonseed.

Section 5 of this petition presents data on the efficacy of the *dCS* RNAi cassette in suppressing transcript levels of the *dCS* genes. Specifically, *dCS* transcript levels were measured by qRT-PCR in developing seed embryos and in root, leaf, bract, floral bud, and axillary bud tissues of TAM66274 and non-transgenic cv. Coker 312 grown in a 2015 TAMU field trial. Results of analysis of embryos collected from unopened bolls showed an 86% reduction in the levels of *dCS* transcripts in embryos of TAM66274 at 31 days post anthesis (dpa), compared to transcript levels in the non-transgenic cv. Coker 312. The result is consistent with reduction of gossypol levels in cottonseed of TAM66274. Measurements of *dCS* gene expression in root, leaf, bract, floral bud, and axillary bud tissues showed no significant differences in the transcript levels between TAM66274 and non-transgenic cv. Coker 312 for any of these non-seed tissues. These results showed the efficacy of the *dCS* RNAi cassette in selectively reducing *dCS* transcript levels in embryos, with no effect on transcript levels in other plant parts. This was as expected, since the *dCS* RNAi construct in TAM66274 is driven by a seed specific promoter and, therefore, gossypol levels remain unchanged in plant tissues other than the cottonseed (refer to Section 3 of this petition).

In addition to determination of *dCS* transcript levels in different plant parts, the safety of RNAi is discussed in general as well as specifically for *dCS* RNAi. Further, the potential impact of suppressing dCS enzyme levels in cotton on other plant metabolic pathways is discussed. Terpenoids in plants are derived from (+)-δ-cadinene. The dCS enzyme catalyzes the first committed step involving the cyclization of FDP to (+)-δ-cadinene (Benedict et al. 2001; Chen et al., 1995; Stipanovic et al., 1999; Townsend et al., 2005). A proposed pathway for the

biosynthesis of gossypol and related terpenoids from FDP in cotton is shown in Section 3 of this petition (Figure 3-1). With reduced levels of the dCS enzyme in TAM66274, the potential for FDP to be diverted to other plant metabolic pathways and accumulation of secondary plant metabolites is addressed.

The *nptII* variant gene introduced into the TAM66274 genome was used to select transformed cotton cells and, therefore, used to generate TAM66274. This gene encodes the NPTII variant protein, which confers resistance to the antibiotics neomycin and other related aminoglycosides (Fraley et al., 1986). The NPTII variant protein functions as a selectable marker in the initial laboratory stages of plant cell selection following transformation. The NPTII variant enzyme uses ATP to phosphorylate neomycin and related aminoglycoside antibiotics, thereby inactivating them. Therefore, cells that produce the NPTII variant protein survive exposure to these aminoglycosides. The purpose of inserting the *nptII* variant gene into cotton cells along with the *dCS* RNAi cassette was to have an efficient method for selecting cells after transformation, and to facilitate the screening of ULGCS events.

Expression levels of the NPTII variant protein were measured by ELISA in leaf, root, pollen and seed tissues of TAM66274 and non-transgenic cv. Coker 312 grown in a 2015 TAMU field trial. Among all the tissues evaluated, NPTII variant protein expression was highest in leaves (253.3 ng/g DW) of TAM66274. NPTII variant expression was lower in the root (58.5 ng/g DW) and was lowest in the seed (41.1 ng/g DW) of TAM66274. The protein was not detected (N.D.) in any tissues of the non-transgenic cv. Coker 312 and was undetected in pollen of TAM66274 at the detectable level of 25 ng/g DW. In addition to measurements of expression levels of the NPTII variant protein in tissues of TAM66274, the characterization and safety of the protein for human and animal consumption, as well as environmental safety, is discussed in this section of the petition.

In addition to the intended expression of the *dCS* RNAi transcripts and the NPTII variant protein in TAM66274, Codex Alimentarius Commission guidelines for the conduct of safety assessments of food derived from recombinant DNA plants include an integrated, stepwise, case-by-case assessment of any newly expressed proteins that could be present in the final food (Codex, 2003a). Such proteins may be expressed from intended ORFs within the inserted DNA or from unintended ORFs created by the inserted DNA and contiguous plant genomic DNA. A component of this assessment is determination of any significant similarity between the amino acid sequence of such proteins and that of known allergens and toxins. Therefore, Section 5 of this petition also presents a comprehensive bioinformatic analysis to investigate similarity between putative amino acid sequences encoded by ORFs within the TAM66274 T-DNA insert and genomic flanking sequences, to amino acid sequences of known allergens and toxins. The DNA sequences of ORFs within the T-DNA insert and its 5' and 3' flanking genomic regions were translated into corresponding putative amino acid sequences and systematically compared

to the amino acid sequences of known allergens or toxins in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database maintained by the University of Nebraska and the NCBI Entrez protein database. The purpose of these analyses was to determine whether the amino acid sequences of these *in silico* translations might have biologically relevant sequence similarity to known or putative protein allergens and toxins should they be expressed *in planta*.

ORF analysis of the TAM66274 insert and genomic flanking sequences identified 33 putative translated ORFs within the T-DNA and no putative ORFs in the genomic flanking sequences. The translated amino acid sequences encoded by the putative ORFs were screened for sequence similarity to known or putative allergens or toxins that would present any safety concerns. Among the 33 putative translated ORFs, there were only six that encoded proteins or peptides greater than 80 amino acids, one of which was the NPTII variant protein. Bioinformatic searches of the AllergenOnline database and the Entrez protein database showed that none of the putative translated ORFs in TAM66274 had sequence similarity to any known or putative protein allergens or toxins.

Details of the studies described above are presented in this Section of the petition, as follows:

5.1. Transcript levels of the *dCS* gene in different plant parts of TAM66274 and non-transgenic cv. Coker 312, safety of *dCS* RNAi, and potential impact of suppressing dCS enzyme levels in cotton on other plant metabolic pathways.

5.2. Expression levels of the NPTII variant protein in different plant parts of TAM66274 compared to levels in non-transgenic Coker 312, and characterization and safety of the NPTII variant protein.

5.3. Bioinformatics analyses of amino acid sequences encoded by intended and unintended ORFs in the T-DNA and flanking genomic DNA of TAM66274 to amino acid sequences of known allergens and toxins.

5.1 Transcript Levels of the *dCS* Gene in Different Plant Parts of TAM66274 and Non-Transgenic cv. Coker 312, Safety of *dCS* RNAi, and Potential Impact of Suppressing dCS Enzyme Levels in Cotton on Other Plant Metabolic Pathways

5.1.1. Transcript levels of the dCS gene in different plant parts of TAM66274 and non-transgenic cv. Coker 312.

dCS gene expression was measured in developing seed embryos, root, leaf, bract, floral bud, and axillary bud tissues of TAM66274 and non-transgenic cv. Coker 312 plants grown in a TAMU field trial (Sommerville, TX) in 2015. Details of plant production, tissue harvest, and materials and methods used to determine *dCS* transcript levels are described in Appendix D.

The level of *dCS* transcripts was measured by qRT-PCR in seed embryos of TAM66274 and non-transgenic cv. Coker 312 (Table 5-1). Results showed an 86% reduction in the levels of *dCS*

transcripts in embryos of TAM66274 compared to non-transgenic cv. Coker 312. The reduction in expression of *dCS* transcripts in TAM66274 embryos compared to levels in non-transgenic cv. Coker 312 shows that the RNAi construct introduced into the genome of TAM66274 effectively inhibits expression of *dCS* transcripts in the cottonseed embryo. This result is consistent with reduction of gossypol levels in cottonseed of TAM66274 to approximately 3% of levels in the non-transgenic cv. Coker 312 cottonseed (refer to compositional analyses of cottonseed in Section 6 of this petition).

The level of *dCS* transcripts was also measured by qRT-PCR in root, leaf, bract, floral bud, and axillary bud tissues of TAM66274 and non-transgenic cv. Coker 312 (Table 5-2). Results showed no significant reduction in the levels of *dCS* transcripts in non-seed tissues of TAM66274 relative to non-transgenic cv. Coker 312. These results are consistent with the seed-specific activity of AGP, the α-globulin B gene promoter that controls transcription of the RNAi-mediated *dCS* gene suppression, in TAM66274 (Sunilkumar et al., 2002; Rathore et al., 2009). Furthermore, these results are consistent with data presented in Section 3 of this petition and Appendix A showing equivalent levels of gossypol and other terpenoids in non-seed tissues of TAM66274 compared to non-transgenic cv. Coker 312. As expected, in field studies with TAM66274 and non-transgenic cv. Coker 312, it was shown that there was no difference in susceptibility to insects and diseases between the two treatments demonstrating that TAM66274 plants retained terpenoid-based defenses under field conditions. These results are presented in detail in Section 7 of this petition.

Table 5-1. Quantification of *dCS* transcripts in embryo tissues of TAM66274 and non-transgenic cv. Coker 312.

Entry	Relative values of *dCS* gene transcripts (mean ± SE, n=8)
	Embryo (31 days post anthesis)
Coker 312	1.00
TAM66274	0.14 ± 0.03 (0.09 - 0.20)

Table 5-2. Quantification of *dCS* transcripts in non-seed tissues of TAM66274 and non-transgenic cv. Coker 312.

Entry	Relative values of *dCS* gene transcripts (mean ± SE, n=9)				
	Root	Leaf	Bract	Floral bud	Axillary bud
Coker 312	1.00	1.00	1.00	1.00	1.00
TAM66274	1.15 ± 0.07 (1.02 - 1.26)	1.17 ± 0.28 (0.63 - 1.60)	0.92 ± 0.22 (0.56 - 1.33)	1.11 ± 0.18 (0.86 - 1.47)	1.34 ± 0.12 (1.12 - 1.55)

5.*1.2. Safety of dCS RNAi.*

As described in Section 3 of this petition, the *dCS* RNAi construct introduced into TAM66274 to suppress *dCS* transcript levels contains a 604-bp internal fragment of the *dCS* gene that was used as the Trigger A sequence and a reverse complement of the Trigger A sequence (Trigger B) to make an ihp RNA construct using the pHANNIBAL/pART27 system. The expression of this cassette results in the formation of a dsRNA transcript containing a fragment of the *dCS* gene in cotton. The dsRNA is recognized by the cotton plant's RNAi machinery, resulting in degradation of the *dCS* transcripts and suppression of expression of the dCS protein. The RNAi machinery is a natural process in eukaryotic organisms for the regulation of endogenous gene expression (Dykxhoorn et al., 2003; Parrott et al., 2010). The dsRNA molecule that activates the mechanism is first processed by a class of RNase III enzymes called Dicers into siRNAs, which are typically 21-25 nucleotides in length (Hammond, 2005; Siomi and Siomi, 2009; Zamore et al., 2000). The resulting siRNA molecules are then incorporated into a multiprotein RNA-induced silencing complex (RISC), which facilitate complementary sequence recognition and mRNA cleavage that leads to specific suppression of the target mRNA (Hammond, 2005; Tomari and Zamore, 2005), which in this case are the *dCS* transcripts.

RNAi-mediated gene suppression has been used in a number of biotechnology-derived food crops that have previously been deregulated by USDA and other regulatory authorities, including virus resistant papaya, squash, potato, common bean, and plum, as well as a delayed ripening tomato, and a soybean with altered oil composition (Parrott et al., 2010). Safety assessments have been conducted (Parrott et al., 2010; Petrick et al., 2013) and global regulatory approvals have been obtained for products employing RNAi-mediated gene suppression. Therefore, there is a history of safe consumption of RNA molecules mediating gene suppression in plants. Additionally, there is no evidence to suggest that dietary consumption of nucleic acids is associated with toxicity (Petrick et al., 2013; FDA, 1992). Furthermore, the U.S. FDA recognizes that all food allergens are proteins (FDA, 1992; 2001) and, therefore, dietary RNA does not pose an allergenicity risk. This lack of toxicity or allergenicity for ingested RNA also extends to RNA molecules associated with dsRNA-mediated gene regulation. Therefore, an extensive history of safe consumption for dietary RNAs, including dsRNAs, has been established, as reviewed (Petrick et al., 2013).

The dCS enzyme is only expressed in plants. Therefore, it is unlikely that the *dCS* gene sequences used in the RNAi construct in TAM66274 have homologues to mRNA sequences expressed in humans or in animals that are likely to consume TAM66274 cottonseed or cottonseed products. Thus, no non-target or adverse effects are expected from expression of the *dCS* RNAi in TAM66274. However, to confirm this expectation, bioinformatic analyses were conducted with the 604 bp *dCS* gene trigger sequence using a BLASTN search of the NCBI database. The sequence was queried against human, cow, pig, chicken, fish, shrimp, dog and cat EST sequences. The results of this search are presented in Appendix D. The search did not show

homology in any 20 bp contiguous stretch, the typical lower limit of siRNA molecules, to the transcripts queried in this database. This analysis further confirms the improbability of adverse non-target effects of *dCS* RNAi on humans and animals that are likely to consume the TAM66274 cottonseed or cottonseed products derived from TAM66274 cottonseed.

5.1.3. Potential impact of suppressing dCS enzyme levels in cotton on other plant biosynthetic pathways.

Terpenoids in plants are derived from (+)-δ-cadinene. The dCS enzyme catalyzes the first committed step involving the cyclization of FDP to (+)-δ-cadinene (Benedict et al., 2001; Chen et al., 1995; Stipanovic et al., 1999; Townsend et al., 2005). A proposed pathway for the biosynthesis of gossypol and related terpenoids from FDP in cotton is shown in Section 3 of this petition (Figure 3-1). With reduced levels of the dCS enzyme in TAM66274, the potential for FDP to be diverted to other plant metabolic pathways is discussed below.

FDP, derived from the cytosolic mevalonate pathway, serves as a common precursor for a diverse set of primary and secondary plant metabolites. These include phytosterols, polyisoprenoids (dolichols and polyprenols), quinones, and sesquiterpenes (Bick and Lange, 2003; Bouvier et al., 2005; Chappell, 1995; Grunler et al., 1994; Laule et al. 2003; Lichtenthaler, 1999; Rodriguez-Concepcion, 2006; Rodriguez-Concepcion and Boronat, 2002). Sterols are found in all eukaryotic organisms and are important membrane components that regulate the fluidity and the permeability of phospholipid bilayers (Benveniste, 1986; Hartmann, 1998; Lindsey et al., 2003; Schaller, 2003; 2004). Unlike animal and fungal cells, which contain only one major sterol, plant cells synthesize a complex array of sterol mixtures in which sitosterol, stigmasterol and 24-methylcholesterol often predominate. Minute amounts of certain sterols, such as campesterols, serve as precursors for brassinosteroids that are involved in various aspects of growth and development (Lindsey et al., 2003; Yokota, 1997). Van Niekerk and Burger (1985) determined the content of various sterols in cottonseed oil and found the major components were campesterol, stigmasterol, β-sitosterol, Δ^5-Avenasterol and Δ^7-Avenasterol at levels of 276, 17.3, 3348, 85.1 and 17.9 mg/kg oil, respectively. Dolichols, which have now been found in several dicot and monocot species, play an important role in the co-translational and post-translational modification of proteins by mediating their glycosylation (Swiezewska and Danikiewicz, 2005; Zhang et al., 2008). Ubiquinone (prenylated benzoquinone coenzyme Q) is present in the mitochondria of most eukaryotic cells, including plants. It is a vital component of the electron transport chain and participates in aerobic cellular respiration, which generates energy in the form of ATP (Ikeda and Kagei, 1979; Tohge et al., 2014).

Protein farnesylation plays an important role in the regulation of plant development and signal transduction (Galichet and Gruissem, 2003; Roskoski Jr., 2003). FDP serves as a substrate for the farnesylation, a type of prenylation, which involves addition of an isoprenyl group to a cysteine residue for post-translational modification of proteins. Such isoprenyl lipid attachment

to the C-termini of proteins serves as an anchor for membrane targeting and is also critical for protein-protein interactions (Galichet and Gruissem, 2003; Maurer-Stroh et al., 2007; Nambara and McCourt, 1999; Rodriguez-Concepcion et al., 1999).

Thus, it is possible that when channeling of FDP into the gossypol pathway is blocked in the cottonseed of TAM66274 by RNAi silencing of the *dCS* gene, more FDP should become available for the production of the primary plant products described above whose biosynthesis pathways are most likely its major consumers. There is also a possibility of a negative feedback inhibition of the FDP synthase activity leading to the reduction in the level of FDP.

Unpublished research from the TAMU Institute for Plant Genomics and Biotechnology (IPGB) on the expression of genes that encode enzymes involved in gossypol biosynthesis suggest that biosynthesis of gossypol from FDP in cottonseed is a minor biosynthetic pathway of this metabolic intermediate compared to biosynthesis of other plant metabolites from FDP. RNAseq analysis was conducted on developing cotton embryos from glanded and glandless (ultra-low gossypol levels in all plant parts) cotton plants at 14, 16, and 32 dpa. Specifically, the expression of genes that encode FDP synthase, dCS and δ-cadinene hydroxylase (dCH; that catalyzes the conversion of (+)-δ-cadinene to 8-hydroxy-(+)-δ-cadinene) were examined. As expected, neither *dCS* nor *dCH* genes are expressed in the 14 and 16 dpa embryos of either glanded or glandless cotton. These genes were found to be active at 32 dpa, but only in the embryos from the glanded cotton plant. Thus, these results are in line with the fact that glandless cottonseeds have ultra-low levels of gossypol and that gossypol biosynthesis begins after 23 dpa in the embryos of glanded cotton (Martin et al., 2003; Meng et al., 1999). It should be noted that the *dCS* genes, which are the target of RNAi-mediated silencing in the ULGCS event TAM66274, were also shown to be transcriptionally inactive in the developing seeds of glandless cotton plants by others (Meng et al., 1999). Also, little or no dCS enzyme activity was detected in the developing seeds or seedling cotyledons of glandless cotton (Davis et al., 1996; Meng et al., 1999).

The tetraploid cotton has four copies of the *FDP synthase* gene, with A and D genomes each having two copies. Unlike *dCS* and *dCH* genes, *FDP synthase* gene expression was observed in the embryos from both glanded and glandless cotton plant, at all three stages of development that were examined. No significant differences were found in the activities of FDP synthase genes between glanded and glandless embryos. These results suggest that FDP is an important compound that is synthesized in the embryos at all stages of development in both glanded and glandless plants. This is to be expected, given its role in so many aspects of plant growth and development. This suggests that a major portion of the FDP pool is used for primary plant product biosynthesis (e.g., phytosterols, polyisoprenoids, quinones, etc.) and only a minute portion is channeled into gossypol biosynthesis in the glanded cotton plant. Therefore, silencing of the *dCS* genes in TAM66274 is unlikely to make a significant difference to the partitioning of FDP into other biosynthetic pathways. The fate of the FDP pool in the TAM66274 seeds will be similar to that in the glandless cottonseed.

Furthermore, there was no evidence of changes to other plant metabolic pathways in TAM66274 as a result of the reduced expression of the dCS enzyme, based on phenotypic, agronomic and seed composition evaluations. Each of the measured phenotypic, agronomic and composition parameters provides an assessment of the cumulative result of numerous biochemical pathways in the plant. Results of these evaluations showed that TAM66274 is phenotypically, agronomically and morphologically equivalent to non-transgenic cv. Coker 312 (refer to Section 7 of the petition). In addition, it was shown that levels of nutrients and antinutrients in TAM66274 cottonseed are comparable to levels in non-transgenic cv. Coker 312 and other conventional cotton varieties, except for the intended reduced levels of gossypol (refer to Section 6 in the petition). Therefore, other than the intended reduction in gossypol levels, cottonseed produced from TAM66274 is compositionally equivalent to non-transgenic cv. Coker 312 as well as other commercial cotton varieties. These data confirm that the intended reduction of expression of the dCS enzyme in TAM66274 results in reduction of levels of gossypol in the cottonseed and has no effect on other plant metabolic pathways associated with the dCS enzyme substrate, FDP. No secondary effects were anticipated and none have been observed or identified. No novel constituents from the intentional modification to TAM66274 have been identified and none were anticipated.

USDA-ARS personnel (Personal communication, R. Stipanovic) have stated that, being an intermediate substrate, FDP is very difficult to accurately measure. It requires radiolabeled substrates and sensitive real-time assays that are difficult to manipulate and are prone to significant variability due to the rapid turnover and minute quantities of substrate involved. Additionally, quantifying the channeling of FDP into the various metabolic pathways in cotton is not a trivial matter. To the best of our knowledge, no one has quantified or determined the percentage of FDP that is used for gossypol biosynthesis, other primary and secondary plant metabolites, or protein farnesylation in cotton. Nevertheless, we continue to believe that given the myriad uses of FDP as a substrate and the lack of demonstrable differences in phenotypic, agronomic, ecological, or compositional characteristics of TAM66274 versus non-transgenic cv. Coker 312, the flux of FDP in TAM66274 is not appreciably altered beyond the intended technical effect on gossypol biosynthesis. We also point out that FDP in the seeds of glandless cotton, which lacks dCS activity, is expected to share the same fate as in the seeds of TAM66274 in which dCS activity is suppressed. Seeds of glandless cotton have been safely used as feed for monogastric animals and as food for human nutrition (Alford et al., 1996; Bressani 1965; Graham et al., 1970). Thus, we believe that food and feed derived from TAM66274 is as safe as that derived from glandless cottonseed.

5.2 Expression Levels of the NPTII Variant Protein in Different Plant Parts of TAM66274 Compared to Levels in Non-Transgenic Coker 312, and Characterization and Safety of the NPTII Variant Protein

5.2.1. Expression levels of the NPTII variant protein in tissues of TAM66274.

NPTII variant protein levels were measured by ELISA in leaf, root, pollen and seed tissues of TAM66274 and non-transgenic cv. Coker 312 grown in a TAMU field trial (Sommerville, TX) in 2015. Details of plant production, tissue harvest, and materials and methods used to determine NPTII variant protein levels are described in Appendix D. NPTII variant levels in TAM66274 were highest in leaves (253.3 ng/g DW), significantly lower in roots and seeds (58.5 ng/g and 41.1 ng/g DW, respectively), and undetected in pollen at the detectable level of 25 ng/g DW (Table 5-3). As expected, NPTII variant protein was not detected in the same tissues of non-transgenic cv. Coker 312, which does not contain the *nptII* variant gene cassette in the plant genome. At the 1:20 (w/v) tissue/buffer ratio used for protein extraction, and using 100 µl of tissue extract in the NPTII ELISA, the assay was able to reliably detect 25 ng of NPTII protein/g DW of plant tissue.

Higher levels of NPTII variant expression in the leaf compared to levels in the cottonseed are consistent with other reports of NPTII protein expression in genetically modified (GM) cotton plants. For example, mean NPTII expression levels in leaves and cottonseed from Bollgard® cotton line 531 grown in multiple field locations were 3.145 µg/g and 2.451 µg/g, respectively, on a fresh weight (FW) basis (Serdy et al., 1995). Similarly, mean expression levels in leaves and cottonseed from Roundup Ready® cotton line 1445 were 45 µg/g and 6.7 µg/g, respectively, on a FW basis (Serdy and Nida, 1995). Although NPTII expression levels in leaf and cottonseed in Bollgard® and Roundup Ready® cotton events are expressed on a FW basis whereas levels in tissues of TAM66274 are expressed on a DW basis (253.3 ng/g DW in leaf and 41 ng/g DW in cottonseed, respectively), it is important to note that NPTII variant expression levels in TAM66274 leaves are approximately 100-fold and 1000-fold less than levels in leaves of Bollgard® and Roundup Ready® cotton events, respectively, using a conservative estimate of 10% dry matter content of leaves. Similarly, NPTII variant expression levels in cottonseed of TAM66274 are approximately 50-fold and 100-fold less than levels in cottonseed of Bollgard® and Roundup Ready® cotton events, respectively, using an estimate of 90% dry matter content of cottonseed. Furthermore, NPTII variant protein represents no more than 0.0000041% of the seed of TAM66274 (41 ng of NPTII variant protein per gram of seed tissue).

Table 5-3. Quantification of NPTII variant protein in tissues of TAM66274 and non-transgenic cv. Coker 312.

Treatments	NPTII variant protein concentration (ng/g DW ± SE, n=4; Ranges)			
	Leaf	Root	Pollen	Seed
Coker 312	N.D.	N.D.	N.D.	N.D.
TAM66274	253.3 ± 45.5 (146.4 - 356.9)	58.5 ± 0.1 (50.45 - 73.45)	N.D.	41.1 ± 5.6 (26.92 - 54.21)

Assay LOD calculated as 25 ng/g DW.
N.D. Not detected.

5.2.2. Characterization and safety of the NPTII variant protein expressed in TAM66274.

The *nptII* gene is the most frequently used selectable marker gene for generating transgenic plants for research purposes and it is found in many of the crops currently approved for commercial production (Miki and McHugh, 2004). International regulatory agencies (CERA, 2017) have approved the commercial release of genetically modified oilseed rape, corn, potato, tomato, flax, chicory, papaya and cotton containing the *nptII* gene, many of which are commercially grown including Genuity® DroughtGard™ corn (MON 87460), YieldGard® Rootworm corn (MON 863), Bollgard® cotton (MON 531), Bollgard®II cotton (MON 15985), Roundup Ready® cotton (MON 1445), and ringspot virus resistant papaya ("Sunset" lines 55-1 and 63-1). Further, NPTII has been approved by the FDA as a food additive for tomato, cotton and oilseed rape (FDA, 1994).

There have been no reports of adverse effects of either NPTII or the *nptII* gene on humans, animals or the environment (European Federation of Biotechnology, 2001; FDA, 1998; Flavell et al., 1992). The food, feed and environmental safety of the NPTII protein has been evaluated extensively in both the peer-reviewed literature (Flavell et al., 1992; Fuchs et al., 1993a; Fuchs et al., 1993b; Nap et al., 1992) and by regulatory authorities of different countries (EFSA 2007; FDA 1998). Generally, the amount of NPTII protein expressed in transgenic plants is low, ranging from approximately 0.00005 to 0.001% FW of cottonseed, potato tuber or tomato fruit (Miki and McHugh, 2004). Fuchs et al. (1993b) showed that the NPTII protein is rapidly digested in simulated mammalian gastric and intestinal fluids, a characteristic of most proteins that are safely consumed in the human diet. Furthermore, it was shown that consumption of exaggerated doses of NPTII protein did not generate ill effects on the health of mice. It was concluded that NPTII is readily degraded like other dietary proteins, does not possess the known attributes of known protein allergens, is not toxic to mammals and, therefore, does not pose a risk for human or animal consumption.

The ecological impact of the use of the *nptII* gene in crops has been reviewed by Nap et al. (1992). The authors concluded that kanamycin resistance will not contribute to enhanced weediness of a *nptII*-expressing plant or its sexually compatible relatives in the absence of selection pressure. Also, enhanced physiological fitness resulting from potential pleiotropic effects of *nptII* gene expression is not likely to occur in plants containing the *nptII* gene (Nap et al., 1992; EFSA, 2009). Additionally, various physical and biological barriers make the likelihood of horizontal gene transfer to other organisms negligible. Therefore, it was concluded that the NPTII protein is safe for use as a selectable marker in transgenic plants and does not pose a toxicity risk to other organisms in the environment.

The binary vector used to produce TAM66274 was pART27-LCT66 (refer to Section 3 of this petition). pART27-LCT66 was based on the binary vector pART27 (Gleave, 1992), which already contains an *nptII* variant expression cassette. In pART27, the first 24 nucleotides of the

nptII gene have been replaced with 51 nucleotides from the *nos* gene. The remaining 768 nucleotides exactly match the *nptII* gene from *E. coli* Tn5. Thus, the NPTII variant protein in TAM66274 is an in-frame translational fusion of a short section of the *nos* gene at the amino terminal end with the *nptII* gene (An et al., 1985; 1988). The amino acid sequence of the NPTII protein expressed in other commercial transgenic crops and the version with amino terminal modification (expressed in TAM66274) is shown in Figure 5-1. The underlined regions at the amino terminal show the difference between the two versions.

Figure 5-1. (A) NPTII amino acid sequence encoded by the *nptII* gene in commercial crop products such as Genuity® DroughtGard™ corn (MON 87460), Bollgard® cotton (MON 531) and (B) the NPTII variant amino acid sequence expressed in TAM66274. The underlined amino acid sequences at the N-terminal end of the protein show the differences between the NPTII proteins.

(A)

MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALN
ELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIM
ADAMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARL
KARMPDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELG
GEWADRFLVLYGIAAPDSQRIAFYRLLDEFF-

(B)

MAITLSATSLPISARIRAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKT
DLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAP
AEKVSIMADAMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAP
AELFARLKARMPDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALAT
RDIAEELGGEWADRFLVLYGIAAPDSQRIAFYRLLDEFF-

There is no reason to believe that the difference in the amino acids at the N-terminal end of the NPTII variant protein expressed in TAM66274 compared to the N-terminal amino acids of the NPTII protein expressed in commercial cotton and corn transgenic crops cited above have any impact on the safety characteristics of the protein. The *nptII* variant cassette in TAM66274 is the same cassette used to generate ringspot virus resistant papaya ("Sunset" lines 55-1 and 63-1) (Gonsalves and Manshardt, 1996). The details of the construction of the transformation plasmid used to produce ringspot virus resistant papaya, transformation method, and integration analysis are presented in various publications (Fitch et al., 1992; Ling et al., 1991; Quemada et al., 1991; Suzuki et al., 2008). The nucleotide sequence located between bp 4791 to 6675 of the T-DNA insert in TAM66274 (Table 4-5) exactly matches the sequence of the *nptII* variant cassette integrated into papaya event 55-1 (Suzuki et al., 2008; Genbank Accession no. FJ467933). The ringspot virus resistant papaya was deregulated by USDA APHIS in 1996, registered by the U.S. EPA in 1997, completed consultations with the FDA in 1997, and was granted regulatory approvals in Canada in 2003 and Japan in 2011 (Fuchs and Gonsalves, 2007). The product has been in commercial production since 1998, so there is a 19-year history of safe use of the same NPTII variant protein produced in TAM66274 as is produced in ringspot virus resistant papaya in food and the environment (Fuchs and Gonsalves, 2007).

The NPTII variant protein expressed in TAM66274 exhibits the same characteristics as the NPTII protein expressed in other commercial transgenic crops. The NPTII variant protein was successfully used to select the TAM66274 event, so it has the same functional activity as the NPTII protein expressed in other commercial transgenic crops. Secondly, the NPTII variant protein in TAM66274 was readily detected and quantified in an ELISA that used antibodies

specific to the NPTII protein, so the protein is immunoreactive with NPTII antibodies. In addition, the NPTII variant protein expressed in TAM66274 lacks characteristics of protein allergens and toxins. A bioinformatics analysis of the amino acid sequence of NPTII variant in TAM66274 was conducted as part of the analysis of ORFs in the T-DNA and flanking genomic sequences in TAM66274 (Section 5.3 below). Bioinformatic searches of databases of all known protein allergens and toxins confirmed that the NPTII variant protein in TAM66274 does not share sequence homology to known protein allergens and toxins. Also, as described above, the NPTII variant protein is expressed at very low levels in the cottonseed of TAM66274. Specifically, the NPTII variant protein is no more than 0.0000041% of the seed of TAM66274 (41 ng of NPTII variant protein per gram DW of seed tissue). Most allergenic proteins are present as major protein components in the specific food and represent from 2-3% to up to 80% of the total protein (Fuchs and Astwood, 1996). In contrast, the NPTII variant protein is present in cottonseed of TAM66274 at extremely low levels. Therefore, the exposure level of NPTII variant in food as well as feed products derived from TAM66274 will be extremely low.

In summary, on the basis of extensive safety studies conducted on the original NPTII protein (Flavell et al., 1992; Fuchs et al., 1993a; Fuchs et al., 1993b; Nap et al., 1992), widespread use of food and feed crops containing this protein, and the commercial cultivation and use of ringspot virus resistant papaya (containing the same NPTII variant protein expressed in TAM66274) for 19 years, as well as the absence of amino acid sequence homology of the NPTII variant in TAM66274 to sequences of known allergens and toxins, it is concluded that the NPTII variant expressed in TAM66274 has the same food, feed and environmental safety characteristics as the NPTII expressed in other commercial transgenic crops.

5.3 Bioinformatics Analyses of Amino Acid Sequences Encoded by Intended and Unintended ORFs in the T-DNA and Flanking Genomic DNA of TAM66274 Compared to Amino Acid Sequences of Known Allergens and Toxins

This section presents a comprehensive bioinformatic analysis to investigate similarity between putative amino acid sequences encoded by ORFs within the TAM66274 cotton T-DNA insert and genomic flanking sequences, to amino acid sequences of known allergens and toxins.

5.3.1. ORF analysis.

The nucleotide sequence of the T-DNA insert and genomic flanking sequences in TAM66274 cotton were determined from overlapping PCR amplicons and were assembled using Sequencher® software (version 5.4.6, Gene Codes, Ann Arbor MI) and SnapGene software (GSL Biotech, Chicago IL), and is presented in Appendix B to this petition (Table B-7). A 6814 bp sequence that includes the 6714 bp sequence of plasmid pART27-LCT66 T-DNA and 50 bp genomic sequences flanking either side of the T-DNA in TAM66274 was used for the ORF analysis using ORF Finder (Stothard, 2000). For this analysis, putative translated ORFs were defined as DNA sequences in any reading frame that is contained between a putative start codon

(ATG) and a putative stop codon (TAG, TAA, or TGA) and have a minimum size of 30 amino acids (aa). Both direct and reverse strands were used to determine the putative ORFs in all three reading frames (1, 2 and 3). A total of 33 putative ORFs were identified in the T-DNA and genomic flanking sequences of TAM66274 cotton: 19 from the direct strand (Table 5-4) and 14 from the reverse strand (Table 5-5). Putative ORFs were tabulated and subjected to various search strategies for homology to allergens and toxins. The location of each putative translated ORFs in the introduced T-DNA in TAM66274 is shown in Figure 5-2.

Thirty amino acids was chosen as the minimum ORF size because it is the default setting on the Sequence Manipulation Suite search tools described by Stothard (2000). The ORF Finder (https://sites.ualberta.ca/~stothard/javascript/orf_find.html) was set to only return ORFs that are at least 30 codons long. This criterion represents a conservative approach to evaluating putative ORFs that could theoretically encode a novel peptide. In light of Codex Alimentarius Commission guidelines that recommend bioinformatic analysis of ORFs of at least 80 aa for potential homology to known allergens or protein toxins, screening putative ORFs of 30 aa or greater is actually more conservative than recommended by these internationally accepted guidelines (Codex, 2003a). When used for allergenicity or toxicity database searches, ORF's below 80 aa, generally result in matches that lack any immunological or biological relevance (FARRP, 2016; Harper et al., 2012).

Of the 33 putative translated ORFs identified by this analysis, two were predicted: TAM66274-1F encoding the 101 aa partial *LacZ* region and TAM66274-12F encoding the 273 aa NPTII variant protein. The partial *LacZ* region is a component of the pART27 plant transformation vector, which was used for cloning purposes. The NPTII variant protein was used as a selectable marker during plant transformation of the recipient organism, *G. hirsutum* cv. Coker 312. The ORF search did not identify any putative translated ORFs that span the junction between the TAM66274 genome and the RB or LB regions of the T-DNA insert (Figure 5-2).

Further, of the 33 putative translated ORFs in the search sequence, only six putative translated ORFs were identified that are at least 80 aa in length: TAM66274-1F (101 aa), TAM66274-5F (80 aa), TAM66274-12F (273 aa), TAM66274-14F (128 aa), TAM66274-3R (178 aa) and TAM66274-9R (89 aa) (Tables 5-4 and 5-5).

Figure 5-2. Location of putative ORFs in the integrated T-DNA and its flanking sequences in TAM66274. Orange arrows indicate putative ORFs on the direct strand and green arrows indicate putative ORFs on the reverse strand. Putative ORFs in all six reading frames are shown.

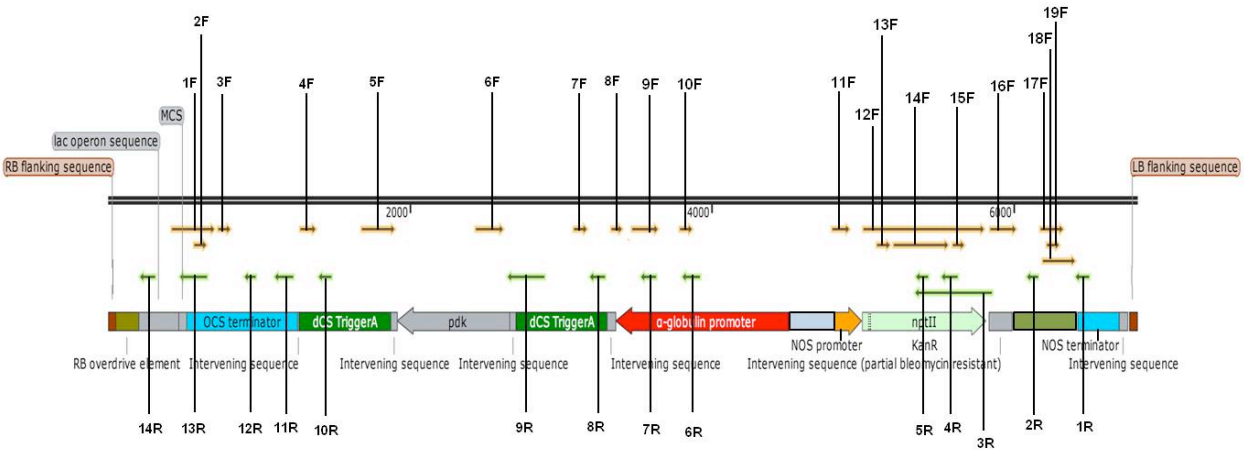


Table 5-4. ORFs in the direct strand of pART27-LCT66 T-DNA insert in TAM66274. Analysis of ORFs in the T-DNA insert in TAM66274. Nineteen putative translated ORFs were identified in all three reading frames of the T-DNA direct strand.

ORF Identification Number	Size of putative transcript (bp)	Size of putative translation (amino acids)	Putative translated ORF
TAM66274-1F	303	101	MTMITPSYLGDTIEYSSYASNALGALPYRPAGGRTSKL ACMPAGPAEPRHVVAKFALDPPNDLSSLSRFDLHFIWG PHTPKKCCIILGAASRLPGRRAGPG
TAM66274-2F	105	35	MLSQNSPWTRPTICRHCQGLTCTSFGAHIHQKNAA
TAM66274-3F	105	35	MVPVTFGRADGQYSTSRNLTHARRRGTGVPFSERY
TAM66274-4F	123	41	MPRTTSTPHPFDSDYSESMDSMFHATYSTSLKTSKGISS HP
TAM66274-5F	240	80	MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEG YFWISGVYFEPQYSLGRKMLTKVIAMASIVEDPSLSISN PASQL
TAM66274-6F	201	67	MLVYHLTCSIKFIKNNILTLNFYLLRLTHHLSYFFTLLC CLCKQYIYKLFFHNYNNYIIIIILINIT
TAM66274-7F	108	36	MVGGERNCFIQYIFPMNPQIGSLVKFQQSSNIACHG
TAM66274-8F	90	30	MDICRIRLGTRIDYDKLCILLLCDGNSKEW
TAM66274-9F	195	65	MQIFINVLKTLTCKMTSVQIKEDEILSLFFFFCIVVGRYR DATSLHMVLARFGQTLLQGGSWLHL
TAM66274-10F	99	33	MSRFTELRVELIEDKSSVHMLLCMVMIYELQEI
TAM66274-11F	135	45	MEIVSVNGFLEFNELSTYVRNHYCAFKSRLRSLSASKY FLSKMLH
TAM66274-12F	819	273	MAITLSATSLPISARIRAGSPAAWVERLFGYDWAQQTI GCSDAAVFRLSAQGRPVLFVKTDLSGALNELQDEAAR LSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLL SSHLAPAEKVSIMADAMRRLHTLDPATCPFDHQAKHRI ERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKAR MPDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGV ADRYQDIALATRDIAEELGGEWADRFLVLYGIAAPDSQ RIAFYRLLDEFF

Table 5-4, continued. ORFs in the direct strand of pART27-LCT66 T-DNA insert in TAM66274. Analysis of ORFs in the T-DNA insert in TAM66274. Nineteen putative translated ORFs were identified in all three reading frames of the T-DNA direct strand.

ORF Identification Number	Size of putative transcript (bp)	Size of putative translation (amino acids)	Putative translated ORF
TAM66274-13F	105	35	MTGHNRQSAALMPPCSGCQRRGARFFLSRPTCPVP
TAM66274-14F	384	128	MNCRTRQRGYRGWPRRAFLAQLCSTLSLKREGTGCYW AKCRGRISCHLTLLLPRKYPSWLMQCGGCIRLIRLPAHS TTKRNIASSEHVLGWKPVLSIRMIWTKSIRGSRQPNCSP GSRRACPTARISS
TAM66274-15F	99	33	MAMPACRISWWKMAAFLDSSTVAGWVWRTAIRT
TAM66274-16F	189	63	MTDQATPNLPSRDFDSTAAFYERLGFGIVFRDAGWMIL QRGDLMLEFFAHPDPTLTFATSKSK
TAM66274-17F	174	58	MHALTTWNIAIFLKNYARWRMSRQLQLLPKSKYPSRM HSSILFMRGKARLIQLANHPA
TAM66274-18F	234	78	MEHRYFSEELCSLEDVAAIAAIAKIEIPLTHAFINIIHAGK GKINPTGKSSSVIGNFSSSDLIRFGATHVFNKDEMVE
TAM66274-19F	102	34	MLVGGCRGNCSYCQNRNTPHACIHQYYSCGERQD

Table 5-5. ORFs in the reverse strand of pART27-LCT66 T-DNA insert in TAM66274. Analysis of ORFs in the T-DNA insert in TAM66274. Fourteen putative translated ORFs were identified in all three reading frames of the T-DNA reverse strand.

ORF Identification Number	Size of putative transcript (bp)	Size of putative translation (amino acids)	Putative translated ORF
TAM66274-1R	114	38	MIIIARPATGFNLKKLYCQMFERSASTHSFFTPPSRPY
TAM66274-2R	99	33	MHDARYEVTVLGSIPSKFHSQYHIHHCIPAREN
TAM66274-3R	534	178	MAGWASLGRSFRTPESRSEELVKKAIEGDALRIGSGDT VKHEEAVSPFAAKLFSNITGSQRYVLIAVRHTQPATVD ESRKAAIFHHDIRQAGIAMGHDEILAVGHARLEPGEQF GWREPLMLFVQIILIDKTGFHPSTCSLDAMFRLVVEWA GSRIKRMQPPHCISHDGYFLGRSKVR
TAM66274-4R	132	44	MNPEKRPFSTMIFGKQASPWVTTRSSPSGMRALSLANS SAGASP
TAM66274-5R	105	35	MRCFAWWSNGQVAGSSVCSRRIASAMMDTFSAGAR
TAM66274-6R	138	46	MSIKRTGYQKYTYLNLFISPATHRSSPCTATCVHLTCPL STQPLTQ
TAM66274-7R	123	41	MNPPATKFVRTLLVPCEGMWHLDIYPPLYKKKKKETIF RLL
TAM66274-8R	123	41	MPRTTSTPHPFDSDYSESMDSMFHATYSTSLKTSKGISS HP
TAM66274-9R	267	89	MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEG YFWISGVYFEPQYSLGRKMLTKVIAMASIVEDPNSVPQ LGKEIIIFFFPFSIK
TAM66274-10R	108	36	MVGGERNCFIQYIFPMNPQIGSLVKFQQSSNIACHG
TAM66274-11R	144	48	MRDAYDRMIFAFNSVVHVVKNLSMCSSDPYRRFRFIL MNISPVTIVFL
TAM66274-12R	99	33	MIERHNNKQLRFIITNPILKKAAEPVKPKRLIT
TAM66274-13R	201	67	MQHFFGVCGPQMKCRSNLDSDDKSLGGSRANFATTCR GSAGPAGMQASLLVRPPAGRYGRAPNALDA
TAM66274-14R	126	42	MSELTHINCVALTARFPVGKPVVPAALMNRPTRGERRF AYWG

5.3.2. Protein databases.

Two protein databases were queried for homology of putative ORFs to known allergens: AllergenOnline and the Entrez protein database. The two-database search strategy was used to ensure queries of the most current and comprehensive databases of known allergens and protein toxins.

AllergenOnline (http://www.allergenonline.org/) (Version 16.0, January 27, 2016) is a curated and peer-reviewed database maintained by the FARRP (2016) and is updated annually. Version 16.0 contains a comprehensive list of 1,956 proteins/peptides that are categorized into 778 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary and contact). All database entries in Version 16.0 are linked to sequences in the NCBI of the National Institutes of Health (NIH, USA).

The NCBI Entrez protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (NCBI, 2016) is a search and retrieval system of a collection of protein sequences compiled from annotated coding regions in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), RefSeq (http://www.ncbi.nlm.nih.gov/refseq/), TPA (http://www.ncbi.nlm.nih.gov/genbank/tpa/), SwissProt (http://www.expasy.org/ sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www.prf.or.jp/index-e.html), PDB (http://www.wwpdb.org/) and all non-redundant GenBank coding sequence translations (CDS). The Entrez database is updated daily. Records from the international collaboration databases DDBJ and EMBL are added daily. For UniProt records, updates are processed when UniProt provides a new "cumulative update" at their FTP site, approximately twice per month. BLASTP (Altschul et al., 1997, Version 2.5.0+) searches were run on the database that includes Genbank CDS, PDB, SwissProt, PIR and PRF excluding environmental samples from whole genome shotgun contigs (WGS) projects.

5.3.2.1. AllergenOnline database.

The FASTA bioinformatics tool (Pearson and Lipman, 1988) provides a quick search and local alignment of sequences contained within a specified database. The AllergenOnline search routine employs three comparative bioinformatics approaches:

1. Search for full-length alignments by FASTA (referred to as “Full FASTA”)
2. Search for 80 amino acid alignments by FASTA (80mer sliding window search with FASTA)
3. Search for 8 amino acid alignments by FASTA (8mer exact match search with FASTA)

All three approaches were used to query the putative translated ORFs in TAM66274 cotton against the database.

Full FASTA. The Full FASTA bioinformatics evaluation of each resulting alignment utilizes the

minimum criterion of at least 80 amino acids of alignment length with greater than 35% shared amino acid identity over the alignment length. Any alignments exceeding these criteria for shared sequence similarity indicate the potential for immunologically relevant sequence similarity (Codex, 2003a). For AllergenOnline, the default scoring matrix for FASTA search is Blocks Substitution Matrix 50 (BLOSUM50, Henikoff and Henikoff, 1992), which is intended to identify highly similar proteins that are likely to have similar overall structure and function, whether of distant evolutionary origin or closely related sequences (Pearson, 1999). Highly similar proteins are considered to be probable homologues that are similar because of the evolutionary relationship of the organisms. The default parameters used for this analysis are "word size" of 2 and an expectation value score (*E*-value) of 1. The *E*-value is a calculated value that reflects the degree of similarity of the query sequence to its corresponding matches. The size of the *E*-value is inversely related to similarity of two proteins. *E* score of 1 is sufficiently large to prevent missing any important alignment, but not so small that even remote homologies are missed. The *E*-value depends on the overall length of joined (gapped) local sequence alignments, the quality (percent identity, similarity) of the overlap, and the size of the database. In general, for a database the size of AllergenOnline, which contains many unrelated as well as related proteins, two sequences might be considered related in evolutionary terms (i.e., diverged from a common ancestor and share common three-dimensional structure), when the *E*-value of the FASTA query is less than 0.02 (Pearson, 1996).

Although a total of 33 putative ORFs were identified in the T-DNA and genomic flanking sequences of TAM66274, there were only six that encoded proteins or peptides greater than 80 amino acids. Therefore, the full FASTA search was only conducted on the amino acid sequences encoded by these six ORFs. These six sequences were subjected to full FASTA search of the AllergenOnline database on October 20, 2016. The results of FASTA searches were tabulated as a list of aligned sequences from best to least similar, *E*-value and percent identity of the overlapping alignment, and the best alignment of the query sequence and aligned protein. Detailed results of these analyses are presented in Appendix D, Table D-7. Full FASTA search of the six putative translated ORFs (which include the NPTII variant protein) in TAM66274 did not reveal any match greater than 35% identity, over a stretch of at least 80 aa, with any immunologically relevant entry in the database.

80mer sliding window search with FASTA. Identification of short regions of high identity shared by a query sequence and an allergen may indicate similarities that could also share IgE binding or cross-reactivity. The rationale to perform a search with every possible 80 amino acid segment of the query protein is based on a recommendation of the FAO/WHO expert panel (2001) that more than 35% identity over any segment of 80 or more amino acids is an indication of possible cross-reactivity for allergens. This recommendation was adopted by the Codex Alimentarius Commission as the primary sequence search criteria for use in identifying proteins of potential concern in genetically modified plants (Codex, 2003b). However, it should be noted that this

specific search has been challenged due to a high level of false positives and, therefore, results should be carefully considered for irrelevant alignments that may have no biological relevance for allergy risk (Goodman et al., 2008, Silvanovich et al., 2006). A FASTA search of every possible 80 amino acid segment of the six putative translated ORFs (which encoded proteins or peptides greater than 80 amino acids) was conducted using the AllergenOnline database on October 20, 2016 using the following criteria. The identity score was adjusted to compensate for segments less than 80 amino acids due to inserted gaps, or aligned segments less than 80 amino acids that calculate to more than 35% identity, if adjusted to 80 amino acids total. The results of the 80mer sliding window search were tabulated and showed that none of the sequential 80-amino acid sequences of the six putative translated ORFs (which include the NPTII variant protein) in TAM66274 share immunologically relevant amino acid sequence segments or structure with known allergens in the AllergenOnline database. Results of these analyses are presented in Appendix D, Table D-8.

8mer exact match search with FASTA. The eight-amino acid search was originally suggested based on the concept that eight or more amino acids are a representative minimal size for an IgE-binding epitope (Metcalfe et al., 1996). However, this search does not detect conformational epitopes that are formed when non-linear amino acids are brought together by the higher-order folding of the protein. For the reasons stated above, results should be carefully considered for irrelevant alignments that may have no biological relevance for allergenicity risk. The 8mer exact match search with FASTA is a precautionary search and an additional indicator of potential cross-reactivity between a query sequence and an allergen.

A FASTA search of every possible 8 amino acid segment of the 33 putative translated ORFs was conducted using the AllergenOnline database on October 20, 2016. The results of the 8mer exact match search were tabulated and showed no exact matches of eight contiguous amino acids between any of the 33 putative translated ORFs (which include the NPTII variant protein) in TAM66274 and any immunologically relevant entry in the AllergenOnline database. Results of these analyses are presented in Appendix D, Table D-9.

5.3.2.2. Entrez database.

Allergens. A BLASTP search was conducted on October 19, 2016 to compare the amino acid sequence of the putative translated ORFs against the Entrez protein database for allergens. The query was restricted to the six putative translated ORFs that encoded at least 80 aa residues: TAM66274-1F (101 aa), TAM66274-5F (80 aa), TAM66274-12F (273 aa), TAM66274-14F (128 aa), TAM66274-3R (178 aa) and TAM66274-9R (89 aa). This bioinformatic query was restricted to ORFs of at least 80 aa residues based on Codex Alimentarius guidelines for the evaluation of potential allergenicity of novel proteins (Codex, 2003a; FAO WHO, 2001). A limit option was selected to query entries for *allergen* to align only with proteins identified as allergens. The purpose of this search was to ensure that a significant match with a newly

identified allergenic sequence not included in the AllergenOnline Version 16 database was not overlooked. On the date of this search, the number of sequences in the database was 98,832,856. With a filter of *allergen* in the Entrez query, the total number of sequences searched was reduced to 41,021. The BLASTP search algorithm parameters were set to default parameters of *E*-value = 1, Word size = 2, Gap costs (Existence = 11, Extension = 1), and Scoring matrix = BLOSUM62. The low complexity filter was turned off and the number of alignments returned was set to 100. According to Codex, a bioinformatics search using the BLASTP algorithm, sequence matches of at least 35% identity over segments of at least 80 aa between the query sequence and an allergen may indicate the possibility of cross-reactivity. The results of BLASTP searches were tabulated into a list of sequences with identities over at least 80 aa of the query sequence to known allergens.

The BLASTP analysis returned 25 alignments for the predicted ORF encoding the 273 aa NPTII variant protein (TAM66274-12F) with 31-33% homology to *Drosophila* spp. venom allergen 5; 29-33% homology to uncharacterized proteins of *Drosophila* spp.; and 24-30% homology to extracellular or hypothetical proteins from *Phytophthora* spp. No alignment exceeded 35% identity over any 80 amino acid of the NPTII variant ORF indicating a lack of meaningful homology to known or putative allergens in the Entrez protein database (Appendix D, Table D-10).

BLASTP analysis returned one alignment for the predicted ORF encoding the 101 aa partial LacZ region (TAM66274-1F) with 19% identity to MD-2-related lipid-recognition protein-like sequence (*Polistes canadensis*) and one alignment for TAM66274-3R with 24% homology to Sar s 27 allergen (*Sarcoptes scabiei*). No alignment exceeded 35% identity over any 80 amino acid segment of these ORFs indicating a lack of meaningful homology to known or putative allergens in the Entrez protein database (Appendix D, Table D-10). Additionally, BLASTP analysis returned no alignment with any known or putative allergens in the Entrez protein database for TAM66274-5F, TAM66274-14F, or TAM66274-9R (Appendix D, Table D-10).

Based on FASTA searches of the AllergenOnline database and BLASTP searches of the Entrez protein databases, no significant homology to known or putative allergens was detected in either of the two intended ORFs (partial LacZ region, NPTII variant) or any of the four unintended ORFs in TAM66274 cottonseed. These results are consistent with other assessments of the LacZ reporter sequence and NPTII coding sequence used as a selectable marker for plant transformation (Miki and McHugh, 2004). These results also support the weight-of-evidence that the introduced T-DNA in TAM66274 cottonseed is unlikely to contain an allergen when used as food for human nutrition.

Toxins. A BLASTP search was also conducted on October 22, 2016 to compare the amino acid sequence of all 33 putative translated ORFs against the Entrez protein database for protein

toxins. A restricted keyword delimiter of *toxin* was used in the query search to align only with proteins identified as toxins. On the date of this search, the number of sequences in the database was 102,256,868. With a filter of *toxin* in the Entrez query, the total number of sequences searched was reduced to 601,590. BLASTP search algorithm parameters were set to *E*-value = 1e-05, Word size = 2, Scoring matrix = BLOSUM62, and Gap costs (Existence = 11, Extension = 1). The low complexity filter was turned off and the number of alignments returned was set at 100. The results of BLASTP searches were tabulated into a list of aligned sequences from best to least similar, *E*-value and a percent identity of the overlapping alignment, and the best alignment between the query sequence and aligned protein for further evaluation.

As expected, the BLASTP search of the 101 aa partial LacZ region (TAM66274-1F) returned sequences with high homology to several cloning vectors containing the *LacZ* sequence. Similarly, TAM66274-12F ORF encoding the 273 aa NPTII variant protein returned sequences with high homology to NPTII protein from various bacterial sources. However, neither of these ORFs showed any significant homology to known or putative protein toxins in the database (Appendix D, Table D-11).

Two BLASTP search results for TAM66274-5F and TAM66274-9R returned sequences with 36% and 33% identity, respectively, with geraniol synthase (GenBank accession #BAM29049.1) from rough lemon (*Citrus jambhiri*). The putative translated TAM66274-5F spans the direct strand of the *dCS* gene and the TAM66274-9R ORF spans the reverse strand of the *dCS* gene of the RNAi cassette in the T-DNA insert (Figure 5-1) and, therefore, when translated would encode the same peptide. Shishido et al. (2012) showed that a host-selective adenylate cyclase toxin (ACT) induces the mRNA for this geraniol synthase. Since the word "toxin" was associated with this Entrez database entry and the keyword delimiter of *toxin* was used in the query search, it was returned in the BLASTP search results. However, the putative translated ORFs, TAM66274-5F and TAM66274-9R, are not otherwise related to any known or putative protein toxins (Appendix D, Table D-11). BLASTP results for the remaining 29 ORFs returned no significant similarity with any known or putative protein toxins in the Entrez database (Appendix D, Table D-11).

5.4 Conclusions of the Characterization and Safety of the Gene Expression Products in TAM66274

Results presented in this section of the petition demonstrated the efficacy of the *dCS* RNAi cassette in suppressing expression of the dCS protein in cottonseed only, but not in other parts of the plant, and thereby inhibiting gossypol levels only in the cottonseed. The food and feed safety of *dCS* RNAi is supported not only from reviews of the safety of dsRNA and nucleic acids in general, but by the specificity of *dCS* RNAi to cotton. Bioinformatic analyses of the 604 bp *dCS* gene trigger sequence using a BLASTN search of human, cow, pig, chicken, fish, shrimp, dog and cat EST sequences showed no homology to any 20 bp contiguous stretch, which confirms

the unlikelihood of adverse non-target effects of *dCS* RNAi on humans or animals that consume TAM66274 cottonseed or cottonseed products. The potential for FDP to accumulate and affect the biosynthesis of other secondary plant products was examined. Analysis suggested that FDP is used for several primary plant metabolites (e.g., phytosterols, polyisoprenoids, and quinones) and only a minute portion is channeled into gossypol biosynthesis. Therefore, silencing of *dCS* genes in TAM66274 is unlikely to make a significant difference in partitioning of FDP into other biosynthetic pathways or for FDP accumulation in seed kernels.

NPTII variant protein levels were measured in leaf, root, pollen and seed tissues of TAM66274 and non-transgenic cv. Coker 312, and were found to be 50-fold to 1000-fold lower than in other genetically engineered cotton cultivars granted non-regulated status. NPTII variant protein was highest in leaves of TAM66274, was lower in the root, was lowest in the seed, and was undetected in pollen at the detectable level of 25 ng/g DW. NPTII variant protein represents no more than 0.0000041% of the seed of TAM66274 (41.1 ng/g DW). The NPTII variant protein in TAM66274 is an in-frame translational fusion of a portion of the *nos* gene at the amino terminal end with the *nptII* gene, which is the same cassette used to generate ringspot virus resistant papaya ("Sunset" lines 55-1 and 63-1). On the basis of extensive safety studies conducted by others on the NPTII protein, widespread use of food and feed crops containing the NPTII protein, a history of safe use of the NPTII protein expressed in ringspot virus resistant papaya as is expressed in TAM66274, as well as the absence of amino acid sequence homology of the NPTII variant in TAM66274 to known allergens and toxins, it is concluded that the NPTII variant expressed in TAM66274 has the same food, feed and environmental safety characteristics as the NPTII expressed in other commercial transgenic crops.

A bioinformatic analysis identified 33 putative ORFs in the T-DNA and genomic flanking sequences of TAM66274, of which only six putative ORFs encoded proteins or peptides greater than 80 amino acids. Bioinformatic analysis of the AllergenOnline and Entrez protein databases using internationally recognized guidance and search criteria revealed no significant similarities to known or putative allergens for any of the six putative translated ORFs, including the NPTII variant protein. These data collectively confirm the lack of both amino acid identity and, hence, immunologically relevant similarities between the putative translated ORFs in TAM66274 and known or putative allergens. These data support the conclusion that the putative translated ORFs in TAM66274 are not potential allergens or protein toxins.

6. COMPOSITION ASSESSMENT OF TAM66274 COTTONSEED

To design a food and feed safety assessment of a genetically modified (GM) crop, it is important to understand the food and feed uses of the crop. Following ginning of seed cotton to separate the fiber, fuzzy cottonseed is processed into four major food and feed products: oil, meal, hulls and linters. Cottonseed oil is primarily used for human food applications. Linters are used to produce cellulose derivatives for both food and industrial applications. The hulls and meal are used for livestock feed, primarily cattle (Anonymous, 2002). Although cottonseed is a rich source of relatively high quality protein (approximately 23% DW of seed) and is used as a feed supplement for ruminant animals, due to the presence of toxic gossypol, cottonseed is not typically consumed by humans or monogastric animals, which are more sensitive to gossypol toxicity (Risco and Chase, 1997; OECD, 2008, 2009). However, cottonseed products modified by mechanical or solvent extraction, or derived from glandless cotton varieties, may be used in human food provided the free gossypol content does not exceed 450 ppm (FDA, 1960; FDA, 1972; FDA, 1976). Examples include dehulled, partially defatted, cooked, ground cottonseed kernels; dehulled, hexane-extracted, ground cottonseed kernels; and roasted glandless cottonseed kernels used as snack food, or in baked goods. Similarly, cottonseed meal modified by mechanical or solvent extraction may be used for monogastric animal feed provided the free gossypol content does not exceed 400 ppm (AAFCO, 1968a; AAFCO, 1968b).

Texas A&M University developed TAM66274, which exhibits ultra-low levels of the anti-nutrient gossypol in the cottonseed, by introducing plasmid pART27-LCT66 into non-transgenic cv. Coker 312 by *A. tumefaciens*-mediated transformation. The reduction of gossypol levels in cottonseed potentially makes cottonseed safe for use as feed for various monogastric animals and as human food. Thus, an important component of the safety assessment of TAM66274 is the comparison of seed gossypol levels to safety standards established for the intended food and feed uses. Provided that seed gossypol levels do not exceed the safety standards for their intended uses, TAM66274 may be legally used in food and feed products. Another important component of the safety assessment of TAM66274 is the comparison of the nutrient and anti-nutrient levels in TAM66274 cottonseed to non-transgenic cv. Coker 312, and to published values for other conventional cotton varieties with a history of safe use in food and feed products. Compositional equivalence confirms the appropriateness of TAM66274 cottonseed for use in conventional food (oil and linters) and livestock feed products.

The cottonseed of TAM66274 and the non-transgenic cv. Coker 312 was produced from plants grown in replicated field trials at three locations in the U.S. during the summer of 2014 (two sites in NC, and one site in MS), and from five field locations in the U.S. during the summer of 2015 (two sites in NC, two sites in MS and one site in TX). Field sites were selected as representative of major cotton-growing regions in the United States. Composition analyses were performed by Covance Laboratories, Inc. (Madison, WI) using standard laboratory methods. The components analyzed were based on the OECD (2009) consensus document on the compositional

considerations for new varieties of cotton and included proximates, fiber (total dietary, crude, acid and neutral detergent fibers), fatty acids, amino acids, minerals, alpha-tocopherol, and anti-nutrients (free and bound gossypol, gossypol isomers, cyclopropenoid fatty acids and phytic acid). Total gossypol content of cottonseed samples was also analyzed by Texas A&M University using a high-performance liquid chromatography (HPLC) method described in Appendix E. Since cottonseed of TAM66274 and non-transgenic cv. Coker 312 were collected separately by treatment and for each location prior to composition analyses, levels of nutrients and anti-nutrients in each treatment were statistically compared across locations, separately for each year. The results of analyses were subjected to statistical analysis, and values for TAM66274 cottonseed were compared to non-transgenic cv. Coker 312. Analyte levels in cottonseed of TAM66274 were considered significantly different from non-transgenic cv. Coker 312 cottonseed if the actual probability of difference (α level) was $\alpha \leq 0.05$. Furthermore, analysis results for TAM66274 were compared to analytical data for cottonseed from conventional cotton varieties in the International Life Sciences Institute Crop Composition Database (ILSI, 2016), as well as to analyte levels in cottonseed from conventional cotton varieties reported in the literature. Details of the cottonseed production, processing, compositional analyses, and statistical analyses are presented in Appendix E.

In addition to the nutrient and anti-nutrient analyses described above, mycotoxin levels in cottonseed of TAM66274 were compared to the non-transgenic cv. Coker 312. Analyses were conducted by Romer Laboratories, Inc. (Union, MO) on cottonseed produced from plants grown in the same replicated field trials at the five locations in the U.S. during the summer of 2015, as described above. The components analyzed included aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, deoxynivalenol, acetyldeoxynivalenol, and zearalenone. Cottonseed production and processing as well as methods for mycotoxin analyses and statistical analyses are presented in Appendix E.

A summary of the results of these compositional comparisons are presented in this section of the petition, and detailed results of cottonseed analyses at each field site location are presented in Appendix E. Results of compositional analyses of cottonseed from 2014 and 2015 field studies demonstrated that introduction of plasmid pART27-LCT66 into the genome of non-transgenic cv. Coker 312 achieved the intended reduction in seed gossypol levels. Mean gossypol levels in cottonseed of TAM66274 were below the maximum allowable level of 450 ppm, considered safe for modified cottonseed products in foods for human consumption and below 400 ppm allowed in animal feed. Further, these compositional analyses demonstrated that introduction of plasmid pART27-LCT66 into the genome of non-transgenic cv. Coker 312 to achieve the ULGCS phenotype did not impact the nutrient composition of TAM66274 cottonseed. Results of these analyses demonstrate that other than the intended reduction in cottonseed gossypol levels, cottonseed from TAM66274 is compositionally equivalent to, and as nutritious as, cottonseed from non-transgenic cv. Coker 312, as well as other conventional cotton varieties.

6.1 Cottonseed Composition Analyses of TAM66274

6.1.1. Proximates.

Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from 2014 and 2015 field trials were analyzed for moisture, protein, total fat, ash, carbohydrates and calorie content. The carbohydrate composition and calorie values were calculated. The means and ranges of analyte values for proximates in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-1. When the cottonseed proximate composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, for samples harvested from 2014 field trials, there were no statistically significant differences observed for any of the proximate analytes between TAM66274 and non-transgenic cv. Coker 312. In the case of cottonseed samples harvested from 2015 field trials, there were no statistically significant differences observed for protein and ash content between TAM66274 and non-transgenic cv. Coker 312, but statistically significant differences were observed for cottonseed content of moisture, total fat, carbohydrates and calories between these two treatments. Since statistically significant differences in moisture, total fat, carbohydrates and calories were not consistently observed between the treatments from 2014 and 2015 field studies, these analyte differences observed in 2015 samples were not considered biologically meaningful. Furthermore, the means and ranges of proximate values for TAM66274 cottonseed harvested from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database and in the literature for conventional cotton varieties (Table 6-1). Therefore, these results show that proximate levels (moisture, protein, total fat, ash, carbohydrates and calories) in cottonseed of TAM66274 are substantially equivalent to levels in non-transgenic cv. Coker 312 and within the same range as cottonseed proximate content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.2. Fiber.

Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from the 2014 and 2015 field trials were analyzed for crude fiber (CF), total dietary fiber (TDF), acid detergent fiber (ADF), and neutral detergent fiber (NDF). The means and ranges of cottonseed composition values for the fiber fractions in TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-2. Fiber levels are expressed on a percent DW basis. When the cottonseed fiber composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, for samples harvested from 2014 and 2015 field trials, there were no statistically significant differences observed for CF between TAM66274 and non-transgenic cv. Coker 312, but statistically significant differences were observed between TAM66274 and non-transgenic cv. Coker 312 for levels of TDF, ADF and NDF. However, the means and ranges of values for the different fiber fractions in cottonseed of TAM66274 harvested from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database and in the literature

for conventional cotton varieties (Table 6-2). Therefore, where differences in amounts of TDF, ADF and NDF were detected between TAM66274 and non-transgenic cv. Coker 312, these were most likely due to small genetic differences resulting from the inherent genetic heterogeneity of the recipient non-transgenic cv. Coker 312. Variation in cottonseed composition among conventional cotton varieties has been well documented (Lawhon et al., 1977; Hamilton et al., 2004; Arackal et al., 2012; Rudgers 2013), and distribution of cottonseed composition parameters within a population of cotton varieties typically follows a normal distribution, with most varieties containing a median value for the cottonseed composition parameter, and fewer varieties exhibiting either lower or higher values than the median (Kohel et al., 1985). Variation in seed composition is also observed among plants of a single variety. For example, variation in cottonseed composition parameters was observed among samples taken from conventional cotton variety Coker 130 grown in a single field location (Arackal et al., 2012). Similarly, data in the current petition for TAM66274 show variation in cottonseed gossypol levels in non-transgenic cv. Coker 312 samples collected from replicate plots within single field sites (gossypol levels measured by TAMU, Appendix E, Table E-10). Thus, the distribution of cottonseed composition parameters within a population of a single variety of cotton is presumed to follow a normal distribution similar to that of a population of different cotton varieties.

TAM66274 cotton was produced from *Agrobacterium tumefaciens*-mediated transformation of a single cell of a non-transgenic cv. Coker 312 cotton plant. Given the inherent variability in cottonseed composition among different plants, it is highly likely that the TAM66274 cotton plant derived from the single cell of non-transgenic cv. Coker 312 produced cottonseed with some compositional parameters that differ from the median values of those same parameters in cottonseed from the broader population of non-transgenic cv. Coker 312. TAM66274 cotton was propagated by self-pollination breeding to generate the T6 and T7 generations used in the 2014 and 2015 cottonseed compositional analysis studies, respectively (Figure 3-5 and Table 3-2 of the petition). Therefore, the differences between TAM66274 and non-transgenic cv. Coker 312 in some compositional parameters were likely perpetuated to the breeding generations used in the compositional studies. This phenomenon of compositional differences between genetically modified cottonseed and its parental variety, which likely resulted from inherent variability in the parental population, has been previously described (Nida et al., 1996). In that example, gossypol levels in cottonseed of Roundup Ready® cotton (MON 1445) were statistically significantly higher than levels in the parental non-transgenic cv. Coker 312. However, when the Roundup Ready® trait was backcrossed in to other cotton varieties, the difference in cottonseed gossypol levels was no longer observed between Roundup Ready® cotton (MON 1445) and the respective control varieties. This demonstrates that there was no linkage between the Roundup Ready® trait and differences in cottonseed gossypol levels between Roundup Ready® cotton (MON 1445) and the non-transgenic cv. Coker 312. Similarly, we conclude that where differences in some cottonseed compositional parameters were observed between TAM66274 and non-transgenic cv. Coker 312, these were the result of inherent genetic differences in the non-transgenic cv. Coker

312 parental population, and are not associated with the ULGCS trait. Overall, these results show that fiber levels (CF, TDF, ADF and NDF) in cottonseed of TAM66274 are comparable to non-transgenic cv. Coker 312 and within the range of other conventional cotton varieties with a history of safe use as food and feed.

6.1.3. Amino acids.

Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from the 2014 and 2015 field trials were analyzed for amino acid content. The means and ranges of amino acid levels in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-3. The levels of amino acids shown in Table 6-3 are expressed as mg/g DW. Because some literature references report levels of amino acids in cottonseed as percent of total amino acids, the levels of amino acids in cottonseed of TAM66274 were converted to percent of total amino acids to compare to other reports in the literature. These comparisons are presented in Table 6-4.

When the cottonseed amino acid composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, for samples harvested from 2014 field trials, there was just one instance of a statistically significant difference observed between TAM66274 and non-transgenic cv. Coker 312, and this was for cystine. There were no statistically significant differences in levels of all the other 17 amino acids between cottonseed of TAM66274 and non-transgenic cv. Coker 312. In the case of cottonseed samples harvested from 2015 field trials, there were no statistically significant differences in levels of amino acids between TAM66274 and non-transgenic cv. Coker 312, except for cystine and tryptophan. However, the means and ranges of amino acid levels in cottonseed of TAM66274 harvested from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database. Further, the means and ranges of amino acid levels in cottonseed of TAM66274 were either within or comparable to values published in the literature for conventional cotton varieties, whether expressed on a mg/g DW basis (Table 6-3) or as percent of total amino acids (Table 6-4). Therefore, where differences in amounts of cystine and tryptophan were detected between TAM66274 and non-transgenic cv. Coker 312, these were not considered biologically meaningful and were most likely due to small genetic differences between TAM66274 and non-transgenic cv. Coker 312 resulting from the inherent genetic heterogeneity of the recipient non-transgenic cv. Coker 312, as explained above. Overall, these results show that amino acid levels in cottonseed of TAM66274 are comparable to levels in non-transgenic cv. Coker 312 and within the same range as cottonseed amino acid content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.4. Fatty acids.

Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from 2014 and 2015 field trials were analyzed for fatty acid content (all fatty acids measured are listed in Appendix E). The means and ranges of fatty acid levels in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-5. Fatty acid levels are expressed as a percent of total fatty acids.

Levels of the following fatty acids in cottonseed of TAM66274 and non-transgenic cv. Coker 312 were below the limits of quantification (LOQ) and are not included in Table 6-5: 8:0 Caprylic, 10:0 Capric, 12:0 Lauric, 14:1 Myristoleic, 15:0 Pentadecanoic, 15:1 Pentadecenoic, 17:1 Heptadecenoic, 18:3 Gamma linolenic, 18:4 Octadecatetraenoic, 20:2 Eicosadienoic, 20:3 Eicosatrienoic, 20:4 Arachidonic, 20:5 Eicosapentaenoic, 22:1 Erucic, 22:5 Docosapentaenoic, and 22:6 Docosahexaenoic. For the fatty acids which were measured above the LOQ in the cottonseed samples (14:0 Myristic, 16:0 Palmitic, 16:1 Palmitoleic, 17:0 Heptadecanoic, 18:0 Stearic, 18:1 Oleic, 18:2 Linoleic, 18:3 Linolenic, 20:0 Arachidic, 20:1 Eicosenoic and 22:0 Behenic), when the cottonseed fatty acid composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, for samples harvested from 2014 field trials, no statistically significant differences were observed between the treatments for levels of 16:1 Palmitoleic, 17:0 Heptadecanoic, and 18:1 Oleic, and statistically significant differences were observed between the treatments for levels of 14:0 Myristic, 16:0 Palmitic, 18:0 Stearic, 18:2 Linoleic, 18:3 Linolenic, 20:0 Arachidic, 20:1 Eicosenoic and 22:0 Behenic (Table 6-5). In the case of cottonseed samples harvested from 2015 field trials, statistically significant differences were observed between TAM66274 and non-transgenic cv. Coker 312 for all fatty acids except for 17:0 Heptadecanoic, 18:0 Stearic, 20:1 Eicosenoic and 22:0 Behenic (Table 6-5). However, the means and ranges of fatty acid levels in cottonseed of TAM66274 harvested from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database and in the literature for conventional cotton varieties (Table 6-5), except in the case of literature values for 20:1 Eicosenoic, which were below the limit of quantification in the one citation (Rudgers et al., 2013) available for this fatty acid. Therefore, where differences in amounts of some fatty acids were detected between the cottonseed of TAM66274 and non-transgenic cv. Coker 312, these were not considered biologically meaningful and were most likely due to small genetic differences between TAM66274 and non-transgenic cv. Coker 312 resulting from the inherent genetic heterogeneity of the recipient non-transgenic cv. Coker 312, as explained above. Overall, these results show that fatty acid levels in cottonseed of TAM66274 are comparable to levels in non-transgenic cv. Coker 312 and within the same range as cottonseed fatty acid content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.5. Minerals.

Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from the 2014 and 2015 field trials were analyzed for mineral content, including copper, iron, manganese, zinc,

calcium, magnesium, phosphorus, potassium and sodium. The means and ranges of mineral levels in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-6. The levels of minerals shown in Table 6-6 are expressed as parts per million (ppm) on a DW basis. When the cottonseed mineral composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, in the case of samples harvested from 2014 field trials, there were no statistically significant differences observed between the treatments for any of the minerals with the exception of potassium, and for cottonseed samples harvested from 2015 field trials, there were no statistically significant differences observed between TAM66274 and Coker 312 for any of the minerals with the exception of zinc (Table 6-6). Since statistically significant differences in potassium and zinc were not consistently observed between the treatments from 2014 and 2015 field studies, these analyte differences were not considered biologically meaningful. Furthermore, the means and ranges of mineral levels in cottonseed of TAM66274 from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database and in the literature for conventional cotton varieties. Therefore, these results show that mineral levels in cottonseed of TAM66274 are comparable to levels in non-transgenic cv. Coker 312 and within the same range as cottonseed mineral content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.6. Alpha-tocopherol.

Tocopherols are naturally present in cottonseed oil and serve as antioxidants that enhance food storage properties. Further, alpha-tocopherol has Vitamin E potency. Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from the 2014 and 2015 field trials were analyzed for alpha-tocopherol content. The means and ranges of alpha-tocopherol levels in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-7. The levels of alpha-tocopherol in cottonseed of the treatments are expressed as mg/100 g DW.

When the cottonseed alpha-tocopherol composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, levels of alpha-tocopherol in cottonseed of TAM66274 were statistically significantly different from levels in cottonseed of non-transgenic cv. Coker 312 in samples collected from both 2014 and 2015 field studies (Table 6-7). However, the means and ranges of alpha-tocopherol levels in cottonseed of TAM66274 from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database and in the literature for conventional cotton varieties. Therefore, where differences in levels of cottonseed alpha-tocopherol were detected between TAM66274 and non-transgenic cv. Coker 312, these were not considered biologically meaningful and were most likely due to small genetic differences between TAM66274 and non-transgenic cv. Coker 312 resulting from the inherent genetic heterogeneity of the recipient non-transgenic cv. Coker 312. Overall, these results show that alpha-tocopherol levels in cottonseed of TAM66274 are comparable to levels in

non-transgenic cv. Coker 312 and within the same range as cottonseed alpha-tocopherol content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.7. Phytic acid.

Phytic acid [myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)] is present in cottonseeds and is a storage form of biological phosphorus. However, phytic acid is considered an anti-nutrient since it is not digestible by monogastric animals and it chelates mineral nutrients in the diet, including phosphorus, calcium, magnesium, potassium, iron, and zinc, making them unavailable to animals consuming cottonseeds and cottonseed meal. Therefore, it is important to measure the phytic acid content of cottonseeds and cottonseed products to evaluate their nutritive value. Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from the 2014 and 2015 field trials were analyzed for phytic acid content. The means and ranges of phytic acid levels in cottonseed of TAM66274 and non-transgenic cv. Coker 312 as well as the ranges published in the ILSI Crop Composition Database are shown in Table 6-8. The levels of phytic acid in cottonseed of the treatments are expressed as a percent on a DW basis.

When the phytic acid composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, levels of phytic acid in cottonseed of TAM66274 were not statistically significantly different from levels in cottonseed of non-transgenic cv. Coker 312 in samples collected from both 2014 and 2015 field trials (Table 6-8). Further, the means and ranges of phytic acid levels in cottonseed of TAM66274 were either within or comparable to the range of values published in the ILSI Crop Composition Database. Therefore, these results show that phytic acid levels in cottonseed of TAM66274 are comparable to levels in non-transgenic cv. Coker 312 cottonseed and within the same range as cottonseed phytic acid content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.8. Cyclopropenoid fatty acids.

Cotton contains several cyclopropenoid fatty acids (CPFA) that are associated with the oil. Those identified that can be measured are malvalic, sterculic and dihydrosterculic acids (OECD, 2009). These CPFAs elevate the melting point of fats in animals fed whole cottonseed and cottonseed meal. The mechanism of action appears to be inhibition of desaturation of saturated fatty acids. Therefore, the CPFAs are considered anti-nutrients and must be minimized in cottonseed products used for feed purposes due to undesirable effects on specific livestock (OECD, 2009). Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from the 2014 and 2015 field trials were analyzed for CPFA content. The means and ranges of CPFA levels in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Table 6-9. The levels of CPFAs in cottonseed of the treatments are expressed as a percent of total fatty acids.

When the cottonseed CPFA composition of TAM66274 was compared to the composition of non-transgenic cv. Coker 312, in the case of samples harvested from 2014 field studies, levels of all three CPFAs in cottonseed of TAM66274 were statistically significantly less than levels in cottonseed of non-transgenic cv. Coker 312. However, in the case of cottonseed samples harvested from 2015 field trials, no statistically significant difference was observed between TAM66274 and non-transgenic cv. Coker 312 for levels of malvalic acid, but levels of sterculic and dihydrosterculic acids in cottonseed of TAM66274 were statistically significantly less than levels in cottonseed of non-transgenic cv. Coker 312. Even though there were statistically significant differences in levels of the CPFAs between TAM66274 and non-transgenic cv. Coker 312, the means and ranges of CPFA levels in cottonseed of TAM66274 from both 2014 and 2015 field trials were within ranges published in the ILSI Crop Composition Database and in the literature for conventional cotton varieties. Therefore, where differences in levels of cottonseed CPFAs were detected between TAM66274 and non-transgenic cv. Coker 312, these were not considered biologically meaningful and were most likely due to small genetic differences between TAM66274 and non-transgenic cv. Coker 312 resulting from the inherent genetic heterogeneity of the recipient non-transgenic cv. Coker 312. Overall, these results show that levels of CPFAs in cottonseed of TAM66274 are comparable to levels in non-transgenic cv. Coker 312 cottonseed and within the same range as cottonseed CPFA content of other conventional cotton varieties with a history of safe use as food and feed.

6.1.9. Gossypol.

Gossypol exists as either free or bound in the plant, and it is the free form of gossypol that is the toxic form of the compound (OECD, 2009). Further, due to steric hindrance between the functional groups of the molecule at the bond connecting the two naphthyl rings (atropisomerism), gossypol has both (+)- and (−)-isomers. In the seed of the commercially important varieties of Upland cotton grown in the United States, the predominant isomer is (+)-gossypol (Cass et al., 1991). Gossypol is physiologically active, with the (−)-isomer appearing to be more active than the (+)-isomer (Yu, 1987). Therefore, it is important to know the relative amounts of the isomers in livestock feeds. Accordingly, total and free gossypol was measured in samples, as well as the isomers of gossypol. Furthermore, total gossypol was measured by two different methods, the aniline and the HPLC methods, both described in Appendix E. The aniline method is a relatively fast method used for measuring gossypol in plant tissues, and is commonly used in the cotton industry. However, because the method also detects impurities and other terpenoids in addition to gossypol, the method can overestimate levels of gossypol in specific plant tissues (Chamkasem, 1988; Stipanovic et al., 1988). The HPLC method measures each terpenoid separately in plant tissues, so is a more accurate method for measuring gossypol in plant tissues. In the case of cottonseed, gossypol is the predominant terpenoid, so there is a good correlation between the aniline and HPLC methods for measuring gossypol (Stipanovic et al., 1988).

Cottonseed samples of TAM66274 and non-transgenic cv. Coker 312 harvested from 2014 and 2015 field trials were analyzed for total and free gossypol content, as well as for levels of the gossypol isomers. The means and ranges of levels of the gossypol fractions in cottonseed of TAM66274 and non-transgenic cv. Coker 312, as well as the ranges published in the ILSI Crop Composition Database and in the literature, are shown in Tables 6-10 and 6-11. Since the intended effect of the ULGCS trait in TAM66274 is ultra-low levels of gossypol in the cottonseed compared to non-transgenic cv. Coker 312, these data show that the ULGCS trait was expressed in TAM66274 and levels of all gossypol fractions in TAM66274 were statistically significantly reduced compared to the levels in non-transgenic cv. Coker 312 in cottonseed harvested from both 2014 and 2015 field trials. Levels of total gossypol in cottonseed of TAM66274 harvested from 2014 and 2015 field trials were 440 ppm and 420 ppm on a DW basis, respectively, compared to levels of 9,630 ppm and 9,410 ppm in cottonseed of non-transgenic cv. Coker 312 harvested from the same field trials, when using the aniline method of measurement. Total gossypol levels in cottonseed of TAM66274 harvested from 2014 and 2015 field trials were 4.57% and 4.46% of levels in non-transgenic cv. Coker 312, respectively. When total gossypol levels were compared between TAM66274 and non-transgenic cv. Coker 312 using the HPLC method of measurement, total gossypol levels in TAM66274 harvested from 2014 and 2015 field trials were 370 ppm and 300 ppm on a DW basis, respectively, compared to levels of 10,300 ppm and 10,000 ppm in cottonseed of non-transgenic cv. Coker 312 harvested from the same field trials. Total gossypol levels in cottonseed of TAM66274 harvested from 2014 and 2015 field trials were 3.61% and 3.00% of levels in non-transgenic cv. Coker 312, respectively. As described above, the HPLC method gives less experimental error and is more accurate than the aniline method for measuring levels of gossypol in cottonseed (Stipanovic et al., 1988). Therefore, total gossypol levels for the treatments measured by HPLC are considered more accurate than the aniline method as presented in Table 6-10. Further, the ultra-low levels of gossypol in cottonseed of TAM66274 were confirmed from total gossypol values calculated from the sum of levels of the isomers which were measured by an HPLC method (total gossypol levels of 256 and 283 ppm for cottonseed of TAM66274 harvested from 2014 and 2015 field trials, respectively, compared to 6,713 ppm and 6,932 ppm for non-transgenic cv. Coker 312 harvested from the same field trials) (Table 6-11).

The ULGCS trait did not have any meaningful effect on the relative levels of free and bound gossypol in the cottonseed, but the percent of the free form of the compound (which is the biologically active form) in total gossypol tended to be lower in TAM66274 compared to non-transgenic cv. Coker 312. Levels of free gossypol relative to total gossypol levels (levels compared using values from the aniline assay) were 80.7% for non-transgenic cv. Coker 312 and 68.2% for TAM66274 for cottonseed from 2014 field trials, and 88.2% for non-transgenic cv. Coker 312, 61.9% for TAM66274 for cottonseed harvested from 2015 field trials (Table 6-10). Furthermore, the ULGCS trait did not have any meaningful effect on the relative levels of the (+)- and (−)-gossypol isomers in the cottonseed. Ratios of levels of the (+)-gossypol isomer to

the (−)-gossypol isomer were 1.38 for non-transgenic cv. Coker 312 and 1.36 for TAM66274 for cottonseed from 2014 field trials, and were 1.54 for non-transgenic cv. Coker 312 and 1.30 for TAM66274 for cottonseed harvested from 2015 field trials (Table 6-11).

In summary, the data presented in Tables 6-10 and 6-11 show that the ULGCS trait was expressed in TAM66274 and, as expected, the level of total gossypol in cottonseed of TAM66274 was reduced to approximately 3% of levels in the non-transgenic cv. Coker 312 cottonseed. Using the more accurate and precise HPLC method for measurement, mean total gossypol levels in cottonseed of TAM66274 harvested from both 2014 and 2015 field trials were below the maximum allowable level of 450 ppm considered safe for modified cottonseed products in foods for human consumption (FDA, 1960; FDA, 1972; FDA, 1976) and below 400 ppm allowed in low-gossypol cottonseed meal used as animal feed (AAFCO, 1968a; AAFCO, 1968b). Also, the ULGCS trait did not have any meaningful effect on either the relative levels of free and bound gossypol in cottonseed, or on relative levels of (+)- and (−)-gossypol isomers in the cottonseed.

6.1.10. Mycotoxins.

Mycotoxins are toxic metabolites produced by certain fungi that can infect and proliferate on various agricultural commodities in the field and/or during storage. The occurrence of these toxins on grains, nuts and other commodities susceptible to mold infestation is influenced by environmental factors such as temperature, humidity, and extent of rainfall during the pre-harvesting, harvesting, and post-harvesting periods. Mycotoxins may exhibit various toxicological manifestations; some are teratogenic, mutagenic and/or carcinogenic in susceptible animal species, and are associated with various diseases in domestic animals, livestock, and humans in many parts of the world (D'Mello and Macdonald, 1997). The mycotoxins of concern in cottonseed are aflatoxins (OECD, 2009). Deoxynivalenol, acetyldeoxynivalenol, and zearalenone were also chosen for analysis based on reports of cottonseed contamination in U.S. production.

Aflatoxins are toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus*, opportunistic pathogens of crops (Klich, 2007). The aflatoxins of concern in cottonseed are designated B1, B2, G1, and G2, with B1 considered the most potent. Cottonseed meal exceeding 300 parts per billion (ppb) aflatoxin may not be used as a feed ingredient for beef cattle, swine, or poultry (FDA, 2000). Foods for human consumption are considered adulterated when aflatoxins exceed 20 ppb. Deoxynivalenol (DON), commonly called vomitoxin, is a natural toxin produced by several molds of the genus *Fusarium*, especially *F. graminearum*. DON has been associated with a number of adverse health effects in humans and animals. Current FDA advisory levels for DON are 10 ppm on grain and grain by-products intended for ruminating beef and feedlot cattle older than 4 months, ruminating dairy cattle older than 4 months, and poultry; 5 ppm on grain and grain by-products intended for swine; and 5 ppm on grain and grain by-products destined for

all other animals (FDA, 2010). Acetyldeoxynivalenol (ADON) is an acetyl derivative of DON, typically a 3-acetyl or 15-acetyl substitution, and is another in the class of trichothecenes that are potent inhibitors of eukaryotic protein synthesis produced by *Fusarium* spp. Zearalenone is a mycotoxin that mimics the reproductive hormone estrogen and affects reproduction. This mycotoxin is produced primarily by *F. graminearum*, the same fungus that produces deoxynivalenol in maize and small grains.

While gossypol has antimicrobial activity, its role in suppressing opportunistic fungal infection of cottonseed has not been reported. Therefore, the purpose of the current study was to determine if the ULGCS trait of TAM66274 conferred any difference in susceptibility to the mycotoxins described above, compared to non-transgenic cv. Coker 312. A single composite sample of each treatment (TAM66274 or non-transgenic cv. Coker 312) was obtained from plants grown in replicated field trials at five locations in the U.S. (two sites in NC, two sites in MS, one site in TX) during the summer of 2015. Location samples were an equal representation of four replicated plots of each treatment at each field location. Accordingly, statistical analysis of variability within each field location was not possible. Mycotoxin analysis was performed by Romer Laboratories, Inc. (Union, MO) using high performance liquid chromatography and liquid chromatography-dual mass spectrometry (described in Appendix E).

The levels of aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 in all samples of TAM66274 and non-transgenic cv. Coker 312 were below the assay limits of detection (LOD). Additionally, no sample exceeded the FDA action level of 20 ppb aflatoxin in food for human consumption (Table 6-12).

In four of five samples of TAM66274, the level of deoxynivalenol was below the assay LOD (<0.6 ppb) and one sample had a detectable level of 1.6 ppb (Table 6-13; Table E-12 in Appendix E). In non-transgenic cv. Coker 312, four of five samples contained deoxynivalenol levels below the assay LOD and one sample had a detectable level of 0.8 ppb (Table 6-13; Table E-12 in Appendix E). The two samples with detectable deoxynivalenol levels were both from plants grown at the same site [NC315 (Table E-12 in Appendix E)], which suggests a localized fungal infection not detected elsewhere. No sample exceeded the FDA advisory level for grain or grain by-products destined for livestock or other animals. A single sample (TAM66274, 1.6 ppb) exceeded the advisory level for finished wheat products intended for human consumption. However, there is currently no FDA action, advisory, or guidance levels established for deoxynivalenol in foods other than finished wheat products, and FDA does not routinely screen foods other than finished wheat products for the presence of deoxynivalenol in either domestic or imported foods (FDA, 2016). The levels of acetyldeoxynivalenol and zearalenone in seed of TAM66274 and non-transgenic cv. Coker 312 were undetectable in all samples (Table 6-13).

More than 80% of the observed values for each analyte in this study were less than the assay LOD. Data sets containing values below the LOD are known as censored data sets and various statistical techniques exist to analyze left-censored data sets (Croghan and Egeghy, 2003; Helsel, 2012). For example, substitution methods that replace values below the LOD with a constant (e.g., zero, LOD/2, LOD/√2) may over- or underestimate both the mean and standard deviation. Parametric distribution estimators are more statistically rigorous methods of handling left-censored data sets, but are valid only if less than 80% of observed values are below LOD. For very large amounts of censoring (>80%), the mean and standard deviation cannot be reliably estimated (Helsel, 2012). Due to these statistical constraints, statistical analysis of the data was not performed.

In summary, mycotoxin analyses showed that levels of aflatoxins G1, G2, B1 and B2, as well as deoxynivalenol, acetyldeoxynivalenol, and zearalenone, in cottonseed of TAM66274 are comparable to non-transgenic cv. Coker 312. The introduction of plasmid pART27-LCT66 into the genome of non-transgenic cv. Coker 312 to achieve the ULGCS trait did not appear to affect mycotoxin levels of cottonseed produced by TAM66274 or alter the susceptibility of TAM66274 cottonseed to mycotoxins relative to non-transgenic cv. Coker 312. However, since more than 80% of the observed values for each analyte were less than the assay limits of detection, statistical analysis was not possible.

6.2 Conclusions of Composition Assessment of TAM66274 Cottonseed

Texas A&M University developed TAM66274 by RNAi-mediated suppression of genes that encode dCS, a key enzyme in gossypol biosynthesis to achieve ultra-low levels of the anti-nutrient gossypol in the cottonseed. Reduction of gossypol levels in TAM66274 cottonseed makes TAM66274 cottonseed safe for use as feed for various monogastric animals and as human food. The purpose of the compositional and nutritional assessment of TAM66274 was two-fold: to confirm the intended technical effect of genetic modification in TAM66274; and to evaluate the nutrient and anti-nutrient levels in cottonseed of TAM66274 to confirm that, other than the intended ULGCS trait, TAM66274 is compositionally equivalent to non-transgenic cv. Coker 312 and other conventional cotton varieties and, therefore, is appropriate for conventional uses of cottonseed in food and feed.

Cottonseed of TAM66274 and non-transgenic cv. Coker 312 was produced from plants grown in replicated field trials at three locations in the U.S. during the summer of 2014, and at five locations in the U.S. during the summer of 2015. The components analyzed included proximates, fiber (total dietary, crude, acid and neutral detergent fibers), fatty acids, amino acids, minerals, alpha-tocopherol, and anti-nutrients (total and free gossypol, gossypol isomers, cyclopropenoid fatty acids and phytic acid). Further, cottonseed harvested from the five field trials in 2015 was analyzed for mycotoxins. Compositional analyses showed that the intended ULGCS trait was expressed in TAM66274, with mean levels of total gossypol in cottonseed of TAM66274

harvested from 2014 and 2015 field trials of 370 and 300 ppm on a DW basis, respectively, compared to levels of 10,300 ppm and 10,000 ppm in cottonseed of non-transgenic cv. Coker 312 harvested from the same field trials (values are based on HPLC analyses which are more accurate and precise than values from the aniline method). Also, the ULGCS trait did not have any meaningful effect on the ratios of free and bound gossypol or the gossypol isomers in cottonseed of TAM66274 compared to non-transgenic cv. Coker 312. Compositional analyses also showed that, other than the intended reduction in gossypol levels, TAM66274 cottonseed is compositionally equivalent to non-transgenic cv. Coker 312. Where statistically significant differences in amounts of individual nutritional constituents were detected between the cottonseed of TAM66274 and non-transgenic cv. Coker 312, the analyte values for TAM66274 were within the range of values for conventional cotton varieties published in the International Life Sciences Institute (ILSI) crop composition database and in the published literature for conventional cotton varieties. Therefore, these instances of differences in analyte levels between cottonseed of TAM66274 and non-transgenic cv. Coker 312 were not considered biologically meaningful, but were most likely due to small genetic differences between TAM66274 and non-transgenic cv. Coker 312 resulting from the inherent genetic heterogeneity of the recipient non-transgenic cv. Coker 312.

Further, the cottonseed harvested from TAM66274 and non-transgenic cv. Coker 312 grown in the 2015 field trials was analyzed for mycotoxins. Results of analyses showed no difference in mycotoxin levels in cottonseed of TAM66274 compared to non-transgenic cv. Coker 312. Therefore, the ULGCS trait does not appear to alter susceptibility of TAM66274 cottonseed to mycotoxins compared to non-transgenic cv. Coker 312.

In summary, these compositional analyses demonstrated that introduction of plasmid pART27-LCT66 into the genome of non-transgenic cv. Coker 312 to produce TAM66274 achieved the intended effect of significantly reducing total seed gossypol levels compared to the non-transgenic cv. Coker 312. The mean total gossypol levels in cottonseed of TAM66274 are also well below established safety standards for modified cottonseed products used in human food (450 ppm) and animal feed (400 ppm). Further, the introduction of plasmid pART27-LCT66 into the non-transgenic cv. Coker 312 genome did not significantly affect the nutritional composition of cottonseed produced by TAM66274. Results of these analyses demonstrate that, other than the intended reduction in cottonseed gossypol levels, cottonseed from TAM66274 is compositionally equivalent to and as nutritious as cottonseed from non-transgenic cv. Coker 312, as well as other conventional cotton varieties, and is appropriate for food and feed uses. The compositional analysis supports the conclusion that TAM66274 cottonseed poses no greater plant pest risk than conventional cottonseed.

Table 6-1. Cottonseed proximate composition.
Comparison of the proximate composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations for each year separately, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Moisture is expressed on a FW basis, and the other proximates on a DW basis.

	Analytes					
	Moisture (% FW)	**Protein (% DW)**	**Total Fat (% DW)**	**Ash (% DW)**	**Carbohydrates (% DW)**	**Calories (Kcal/100 g DW)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**					
Coker 312	7.47 ± 0.13 (7.02-7.80)	27.8 ± 0.5 (26.7-29.5)	23.0 ± 0.3 (21.9-23.6)	3.90 ± 0.05 (3.71-4.25)	45.4 ± 0.4 (44.8-46.0)	499 ± 2 (495-503)
TAM66274	7.47 ± 0.13 (6.99-7.92)	27.5 ± 0.5 (26.9-28.1)	22.5 ± 0.3 (21.4-23.5)	3.88 ± 0.05 (3.62-4.16)	46.2 ± 0.4 (45.5-46.8)	497 ± 2 (493-501)
Significance (p-value) of TAM66274 vs. Coker 312	1.0000	0.6905	0.2906	0.8639	0.2179	0.3340
Treatments	**2015 Studies Mean ± S.E.M. (Range)**					
Coker 312	7.91 ± 0.06 (7.52-8.30)	29.0 ± 0.3 (26.8-30.8)	23.0 ± 0.1 (22.0-23.8)	4.26 ± 0.05 (3.98-4.43)	43.7 ± 0.4 (42.5-45.1)	499 ± 1 (493-502)
TAM66274	8.28 ± 0.06 (7.93-8.59)	28.0 ± 0.3 (25.8-29.2)	21.7 ± 0.1 (20.5-22.6)	4.24 ± 0.05 (4.07-4.40)	46.0 ± 0.4 (45.3-47.6)	491 ± 1 (486-496)
Significance (p-value) of TAM66274 vs. Coker 312	0.0027†	0.6905	0.0001†	0.8408	0.0016†	0.0001†
ILSI CCDB range of analyte values						
Min	2.30	19.19	15.05	3.01	39.00	407.41
Max	11.2	32.97	27.90	5.48	59.20	520.68
Literature range of analyte values						
Min	2.25[a]	12.00[c]	14.40[d]	3.53[e]	41.00[f]	466.09[a]
Max	15.9[b]	32.00[c]	27.90[e]	5.29[a]	54.90[g]	512.65[g]

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Berberich et al. (1996); [c]Kohel et al. (1985); [d]Bertrand et al. (2005); [e]Rudgers (2013); [f]Nida et al. (1996); [g]Arackal et al. (2012).

Table 6-2. Cottonseed fiber composition.
Comparison of the fiber composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations for each year separately, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Fiber levels are expressed on a percent DW basis.

	Analytes			
	Crude Fiber (% DW)	**Total Dietary Fiber (% DW)**	**Acid Detergent Fiber (% DW)**	**Neutral Detergent Fiber (% DW)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**			
Coker 312	20.2 ± 0.5 (19.5-20.8)	41.9 ± 0.5 (40.3-43.3)	26.3 ± 0.3 (25.8-27.2)	34.9 ± 0.2 (32.9-35.9)
TAM66274	21.4 ± 0.5 (20.9-22.1)	44.3 ± 0.5 (43.5-45.7)	27.9 ± 0.3 (26.7-29.0)	36.7 ± 0.2 (34.5-38.1)
Significance (p-value) of TAM66274 vs. Coker 312	0.1323	0.0193†	0.0182†	0.0015†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**			
Coker 312	19.6 ± 0.3 (17.6-21.0)	38.6 ± 0.6 (37.4-40.5)	26.0 ± 0.3 (24.6-27.0)	32.3 ± 0.5 (30.6-34.6)
TAM66274	20.7 ± 0.3 (18.9-21.8)	41.6 ± 0.6 (39.1-44.2)	28.4 ± 0.3 (26.6-29.1)	37.0 ± 0.5 (34.7-38.2)
Significance (p-value) of TAM66274 vs. Coker 312	0.0583	0.0069†	0.0009†	0.0001†
ILSI CCDB range of analyte values				
Min	13.86	33.69	19.74	25.56
Max	24.50	53.50	38.95	51.87
Literature range of analyte values				
Min	13.85[a]	37.29[d]	20.40[c]	27.20[c]
Max	23.50[c]	51.30[c]	40.50[b]	53.60[b]

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Bertrand et al. (2005); [c]Rudgers (2013); [d]Arackal et al. (2012).

Table 6-3. Cottonseed amino acid composition.
Comparison of the amino acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations for each year separately, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Amino acid levels are expressed on a DW basis.

	Analytes				
	Alanine (mg/g DW)	**Arginine (mg/g DW)**	**Aspartic Acid (mg/g DW)**	**Cystine (mg/g DW)**	**Glutamic Acid (mg/g DW)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**				
Coker 312	10.3 ± 0.1 (9.95-10.7)	30.5 ± 0.5 (28.8-32.4)	24 ± 0.3 (22.8-25.2)	4.52 ± 0.06 (4.42-4.71)	51.1 ± 0.9 (48.6-53.6)
TAM66274	10.7 ± 0.1 (10.3-11.2)	30.4 ± 0.5 (29.2-32.3)	24.6 ± 0.3 (23.7-25.9)	4.82 ± 0.06 (4.55-5.01)	52.3 ± 0.9 (50.2-55.0)
Significance (p-value) of TAM66274 vs. Coker 312	0.0775	0.8860	0.2211	0.0261†	0.3710
Treatments	**2015 Studies Mean ± S.E.M. (Range)**				
Coker 312	11.2 ± 0.2 (10.6-12.4)	33.2 ± 0.6 (30.6-37.6)	25.9 ± 0.4 (23.7-28.6)	4.89 ± 0.07 (4.40-5.34)	54.0 ± 0.8 (49.4-59.1)
TAM66274	11.1 ± 0.2 (10.3-11.7)	31.6 ± 0.6 (29.1-33.1)	25.0 ± 0.4 (23.1-26.0)	4.62 ± 0.07 (4.29-4.95)	51.5 ± 0.8 (47.9-54.6)
Significance (p-value) of TAM66274 vs. Coker 312	0.6305	0.1002	0.1466	0.0256†	0.0510
ILSI CCDB range of analyte values					
Min	6.9	17.6	15.1	2.9	30.4
Max	12.9	39.3	32.1	5.6	67.2
Literature range of analyte values					
Min[a]	8.30	23.0	17.9	2.90	33.9
Max[a]	12.2	35.5	27.2	4.70	54.5

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Arackal et al. (2012).

Table 6-3, continued. Cottonseed amino acid composition.
Comparison of the amino acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations for each year separately, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Amino acid levels are expressed on a DW basis.

	Analytes				
	Glycine (mg/g DW)	**Histidine (mg/g DW)**	**Isoleucine (mg/g DW)**	**Leucine (mg/g DW)**	**Lysine (mg/g DW)**
Treatments	**2014 Studies** **Mean ± S.E.M. (Range)**				
Coker 312	10.9 ± 0.1 (10.5-11.3)	7.29 ± 0.16 (6.69-7.68)	8.61 ± 0.12 (8.26-8.90)	15.3 ± 0.2 (14.6-15.9)	10.9 ± 0.2 (10.2-11.3)
TAM66274	11.3 ± 0.1 (11.0-11.8)	7.67 ± 0.16 (7.46-8.02)	8.96 ± 0.12 (8.62-9.29)	15.9 ± 0.2 (15.5-16.5)	11.4 ± 0.2 (11.2-11.8)
Significance (p-value) of TAM66274 vs. Coker 312	0.0782	0.1722	0.1122	0.0973	0.1233
Treatments	**2015 Studies** **Mean ± S.E.M. (Range)**				
Coker 312	11.4 ± 0.2 (10.7-12.4)	7.70 ± 0.13 (7.25-8.46)	9.14 ± 0.12 (8.59-9.95)	16.2 ± 0.2 (15.3-17.5)	12.0 ± 0.2 (11.4-13.0)
TAM66274	11.1 ± 0.2 (10.5-11.4)	7.37 ± 0.13 (6.94-7.67)	8.88 ± 0.12 (8.22-9.26)	15.7 ± 0.2 (14.6-16.1)	11.7 ± 0.2 (11.0-12.1)
Significance (p-value) of TAM66274 vs. Coker 312	0.2237	0.1090	0.1580	0.1304	0.2177
ILSI CCDB range of analyte values					
Min	7.3	4.52	5.81	10.1	8.37
Max	13.3	9.85	10.5	18.6	14.6
Literature range of analyte values					
Min[a]	8.50	5.70	7.20	12.0	9.90
Max[a]	12.3	8.40	10.3	17.2	14.4

†Mean analyte values of event TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Arackal et al. (2012).

Table 6-3, continued. Cottonseed amino acid composition.
Comparison of the amino acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations for each year separately, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Amino acid levels are expressed on a DW basis.

	Analytes			
	Methionine (mg/g DW)	**Phenylalanine (mg/g DW)**	**Proline (mg/g DW)**	**Serine (mg/g DW)**
Treatments	**2014 Studies** **Mean ± S.E.M. (Range)**			
Coker 312	4.10 ± 0.12 (3.83-4.27)	14.5 ± 0.2 (13.8-15.1)	9.74 ± 0.11 (9.25-10.2)	11.3 ± 0.2 (10.8-11.8)
TAM66274	4.16 ± 0.12 (3.97-4.33)	15.0 ± 0.2 (14.5-15.8)	10.14 ± 0.11 (9.88-10.6)	11.7 ± 0.2 (11.3-12.2)
Significance (p-value) of TAM66274 vs. Coker 312	0.7217	0.1492	0.0617	0.1328
Treatments	**2015 Studies** **Mean ± S.E.M. (Range)**			
Coker 312	4.17 ± 0.07 (3.91-4.47)	15.3 ± 0.2 (14.4-16.8)	10.55 ± 0.19 (9.86-11.8)	12.3 ± 0.2 (11.4-13.3)
TAM66274	4.16 ± 0.07 (3.95-4.29)	14.7 ± 0.2 (13.5-15.2)	10.50 ± 0.19 (9.49-11.5)	12.0 ± 0.2 (11.4-12.5)
Significance (p-value) of TAM66274 vs. Coker 312	0.9547	0.1098	0.8422	0.2289
ILSI CCDB range of analyte values				
Min	2.9	8.79	6.0	7.4
Max	4.9	17.6	13.7	13.9
Literature range of analyte values				
Min[a]	2.90	11.0	7.90	8.10
Max[a]	4.90	16.3	11.7	12.4

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Arackal et al. (2012).

Table 6-3, continued. Cottonseed amino acid composition.
Comparison of the amino acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations for each year separately, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Amino acid levels are expressed on a DW basis.

	Analytes			
	Threonine (mg/g DW)	**Tryptophan (mg/g DW)**	**Tyrosine (mg/g DW)**	**Valine (mg/g DW)**
Treatments	**2014 Studies** **Mean ± S.E.M. (Range)**			
Coker 312	8.52 ± 0.12 (8.16-8.80)	3.59 ± 0.18 (3.50-3.67)	8.16 ± 0.12 (7.77-8.18)	11.9 ± 0.2 (11.4-12.3)
TAM66274	8.94 ± 0.12 (8.62-9.29)	3.57 ± 0.18 (3.38-3.69)	8.54 ± 0.12 (8.26-8.92)	12.4 ± 0.2 (12.1-13.1)
Significance (p-value) of TAM66274 vs. Coker 312	0.0611	0.8645	0.0801	0.1052
Treatments	**2015 Studies** **Mean ± S.E.M. (Range)**			
Coker 312	9.09 ± 0.13 (8.64-9.81)	4.01 ± 0.06 (3.83-4.30)	8.78 ± 0.12 (8.26-9.51)	12.3 ± 0.2 (11.6-13.5)
TAM66274	8.95 ± 0.13 (8.35-9.19)	3.76 ± 0.06 (3.51-4.02)	8.51 ± 0.12 (7.92-8.90)	11.9 ± 0.2 (11.2-12.3)
Significance (p-value) of TAM66274 vs. Coker 312	0.4649	0.0138†	0.1527	0.1282
ILSI CCDB range of analyte values				
Min	5.5	1.62	4.66	7.6
Max	10.6	5.19	9.98	14.9
Literature range of analyte values				
Min[a]	6.70	3.10	6.30	9.70
Max[a]	9.60	4.60	9.10	13.6

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Arackal et al. (2012).

Table 6-4. Cottonseed amino acid composition expressed as percent of total amino acids for TAM66274 compared to literature values.

Amino acid composition of cottonseed from TAM66274 grown in three U.S. field locations in 2014 and five U.S. five locations in 2015 compared to literature values. Analyte levels in TAM662274 are presented as the means and the range across field locations, and levels are compared with the range of analyte levels reported in the literature for cottonseed from conventional cotton varieties. Amino acid levels are expressed on a percent of total amino acids.

	Analytes (% of total amino acids)								
	Alanine	**Arginine**	**Aspartic Acid**	**Cystine**	**Glutamic Acid**	**Glycine**	**Histidine**	**Isoleucine**	**Leucine**
Treatment	**2014 Studies Means (Ranges)**								
TAM66274	4.25 (4.22-4.29)	12.0 (11.9-12.2)	9.76 (9.71-9.78)	1.90 (1.86-1.96)	20.7 (20.5-20.8)	4.48 (4.45-4.51)	3.03 (3.01-3.06)	3.54 (3.51-3.59)	6.29 (6.24-6.35)
Treatment	**2015 Studies Means (Ranges)**								
TAM66274	4.37 (4.14-4.68)	12.5 (12.3-12.6)	9.87 (9.78-9.98)	1.83 (1.77-1.89)	20.3 (20.0-20.8)	4.39 (4.31-4.46)	2.91 (2.79-2.97)	3.51 (3.47-3.58)	6.21 (6.13-6.30)
Literature range of analyte values									
Min	4.16[b]	10.9[c]	8.80[c]	1.59[a]	19.4[a]	3.80[c]	2.60[c]	3.10[b]	6.04[a]
Max	4.65[a]	13.6[a]	11.4[a]	3.40[c]	22.4[c]	4.59[a]	3.12[b]	3.82[a]	6.65[b]

Literature ranges of analyte values: [a]Rudgers (2013); [b]Hamilton et al. (2004); [c]Lawhon et al. (1977).

Table 6-4, continued. Cottonseed amino acid composition expressed as percent of total amino acids for TAM66274 compared to literature values.

Amino acid composition of cottonseed from TAM66274 grown in three U.S. field locations in 2014 and five U.S. five locations in 2015 compared to literature values. Analyte levels in TAM662274 are presented as the means and the range across field locations, and levels are compared with the range of analyte levels reported in the literature for cottonseed from conventional cotton varieties. Amino acid levels are expressed on a percent of total amino acids.

	Analytes (% of total amino acids)								
	Lysine	**Methionine**	**Phenylalanine**	**Proline**	**Serine**	**Threonine**	**Tryptophan**	**Tyrosine**	**Valine**
Treatment	**2014 Studies Means (Ranges)**								
TAM66274	4.51 (4.49-4.59)	1.65 (1.58-1.74)	5.92 (5.81-5.98)	4.01 (3.99-4.04)	4.64 (4.62-4.69)	3.54 (3.51-3.57)	1.41 (1.35-1.49)	3.38 (3.37-3.38)	4.93 (4.85-4.96)
Treatment	**2015 Studies Means (Ranges)**								
TAM66274	4.65 (4.57-4.73)	1.65 (1.60-1.71)	5.81 (5.75-5.88)	4.14 (4.03-4.44)	4.64 (4.66-4.82)	3.54 (3.50-3.62)	1.49 (1.35-1.62)	3.36 (3.33-3.39)	4.69 (4.60-4.74)
Literature range of analyte values									
Min	4.27[a]	1.34[a]	5.44[a]	3.78[a]	3.90[c]	3.19[a]	0.97[b]	2.65[b]	4.30[c]
Max	5.37[b]	1.88[b]	6.02[a]	4.28[b]	5.05[a]	3.75[b]	1.68[a]	3.46[a]	5.14[b]

Literature ranges of analyte values: [a]Rudgers (2013); [b]Hamilton et al. (2004); [c]Lawhon et al. (1977).

Table 6-5. Cottonseed fatty acid composition.
Comparison of the fatty acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Fatty acid levels are expressed as a percent of total fatty acids.

	Analytes (% of total fatty acids)			
	14:0 Myristic (%)	**16:0 Palmitic (%)**	**16:1 Palmitoleic (%)**	**17:0 Heptadecanoic (%)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**			
Coker 312	0.700 ± 0.010 (0.682-0.727)	22.6 ± 0.1 (22.3-22.9)	0.462 ± 0.008 (0.455-0.467)	0.084 ± 0.001 (0.083-0.086)
TAM66274	0.541 ± 0.10 (0.531-0.555)	21.0 ± 0.1 (20.7-21.4)	0.459 ± 0.008 (0.454-0.467)	0.083 ± 0.001 (0.081-0.085)
Significance (p-value) of TAM66274 vs. Coker 312	0.0003†	0.0009†	0.8202	0.6531
Treatments	**2015 Studies Mean ± S.E.M. (Range)**			
Coker 312	0.897 ± 0.11 (0.772-1.15)	25.1 ± 0.1 (23.3-28.0)	0.547 ± 0.006 (0.503-0.619)	0.085 ± 0.002 (0.078-0.089)
TAM66274	0.651 ± 0.11 (0.539-0.839)	22.4 ± 0.1 (21.0-24.9)	0.495 ± 0.006 (0.467-0.559)	0.089 ± 0.002 (0.086-0.092)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†	0.0001†	0.0003†	0.2203
ILSI CCDB range of analyte values				
Min	0.426	15.1	0.375	0.077
Max	2.40	27.9	1.190	1.12
Literature range of analyte values				
Min	0.432[b]	18.7[b]	0.378[b]	<LOQ[b]
Max	2.40[a]	27.9[a]	1.16[a]	0.108[b]

LOQ. Limit of Quantification
†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Rudgers (2013).

Table 6-5, continued. Cottonseed fatty acid composition.
Comparison of the fatty acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Fatty acid levels are expressed as a percent of total fatty acids.

	Analytes (% of total fatty acids)			
	18:0 Stearic (%)	**18:1 Oleic (%)**	**18:2 Linoleic (%)**	**18:3 Linolenic (%)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**			
Coker 312	2.19 ± 0.02 (2.13-2.23)	13.7 ± 0.2 (13.3-14.1)	58.7 ± 0.3 (58.3-58.9)	0.150 ± 0.001 (0.148-0.152)
TAM66274	2.07 ± 0.02 (2.00-2.12)	14.0 ± 0.2 (13.8-14.3)	60.4 ± 0.3 (60.3-60.5)	0.168 ± 0.001 (0.165-0.172)
Significance (p-value) of TAM66274 vs. Coker 312	0.0080†	0.2720	0.0099†	0.0003†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**			
Coker 312	2.27 ± 0.03 (2.16-2.38)	14.1 ± 0.2 (13.3-15.6)	55.6 ± 0.2 (51.0-57.9)	0.150 ± 0.003 (0.123-0.166)
TAM66274	2.24 ± 0.03 (2.13-2.30)	14.8 ± 0.2 (13.8-16.2)	58.0 ± 0.2 (53.9-60.4)	0.178 ± 0.003 (0.137-0.202)
Significance (p-value) of TAM66274 vs. Coker 312	0.4643	0.0138†	0.0001†	0.0001†
ILSI CCDB range of analyte values				
Min	0.20	12.8	42.5	0.100
Max	3.54	25.4	63.0	0.640
Literature range of analyte values				
Min	1.80[b]	12.9[b]	46.0[a]	0.050[a]
Max	3.11[a]	20.7[c]	63.9[b]	0.290[d]

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Rudgers (2013); [c]Lawhon et al. (1977); [d]Arackal et al. (2012).

Table 6-5, continued. Cottonseed fatty acid composition.
Comparison of the fatty acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Fatty acid levels are expressed as a percent of total fatty acids.

	Analytes (% of total fatty acids)		
	20:0 Arachidic (%)	**20:1 Eicosenoic (%)**	**22:0 Behenic (%)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**		
Coker 312	0.256 ± 0.001 (0.254-0.259)	0.064 ± 0.001 (0.061-0.067)	0.128 ± 0.002 (0.125-0.133)
TAM66274	0.228 ± 0.001 (0.223-0.235)	0.071 ± 0.001 (0.069-0.072)	0.117 ± 0.002 (0.112-0.121)
Significance (p-value) of TAM66274 vs. Coker 312	0.0008†	0.0082†	0.0149†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**		
Coker 312	0.290 ± 0.005 (0.259-0.331)	0.062 ± 0.002 (<LOQ-0.067)	0.141 ± 0.006 (0.109-0.164)
TAM66274	0.273 ± 0.005 (0.247-0.298)	0.072 ± 0.002 (<LOQ-0.077)	0.123 ± 0.006 (0.093-0.154)
Significance (p-value) of TAM66274 vs. Coker 312	0.0461†	0.1839	0.0631
ILSI CCDB range of analyte values			
Min	0.149	0.095	0.099
Max	0.484	0.100	0.295
Literature range of analyte values			
Min	0.185[a]	<LOQ[a]	0.051[b]
Max	0.360[b]	<LOQ[a]	0.190[b]

LOQ. Limit of Quantification
†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Rudgers (2013); [b]Arackal et al. (2012).

Table 6-6. Cottonseed mineral composition.
Comparison of the mineral composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Mineral levels are expressed as parts per million (ppm) on a DW basis.

	Analytes				
	Copper (ppm)	**Iron (ppm)**	**Manganese (ppm)**	**Zinc (ppm)**	**Calcium (ppm)**
Treatments	**2014 Studies** **Mean ± S.E.M. (Range)**				
Coker 312	8.69 ± 0.20 (8.54-8.97)	45.8 ± 1.1 (42.2-51.9)	12.5 ± 0.2 (10.8-14.1)	42.3 ± 0.4 (38.6-47.9)	1057 ± 40 (1030-1090)
TAM66274	9.20 ± 0.20 (8.84-9.55)	45.9 ± 1.1 (42.7-49.8)	12.9 ± 0.2 (11.5-14.0)	42.3 ± 0.4 (39.4-47.4)	1046 ± 40 (999-1090)
Significance (p-value) of TAM66274 vs. Coker 312	0.1486	0.9379	0.3937	1.0000	0.8626
Treatments	**2015 Studies** **Mean ± S.E.M. (Range)**				
Coker 312	8.97 ± 0.23 (5.15-11.5)	50.0 ± 0.9 (46.3-53.7)	13.6 ± 0.4 (11.7-16.0)	49.3 ± 1.3 (39.2-57.4)	1437 ± 56 (984-1980)
TAM66274	9.20 ± 0.23 (5.93-11.9)	50.3 ± 0.9 (44.9-54.6)	14.6 ± 0.4 (11.6-18.2)	44.7 ± 1.3 (37.3-53.0)	1546 ± 56 (1120-2160)
Significance (p-value) of TAM66274 vs. Coker 312	0.4901	0.8467	0.0984	0.0396†	0.2042
ILSI CCDB range of analyte values					
Min	2.62	27.2	8.61	20.0	698
Max	24.6	318	24.8	64.5	3258
Literature range of analyte values					
Min	3.54[a]	34.3[b]	9.07[c]	25.1[c]	789[b]
Max	14.4[b]	114[c]	22.8[b]	48.5[c]	3300[a]

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Rudgers (2013); [c]Arackal et al. (2012).

Table 6-6, continued. Cottonseed mineral composition.
Comparison of the mineral composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Mineral levels are expressed as parts per million (ppm) on a DW basis.

	Analytes			
	Magnesium (ppm)	**Phosphorus (ppm)**	**Potassium (ppm)**	**Sodium (ppm)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**			
Coker 312	4053 ± 57 (3850-4390)	6357 ± 148 (5450-7570)	10800 ± 111 (10500-11200)	967 ± 34 (796-1110)
TAM66274	3843 ± 57 (3630-4030)	6310 ± 148 (5600-7110)	11267 ± 111 (10900-11500)	873 ± 34 (824-923)
Significance (p-value) of TAM66274 vs. Coker 312	0.0596	0.8345	0.0405†	0.1224
Treatments	**2015 Studies Mean ± S.E.M. (Range)**			
Coker 312	3980 ± 61 (3590-4360)	6440 ± 161 (5630-7440)	11078 ± 70 (9890-12200)	925 ± 54 (793-1100)
TAM66274	3816 ± 61 (3660-4080)	6490 ± 161 (5650-7150)	10940 ± 70 (10000-11700)	878 ± 54 (800-917)
Significance (p-value) of TAM66274 vs. Coker 312	0.0950	0.8320	0.2036	0.5514
ILSI CCDB range of analyte values				
Min	2625	3842	7894	112
Max	4931	9916	14483	7355
Literature range of analyte values				
Min	2850[b]	4600[b]	9000[c]	54[a]
Max	4700[b]	9010[b]	12900[b]	7400[a]

†Mean analyte values of TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Rudgers (2013); [c]Arackal et al. (2012).

Table 6-7. Cottonseed alpha-tocopherol composition.
Comparison of the alpha tocopherol composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Alpha tocopherol levels are expressed as mg/100 g DW.

	Analyte
	Alpha-tocopherol (mg/100 g DW)
Treatments	**2014 Studies** **Mean ± S.E.M. (Range)**
Coker 312	13.4 ± 0.5 (12.0-15.4)
TAM66274	11.1 ± 0.5 (10.3-12.3)
Significance (p-value) of TAM66274 vs. Coker 312	0.0232†
Treatments	**2015 Studies** **Mean ± S.E.M. (Range)**
Coker 312	17.1 ± 0.2 (15.6-18.4)
TAM66274	14.8 ± 0.2 (13.4-15.8)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†
ILSI CCDB range of analyte values	
Min	2.66
Max	19.7
Literature range of analyte values	
Min	3.11[b]
Max	16.2[a]

†Mean analyte values for TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Arackal et al. (2012); [b]Rudgers (2013).

Table 6-8. Cottonseed phytic acid composition.
Comparison of the phytic acid composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Analyte levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of analyte levels reported in the ILSI Crop Composition Database (ILSI, 2016). Phytic acid levels are expressed as a percent on a DW basis.

	Analyte
	Phytic Acid (% DW)
Treatments	**2014 Studies** **Mean ± S.E.M. (Range)**
Coker 312	1.79 ± 0.07 (1.49-2.19)
TAM66274	1.75 ± 0.07 (1.51-2.01)
Significance (p-value) of TAM66274 vs. Coker 312	0.6937
Treatments	**2015 Studies** **Mean ± S.E.M. (Range)**
Coker 312	1.73 ± 0.06 (1.51-1.93)
TAM66274	1.75 ± 0.06 (1.49-1.88)
Significance (p-value) of TAM66274 vs. Coker 312	0.8633
ILSI CCDB range of analyte values	
Min	1.64
Max	1.94
Literature range of analyte values	
Min	N.R.
Max	N.R.

†Mean analyte values for TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
N.R. Not reported.

Table 6-9. Cottonseed cyclopropenoid fatty acid (CPFA) composition.
Comparison of the CPFA composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. CPFA levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of CPFA levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. CPFA levels are expressed as percent of total fatty acids.

	Analytes (% of total fatty acids)		
	Malvalic Acid (%)	**Sterculic Acid (%)**	**Dihydrosterculic Acid (%)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**		
Coker 312	0.561 ± 0.013 (0.543-0.574)	0.259 ± 0.003 (0.246-0.266)	0.163 ± 0.003 (0.159-0.168)
TAM66274	0.475 ± 0.013 (0.467-0.486)	0.227 ± 0.003 (0.217-0.232)	0.118 ± 0.003 (0.114-0.124)
Significance (p-value) of TAM66274 vs. Coker 312	0.0085†	0.0014†	0.0002†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**		
Coker 312	0.473 ± 0.010 (0.294-0.569)	0.242 ± 0.006 (0.176-0.294)	0.174 ± 0.005 (0.153-0.183)
TAM66274	0.434 ± 0.010 (0.329-0.530)	0.212 ± 0.006 (0.171-0.260)	0.115 ± 0.005 (0.106-0.130)
Significance (p-value) of TAM66274 vs. Coker 312	0.0867	0.0057†	0.0001†
ILSI CCDB range of analyte values			
Min	0.112	0.061	0.031
Max	0.854	0.556	0.325
Literature range of analyte values			
Min	0.110[c]	0.061[c]	0.038[c]
Max	0.854[b]	0.560[a]	0.325[b]

†Mean analyte values for TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Hamilton et al. (2004); [b]Rudgers (2013); [c]Arackal et al. (2012).

Table 6-10. Cottonseed total and free gossypol composition.
Comparison of the total and free gossypol composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Total gossypol was measured by two different methods, the aniline method and by HPLC, as described in Appendix E. Total and free gossypol levels for each treatment are presented as the means, standard error of the means, and the range across field locations, and levels are compared with the range of levels reported in the ILSI Crop Composition Database (ILSI, 2016), and with ranges of levels reported in the literature for cottonseed from conventional cotton varieties. Gossypol levels are expressed as a percent on a DW basis.

	Analytes		
	Total Gossypol (%) (by aniline)	**Total Gossypol (%) (by HPLC)**	**Free Gossypol (%) (by aniline)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**		
Coker 312	0.963 ± 0.009 (0.930-0.988)	1.03 ± 0.035 (0.880-1.14)	0.777 ± 0.004 (0.763-0.789)
TAM66274	0.044 ± 0.009 (0.040-0.050)	0.037 ± 0.035 (0.028-0.048)	0.030 ± 0.004 (0.027-0.033)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†	0.0001†	0.0001†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**		
Coker 312	0.941 ± 0.025 (0.781-1.04)	1.00 ± 0.042 (0.731-1.28)	0.830 ± 0.020 (0.701-0.905)
TAM66274	0.042 ± 0.025 (0.035-0.051)	0.030 ± 0.002 (0.018-0.047)	0.026 ± 0.020 (0.021-0.029)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†	0.0001†	0.0001†
ILSI CCDB range of analyte values			
Min	0.350	N.R.	0.384
Max	1.61	N.R.	1.42
Literature range of analyte values			
Min	0.550[a]	N.R.	0.492[b]
Max	1.61[c]	N.R.	1.41[c]

†Mean analyte values for TAM66274 compared to values for Coker 312 are statistically significantly different at $P<0.05$.
Literature ranges of analyte values: [a]Bertrand et al. (2005); [b]Rudgers (2013); [c]Arackal et al. (2012).
N.R. Not reported.

Table 6-11. Cottonseed (+)- and (−)-gossypol isomers and total gossypol composition.
Comparison of the gossypol isomers and total gossypol composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014, and five U.S. field locations in 2015. Total gossypol content of the cottonseed was calculated as the sum of the content of the (+)- and (−)-gossypol isomers. Levels of the gossypol isomers were measured by an HPLC method described in Appendix E. Levels of the gossypol isomers and total gossypol for each treatment are presented as the means, standard error of the means, and the range across field locations. Gossypol levels are expressed on a DW basis.

	Analytes		
	(+)-gossypol (µg/g)	**(−)-gossypol (µg/g)**	**Total gossypol (µg/g)**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**		
Coker 312	3,893 ± 37 (3,800-4,010)	2,820 ± 43 (2,670-2,920)	6,713 ± 76 (6,470-6,930)
TAM66274	148 ± 37 (141-158)	109 ± 43 (104-118)	256 ± 76 (245-276)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†	0.0001†	0.0001†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**		
Coker 312	4,204 ± 86 (3,870-4,600)	2,728 ± 88 (2,220-3,090)	6,932 ± 170 (6,090-7,610)
TAM66274	160 ± 86 (122-192)	123 ± 88 (97.6-146)	283 ± 170 (220-338)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†	0.0001†	0.0001†
ILSI CCDB range of analyte values			
Min	N.R.	N.R.	N.R.
Max	N.R.	N.R.	N.R.
Literature range of analyte values			
Min	N.R.	N.R.	N.R.
Max	N.R.	N.R.	N.R.

†Mean analyte values for TAM66274 compared to values for non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$.
N.R. Not reported.

Table 6-12. Cottonseed aflatoxin composition.
Comparison of the aflatoxin composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in five field locations in the U.S. in 2015. Aflatoxins were measured by HPLC as described in Appendix E. Aflatoxin levels for each treatment are presented as the means of samples across all field locations (n=5). Aflatoxin concentrations are reported per FW of seed tissue. FDA action levels are the levels of aflatoxins at which foods for human consumption are considered adulterated, and at which cottonseed meal may not be used as a feed ingredient for beef cattle, swine, or poultry.

	Aflatoxin B1 (ppb)	**Aflatoxin B2 (ppb)**	**Aflatoxin G1 (ppb)**	**Aflatoxin G2 (ppb)**
Treatments	**Mean ± SE (Range)**	**Mean ± SE (Range)**	**Mean ± SE (Range)**	**Mean ± SE (Range)**
Coker 312	<0.7* (NC)	<0.9* (NC)	<0.7* (NC)	<0.8* (NC)
TAM66274	<0.7* (NC)	<0.9* (NC)	<0.7* (NC)	<0.8* (NC)
FDA Action Levels				
Human foods			**Action Level (ppb)**	**Reference**
Brazil nuts			20	CPG 570.200
Foods			20	CPG 555.400
Milk (Aflatoxin M1)			0.5	CPG 527.400
Peanuts and peanut products			20	CPG 570.375
Pistachio nuts			20	CPG 570.500
Animal feeds				
Corn, peanut products, cottonseed meal, and other animal feeds and feed ingredients intended for dairy animals, for animal species or uses not otherwise specified, or when the intended use is not known.			20	CPG 683.100
Cottonseed meal intended for beef cattle, swine, or poultry (regardless of age or breeding status).			300	CPG 683.100

*Mean analyte values were below limit of detection (LOD) in all samples. Standard error (SE) and range could not be calculated (NC).

Table 6-13. Cottonseed deoxynivalenol, acetyldeoxynivalenol and zearalenone composition. Comparison of the deoxynivalenol, acetyldeoxynivalenol, and zearalenone composition of cottonseed from TAM66274 and non-transgenic cv. Coker 312 grown in five field locations in the U.S. in 2015. Analytes were measured by HPLC and LC-MS/MS as described in Appendix E. Mycotoxin levels for each treatment are presented as the means of samples across all field locations (n=5). Mycotoxin concentrations are reported per FW of seed tissue. FDA advisory levels are levels of deoxynivalenol and acetyldeoxynivalenol in grain that are considered unsafe for different feed uses.

	Deoxynivalenol (ppm)	**Acetyldeoxynivalenol (ppm)**	**Zearalenone (ppb)**
Treatments	**Mean ± SE (Range)**	**Mean ± SE (Range)**	**Mean ± SE (Range)**
Coker 312	(<0.6 – 0.8)§ (NC)	<0.8* (NC)	<43.1* (NC)
TAM66274	(<0.6 – 1.6)§ (NC)	<0.8* (NC)	<43.1* (NC)

FDA Advisory Levels		
Animal feed	**Advisory Level † (ppm)**	**Reference**
Grain, grain by-products destined for ruminating beef and feedlot cattle older than 4 months and for chickens with an added recommendation that these ingredients not exceed 50% of the diet for cattle or chickens	10	CPG 7371.003
Grains and grain by-products destined for swine with the added recommendation that these ingredients not exceed 20% of their diet	5	CPG 7371.003
Grains and grain by-products destined for all other animals with the added recommendation that these ingredients not exceed 40% of their diet	5	CPG 7371.003

§Mean analyte values were below limit of detection (LOD) in 80% of samples. Means and standard deviation (SD) could not be calculated (NC).
*Mean analyte values were below limit of detection (LOD) in all samples. Standard error (SE) and range could not be calculated (NC).
†Advisory level for deoxynivalenol (DON); no FDA advisory levels for acetyldeoxynivalenol or zearalenone.

7. PHENOTYPIC, AGRONOMIC AND ECOLOGICAL CHARACTERISTICS OF TAM66274

In order to determine whether genetically engineered cotton has unanticipated effects on U.S. agriculture production or the natural environment, USDA APHIS requires a detailed description of the phenotype of the modified cotton relative to its unmodified progenitor. USDA APHIS uses this information to assess whether there are differences that may affect plant pest risk or weed potential. USDA APHIS recommends that phenotypic characterization of cotton include agronomic performance data on the growth habit, germination and seedling emergence, overwintering capacity, vegetative vigor, flowering, maturity, reproductive potential, and fiber quality from field sites that represent the major cotton growing regions of the United States (USDA APHIS, 2016). Such data is typically collected from small-scale, replicated field trials over one or more growing seasons to ensure exposure to a wide range of environmental conditions.

The environmental safety of TAM66274 was shown to be comparable to non-transgenic cv. Coker 312 through the evaluation of various phenotypic, agronomic and ecological interaction characteristics. The evaluations included:

A) Seed germination and dormancy characteristics conducted under controlled environment (laboratory) conditions;
B) Phenotypic, agronomic and ecological characteristics conducted under small-scale field (environmental release) conditions.

7.1 Seed Germination and Dormancy Characteristics

Seed germination is an important agronomic characteristic used to compare different varieties within a crop species, and seed dormancy is an important characteristic often associated with plants that are weeds (Anderson, 1996; Lingenfelter and Hartwig, 2003). An assessment of seed dormancy is often used to assess the weediness potential of different plant species (Baker, 1974). Therefore, USDA APHIS recommends that phenotypic characterization of cotton include seed germination and dormancy, among other characteristics, in a comprehensive characterization (USDA APHIS, 2016). Such data is typically collected in laboratory studies on seed harvested from multi-location field trials over one or more growing seasons to ensure seed produced for these studies was subject to a wide range of environmental conditions. Although cotton seeds can have a natural capability of two to three months of innate or induced dormancy, dormant seeds are undesirable for crop production, and seed dormancy has been minimized or completely eliminated in modern cultivars through domestication and selective breeding (OECD, 2008). Therefore, even though modern cultivars of cotton do not typically display seed dormancy characteristics, assessments of seed germination and seed dormancy were conducted comparing TAM66274 and non-transgenic cv. Coker 312.

TAM66274 and non-transgenic cv. Coker 312 were grown in three U.S. locations in 2014 (MS114, Washington County, MS; NC114, Perquimans County, NC; NC214, Perquimans County, NC) and five U.S. locations in 2015 (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX). Field sites were selected as representative of major cotton-growing regions in the United States. The plants were grown under standard agronomic practices in a complete randomized block design with four replicated plots per location. Details of the field trials and agronomic practices for plant growth and production of seed of each treatment are described in Appendix F. Briefly, seed cotton was hand-harvested from replicated plots of each treatment at each location for a total of 64 samples. Samples were individually packed and shipped to Cotton Incorporated (Cary, NC), where samples were ginned to separate lint and fuzzy seed. Ginned samples were labeled and shipped to Texas A&M University (College Station, TX) for processing and analysis. Fuzzy seed of each treatment was acid de-linted and sub-sampled for germination analysis in accordance with the Association of Official Seed Analysts (AOSA) guidelines. The germination assays were conducted under warm (30°C) and cool (18°C) conditions using methods adapted from the AOSA Seed Vigor Testing Handbook (AOSA, 2009). Details of the seed production and germination assays are presented in Appendix F, and the results of analyses are presented below.

Results of percent germination of seed of TAM66274 and non-transgenic cv. Coker 312 harvested from each field site are presented in Table 7-1, and results of analysis across locations are presented in Table 7-2. There were no statistically significant differences in percent germination under cool conditions between TAM66274 and non-transgenic cv. Coker 312 for seed collected from any of the eight field sites (Table 7-1). Similarly, there were no statistically significant differences in percent germination under warm conditions between TAM66274 and non-transgenic cv. Coker 312 for seed collected from seven of the eight field sites. However, percent germination of TAM66274 seed was statistically greater than that of non-transgenic cv. Coker 312 under warm conditions for seed collected from a single (NC115) field site (Table 7-1). Therefore, by definition, there were fewer non-germinated seeds, including dormant seeds, harvested from the NC site in 2015 for TAM66274 compared to non-transgenic cv. Coker 312.

There was considerable variation in percent germination of seed of both treatments harvested from the eight field locations. The average percent germination for both treatments and for both cool and warm germination conditions was 84.25% (NC114), 90.56% (NC214), 87.70% (MS114), 75.50% (MS115), 71.37% (MS315) and 92.12% (TX515), but average percent germination for seed harvested from the two NC sites in 2015 were significantly lower (62.19% for NC115, and 62.19% for NC315). These differences in percent germination were most likely due to differences in seed quality from the different field sites, with poorer quality seed harvested from NC115 and NC315, which may have resulted from higher than normal rainfall during boll ripening at these sites (Appendix F).

When percent seed germination and percent non-germinated seeds of TAM66274 were compared to non-transgenic cv. Coker 312 by analysis of data across all eight field locations in 2014 and 2015, there was no statistically significant difference observed between the treatments under cool germination conditions (Table 7-2). The mean cool germination rate of TAM66274 was 80.25% compared to 78.31% for non-transgenic cv. Coker 312. Cool germination rates ranged from 63.25% to 93.75% for TAM66274 and from 60.75% to 93.0% for Coker 312. However, percent germination of TAM66274 seed was statistically greater, and non-germinated seeds lower, compared to non-transgenic cv. Coker 312 under warm germination conditions (Table 7-2). The mean warm germination rate of TAM66274 was 79.06% compared to 75.31% for non-transgenic cv. Coker 312. Warm germination rates ranged from 63.25% to 93.50% for TAM66274 and from 52.0% to 91.50% for Coker 312.

Although there was high variability of percent germination of both treatments harvested from the eight field sites, the 32 data values per treatment used to conduct the across field site statistical analyses were sufficient to show that percent germination of TAM66274 seed was not statistically different from non-transgenic cv. Coker 312 under cool germination conditions, but was significantly greater than that of non-transgenic cv. Coker 312 under warm germination conditions. Further, at individual locations, germination rates of TAM66274 seed were generally higher (in 13 of 16 measurements) than non-transgenic cv. Coker 312 at both warm and cool temperatures (Table 7-1). Considering the germinated seed data from both the individual field sites and across field sites, the percent germination of TAM66274 is equal to or greater than non-transgenic cv. Coker 312. Conversely, the percent non-germinated seed of TAM66274 is less than or equal to non-transgenic cv. Coker 312 and, therefore, exhibits no greater seed dormancy than non-transgenic cv. Coker 312. It is, therefore, concluded that TAM66274 poses no greater weediness potential than non-transgenic cv. Coker 312 and is, therefore, unlikely to pose a greater weediness potential than other conventional cotton varieties.

Table 7-1. Percent germination of TAM66274 and non-transgenic cv. Coker 312 seed harvested from field trials in 2014 and 2015.

Comparison of seed germination of TAM66274 and non-transgenic cv. Coker 312. Seed was harvested from plants grown at three field site locations in 2014 (Perquimans County, NC [NC114 and NC214], and Washington County, MS [MS114]) and five field trial sites in 2015 (Perquimans County, NC [NC115 and NC315], Washington County, MS [MS115 and MS315] and Tom Green County, TX [TX515]). Percent germination at warm and cool temperatures was determined according to AOSA methods. Values for each treatment are presented as the mean, standard error of the means, and range (n=4) of individual field locations.

		Percent Germination	
		Cool (18°C)	**Warm** (30°C)
Site	**Treatments**	**Mean ± S.E.M. (Range)**	
NC114	TAM66274	82.25 ± 2.49 (78-88)	85.00 ± 2.41 (80-91)
	Coker 312	84.25 ± 2.49 (76-91)	85.50 ± 2.41 (80-94)
NC214	TAM66274	93.75 ± 1.32 (91-97)	91.50 ± 3.19 (90-93)
	Coker 312	93.00 ± 1.32 (90-96)	84.00 ± 3.19 (75-92)
MS114	TAM66274	88.25 ± 1.70 (85-93)	89.50 ± 2.5 (85-93)
	Coker 312	87.00 ± 1.70 (82-94)	86.00 ± 2.5 (80-92)

† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites are statistically significantly different at $P<0.05$.

Table 7-1, continued. Percent germination of TAM66274 and non-transgenic cv. Coker 312 seed harvested from field trials in 2014 and 2015.

Comparison of seed germination of TAM66274 and non-transgenic cv. Coker 312. Seed was harvested from plants grown at three field site locations in 2014 (Perquimans County, NC [NC114 and NC214], and Washington County, MS [MS114]) and five field trial sites in 2015 (Perquimans County, NC [NC115 and NC315], Washington County, MS[(MS115 and MS315] and Tom Green County, TX [TX515]). Percent germination at warm and cool temperatures was determined according to AOSA methods. Values for each treatment are presented as the mean, standard error of the means, and range (n=4) of individual field locations.

		Percent Germination	
		Cool (18°C)	**Warm** (30°C)
Site	**Treatments**	**Mean ± S.E.M. (Range)**	
NC115	TAM66274	69.75 ± 3.14 (62-76)	66.25 ± 3.17† (55-72)
	Coker 312	60.75 ± 3.14 (56-66)	52.00 ± 3.17 (45-56)
NC315	TAM66274	63.25 ± 2.69 (60-66)	63.25 ± 3.31 (57-66)
	Coker 312	62.50 ± 2.69 (56-71)	59.75 ± 3.31 (51-72)
MS115	TAM66274	79.00 ± 2.23 (74-82)	72.50 ± 4.12 (66-78)
	Coker 312	78.75 ± 2.23 (73-83)	71.75 ± 4.12 (63-80)
MS315	TAM66274	73.25 ± 3.74 (66-80)	71.00 ± 2.44 (68-73)
	Coker 312	69.25 ± 3.74 (62-76)	72.00 ± 2.44 (68-81)
TX515	TAM66274	92.50 ± 1.37 (86-96)	93.50 ± 1.75 (87-96)
	Coker 312	91.00 ± 1.37 (88-95)	91.50 ± 1.75 (87-94)

† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites are statistically significantly different at $P<0.05$ ($p=0.0191$).

Table 7-2. Percent germination of TAM66274 and non-transgenic cv. Coker 312 seed across eight field locations.

Comparison of seed germination of TAM66274 and non-transgenic cv. Coker 312 harvested from three field locations in the U.S. in 2014 and from five field locations in the U.S. in 2015. Percent normal germination at warm and cool temperatures determined according to AOSA methods. Values for each treatment are presented as the mean, standard error of the means, and range across all eight field locations.

	Percent Germination	
	Cool (18°C**)**	**Warm (**30°C**)**
Treatments	**Mean ± S.E.M. (Range)**	
TAM66274	80.25 ± 0.85 (63.25-93.75)	79.06 ± 1.04† (63.25-93.50)
Coker 312	78.31 ± 0.85 (60.75-93.00)	75.31 ± 1.04 (52.00-91.50)

† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 are statistically significantly different at $P<0.05$ ($p=0.0127$).

7.2 Field Evaluations of Phenotypic, Agronomic and Ecological Characteristics

Upland cotton (*G. hirsutum* L.) is one of the four major crops that are grown in the United States, but one that is more limited geographically than other crops (Fryxell, 1979). Cotton can be grown only in those regions in which there are more than 180 frost-free days per year (Fryxell, 1979; OECD, 2008). Although cotton is basically a perennial tropical crop, breeding selection has produced an annual crop able to produce quality fibers in a temperate climate. In order to determine whether genetically engineered cotton has unanticipated effects on U.S. agriculture or the natural environment, USDA APHIS requires a detailed description of the phenotype of the modified cotton relative to its unmodified progenitor. Phenotypic, agronomic and ecological characteristics were evaluated under field conditions as part of the plant characterization of TAM66274. These data were developed to provide USDA APHIS with a detailed description of TAM66274 relative to the non-transgenic cv. Coker 312.

Forty characteristics were measured at six in-season time points and at harvest comparing TAM66274 and non-transgenic cv. Coker 312 grown at eight field sites representative of major U.S. cotton growing regions during the 2014 and 2015 cotton growing seasons. These data were collected to support the plant pest risk and environmental assessment of TAM66274 in accordance with 7 CFR §340.6. These studies also generated samples for compositional studies. Details of field site characteristics, field trial management practices, methods of data collection, weather data for each field site, and results of individual field site evaluations are presented in Appendix F. A list of trials conducted with TAM66274 under USDA notifications and the status of the final reports for these trials are provided in Appendix G.

Phenotypic, agronomic and ecological data were collected by field study personnel at three field sites in 2014, one in Mississippi (Washington County, designated as MS114 in the Tables) and two separate field trials in North Carolina (Perquimans County, designated as NC114 and NC214 in the Tables)[2] and at five field sites in 2015, two separate field sites in North Carolina (Perquimans County, designated as NC115 and NC315 in the Tables), two in Mississippi (Washington County, designated MS115 and MS315 in the Tables), and one in Texas (Tom Green County, designated as TX515 in the Tables). Field sites were selected as representative of major cotton-growing regions of the United States. Each field trial study was designed as a randomized complete block with four replications per treatment.

[2] A total of six field trials with TAM66274 and non-transgenic cv. Coker 312 were planted in 2014. However, two field sites (TX114, Hale County, TX; TX214, Hale County, TX) were terminated early due to weather damage that rendered the field sites impractical to continue. Termination of TX114 and TX214 occurred nine and 14 weeks after planting, respectively. No plant material was harvested from either field site. One field site (MS314, Washington County, MS) was terminated early due to loss of reproductive isolation after inadvertent destruction of border rows. Termination of MS314 occurred seven weeks after planting. No plant material was harvested from this field site.

The field trials were monitored from stand establishment through harvest by agronomists experienced in cotton production and research. Phenotypic, agronomic and ecological characteristics were evaluated comparing TAM66274 to non-transgenic cv. Coker 312. Data collection encompassed six general categories: 1) seedling emergence and stand count; 2) vegetative growth (plant vigor, height and lodging); 3) reproductive development (days to bloom, seeds per boll, seed index (g/100 seed), lint percent, lint yield and seed yield); 4) fiber quality (micronaire, elongation, strength, length, short fiber content and uniformity); 5) plant mapping (total nodes, height to node ratio, total bolls, number of first and second position bolls and boll type); and 6) plant susceptibility to diseases and insect pests, as well as to rodents. The phenotypic, agronomic and ecological characteristics evaluated in field and laboratory studies are listed in Table 7-3. The collected data was subjected to statistical analysis (described in Appendix F) across the three field sites in 2014 and the five field sites in 2015 to detect significant differences between TAM66274 and non-transgenic cv. Coker 312 ($P<0.05$), and these results are presented in Tables 7-4 through 7-10. Statistical analyses were also conducted on data collected for the treatments at each field site to detect significant differences between TAM66274 and non-transgenic cv. Coker 312 ($P<0.05$), and these results are presented in Appendix F.

Table 7-3. Phenotypic, agronomic and ecological characteristics evaluated in field and laboratory studies.

Forty phenotypic, agronomic and ecological characteristics were evaluated by field and laboratory personnel comparing TAM66274 to non-transgenic cv. Coker 312, in each of four replicated plots at each field site.

Data category	Characteristics measured	Evaluation timing (evaluation setting)	Evaluation description (measurement endpoint)
Seedling emergence and stand count	Stand count	Approximately 7 and 14 DAP[1] and within 7-10 days of harvest (Field)	Number of emerged plants in two rows, standardized to 30 ft rows.
	Final stand count	Within 7-10 days of harvest (Field)	Number of plants in two rows, standardized to 30 ft rows.
Vegetative growth	Plant vigor	Approximately 28, 56, and 84 DAP (Field)	Rated entire plot on a 1-9 scale: 1 = short plants with small leaves; 9 = tall plants with large, robust leaves.
	Plant height (inch)	Approximately 28, 56, 84 DAP and within 7-10 days of harvest (Field)	Distance from the cotyledon leaf scar to the tip of terminal meristem on 10 plants in two rows.
	Lodging	Within 7-10 days of harvest (Field)	Rated 10 plants in two rows on a 1-9 scale: 1 = fully upright, no leaning, 5 = leaning 45 degrees from ground, 9 = laying on soil surface.
Reproductive development	Days to bloom	Flowering (Field)	Number of days from planting to appearance of 5 white flowers in two rows.
	Total seeds per boll	At harvest (Laboratory)	Average number of mature seeds per boll from a 25-boll sample.
	Seed index (g/100 seed)	At harvest (Laboratory)	Mass of 100 ginned, fuzzy seed.
	Lint percent	At harvest (Laboratory)	Lint weight divided by seed cotton weight expressed as a percentage.
	Lint yield (lb/A)	At harvest (Laboratory)	Weight of lint harvested from two middle rows, standardized to one acre.
	Seed yield (lb/A)	At harvest (Laboratory)	Weight of seed harvested from two middle rows, standardized to one acre.

[1] Days after planting

Table 7-3, continued. Phenotypic, agronomic and environmental characteristics evaluated in field and laboratory studies.

Data category	Characteristics measured	Evaluation timing (evaluation setting)	Evaluation description (measurement endpoint)
Fiber quality	Fiber micronaire (mic units)	At harvest (Laboratory)	Measure of fiber fineness and maturity (expressed in dimensionless micronaire (mic) units.
	Fiber elongation (%)	At harvest (Laboratory)	Measure of tensile-elastic behavior of the fiber.
	Fiber strength (g/tex)	At harvest (Laboratory)	Forces in grams required to break a bundle of fibers one tex unit in size. One tex unit is the mass in grams of 1,000 meters of fiber.
	Fiber length (inches)	At harvest (Laboratory)	Mean length of the longer half of the fibers, the upper half mean length.
	Short fiber content (%)	At harvest (Laboratory)	Percentage of fibers shorter than one-half inch.
	Fiber uniformity (%)	At harvest (Laboratory)	Ratio between the mean length and the longer half mean length of fibers.
Plant mapping characteristics	Total nodes	Within 7-10 days of harvest (Field)	Number of nodes on mainstem of 10 plants in two rows.
	Height to node ratio	Within 7-10 days of harvest (Field)	Plant height divided by number of nodes on 10 plants in two rows.
	Total bolls	Within 7-10 days of harvest (Field)	Number of fruiting and vegetative bolls on 10 plants in two rows.
	Number of first position bolls	Within 7-10 days of harvest (Field)	Number of bolls on 10 plants in two rows.
	Number of second position bolls	Within 7-10 days of harvest (Field)	Number of bolls on 10 plants in two rows.
	Boll type	Within 7-10 days of harvest (Field)	Rated 10 plants in two rows on a 1-9 scale: 1 = loose, 5 = intermediate, 9 = stormproof.
Plant susceptibility to diseases and insects; rodent damage	Disease incidence	14, 28, 56, 84, and 112 DAP (Field)	Rated 10 plants in two rows on a 1-9 scale: 1 = no symptoms, 5 = intermediate symptoms, 9 = severe disease.
	Insect damage	14, 28, 56, 84, and 112 DAP (Field)	Rated 10 plants in two rows on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage.
	Rodent damage	Within 7-10 days of harvest (Field)	Rated seed damage on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage.

7.2.1. Seedling emergence and plant stand count.

Seedling emergence and plant stand was evaluated at 7 and 14 days after planting (DAP), and at harvest. The total number of emerged plants in two rows were counted and standardized to 30-foot rows. In both 2014 and 2015 field studies, there were no statistically significant differences detected in seedling emergence or stand count of TAM66274 compared to non-transgenic cv. Coker 312 at any stage of plant development across all three locations in 2014 or five locations in 2015 (Table 7-4). A significant increase in emergence was detected for TAM66274 compared to non-transgenic cv. Coker 312 at 7 DAP at one location (NC315), however this difference was not considered agronomically meaningful since it was no longer detectable by 14 DAP (see Table F-10 in Appendix F). Stand establishment in cotton is well known to be sensitive to extremes of temperature, moisture, soil texture, and seed placement (Hake-Johnson et al., 1996). Thus, it is not unexpected that at one location (NC315), a statistically significant difference was observed in plant stand at one sampling date. Results of the across location analysis of field studies in 2014 and 2015 on seedling emergence and plant stand support conclusions of the laboratory seed germination studies described above, which showed that seed germination and dormancy of TAM66274 is equivalent to that of non-transgenic cv. Coker 312.

7.2.2. Vegetative growth.

Vegetative growth was evaluated by rating plant vigor, plant height, and lodging at four stages of plant development. Plant vigor was evaluated at 28, 56, and 84 DAP by rating all plants of each plot and assigning an average rating on a 1-9 scale, where 1 = short plants with small leaves and 9 = tall plants with robust leaves. Plant height was evaluated at 28, 56, and 84 DAP and at harvest by measuring the distance in inches from the cotyledon leaf scar to the tip of the terminal meristem on 10 plants in two rows of each replicated plot. Additionally, plant lodging was evaluated at harvest by rating 10 plants in two rows in each replicated plot on a 1-9 scale, where 1 = fully upright, no leaning; 5 = plant leaning 45 degrees from the ground; and 9 = plant laying on the soil surface.

No statistically significant differences were detected in plant vigor of TAM66274 compared to non-transgenic cv. Coker 312 across all three locations in 2014 or five locations in 2015 at any of the observed stages of plant development (Table 7-5). Vigor ratings of TAM66274 were statistically significantly lower than non-transgenic cv. Coker 312 at two locations in 2015 (NC115 and NC315) at 28 DAP (Table F-11 in Appendix F). However, plant vigor differences between TAM66274 and non-transgenic cv. Coker 312 were undetectable at this location at 56 and 84 DAP and, therefore, the difference at 28 DAP was not considered agronomically meaningful.

There were no statistically significant differences in plant height between TAM66274 and non-transgenic cv. Coker 312 at 28, 56, and 84 DAP or at harvest across all three locations in 2014 (Table 7-6). In 2015 field studies, a statistically significant difference was detected in plant

height of TAM66274 compared to non-transgenic cv. Coker 312 at 28 DAP, with non-transgenic cv. Coker 312 being taller than TAM66274 by approximately one inch (Table 7-6). However, this difference was not considered agronomically meaningful because no statistically significant differences in plant height were observed between the treatments at any subsequent stage of plant development (56 DAP through harvest). The reason for the statistically significant difference in height between the treatments at 28 DAP in the across location analysis was most likely because TAM66274 plants were statistically significantly shorter than non-transgenic cv. Coker 312 at the NC sites in 2015 (NC115 and NC315) at 28 DAP, but were not significantly different at any later stage of plant development at these locations (Table F-12 in Appendix F). The soil texture at the NC sites contained more sand than at the other sites (80% and 70% sand at NC115 and NC315, respectively compared to 30%, 31% and 17% at MS115, MS315 and TX515, respectively as shown in Appendix F). Sandier soils hold less available moisture and have reduced unsaturated hydraulic conductivity. These two features can result in drought stress in summer grown crops, which manifests itself in early season short plant stature. In contrast, at locations with the lowest sand content (MS115, MS315 and TX515), there were no statistically significant differences in plant height between the treatments at 28 DAP or at any other stage of plant development. There were no statistically significant differences in lodging between TAM66274 and non-transgenic cv. Coker 312 across locations in either 2014 or 2015 field studies (Table 7-6), and no statistically significant differences between TAM66274 and non-transgenic cv. Coker 312 at any individual location in the two years of field trials (Table F-12 in Appendix F).

7.2.3. Reproductive development.

Reproductive development was evaluated by rating flowering time, seed production, lint production, and lint percent in each replicated plot. Flowering time was evaluated by counting the number of days from the planting date to the appearance of five white flowers in two rows. Seed production was evaluated by counting the average number of seed produced per boll in a 25-boll sample and by determining the mass of 100 ginned, fuzzy seed (seed index). Lint yield was calculated by determining the weight of lint as a percentage of seed cotton harvested from two rows (lint percent) and multiplying lint percent by the weight of seed cotton harvested from two rows, standardized to one acre. Seed yield was calculated by determining the weight of seed harvested from two rows, standardized to one acre.

In 2014 field studies, no statistically significant difference in flowering time (days to bloom), seed index or lint yield was detected for TAM66274 compared to non-transgenic cv. Coker 312 across all three locations (Table 7-7). On the other hand, seeds per boll were slightly greater (4.3%) and seed yield was slightly reduced (6%) in TAM66274, but statistically significant, relative to non-transgenic cv. Coker 312 across all three locations (Table 7-7). Lint percent was marginally greater, but statistically significant in TAM66274 compared to non-transgenic cv. Coker 312 (Table 7-7). In 2015 field studies, no statistically significant difference in flowering

time, lint yield or seed yield was detected for TAM66274 compared to non-transgenic cv. Coker 312 across all five locations (Table 7-7). However, seeds per boll were less (7.9%) and seed index was slightly reduced (4.3%) in TAM66274, but statistically significant, relative to non-transgenic cv. Coker 312 across all five locations (Table 7-7). Lint percent was also slightly lower, but statistically significant for TAM66274 compared to non-transgenic cv. Coker 312 (Table 7-7). Where statistically significant differences in reproductive parameters were observed, the differences between the treatments were not consistent across the two years of field trials (Table F-13 in Appendix F) and are, therefore, not considered agronomically meaningful.

7.2.4. Fiber quality.

After ginning, which separates the fiber from the seed, fiber quality analysis was conducted using an industry standard HVI Uster 9000 calibrated using USDA Agricultural Marketing Service (AMS) fiber samples. Six fiber quality parameters were assessed (micronaire, elongation, strength, length, short fiber content and uniformity), all of which are impacted by genotype, plant height, boll retention pattern, boll size, and the field environment during boll maturation.

There were no statistically significant differences for fiber elongation, strength and uniformity between TAM66274 and non-transgenic cv. Coker 312 across all three field sites in 2014 (Table 7-8). Fiber micronaire was statistically higher in TAM66274 compared to non-transgenic cv. Coker 312 across all locations in 2014 (Table 7-8) and was consistently higher, although not statistically significant, at individual field locations in 2014 (Table F-14 in Appendix F). Micronaire of a fiber sample is determined by forcing air through a chamber containing a known weight of fiber. High airflow is reported as high micronaire and is indicative of high fiber maturity and/or high fiber fineness. Although TAM66274 exhibited significantly higher fiber micronaire than non-transgenic cv. Coker 312, these values are still considered commercially acceptable. Fibers were statistically significantly shorter in TAM66274 compared to non-transgenic cv. Coker 312 (Table 7-8), as also observed in two of the three locations (NC114, NC214) in 2014 (Table F-14 in Appendix F). Short fiber content was statistically significantly less in TAM66274 compared to non-transgenic cv. Coker 312 across all three locations (Table 7-8), which was detected in two of the three locations (MS114, NC214) in 2014 (Table F-14 in Appendix F).

When fiber quality of TAM66274 was compared to non-transgenic cv. Coker 312 in 2015 field studies, fiber micronaire, strength, percent short fibers and percent uniformity were not statistically significantly different between the two treatments across all five locations (Table 7-8). Fiber length was statistically significantly lower in TAM66274 relative to non-transgenic cv. Coker 312, whereas percent elongation was significantly greater in TAM66274 compared to non-transgenic cv. Coker 312 across 2015 field locations (Table 7-8). Where statistically significant differences in fiber quality parameters were observed between the treatments, except

for fiber length, the differences between treatments were not consistent across the two years of field trials (Table F-14 in Appendix F) and, therefore, were not considered agronomically meaningful. Although TAM66274 fibers were slightly shorter than those of non-transgenic cv. Coker 312 (mean lengths were 3.3% shorter in 2014 field studies and 6.3% in 2015 field studies), TAM66274 fiber length is within acceptable commercial limits, and this parameter does not pose a weediness or plant pest risk for TAM66274.

7.2.5. Plant mapping.

Plant mapping provides a system to evaluate the growth and development of cotton plants throughout the season (Guthrie and Kerby, 1993). In this study, a final or terminal plant map was performed to assess the environmental, biological and production inputs that affected crop development and harvestable yield of TAM66274 relative to non-transgenic cv. Coker 312. Plant data was mapped within 7-10 days of harvest on 10 plants in two rows of each replicated plot of each treatment in 2014 and 2015 field studies. Mapping data included plant height, total nodes, height to node ratio, total bolls, number of first position bolls, number of second position bolls, and boll type. These characteristics and their interpretation are described below.

Plant height at harvest is the easiest growth index to measure, but the most difficult to interpret (Guthrie and Kerby, 1993). Plant height was measured in inches from the cotyledons leaf scar to the tip of the terminal meristem. In general terms, if the plant height is significantly less than the row spacing, stress or good boll retention has limited the crop growth potential. If plant height is significantly greater than row spacing, inadequate boll set and/or generous fertilization may be the cause. Internode length can provide additional clues to the cause of plant height extremes, however no attempts were made to record internode length in the present studies comparing TAM66274 and non-transgenic cv. Coker 312.

Total nodes suggest the length of season, boll loading dynamics and severity of late season second growth. Total nodes were counted on the main stem at plant maturity beginning at the first true leaf and continuing to the terminal. As season length increases, the number of potential nodes increases. In fields with similar production inputs, significant differences in total nodes can often be traced to differences in boll retention. Late season second growth can result in additional nodes without productive value, which may indicate premature cutout and/or excessive fertility.

The ratio of plant height to nodes (HNR) was calculated by dividing plant height by the number of main-stem nodes. This ratio indicates the amount of stress that a cotton plant has encountered and will vary according to variety and time of season (Guthrie et al., 1993). Height-to-node ratios reflect the sum total of a particular plant's environmental experiences during the growing season, such as the availability of water, nutrients, heat, sunlight, insect damage, and disease. As these biotic and abiotic factors vary, so does the HNR. Attempts have been made to develop HNR

guidelines that describe desirable crop vigor. These HNR guidelines were derived from non-stressed fields with excellent final yields (Kerby and Hake, 1996). In typical cotton plants at harvest, optimal HNR ratios range from 2.0 to 2.2 (Kerby and Hake, 1996). A sub-optimal HNR indicates low relative vigor and suggests that efforts to enhance crop growth would be needed to relieve the stress. A high relative HNR indicates robust growth, but growth that can render plants more attractive and susceptible to late season insects, more susceptible to boll rot, and more difficult to defoliate. While the HNR provides a good average indication of overall plant vigor and growth potential, it has one main limitation. As an average, the HNR integrates the entire growth history into a single number and is relatively insensitive to recent changes in growth that can indicate the need for crop management intervention. In the present studies comparing TAM66274 and non-transgenic cv. Coker 312, HNR was used as one indication of relative overall plant growth and development.

The distribution of bolls by node and position is the backbone of final plant mapping (Guthrie and Kerby, 1993). The presence or absence of bolls at the various positions affects all aspects of crop and yield development. The proportion of yield from first position bolls increases with higher plant populations. Boll retention at the first position indicates crop health in moderate densities (3 or 4 plants per foot in 38" rows). Boll retention above 60% at the first position indicates excellent environmental conditions for yield development in moderate to high plant populations. In the present studies comparing TAM66274 and non-transgenic cv. Coker 312, the number of bolls produced on the first position and second position of fruiting branches were recorded. Since the majority of productive yield derives from these two positions, no effort was made to record the number of bolls beyond the second position. The total number of bolls on fruiting and vegetative branches at harvest was also recorded.

In 2014 field studies, no significant differences were detected in any final plant map characteristics between TAM66274 and non-transgenic cv. Coker 312 at harvest across the three field locations (Tables 7-6 and 7-9). Plant height (Table 7-6), total nodes, HNR, total bolls, first and second position bolls, and boll type were comparable between the treatments across all three locations. Plant height of both treatments at one location (MS114) was 35-40% greater than plant row spacing, which was also reflected in reduced boll set compared to other locations (Table F-15 in Appendix F). However, no significant differences in plant height and total bolls were detected between TAM66274 and non-transgenic cv. Coker 312 at MS114. In 2015 field studies, no statistically significant differences were detected in total bolls, number of first position bolls, second position bolls or boll type between TAM66274 and non-transgenic cv. Coker 312 across field locations (Table 7-9). However, across all locations, TAM66274 exhibited significantly more nodes and decreased HNR relative to non-transgenic cv. Coker 312, although plant height at harvest was not significantly different between the treatments (Table 7-6). The statistically significant differences in total nodes and HNR between the two treatments were not consistent

across the two years of field trials (Table F-15 in Appendix F) and, therefore, were not considered agronomically meaningful.

7.2.6. Disease, insect and rodent feeding susceptibility.

Gossypol is one of many secondary plant metabolites expressed in cotton that exhibit insecticidal and antimicrobial properties to protect the plant from insects and disease (Bell, 1967; Hedin et al., 1992; Stipanovic et al., 1975; Stipanovic et al., 1999). In TAM66274, gossypol production was intentionally reduced selectively in seed kernels, while leaving levels unchanged in other plant tissues (e.g., roots, stems, leaves) where it retains its pesticidal activities (Palle et al., 2013; Rathore et al., 2012; Sunilkumar et al., 2006). In contrast, glandless cottonseed is a naturally occurring mutant that does not produce gossypol in any plant tissues, which renders the plant susceptible to insect predation and plant diseases, and limits commercial utility (Benedict et al., 1977; Bottger et al., 1964; Jenkins et al., 1966; Jenkins et al., 1967). Further, gossypol expressed in glanded cottonseed is toxic to non-ruminant animals (Gadelha et al., 2014; Risco and Chase, 1997), whereas glandless cottonseed, which does not accumulate gossypol, is susceptible to predation by foraging mammals in cotton fields prior to harvest (T. Wedegaertner, personal communication). Therefore, a critical element of characterizing TAM66274 was evaluation of plant susceptibility to disease and insect pressure and rodent feeding under typical cultivation conditions, in order to determine if TAM66274 exhibited disease, insect and rodent feeding susceptibilities comparable to non-transgenic cv. Coker 312. Any significant increase in susceptibility of TAM66274 relative to non-transgenic cv. Coker 312 would constitute a phenotypic change that could affect cultivation practices or have unintended environmental effects on the agricultural ecology.

Plant susceptibility to disease and insect pressure was evaluated at 14, 28, 56, 84, and 112 DAP by rating 10 plants in two rows of each replicated plot on a 1-9 scale where disease ratings were 1 = no symptoms, 5 = intermediate symptoms, and 9 = severe damage, and insect damage was rated on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage. Additionally, rodent feeding damage on mature cottonseed was evaluated at harvest by rating plots on a 1-9 scale where 1 = no damage, 5 = intermediate damage, and 9 = severe damage.

In both 2014 and 2015 field studies, no statistically or agronomically significant differences in plant disease susceptibility were observed in TAM66274 compared to non-transgenic cv. Coker 312 across locations or in any individual location (Table 7-10 and Table F-16 in Appendix F). Plant diseases observed during this field study were typical of those found in commercial cotton cultivation: leaf spot (*Alternaria* spp., *Cercospora* spp., *Stemphyllium* spp., *Colletrotrichum* spp.) and boll rot (*Fusarium* spp., *Diplodia* spp., *Glomerella gossypii*, *Xanthomonas* spp., *Rhizoctonia* spp., *Alternaria* spp.) (Appendix F). Pesticides were uniformly applied to all treatments to manage plant disease (Appendix F). No differences in plant response to these crop management practices were observed in TAM66274 compared to non-transgenic cv. Coker 312.

Similarly, no statistically or agronomically significant differences in insect damage were observed in TAM66274 compared to non-transgenic cv. Coker 312 in 2014 and 2015 field studies either across locations or in any individual location (Table 7-11 and Table F-17 in Appendix F). Insects observed during this field study were typical of those found in commercial cotton cultivation: thrips (*Frankiella fusca*), tarnished plant bug (*Lygus lineolaris*), stinkbug (*Halyomorpha halys*), cotton bollworm (*Helicoverpa armigera*), and spider mites (*Tetranychidae* spp.) (Appendix F). Pesticide applications were uniformly applied to all treatments to manage insects (Appendix F). No differences in plant response to these crop management practices were observed in TAM66274 compared to non-transgenic cv. Coker 312.

No evidence of rodent feeding was observed in TAM66274 or non-transgenic cv. Coker 312 at any field location in either 2014 or 2015 field studies (Table 7-12 and Table F-18 in Appendix F).

7.3 Conclusions of Phenotypic, Agronomic and Ecological Characteristics of TAM66274

The 2014 and 2015 replicated field trials conducted in the United States evaluated the agronomic performance and environmental safety of TAM66274 compared to non-transgenic cv. Coker 312. Specifically, the purpose of these studies was to evaluate agronomic and phenotypic characteristics of TAM66274 cotton relative to non-transgenic cv. Coker 312, and to determine the ecological impact (interaction with diseases, insects and rodents) of growing TAM66274 relative to non-transgenic cv. Coker 312 in typical U.S. cotton production environments. Field studies were conducted during the 2014 U.S. cotton growing season at three locations (two in NC and one in MS) and at five locations in 2015 (two in NC, two in MS and one in TX), which are representative of U.S. commercial cotton production. Agronomic and ecological characteristics encompassed six general categories: 1) seed germination, dormancy, and stand count; 2) vegetative growth; 3) reproductive development; 4) fiber quality; 5) plant mapping and 6) plant susceptibility to diseases and insect pests, as well as to rodents. Forty characteristics were measured at six in-season time points and at harvest.

No statistically significant or biologically meaningful differences were detected in seed germination and stand count, vegetative growth, or plant susceptibility to disease and insect pests or rodents for TAM66274 relative to non-transgenic cv. Coker 312 in either 2014 or 2015 field studies. Further, there were no statistically significant differences for the majority of the reproductive development, fiber quality and plant mapping parameters in both field trial seasons. Overall, statistical differences were detected in only 11.9% of all comparisons at individual locations over two years (i.e., 40 of 336 agronomic and germination comparisons). In the few instances where statistically significant differences were observed between the treatments, these differences were inconsistent between the two field trial seasons and, therefore, were not considered agronomically meaningful. The only parameter that was consistently statistically different between the treatments over the two field trial seasons was fiber length. Although fiber

length of TAM66274 was slightly shorter than non-transgenic cv. Coker 312, it was within commercially acceptable limits, and this parameter does not pose an increased risk of weediness or plant pest characteristics.

Results of these studies showed the lack of biologically meaningful differences in phenotypic, agronomic and ecological characteristics between TAM66274 and non-transgenic cv. Coker 312. These data demonstrated that TAM66274 is phenotypically, agronomically and ecologically equivalent to non-transgenic cv. Coker 312 and, therefore, is likely comparable to other conventional cotton varieties. Overall, the results demonstrate that the cultivation of TAM66274 poses no greater risk of weediness or plant pest characteristics than does the cultivation of non-transgenic cv. Coker 312 and, therefore, is unlikely to pose greater risk ecological or environmental impacts than other conventional cotton varieties.

Table 7-4. Seedling emergence and plant stand count.
Comparison of seedling emergence and plant stand of TAM66274 and non-transgenic cv. Coker 312 grown in three U.S. field locations in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Stand count was the number of emerged plants in two rows, standardized to 30 ft rows.

	Plant Stand Count		
	7 DAP	**14 DAP**	**Harvest**
Treatments	**2014 Studies Mean ± S.E.M. (Range)**		
Coker 312	96.3 ± 1.0 (90.1-108.1)	96.3 ± 1.0 (89.5-109.5)	93.5 ± 1.0 (85.9-107.5)
TAM66274	96.8 ± 1.0 (88.1-108.9)	94.7 ± 1.0 (84.6-108.7)	94.0 ± 1.0 (84.3-107.5)
Significance (p-value) of TAM66274 vs. Coker 312	0.6335	0.2686	0.7385
Treatments	**2015 Studies Mean ± S.E.M. (Range)**		
Coker 312	89.3 ± 1.6 (74.4-101.8)	94.3 ± 1.3 (74.9-109.4)	87.8 ± 1.2 (74.4-105.0)
TAM66274	90.4 ± 1.6 (71.9-105.6)	92.2 ± 1.3 (71.5-107.8)	87.8 ± 1.2 (72.1-113.4)
Significance (p-value) of TAM66274 vs. Coker 312	0.6334	0.2476	1.0000

† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-5. Plant vigor.
Comparison of plant vigor of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Plant vigor was rated on a 1-9 scale: 1 = short plants with small leaves, 9 = tall plants with robust leaves.

	Plant Vigor		
	28 DAP (1-9 scale)	**56 DAP** (1-9 scale)	**84 DAP** (1-9 scale)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**		
Coker 312	7.8 ± 0.0 (7.0-8.3)	7.7 ± 0.03 (7.0-9.0)	8.1 ± 0.1 (7.5-9.0)
TAM66274	7.8 ± 0.0 (7.0-8.3)	7.7 ± 0.03 (7.0-9.0)	8.0 ± 0.1 (7.5-9.0)
Significance (p-value) of TAM66274 vs. Coker 312	1.000	1.000	0.6240
Treatments	**2015 Studies Mean ± S.E.M. (Range)**		
Coker 312	7.3 ± 0.17 (6.8-8.0)	7.7 ± 0.09 (7.0-9.0)	7.7 ± 0.1 (7.0-9.0)
TAM66274	6.8 ± 0.17 (6.0-8.0)	7.8 ± 0.09 (6.9-9.0)	7.7 ± 0.1 (7.0-9.0)
Significance (p-value) of TAM66274 vs. Coker 312	0.0577	0.8515	0.5259

† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-6. Plant height and lodging.
Comparison of plant height of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Plant height was measured as the distance in inches from the cotyledon leaf scar to the tip of the terminal meristem. Plant lodging was rated on a 1-9 scale: 1 = plants fully upright, 5 = plants leaning 45 degrees from ground, 9 = plants laying on soil surface.

	Plant Height				**Lodging**
	28 DAP (inches)	**56 DAP** (inches)	**84 DAP** (inches)	**Harvest** (inches)	**Harvest** (1-9 scale)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**				
Coker 312	5.8 ± 0.1 (4.4-8.2)	27.1 ± 0.4 (25.6-28.1)	40.4 ± 0.5 (34.3-51.0)	40.1 ± 0.5 (34.0-50.8)	1.6 ± 0.1 (1.3-2.3)
TAM66274	5.5 ± 0.1 (4.1-7.5)	26.5 ± 0.4 (24.9-28.0)	39.8 ± 0.5 (34.2-49.8)	40.1 ± 0.5 (34.0-51.2)	1.5 ± 0.1 (1.3-2.0)
Significance (p-value) of TAM66274 vs. Coker 312	0.0500	0.3228	0.3688	0.9381	0.7212
Treatments	**2015 Studies Mean ± S.E.M. (Range)**				
Coker 312	11.4 ± 0.3 (7.4-18.9)	29.8 ± 0.5 (27.4-34.9)	35.7 ± 0.6 (29.2-41.0)	34.5 ± 0.6 (27.9-40.2)	1.3 ± 0.1 (0.8-1.6)
TAM66274	10.3 ± 0.3 (6.7-17.4)	28.3 ± 0.5 (24.4-31.7)	34.2 ± 0.6 (30.5-37.3)	33.8 ± 0.6 (29.7-37.6)	1.3 ± 0.1 (1.0-1.6)
Significance (p-value) of TAM66274 vs. Coker 312	0.0187†	0.0521	0.0883	0.4120	0.9023

† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-7. Reproductive development.
Comparison of reproductive development of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Days to bloom were the number of days after planting to the appearance of five white flowers in two rows. Seeds per boll was the average number of mature seeds per boll in a 25-boll sample. Seed index was the mass of 100 ginned, fuzzy seed from a 25-boll sample. Lint percent was determined by dividing lint weight by weight of seed cotton hand harvested from two rows. Yields (lb/A) were calculated based on the weight of seed cotton hand harvested from two rows, standardized to one acre.

	Reproductive Development					
	Days to Bloom	**Seeds per Boll**	**Seed Index** (g/100 seed)	**Lint Percent**	**Lint Yield** (lb/A)	**Seed Yield** (lb/A)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**					
Coker 312	56.6 ± 0.2 (55.5-57.3)	27.8 ± 0.6 (25.5-29.1)	9.1 ± 0.1 (8.5-12.4)	39.9 ± 0.2 (36.9-41.9)	1379 ± 27 (949-1612)	2045 ± 34 (1620-2274)
TAM66274	56.8 ± 0.2 (55.5-57.8)	29.0 ± 0.6 (19.9-29.8)	9.2 ± 0.1 (8.8-12.0)	40.6 ± 0.2 (37.1-42.6)	1339 ± 27 (853-1592)	1916 ± 34 (1442-2164)
Significance (p-value) of TAM66274 vs. Coker 312	0.4621	0.0142†	0.4042	0.0347†	0.3099	0.0142†
Treatments	**2015 Studies Mean ± S.E.M. (Range)**					
Coker 312	50.7 ± 0.2 (46.0-54.8)	25.3 ± 0.6 (22.6-27.5)	11.5 ± 0.2 (10.1-12.5)	38.7 ± 0.04 (35.7-41.0)	1174 ± 28 (535-1484)	1840 ± 48 (965-2476)
TAM66274	51.1 ± 0.2 (46.0-54.8)	23.3 ± 0.6 (19.9-25.9)	11.0 ± 0.2 (9.7-12.0)	36.7 ± 0.04 (32.3-39.5)	1111 ± 28 (469-1424)	1887 ± 48 (983-2639)
Significance (p-value) of TAM66274 vs. Coker 312	0.0883	0.0142†	0.0478†	0.0001†	0.1213	0.4941

† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-8. Fiber quality.
Comparison of fiber quality of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Fiber quality was measured by HVI instrumentation calibrated using USDA AMS fiber samples.

	Fiber Quality					
	Micronaire (mic units)	**Elongation** (%)	**Strength** (g/tex)	**Length** (inch)	**Short Fiber Content** (%)	**Uniformity** (%)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**					
Coker 312	3.93 ± 0.08 (3.71-4.08)	4.97 ± 0.07 (4.58-5.20)	28.22 ± 0.39 (27.6-28.8)	1.20 ± 0.01 (1.19-1.22)	7.04 ± 0.11 (6.90-7.25)	85.58 ± 0.27 (85.48-85.75)
TAM66274	4.35 ± 0.08 (4.11-4.53)	5.13 ± 0.07 (4.85-5.33)	28.69 ± 0.39 (28.1-29.7)	1.16 ± 0.01 (1.13-1.22)	6.65 ± 0.11 (6.48-6.80)	85.93 ± 0.27 (85.18-86.80)
Significance (p-value) of TAM66274 vs. Coker 312	0.0006†	0.1004	0.3981	0.0098†	0.0202†	0.3623
Treatments	**2015 Studies Mean ± S.E.M. (Range)**					
Coker 312	4.13 ± 0.06 (3.51-4.75)	5.17 ± 0.06 (4.88-5.28)	30.31 ± 0.26 (28.83-31.93)	1.26 ± 0.01 (1.20-1.29)	6.53 ± 0.09 (6.43-6.75)	86.11 ± 0.18 (85.30-86.50)
TAM66274	4.27 ± 0.06 (4.11-4.68)	5.55 ± 0.06 (5.13-5.73)	30.11 ± 0.26 (28.28-32.03)	1.18 ± 0.01 (1.13-1.22)	6.52 ± 0.09 (6.35-6.65)	86.25 ± 0.18 (85.60-86.53)
Significance (p-value) of TAM66274 vs. Coker 312	0.1117	0.0002†	0.5821	0.0001†	0.9027	0.5918

† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-9. Plant mapping characteristics.
Comparison of plant mapping characteristics of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Total nodes represent the total number of nodes on the main stem of the plant at maturity. Height to node ratio was calculated by dividing plant height by the total number of nodes. Total bolls represent the total number of fruiting and vegetative bolls. First and second position bolls represent the total number of bolls set on the first and second position, respectively, of fruiting branches. Boll type was rated on a 1-9 scale: 1 = loose bolls, 5 = intermediate tightness, 9 = stormproof bolls.

	Plant Mapping Characteristics					
	Total Nodes	**Height to Node Ratio**	**Total Bolls**	**No. of First Position Bolls**	**No. of Second Position Bolls**	**Boll Type** (1-9 scale)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**					
Coker 312	18.2 ± 0.3 (16.0-22.0)	2.2 ± 0.04 (2.1-2.3)	8.2 ± 0.5 (5.3-9.6)	3.6 ± 0.2 (0.9-5.0)	2.4 ± 0.1 (2.0-2.7)	6.9 ± 0.2 (4.5-8.5)
TAM66274	18.8 ± 0.3 (17.3-21.9)	2.1 ± 0.04 (2.0-2.4)	7.9 ± 0.5 (4.2-10.2)	4.2 ± 0.2 (0.6-6.4)	2.2 ± 0.1 (2.0-2.5)	6.4 ± 0.2 (3.8-8.0)
Significance (p-value) of TAM66274 vs. Coker 312	0.1377	0.1215	0.6880	0.0979	0.4556	0.0914
Treatments	**2015 Studies Mean ± S.E.M. (Range)**					
Coker 312	16.4 ± 0.2 (14.7-18.3)	2.1 ± 0.03 (1.75-2.26)	9.4 ± 0.3 (7.8-11.7)	4.4 ± 0.2 (3.2-5.4)	2.2 ± 0.1 (1.6-3.2)	5.7 ± 0.1 (4.8-7.5)
TAM66274	18.0 ± 0.2 (15.9-20.0)	1.9 ± 0.03 (1.57-2.10)	9.6 ± 0.3 (7.9-12.0)	4.5 ± 0.2 (2.9-6.3)	2.1 ± 0.1 (1.6-3.2)	5.4 ± 0.1 (4.5-7.7)
Significance (p-value) of TAM66274 vs. Coker 312	0.0001†	0.0001†	0.6673	0.7665	0.5719	0.1070

† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-10. Disease incidence.
Comparison of plant disease susceptibility of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Disease severity was rated on a 1-9 scale: 1 = no symptoms, 5 = intermediate symptoms, 9 = severe disease.

	Disease Incidence*				
	14 DAP (1-9 scale)	**28 DAP** (1-9 scale)	**56 DAP** (1-9 scale)	**84 DAP** (1-9 scale)	**112 DAP** (1-9 scale)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**				
Coker 312	1.0 (N.A.)	1.0 (N.A.)	1.0 (N.A.)	1.8 ± 0.1 (1.5-2.0)	2.0 (N.A.)
TAM66274	1.0 (N.A.)	1.0 (N.A.)	1.0 (N.A.)	1.6 ± 0.1 (1.3-2.0)	2.0 (N.A.)
Significance (p-value) of TAM66274 vs. Coker 312	1.0000	1.0000	1.0000	0.3240	1.0000
Treatments	**2015 Studies Mean ± S.E.M. (Range)**				
Coker 312	1.0 (N.A.)	1.0 (N.A.)	1.0 (N.A.)	1.6 ± 0.0 (1.0-3.0)	1.4 (1.0-2.0)
TAM66274	1.0 (N.A.)	1.0 (N.A.)	1.0 (N.A.)	1.6 ± 0.0 (1.0-3.0)	1.4 (1.0-2.0)
Significance (p-value) of TAM66274 vs. Coker 312	1.0000	1.0000	1.0000	1.0000	1.0000

* Plant diseases observed during the field studies were typical of those found in commercial cotton cultivation: leaf spot (*Alternaria* spp., *Cercospora* spp., *Stemphyllium* spp., *Colletrotrichum* spp.) and boll rot (*Fusarium* spp., *Diplodia* spp., *Glomerella gossypii*, *Xanthomonas* spp., *Rhizoctonia* spp., *Alternaria* spp.)
N.A. All treatments and replications had the same value; therefore, there is no variation.
† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-11. Insect damage.
Comparison of insect damage of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Insect damage was rated on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage.

	Insect Damage*				
	14 DAP (1-9 scale)	**28 DAP** (1-9 scale)	**56 DAP** (1-9 scale)	**84 DAP** (1-9 scale)	**112 DAP** (1-9 scale)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**				
Coker 312	3.7 ± 0.0 (3.0-4.0)	2.3 ± 0.0 (2.0-3.0)	2.7 ± 0.03 (2.0-4.0)	1.73 ± 0.0 (1.0-3.2)	1.7 ± 0.0 (1.0-3.0)
TAM66274	3.7 ± 0.0 (3.0-4.0)	2.3 ± 0.0 (2.0-3.0)	2.7 ± 0.03 (2.0-4.0)	1.80 ± 0.0 (1.0-3.1)	1.7 ± 0.0 (1.0-3.0)
Significance (p-value) of TAM66274 vs. Coker 312	1.0000	1.0000	1.0000	0.4790	1.0000
Treatments	**2015 Studies Mean ± S.E.M. (Range)**				
Coker 312	2.39 ± 0.01 (1.0-3.0)	2.07 ± 0.05 (1.1-3.3)	2.48 ± 0.03 (1.3-3.1)	2.08 ± 0.01 (1.0-3.3)	1.90 ± 0.01 (1.0-2.5)
TAM66274	2.38 ± 0.01 (1.0-3.0)	2.12 ± 0.05 (1.1-3.5)	2.43 ± 0.03 (1.0-3.1)	2.08 ± 0.01 (1.0-3.2)	1.90 ± 0.01 (1.0-2.5)
Significance (p-value) of TAM66274 vs. Coker 312	0.3482	0.5608	0.2422	0.6615	1.0000

***** Insects observed during the field studies were typical of those found in commercial cotton cultivation: thrips (*Frankiella fusca*), tarnished plant bug (*Lygus lineolaris*), stinkbug (*Halyomorpha halys*), cotton bollworm (*Helicoverpa armigera*), and spider mites (*Tetranychidae* spp.)
† Mean values of TAM66274 and non-transgenic cv. Coker 312 compared across all locations separately by year are statistically significantly different at $P<0.05$.

Table 7-12. Rodent damage.
Comparison of rodent damage of TAM66274 and non-transgenic cv. Coker 312 grown in three field locations in the U.S. in 2014 and five U.S. field locations in 2015. Reported values for each treatment are the mean, standard error of the means, and range across field locations for each year separately. Rodent feeding on mature seed was rated on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage.

	Rodent Damage
	Harvest (1-9 scale)
Treatments	**2014 Studies Mean ± S.E.M. (Range)**
Coker 312	1.0 (N.A.)
TAM66274	1.0 (N.A.)
Significance (p-value) of TAM66274 vs. Coker 312	1.000
Treatments	**2015 Studies Mean ± S.E.M. (Range)**
Coker 312	1.0 (N.A.)
TAM66274	1.0 (N.A.)
Significance (p-value) of TAM66274 vs. Coker 312	1.0000

N.A. All treatments and replications had the same value; therefore, there is no variation.
† Mean values compared across all locations separately by year (orthogonal contrast) are statistically significantly different at $P<0.05$.

8. AGRONOMIC PRACTICES AND ENVIRONMENTAL IMPACTS

As part of the plant pest risk assessment required by 7 CFR §340.6(c)(4), detailed descriptions of known and potential differences from the unmodified recipient organism must be included that would substantiate that a regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived. This section provides a summary of current agronomic practices in the U.S. for producing cotton and describes potential impacts of cultivation of TAM66274 on these practices. In addition, this section describes the weediness of TAM66274, the impact on the weediness of any other plant with which it can interbreed, effects of TAM66274 on NTOs, indirect plant pest effects on other agricultural products, and transfer of genetic information to organisms with which it cannot interbreed.

With the exception of the reduced seed gossypol, TAM66274 is phenotypically and agronomically comparable to non-transgenic cv. Coker 312. The ULGCS trait does not alter the weediness characteristics of TAM66274 and is unlikely to confer a selective advantage to any other plant with which it can interbreed. The genetic material introduced into TAM66274 is not toxic and does not encode a substance toxic to NTOs beneficial to agriculture. The ULGCS trait does not increase the insect pest or disease susceptibility of TAM66274 and is unlikely to have indirect plant pest effects on other agricultural products. We are not aware of any reports of unintended transfer of genetic material from cotton to sexually incompatible species, but in the unlikely event that such a transfer were to occur, the ULGCS trait is unlikely to present a human health or plant pest risk. Therefore, unconfined environmental release of TAM66274 will likely have no significant impact on U.S. cotton agronomic practices, except for implementation of an identity preservation system to capture the increased value of the cottonseed. Similarly, unconfined release of TAM66274 will likely have no significant plant pest risks to U.S. agriculture or the natural environment.

8.1 Agronomic Practices

8.1.1. Cotton production.

Cotton is one of the world's most important natural textile fibers, accounting for about 30% of the total world fiber use (USDA ERS, 2017d). The United States, which ranks third in production behind India and China, is the leading exporter, accounting for nearly 30% of global trade in raw cotton (USDA ERS, 2017d). Cotton is an important commodity in the agricultural economy of the United States. In 2012, there were 18,155 cotton farms across the U.S. Cotton Belt from Virginia to California (USDA NASS, 2014). Beyond the farm gate, the distribution and processing of cotton includes cotton gins, merchants, warehouses, cottonseed distributors and processors, and textile mills (Adams, 2015). The U.S. cotton industry accounts for more than $25 billion in products and services annually, generating approximately 200,000 jobs among various industry sectors from farm to textile mill (USDA ERS, 2017c). Two species of cotton

account for 95% of world cotton production: *G. hirsutum*, known as upland cotton; and *G. barbadense*, known as Pima or Egyptian cotton.

In the U.S., cotton is grown in 17 southern States, with major concentrations in the following areas: Texas High and Rolling Plains; the Mississippi, Arkansas and Louisiana Delta; Southern Georgia; and California's San Joaquin Valley. Upland cotton is grown in all cotton-producing states, and accounts for over 95% of planted acreage. Pima cotton accounts for the remaining acreage and is grown only in California, Texas, Arizona and New Mexico. Table 8-1 shows cotton acreage planted by state and type for 2017. Figures 8-1 and 8-2 show acreage planted to upland and Pima cotton by county in 2015 for selected states.

Yields vary by region and from year to year. Average lint yields ranged from a low of 665 lbs/acre in North Carolina to a high of 1,846 in California in 2016 for upland cotton production, and from 720 lbs/acre in Arizona to 1,527 lbs/acre in California in 2016 for Pima cotton production. Across the Cotton Belt, average lint yields in 2016 were 844 lbs/acre and 1,411 lbs/acre for upland and Pima cotton production, respectively (Table 8-2). Cottonseed yields vary accordingly, from a low of 800 lbs/acre in North Carolina to 2,541 lbs/acre in California and 1,122 lbs/acre on average, for 2016 (Table 8-3).

Acreage devoted to cotton production fluctuates over time. Since 2007, acres planted to upland cotton have varied between a low of 8.4 million in 2015 and a high of 14.4 million in 2011. Similarly, acres planted to Pima cotton have varied from a low of 141,000 in 2009 to a high of 307,000 in 2011 (Figures 8-3 and 8-4).

Texas devotes more area to cotton production and produces more cotton than any other state, producing nearly seven million bales in 2016, and accounting for 44% of all U.S. cotton production (Table 8-2). Other states producing over one million bales in 2016 included Georgia and Mississippi (Table 8-2). In 2016, 9.5 million acres of cotton were harvested in the U.S. with production value estimated at approximately $5.7 billion (USDA NASS, 2017c).

Prospective estimates of all cotton planted in the U.S. for 2017 are 12.2 million acres, with 12.0 million acres estimated for upland cotton and the remaining acreage dedicated to Pima cotton (USDA NASS, 2017b).

Cotton is cultivated primarily for fiber. However, cottonseed is an economically important secondary product of cotton production that accounts for between 13-24% of crop value, depending on relative prices of fiber and various seed products (USDA ERS, 2017b). For every 100 pounds of fiber, the cotton plant also produces 145 to 165 pounds of cottonseed. The ginning process separates fiber for textile use from the seed. The resulting cottonseed can either be further processed or be used directly as cattle feed (OECD, 2009). Cottonseed is processed into

meal (45% by weight), hulls (27%), crude oil (16%), linters (8%) and waste (4%) (NCPA, 2017). The oil is the most valuable product of cottonseed, a major oilseed crop in the U.S. Cottonseed oil makes up approximately 5-6% of the total U.S. domestic fat and oil supply, ranking third behind soybean and corn oil for human consumption (OECD, 2009).

Cottonseed is a valuable foodstuff for cattle, combining high energy, high fiber and high protein, and is used as whole seed, hulls, flour and cake (OECD, 2008). Cottonseed meal or flour is also sometimes used for human consumption when derived from gossypol-free varieties, or if the gossypol has been extracted or is present in the food at low levels (OECD, 2008). Linters are short cellulose fibers removed from fuzzy seed before crushing, which are then highly processed into cellulose for industrial chemical and human food uses (OECD, 2009).

Table 8-1. Cotton acres planted by state and type (2017).

State	Upland cotton (1000 acre)	Pima cotton (1000 acre)	Total (1000 acre)
Alabama	450		450
Arizona	165	15	180
Arkansas	440		440
California	81	215	296
Florida	90		90
Georgia	1,350		1,350
Kansas	56		56
Louisiana	200		200
Mississippi	550		550
Missouri	300		300
New Mexico	56	5	61
North Carolina	360		360
Oklahoma	470		470
South Carolina	240		240
Tennessee	320		320
Texas	6,600	17	6,617
Virginia	75		75
Total U.S.	**11,803**	**252**	**12,055**

Source: USDA NASS, 2017c

Table 8-2. Cotton area planted and harvested, yield and production by type and state (2016).

State	Acres Planted (1000)	Acres Harvested (1000)	Yield (lb/acre)	Production (1000 bales)
Upland				
Alabama	345	343	987	690
Arizona	120	118	1,586	360
Arkansas	380	375	1,075	830
California	66	65	1,846	250
Florida	102	100	960	180
Georgia	1,180	1,170	903	2,250
Kansas	32	31	1,099	60
Louisiana	140	137	911	280
Mississippi	435	430	1,228	1,100
Missouri	280	266	1,029	600
New Mexico	47	41	1,171	75
North Carolina	280	260	665	430
Oklahoma	305	290	1,026	565
South Carolina	190	184	678	340
Tennessee	255	250	1,104	560
Texas	5,650	5,200	720	6,900
Virginia	73	72	667	130
U.S. Total Upland	**9,880**	**9,332**	**844**	**15,600**
Pima				
Arizona	14.5	13	720	20
California	155	154	1,527	490
New Mexico	8	7.7	997	16
Texas	17	15	1,024	32
U.S. Total Pima	**194.5**	**189.7**	**1,411**	**558**

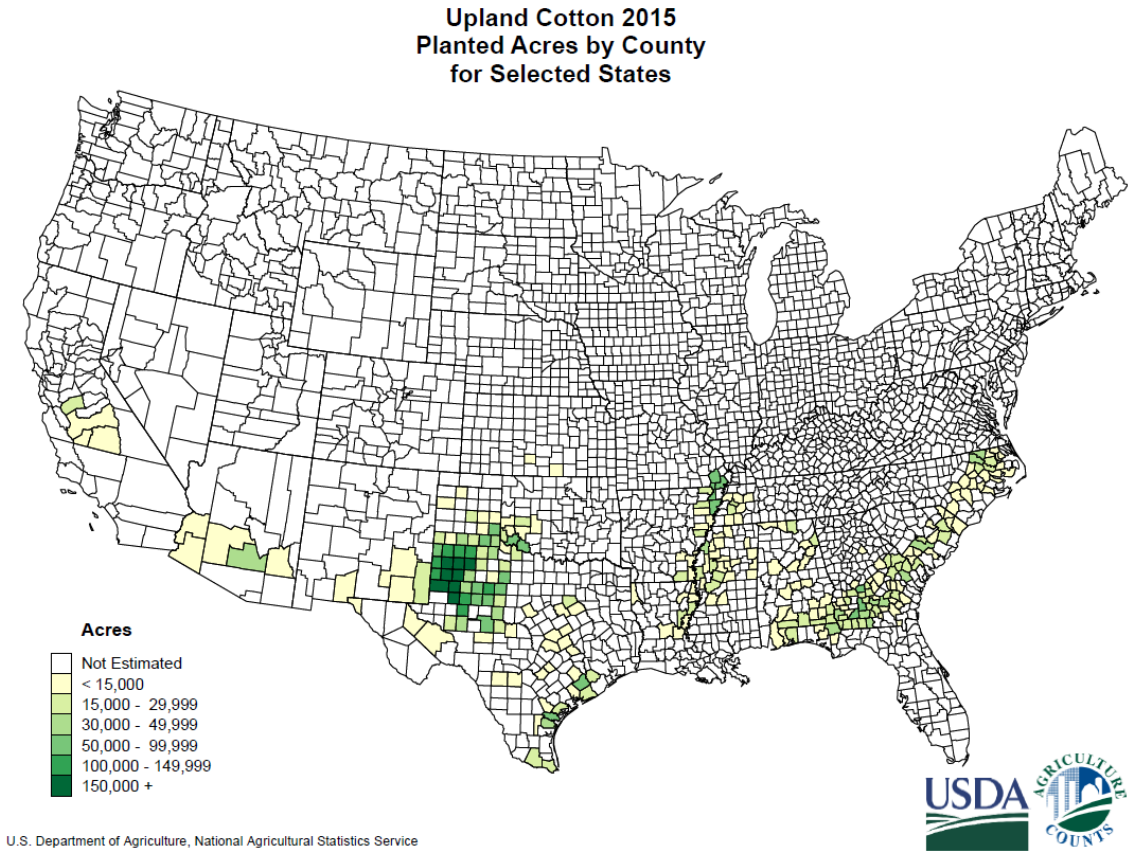
Source: USDA NASS, 2017c

Table 8-3. Cottonseed production by state (2016).

State	Total Harvested Acres[1]	Yield (lbs/acre)[2]	Production (1000 tons)
Alabama	342	1,193	204
Arizona	129	2,155	139
Arkansas	375	1,541	289
California	218	2,541	277
Florida	100	1,140	57
Georgia	1,180	1,063	627
Kansas	31	1,419	22
Louisiana	140	1,271	89
Mississippi	435	1,605	349
Missouri	271	1,587	215
New Mexico	48	1,625	39
North Carolina	275	800	110
Oklahoma	285	1,432	204
South Carolina	189	825	78
Tennessee	250	1,504	188
Texas	5,316	941	2,502
Virginia	72	806	29
Total U.S.	**9,655**	**1,122**	**5,418**

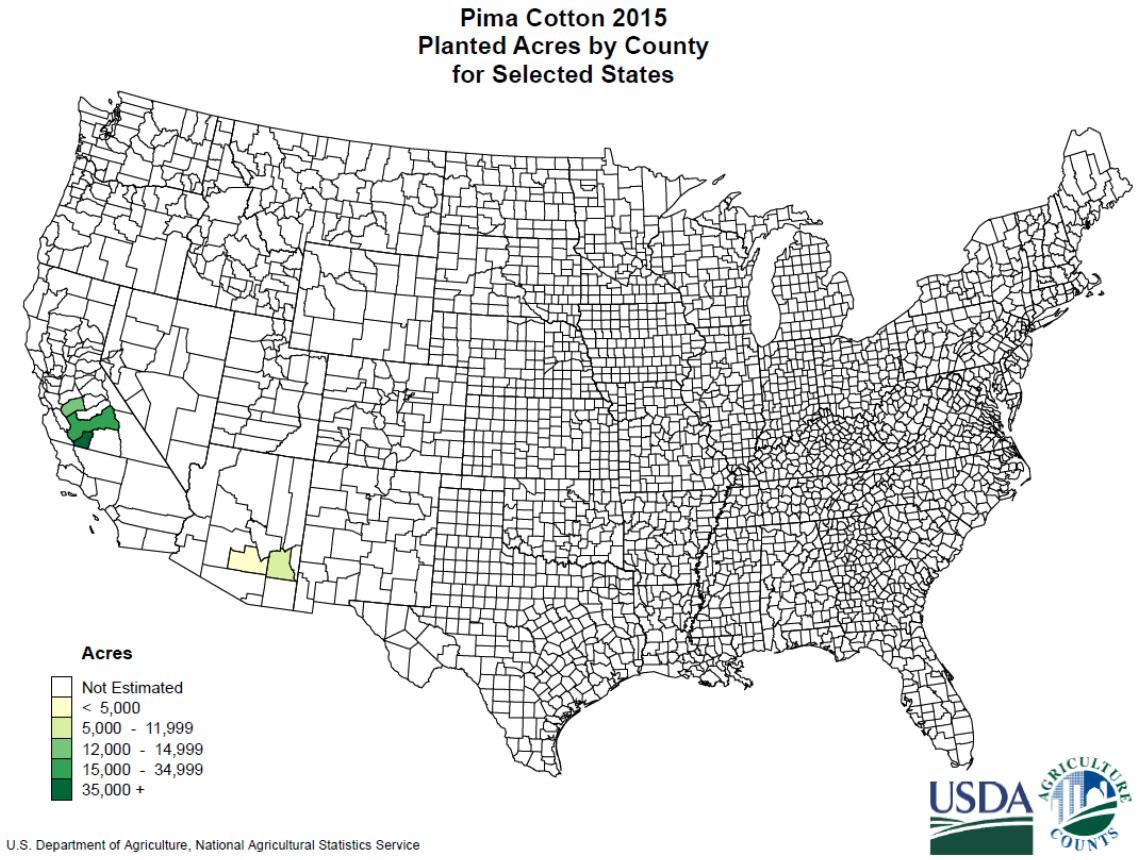
[1] Total of upland and Pima cotton acres harvested.
[2] Calculated.
Source: USDA NASS, 2017c

Figure 8-1. Upland cotton planted acres by county in the U.S. (2015).



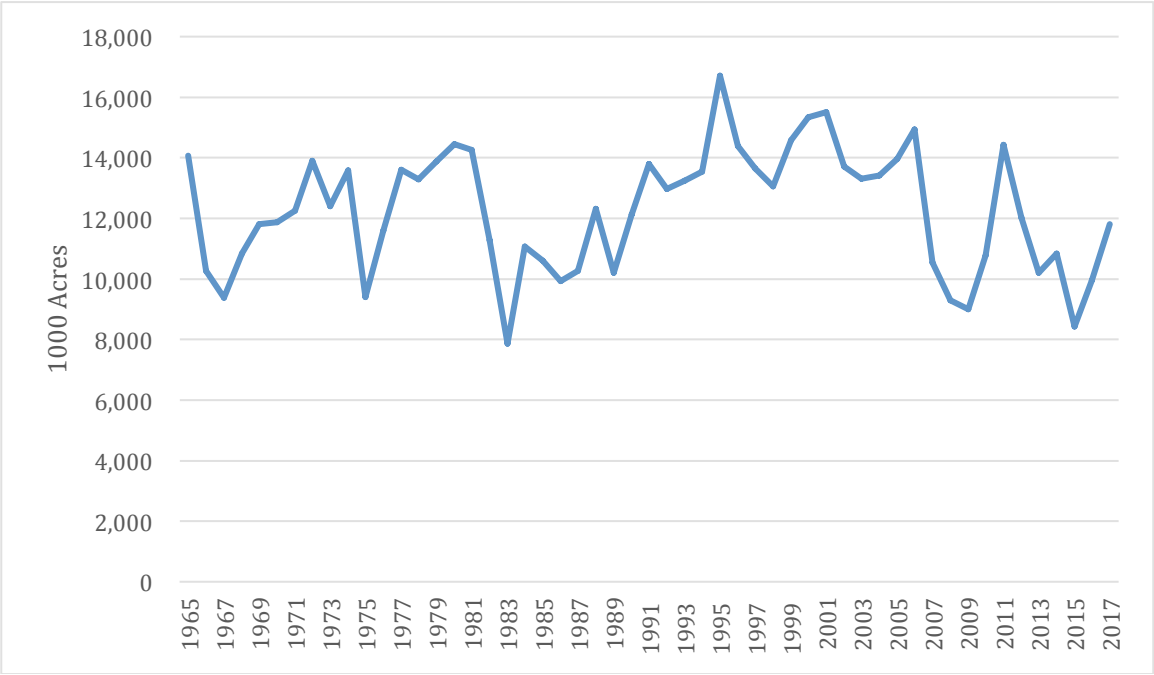
Source: USDA NASS, 2017d

Figure 8-2. Pima cotton planted acres by county in the U.S. (2015).



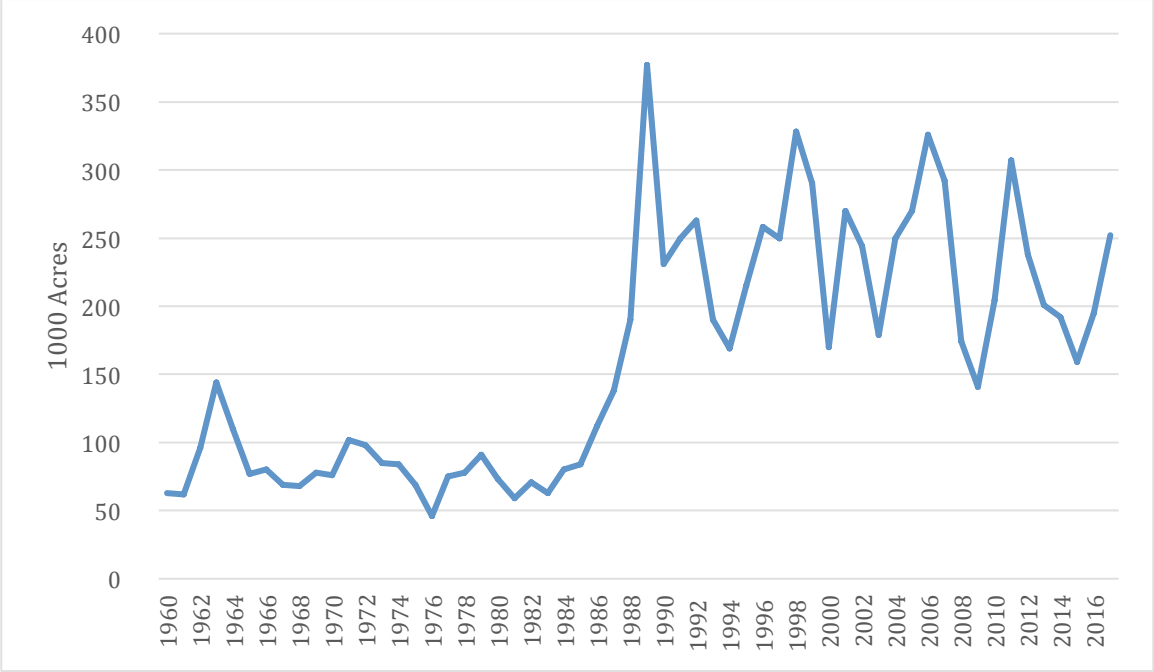
Source: USDA NASS, 2017a

Figure 8-3. Upland cotton acres planted in the U.S. (1965-2017).



Source: USDA ERS, 2017e

Figure 8-4. Pima cotton acres planted in the U.S. (1960-2017).



Source: USDA ERS, 2017e

8.1.2. General production practices.

Both upland and Pima cotton species are potentially perennial, but are grown commercially in the U.S. as annual crops. Planting dates vary by region and may begin as early as mid-March in Arizona and continue into late June in Kansas, with most active planting taking place from April 1 through mid-June. Harvest also varies by region, beginning as early as August 10 in Texas and continuing into late December in Oklahoma, with most active harvest periods from mid-September to mid-late December (USDA NASS, 2010).

Cotton is generally sensitive to temperature, as germination and seedling development is prevented by temperatures below 15°C and delayed by temperatures above 38°C (Hake-Johnson et al., 1996; OECD, 2008). Between 180 and 200 frost-free days after planting are necessary for the normal development of upland cotton, with an average of 150 days of sufficient temperatures (i.e., 1200 heat units above 15.5°C accumulated). For Pima cotton, 200-250 days are needed. Cotton develops best in deep arable soils with good drainage, high organic matter and high moisture retention capacity, but may be cultivated in a wide variety of soils (OECD, 2008).

Optimal seeding rates for a given field depend on climate, production practices and soil. Recommended rates vary for irrigated and non-irrigated acreage as well as temperate versus tropical areas (Bauer, 2015).

Cultivation is used to prepare fields for planting, controlling weeds, preparing seedbeds and improving the physical condition of the soil. However, conventional tillage results in soils that are susceptible to erosion. Conservation tillage practices that leave at least 30% of the soil surface covered by plant residues after planting reduce erosion, increase soil organic matter near the soil surface and reduce water losses. Cotton farmers have increased their adoption of conservation tillage practices over the past 25 years. Adoption of conservation tillage practices, including no-till, ridge till and mulch till, has increased from 0.2% of total U.S. cotton acreage in 1989 to 21.4% in 2004, while decreasing conventional tillage (0-15% residue) from 90.3% to 65.5% (CTIC, 2017). Using slightly different definitions, surveys conducted in 2008 and 2015 showed a decrease in use of conservation tillage (15-30% residue) from 31% to 19%, while no-till and strip till increased from 36% to 45% (Daystar et al., 2017). Adoption of no-till and strip-till practices varies across cotton production regions, from 18% in the Fruitful Rim, 24% in the Prairie Gateway, 31% in the Heartland and Mississippi Portal and 60% in the Southern Seaboard in 2007 (Wade et al., 2015).

Cover crops can also improve soil health by reducing soil erosion, trapping nitrogen and other nutrients, increasing biomass, reducing weeds and loosening the soil to reduce compaction and improve water infiltration. In a 2010-11 survey of corn, soybean, cotton and wheat farmers, 4% of farmers adopted cover crops on some portion of their fields, accounting for 1.7% of cropland in 2010-11. Planting of cover crops varies by region, with highest adoption in the Southern

Seaboard, followed by the Mississippi Portal (Wade et al., 2015). Surveys conducted in 2008 and 2015 indicated an increase in the use of winter cover crops by cotton farmers, from 39% to 48% (Daystar et al., 2017).

The majority of cotton acreage, over 60%, is planted to continuous cotton. However, a significant proportion of cotton is rotated to other crops or small grains, with a small percentage left fallow (Ebel, 2012).

8.1.3. Insect pest management.

Cotton is susceptible to a wide range of insect pests. Among the most destructive are the cotton bollworm, plant bugs, stink bugs, aphids, thrips and spider mites (Cotton Incorporated, 2017). Each year, experts in each state of the Cotton Belt estimate the extent and damage caused by insect pests. In 2016, lygus and stink bugs caused the greatest amount of damage to the U.S. cotton crop (Table 8-4). During the past three decades, there have been major shifts in the relative abundance and importance of major insect pests. Significantly, overall crop losses from insects and mites declined from 7.6% of the harvestable crop in 1983 to 2.6% in 2013 (Luttrell et al., 2015).

Insect pest management in cotton has seen several advances in the past 30 years, including the near-complete eradication of boll weevil and the availability of genetically engineered insect resistant cotton, among the most notable. Adoption of insect resistant varieties ranges from 36% in California to 98% in Tennessee (USDA ERS, 2017a). The extent of use of conventional insecticides also varies, from just 15% of cotton acres treated in Texas in 2015 to 99% in Arkansas, and 40% for the U.S. (USDA NASS, 2017c).

Eradication of boll weevil and the introduction of insect-protected cotton correspond with large expansions of area planted to cotton in several states between 1983 and 2013, including Florida (13-fold), Georgia (11-fold), North Carolina (8-fold), South Carolina (3-fold) and Missouri (2-fold) (Luttrell et al., 2015).

Table 8-4. Cotton insect losses (2016).

Pest	Acres Infested	Acres Treated	Bales Lost
Bollworm/Budworm	3,709,377	1,480,156	117,118
Beet Armyworm	130,088	934	29
Fall Armyworm	571,926	140,556	21,604
Loopers	122,325	0	0
Cutworms	305,759	494,839	100
Cotton Leaf Perforator	6,648	0	0
Saltmarsh Caterpillar	32,104	982	68
Verde Plant Bugs	157,780	75,460	52
Cotton Fleahopper	6,229,625	1,355,471	16,439
Lygus	4,906,100	2,374,603	260,154
Stink Bugs	4,390,201	2,623,231	164,558
Clouded Plant Bugs	363,648	104,340	8,141
Brown Stink Bug	705,959	76,415	1,962
Bagrada Bugs	0	0	0
Leaf Footed Bugs	252,778	7,140	42
Spider Mites	2,066,204	687,779	40,813
Thrips	9,477,763	3,340,547	120,286
Aphids	3,054,545	498,525	5,949
Grasshoppers	1,597,856	81,983	19
Banded Winged Whitefly	495,141	5,621	5,631
Silverleaf Whitefly	465,999	214,807	12,338
Darkling Beetle	2,906	0	0
Pale-striped Flea Beetles	14,475	3,244	85
Mealybugs	0	0	0
Crickets	982	0	0
Boll Weevils	68,600	0	0
Total			**775,389**

Source: Williams, 2017

8.1.4. Management of diseases and other pests.

Cotton is affected by a number of important diseases and other pests such as nematodes that limit production. It is estimated that between 8 and 17% of yields are lost from diseases and 4% of yields are lost from nematode feeding in the U.S. (Rothrock et al., 2015; Weaver, 2015).

Consistent occurrence of seedling disease, boll rots and Fusarium wilt reduce cotton yields. The four primary agents of seedling disease worldwide are *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola*. Seedling diseases affect germination of cottonseed and emergence, survival and development of seedlings. Boll rot is an important problem in cotton production, with losses as high as 33% reported in certain fields in the southeast. While many pathogens may cause boll rot, the most important of these are "primary" pathogens, particularly the bacterial blight pathogen, *Xanthomonas citri* subsp. *malvacearum*. Fusarium wilt is capable of causing significant yield reductions in cotton, infecting plants through the roots, and later colonizing the xylem elements of the vascular system (Rothrock et al., 2015).

Cotton seedling disease control relies on avoidance by delaying planting until soil temperatures are favorable for germination and growth; planting high quality seed; planting at the proper depth; and planting a well-prepared raised seedbed in well-drained soils. Fungicide seed treatments are also frequently used (Rothrock et al., 2015). Bacterial blight is managed by limiting carryover through appropriate cultural practices and processing planting seed, as the pathogen is unlikely to survive for more than one growing season. Fusarium wilt is often associated with the nematode *Meloidogyne incognita*, the use of cultivars with nematode resistance or nematicides can reduce disease severity. Crop rotation is also often recommended for the impact on *M. incognita*.

Two main species of nematodes cause economic yield loss in cotton: the southern root-knot nematode (*Meloidogyne incognita*) and the reniform nematode (*Rotylenchulus reniformis*). Of lesser importance are the Columbia lance (*Hoplolaimus columbus*) and sting nematodes (*Belonolaimus longicaudatus*). Data presented in Weaver (2015) estimate cotton yield losses due to nematodes at approximately 4.25% in 2012, the most recent year reported in that publication. Data from the National Cotton Council estimates cotton yield losses due to nematodes at approximately 4% in 2010, a drop from the highest reported losses of 5.32% in 2006. According to the National Cotton Council, Beltwide average annual cotton yield losses due to nematodes for the 50-year period is 2.47% (National Cotton Council, 2017).

Nematicides applied at planting time can reduce soil populations of root-knot nematode and improve yields in fields infected with reniform nematodes. The use of crop rotation is limited by a general lack of suitable non-host rotation crops as well as economic factors. Cultivars with high levels of genetic resistance to root-knot nematode are available, and cultivars with resistance to reniform nematode are under development and expected to be available soon (Weaver, 2015).

8.1.5. Weed management.

Weeds are a major problem in cotton production, as cotton emerges and grows slowly during the first few weeks after planting. During this period of crop establishment, which may last for 9 to 10 weeks after planting, weed control is necessary in cotton. It is estimated that over 30 genera of plants include important species of weeds in U.S. cotton production, including both annual and perennial as well as grass, sedge and broadleaf species (Buchanan, 1992). The prevalence and importance of different weed species varies across the Cotton Belt. For the southern states, a 2013 weed survey identifies the 10 most common and 10 most troublesome weeds in cotton across eight states. Palmer amaranth (*Amaranthus palmeri*) is ranked the number one most troublesome weed in seven of the eight states. Of those rankings, glyphosate-resistant biotypes were ranked number one in two states (AL, FL), and glyphosate/acetolactate synthase (ALS)-resistant biotypes were ranked number one in two states (GA, NC) (Webster, 2013).

Weed management in cotton changed with the introduction of herbicide-tolerant cultivars in the mid-1990s. The adoption of herbicide tolerant cotton varieties ranges from 61% in California to 98% in Mississippi in 2016 (USDA ERS, 2017a). Herbicides are widely used, on 92% of total U.S. cotton acreage, ranging from 85% of cotton acres treated in Arizona to 100% in Alabama, Georgia, Mississippi, Missouri, South Carolina and Tennessee (USDA NASS, 2017c). Commercial introduction of glyphosate-resistant cotton in 1997 has been associated with an increase in the area of monoculture cotton and conservation tillage, as well as a reduction in non-glyphosate and pre-emergence herbicides. Monoculture and reliance on a single herbicide are commonly linked to the evolution of herbicide resistant weeds (National Academy of Sciences, 2016). Glyphosate resistant weeds have become an increasing concern for cotton growers in recent years, leading to the increased use of more diverse herbicides and physical weed control practices such as tillage and hand weeding (Sosnoskie et al., 2014).

8.1.6. Potential impacts of TAM66274 on agronomic practices.

TAM66274 does not significantly differ from non-transgenic cv. Coker 312 in agronomic or ecological characteristics (Section 7) and is not expected to significantly impact U.S. cotton or cottonseed production practices.

Once deregulated, TAM66274 will be combined with public sector cotton varieties using traditional breeding techniques for release into an identity preserved production system. Initially, the ULGCS trait in TAM66274 will be introduced as a stand-alone trait in public sector upland cotton varieties. Eventually, it is anticipated that the ULGCS trait will be incorporated into private sector breeding programs as well, and will be made available in a wide range of cotton varieties with other currently available (e.g., herbicide tolerance, insect resistance) and/or forthcoming traits.

The vast majority of upland cotton acreage in the U.S. is planted to genetically engineered varieties with insect resistance and/or herbicide tolerance traits. In 2016, 93% of upland cotton acreage was planted to genetically engineered varieties: 4% were insect resistant only varieties, 9% were herbicide tolerant only varieties, and 80% were stacked varieties containing both herbicide tolerance and insect resistance (USDA ERS, 2017a). Approximately 674,000 acres of upland cotton were planted to non-genetically engineered varieties in 2016 (Table 8-5). Agronomic practices of growers planting varieties with TAM66274 are expected to be similar to those currently used by growers of non-genetically engineered varieties.

Except for ultra-low levels of seed gossypol, TAM66274 is phenotypically and agronomically comparable to non-transgenic cv. Coker 312. No statistically significant or biologically meaningful differences were detected in seed germination and stand count, vegetative growth, or plant susceptibility to disease and insect pests or rodents for TAM66274 relative to non-transgenic cv. Coker 312. Further, there were no statistically significant differences for the majority of the reproductive development, fiber quality and plant mapping parameters for TAM66274 compared to non-transgenic cv. Coker 312. In the few instances where statistically significant differences were observed between the treatments for some agronomic and germination comparisons, these differences were inconsistent over the two field trial seasons and, therefore, were not considered agronomically meaningful. Fiber length of TAM66274 was consistently shorter than non-transgenic cv. Coker 312, but within commercially acceptable limits and does not pose a risk of increased weediness or plant pest characteristics. Therefore, unconfined environmental release of TAM66274 is unlikely to significantly impact U.S. cotton agronomic practices, except for implementation of an identity preservation system to capture the increased value of the cottonseed.

Table 8-5. Upland cotton acres planted to non-GE cotton (2017).

State	Acres Planted	Percent Non-GE	Non-GE Acres Planted[1]
Alabama	450,000	2	9,000
Arkansas	440,000	1	4,400
California	81,000	28	22,680
Georgia	1,350,000	1	13,500
Louisiana	200,000	1	2,000
Mississippi	550,000	1	5,500
Missouri	300,000	1	3,000
North Carolina	360,000	4	14,400
Tennessee	320,000	1	3,200
Texas	6,600,000	6	396,000
Other States[2]	1,152,000	3	34,560
U.S.	**11,803,000**	**7**	**472,120**

[1]Calculated
[2]Acres planted for other states includes Arizona, Florida, Kansas, New Mexico, Oklahoma, South Carolina and Virginia
Sources: USDA ERS, 2017a; USDA NASS, 2017c

8.2 Environmental Impacts

8.2.1. Weediness of TAM66274.

Upland cotton is not considered to have weedy characteristics and USDA APHIS has previously determined that cotton is not a plant pest in the U.S. (USDA petition 13-262-01p, 12-185-01p, 12-033-01p, 08-340-01p, 07-108-01p, 06-332-01p, 06-332-01p, 04-086-01p, 03-155-01p, 03-036-01p, 02-042-01p, 00-342-01p, 97-013-01p, 95-256-01p, 95-045-01p, 94-308-01p, 93-196-01p) (USDA APHIS, 2017). Additionally, cotton is not listed as a Federal noxious weed species (7 CFR Part 360), nor does it possess attributes commonly associated with weeds. Commercial cotton varieties rarely display any dormancy characteristics, but may grow as a volunteer under favorable conditions (OECD, 2008; OGTR, 2008). Volunteer cotton is readily controlled by two primary methods: tillage or herbicide treatment (Morgan et al., 2011a; Morgan et al., 2011b).

The introduction of the ULGCS trait into cotton does not alter its weediness characteristics. Agronomic properties of TAM66274 related to weediness, such as germination, emergence, seedling vigor, and response to environmental conditions have been shown to be substantially identical to non-transgenic cv. Coker 312 (Section 7). If individual TAM66274 plants were to overwinter, they can still be effectively controlled by tillage or herbicide treatment.

8.2.2. Impact on the weediness of any other plant with which TAM66274 can interbreed.

Two cultivated and two wild species of cotton grow in the United States and its territories. *G. hirsutum* (upland cotton) is the most widely cultivated species. Native or naturalized populations of *G. hirsutum* occur in Florida, Puerto Rico, and the Virgin Islands, while naturalized populations grow in some of the Hawaiian Islands. The second cultivated species, *G. barbadense* (Pima or Egyptian cotton), is grown in Arizona, California, New Mexico, and Texas. Naturalized populations of *G. barbadense* grow in Puerto Rico, the Virgin Islands and most of the Hawaiian Islands, but it is no longer widely grown as an agricultural commodity in Hawaii. Two wild species of cotton are native to the United States, *G. thurberi* and *G. tomentosum*, which grow in Arizona and Hawaii respectively (USDA NRCS, 2017).

The reproductive biology and pollination characteristics of cotton are well known and have previously been described (OECD, 2008). TAM66274 (*G. hirsutum*) is tetraploid and thus effectively incompatible with diploid species such as *G. thurberi*. Plants from these two groups do not normally hybridize in natural settings and produce fertile offspring, and experimental crosses are difficult (OECD, 2008). In contrast, *G. hirsutum* is sexually compatible with the tetraploids *G. barbadense* and *G. tomentosum* and can form viable progeny with both species (OECD, 2008). Thus, unassisted outcrossing and gene introduction could potentially occur in areas where these species are co-located.

Cotton is considered predominately self-pollinating. Pollen grains are large, heavy and somewhat sticky, which makes dissemination by wind negligible (Jenkins, 1993; McGregor, 1976; OECD, 2008). However, in the presence of suitable insect pollinators cotton is also cross-pollinating at generally low levels (McGregor, 1976; OECD, 2008; Van Deynze, et al., 2005). The extent of spontaneous (unaided) or natural outcrossing depends greatly on the species pool, preferences, and abundance of pollinators, which can vary according to region, location, season, time of day, and use of insecticides (OECD, 2008). Additionally, gene introgression will decrease with increasing spatial isolation between the source and recipient plant populations and physical barriers; intermediate pollinator-attractive plants can reduce the potential for pollen movement (Green and Jones, 1953; Llewellyn et al., 2007; McGregor, 1976; OECD, 2008; Umbeck et al., 1991; Van Deynze et al., 2005; Zhang et al., 2005). Farm scale studies with upland cotton indicate that outcrossing declines sharply with distance from the pollen source, typically below 1% beyond 10 meters (Van Deynze et al., 2005).

Native and feral populations of *G. hirsutum* have become very rare in the major U.S. cotton growing areas due to eradication efforts to control pink bollworm; it has been listed as endangered by the state of Florida (USDA FS, 2013). Naturalized populations of *G. hirsutum* are known to occur in South and Central Florida, but are separated by over 120 miles from the nearest commercial cotton production areas in the Florida panhandle (Calhoun County, FL) (Wunderlin et al., 2017; USDA NASS, 2017d). Thus, outcrossing from TAM66274 to naturalized *G. hirsutum* is highly unlikely.

G. hirsutum is cultivated in many areas where *G. barbadense* is also grown (USDA NASS, 2017b and 2017d). Native or naturalized populations of both species are also present in Hawaii, Puerto Rico, and the Virgin Islands. Although cultivated varieties of both species are predominantly self-pollinating, insect-mediated cross-pollination can occur both within and between the species (Brubaker et al., 1993; Llewellyn et al., 2007; OECD, 2008; Van Deynze et al., 2005). Bumble bees (*Bombus* spp.), *Melissodes* and *Halictus* bees, honey bees (*Apis mellifera*), and *Scolia* wasps are the primary pollinators (McGregor, 1976). Published studies indicate there has been relatively little gene introgression from *G. hirsutum* into native or naturalized *G. barbadense* in Central America and the Caribbean (Fryxell, 1979), while introgression from *G. barbadense* to native or naturalized *G. hirsutum* is relatively common (Brubaker et al., 1993; Wendel et al., 1992). While various mechanisms have been suggested to account for this asymmetry (Brubaker et al., 1993; Jiang et al., 2000; OGTR, 2008; Percy and Wendel, 1990), none leads to complete isolation of the two species (USDA APHIS, 2015). The reported asymmetry in gene flow from *G. hirsutum* to *G. barbadense*, and the lack of commercial cotton production in Hawaii, Puerto Rico, or the Virgin Islands suggests that gene introgression from cultivated TAM66274 to native or naturalized *G. barbadense* is highly unlikely.

In contrast, gene introgression from cultivated *G. hirsutum* to cultivated *G. barbadense* may be more likely due to asymmetric gene flow that is directionally opposite from that observed between native and naturalized populations (Brubaker et al., 1993; Van Deynze et al., 2011; Wendel et al., 1992). However, outcrossing rates from TAM66274 to cultivated *G. barbadense* are likely to be the same as that observed between any cultivated cotton varieties, which depends on spatial isolation during seed production to maintain genetic purity.

Although outcrossing from *G. hirsutum* to *G. tomentosum* is theoretically possible, *G. tomentosum* population are limited to the Hawaiian Islands and the lack of commercial cotton production suggests that gene introgression from TAM66274 to native populations of *G. tomentosum* is highly unlikely.

Overall, the likelihood of TAM66274 hybridizing with cultivated, wild or feral cotton is low due to the predominance of self-pollination, geographic isolation, and other reproductive barriers. If such crosses did occur, the ULGCS trait is unlikely to confer a selective advantage to or enhance the persistence of resulting progeny. Accordingly, the environmental consequence of gene flow from TAM66274 to sexually compatible species is considered to be negligible.

8.2.3. Effects of TAM66274 on non-target organisms beneficial to agriculture.

TAM66274 cotton is genetically engineered for improved product quality and, therefore, has neither target nor non-target species. Rather, the RNAi construct in TAM66274 interferes with expression of *dCS* genes that encode a key enzyme in gossypol biosynthesis in cottonseed, while

leaving gossypol levels unchanged in other plant tissues. The potential for direct or indirect adverse impact on species beneficial to agriculture are considered here.

The genetic material inserted into TAM66274 is not toxic and does not produce any substance that would be considered toxic (Section 5). The trigger sequences for RNAi-mediated suppression are highly specific to *dCS* genes in cotton and share no significant homology to genes in other plant or animal species, nor do they encode a protein toxin or allergen (Section 5). Additionally, TAM66274 contains the *nptII* gene, which is widely distributed in nature and has previously been evaluated for human and environmental safety (EFSA, 2007; Fuchs et al., 1993b). Similarly, USDA APHIS has identified no human or environmental safety issues for use of *nptII* in genetically engineered plants and plant products (APHIS petitions 10-161-01p, 04-337-01p, 04-264-01p, 01-206-02p, 01-137-01p, 96-051-01p, 95-352-01p, 95-045-01p, 94-308-01p, 94-228-01p) (USDA APHIS, 2017).

Based on the specificity of the RNAi-mediated suppression and the absence of demonstrable human or environmental harm from exposure to the NPTII protein, no effects on NTOs beneficial to agriculture are likely from the unconfined environmental release of TAM66274.

8.2.4. Indirect plant pest effects of TAM66274 on other agricultural products.

Field data indicate that in a highly managed cotton cultivation environment, the ULGCS phenotype does not increase the incidence of insect pests or diseases on TAM66274 relative to non-transgenic cv. Coker 312. TAM66274 is no more susceptible to the insect pests and plant pathogens than non-transgenic cv. Coker 312. It follows that there are likely to be no indirect plant pest effects on other agricultural products that are grown or stored in proximity to TAM66274.

8.2.5. Transfer of genetic information to organisms with which TAM66274 cannot interbreed.

Texas A&M University is not aware of any reports regarding the unintended transfer of genetic material from cotton to other sexually incompatible species. In the unlikely event that such a transfer were to occur, the ULGCS trait is unlikely to present a human health or plant pest risk based on safety data presented in this petition.

8.3 Summary of Potential Impacts of TAM66274 on Agronomic Practices and the Environment

Field and laboratory studies confirm that, except for reduced seed gossypol, TAM66274 is phenotypically and agronomically comparable to non-transgenic cv. Coker 312. Therefore, unconfined environmental release of TAM66274 is unlikely to significantly impact U.S. cotton agronomic practices, except for implementation of an identity preservation system to capture the

increased value of the cottonseed. No significant impact is expected on general production practices, insect pest management, disease and other pest management or weed management.

The introduction of the ULGCS trait into TAM66274 does not alter its weediness characteristics. Agronomic properties of TAM66274 related to weediness, such as germination, emergence, seedling vigor, and response to environmental conditions were shown to be substantially identical to non-transgenic cv. Coker 312.

The potential for TAM66274 to hybridize with cultivated, wild or feral cotton and persist in the environment is low due to the predominance of self-pollination, geographic isolation, and other reproductive barriers. If such crosses did occur, the ULGCS trait is unlikely to confer a selective advantage to or enhance the persistence of resulting progeny. Accordingly, the environmental consequences of gene flow from TAM66274 to sexually compatible species are considered to be negligible.

The genetic material inserted into TAM66274 is not toxic and does not produce any substance that would be considered toxic. Based on the specificity of the RNAi-mediated suppression of *dCS* genes in TAM66274 and the absence of demonstrable human or environmental harm from exposure to the NPTII variant protein, no effects on NTOs beneficial to agriculture are likely from unconfined environmental release of TAM66274.

The ULGCS trait does not increase the insect pest or disease susceptibility of TAM66274 relative to non-transgenic cv. Coker 312 and is unlikely to have indirect plant pest effects on other agricultural products that are grown or stored in proximity to TAM66274.

The potential for transfer of genetic material from TAM66274 to sexually incompatible organisms is remote. If such a transfer were to occur, the ULGCS trait is unlikely to present a human health or plant pest risk based on safety data presented in this petition.

Based on the data and information presented in this petition, Texas A&M AgriLife Research submits that TAM66274 is unlikely to pose a plant pest risk to U.S. agriculture or the natural environment.

9. ADVERSE CONSEQUENCES OF INTRODUCTION

Field and laboratory testing have demonstrated that TAM66274 is substantially equivalent to non-transgenic cv. Coker 312 apart from the intended change of ultra-low gossypol levels in the cottonseed. TAMU knows of no study results or other observations indicating that there would be adverse consequences from the unconfined introduction of TAM66274.

10. LIST OF APPENDICES

Appendix A. – Development of TAM66274 and Demonstration of Seed Specificity of the α-Globulin B Gene Promoter (AGP)

A. Methods and Results of β -glucuronidase (GUS) Expression in Cotton Plants Transformed with a Construct Containing the AGP Fused to the *gusA* Reporter Gene
B. Methods and Results of Measurements of Levels of Gossypol and Related Terpenoids in Independent ULGCS Events

Appendix B. – Materials and Methods for Molecular Characterization of TAM66274

Appendix C. – Gel-based, Event-specific Polymerase Chain Reaction Method for Detection of TAM66274 Cottonseed and Certificates of Analysis for Test and Control Seed Used in Safety Assessment Studies

Appendix D. – Materials and Methods for Gene Expression and Bioinformatic Analysis of TAM66274

A. Methods to Measure Levels of *dCS* Transcripts in Tissues of TAM66274 and Non-Transgenic cv. Coker 312, and Bioinformatics Analysis of Potential Non-Target Effects of *dCS* RNAi
B. Methods to Measure Levels of the NPTII Variant Protein in Tissues of TAM66274 and Non-Transgenic cv. Coker 312
C. Results of Bioinformatics Analyses of Amino Acid Sequences Encoded by Putative Open Reading Frames (ORFs) in the T-DNA and Flanking Genomic DNA of TAM66274 Compared to Amino Acid Sequences of Known Allergens and Toxins

Appendix E. – Methods and Results of Cottonseed Composition Analyses

Appendix F. – Materials and Methods of Phenotypic, Agronomic and Ecological Characteristics of TAM66274

Appendix G. – USDA Notifications for TAM66274

11. REFERENCES

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Appendix A.

Development of TAM66274 - Demonstration of Seed Specificity of the α-Globulin B Gene Promoter (AGP)

A. Methods and Results of β-glucuronidase (GUS) Expression in Cotton Plants Transformed with a Construct Containing the AGP Fused to the *gusA* Reporter Gene

B. Methods and Results of Measurements of Levels of Gossypol and Related Terpenoids in Independent ULGCS Events

The ultra-low gossypol cottonseed (ULGCS) phenotype derives from RNAi-mediated suppression of δ-cadinene synthase (*dCS*) genes that encode a key enzyme in gossypol biosynthesis. The *dCS* RNAi construct silences endogenous *dCS* genes in cottonseed through transcriptional control of a highly seed-specific α-globulin B gene promoter (AGP) derived from cotton (*G. hirsutum*) (Sunilkumar et al., 2002). As part of the development of TAM66274 and other ULGCS events, it was critical to demonstrate the seed specificity of the AGP. Therefore, the AGP region was fused to the β-glucuronidase (*gusA*) reporter gene in binary vector pBI101.3 (Clontech), which was used to characterize the promoter activity in transgenic cotton, *Arabidopsis*, and tobacco plants (Sunilkumar et al., 2002). Section A of this Appendix describes the materials and methods for functional characterization of the AGP in cotton, as well as a summary of key results of these reporter gene studies.

After demonstrating the seed-specificity of AGP in the studies described above, the AGP was fused to the *dCS* RNAi construct and was transformed into cotton to create ULGCS events. Quantitative analysis of gossypol and related terpenoids was performed on multiple ULGCS events to confirm the specificity, efficacy and stability of the ULGCS phenotype under field conditions. Section B of this Appendix describes the materials and methods for terpenoid analysis, as well as results of terpenoid analyses in vegetative and reproductive tissues of ten independent ULGCS events grown at the Texas A&M University (TAMU) field site over seven years, including TAM66274.

A. GUS Expression in Cotton Plants Transformed with a Construct Containing AGP Fused to the β-glucuronidase (*gusA*) Reporter Gene

Hypocotyl segments of cotton (*G. hirsutum* cv. Coker 312) seedlings were transformed with an AGP::*gusA* reporter gene construct in *Agrobacterium tumefaciens* strain LBA4404 following the method described by Sunilkumar and Rathore (2001). Plants were regenerated from kanamycin-resistant transgenic calli and grown to maturity in a TAMU greenhouse.

Histochemical and fluorometric GUS analysis was performed in various cotton tissues according to methods described by Jefferson et al. (1987). Briefly, the fluorogenic reaction was carried out in 1 mM 4-methyl umbelliferyl glucuronide (MUG) extraction buffer with a reaction volume of 1 ml. The extracted β-glucuronidase hydrolyzes MUG to the fluorescent compound 4-methylumbelliferone (4-MU) and glucuronic acid. The reaction was incubated at 37°C, aliquots were removed at zero time and subsequent time points, and the reaction quenched with the addition of 0.2 M sodium carbonate. Fluorescence was measured with excitation at 365 nanometers (nm) and detection at 455 nm on a spectrofluorometer calibrated with appropriate 4-MU standards. Protein concentrations of plant extracts were determined by the dye-binding method of Bradford (1976). GUS activity was normalized to the total protein and the results are

presented as GUS specific activity (nanomole of 4-MU released per milligram protein per minute) in various plant tissues.

The results of histochemical and quantitative fluorometric analyses of GUS activity during various stages of transgenic cotton seed and plant development are described in Sunilkumar et al. (2002). A key result from this analysis shows that a high level of GUS activity was detected only in the seeds, while no measurable GUS activity was present in stem, leaf, floral bud, pollen, and root (Table A-1). These results suggested that AGP-driven transgene activity is tightly controlled and is specific to the seed.

Table A-1. GUS activity in tissues of a T1 homozygous AGP::*gusA* transgenic cotton plant. Fluorometric GUS activity in tissues of a T1-homozygous transgenic cotton plant and non-transgenic control cottonseed. Cotton was transformed with a *gusA* reporter gene under transcriptional control of a putative seed-specific promoter of the cotton α-globulin B gene (AGP).

Tissue type	GUS activity[a] (nmol 4-MU/mg protein/min)
Stem	0.018 ± 0.002
Leaf	0.014 ± 0.005
Root	0.12 ± 0.006
Floral bud	0.11 ± 0.05
Pollen	0.024[b]
Transgenic seed[c]	349.9 ± 55
Control seed[c]	0.002 ± 0.0004

[a] Values are mean GUS activity ± SE from three replicates.

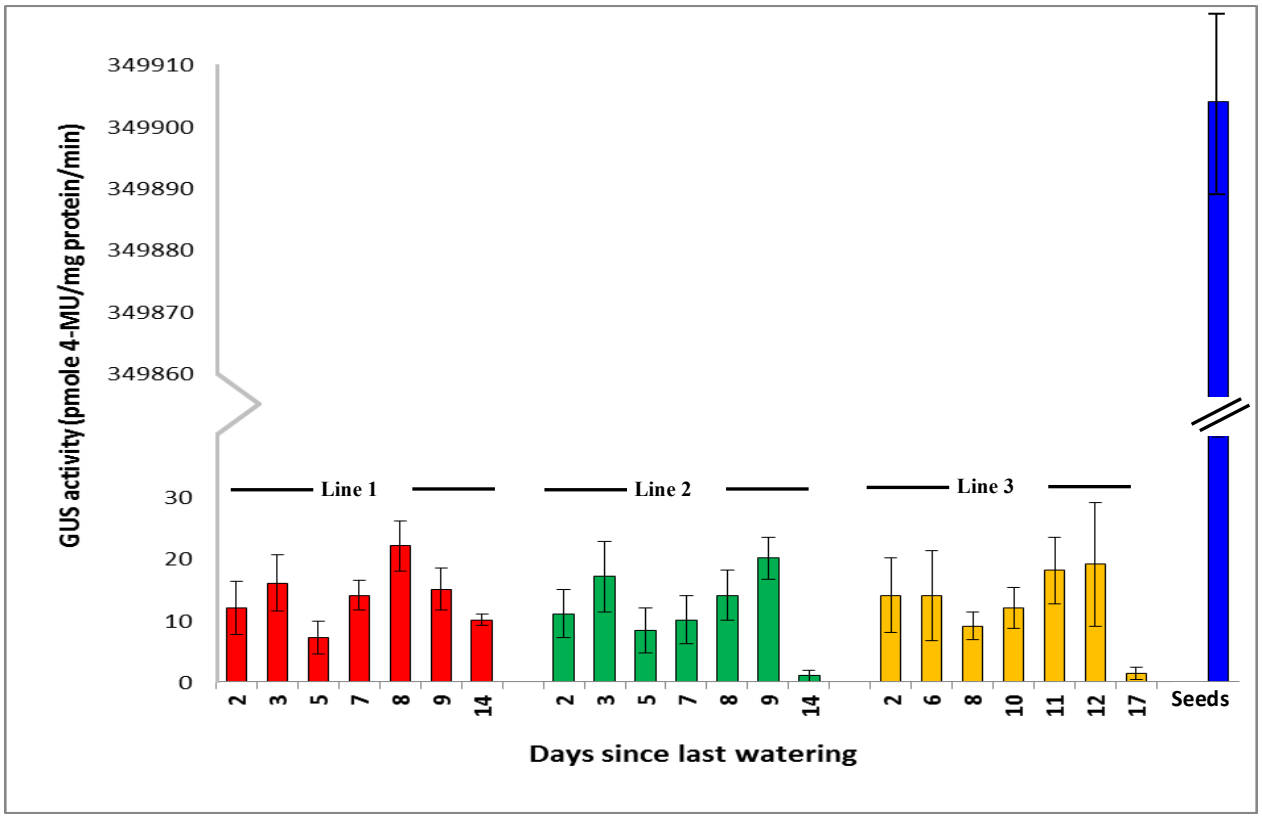
[b] The number of replicates were not sufficient to calculate SE (5.7 mg pollen was used in the assay).

[c] Assay was performed in embryos collected from 10 seeds for each replicate.

Source: Sunilkumar et al., 2002

Certain seed-specific promoters have been shown to be activated in the vegetative parts under water stress conditions (Vivekananda et al., 1992; Siddiqui et al., 1998). In order to explore the possibility of AGP activation under water deficit conditions, fluorometric GUS analysis was performed on the leaves of three independent transgenic cotton plants that were also found to produce GUS-positive seeds. Plants were subjected to drought stress in the greenhouse by withholding water. Leaf samples were analyzed for GUS activity beginning 48 hours after the last watering until they showed complete wilting. No measurable GUS activity was detected in any of the leaf samples from three transgenic plants even after they were completely wilted (Figure A-1). These results suggested that the AGP-based seed-specificity of the ULGCS phenotype would be maintained under field conditions where the plants are likely to experience water stress.

Figure A-1. GUS activity in the leaves of water-stressed AGP::*gusA* transgenic cotton plants obtained from three different events.
Watering was withheld until the leaves showed complete wilting. The seeds were obtained from a transgenic plant expressing the AGP::*gusA* construct, grown under normal greenhouse conditions. [The graph was redrawn based on results presented in U.S. Patent #7,626,081 (Rathore et al., 2009) and Sunilkumar et al., 2002].



B. Terpenoid Analyses in Vegetative and Reproductive Tissues of Multiple ULGCS Events, including TAM66274

To assess the specificity, efficacy and stability of the ULGCS phenotype under field conditions, the levels of gossypol and related plant-defense terpenoids were measured in seed and six non-seed tissues obtained from multiple ULGCS events and non-transgenic cv. Coker 312. Plants were grown at the TAMU Field Laboratory (Sommerville, TX) during the 2009–2015 growing seasons. Ten independent ULGCS events were tested: 66-49B, 66-81, 66-103, 66-163C, 66-193B, 66-239, 66-250, 66-274, 66-316, and 66-317. Not all ULGCS events were planted each year (Table A-2). TAM66274, which is the subject of this petition, is synonymous with event 66-274 and was evaluated during the 2012 and 2015 growing seasons.

Each year, six different tissues (leaves, bracts, terminal ends of axillary branches, floral buds, petals and 2-day old bolls) were harvested 8–10 weeks after sowing, freeze-dried and ground to a fine powder. Terpenoid analyses were performed on these samples and cottonseed kernels using HPLC, following the methods described by Stipanovic et al. (1988) and Benson et al. (2001). Briefly, the finely ground green tissue/petal sample (approximately 100 mg) was extracted by ultrasonication (10 minutes) in 5 ml of solvent containing acetonitrile/water/phosphoric acid (80:20:0.1) in a 15 ml polypropylene tube. Following centrifugation at 2800 x *g* for 5 minutes, a 50-µl fraction of the extract was analyzed on an Agilent Technologies (Palo Alto, CA) 1200 liquid chromatograph, equipped with a diode array detector for compound spectral identification (Stipanovic et al., 1988; Sunilkumar et al., 2006).

A slightly different procedure was used for extracting terpenoids from seeds. Twelve to 15 seeds from each plant were dehulled, and the kernels were ground to a fine powder. Approximately, 500 mg of ground seed was mixed with 50 ml of solvent containing ethanol/ether/water/glacial acetic acid (59:17:24:0.2). The suspension was agitated on a shaker at room temperature for 1 hour to facilitate extraction of terpenoids. The final sample volume was adjusted to 50 ml to account for evaporation and centrifuged for 15 minutes at 2800 x *g*. A 50-µl fraction of the extract was analyzed using the HPLC as described above.

Gossypol, hemigossypolone, and heliocides H1-H4 were reported as µg/mg dry weight of tissue (mean ± SE; n=3 in 2009, 2010, 2011 and 2012; n=4 in 2013, 2014 and 2015). The results demonstrate the tissue-specificity of the AGP promoter that drives RNAi-mediated suppression of *dCS* in seed tissues while leaving levels of gossypol and related terpenoids unchanged in non-seed tissue (Table A-3). Additionally, these results demonstrate the efficacy and stability of the ULGCS phenotype under field conditions in seven years of field trials, in multiple independent cotton events and across multiple seed generations.

Table A-2. ULGCS events planted for terpenoid analysis in various plant tissues.
Selected ULGCS events and non-transgenic cv. Coker 312 were planted at Texas A&M Field Laboratory (Sommerville, TX) during the 2009-2015 growing seasons for analysis of gossypol and related terpenoids in various tissues. Not all ULGCS events were planted in each field trial year.

Event No.	Field Trial Year						
	2009[a]	2010[a]	2011[a]	2012	2013	2014	2015
66-49B	X	X	X				X
66-81	X	X	X				
66-103					X		
66-163C					X		
66-193B					X		
66-239						X	
66-250			X				
66-274[b]				X			X
66-316				X			
66-317				X			
Coker 312 (control)	X	X	X	X	X	X	X

[a] Results previously reported in Palle et al. (2013)
[b] Synonymous with TAM66274

Table A-3. Levels of gossypol and related terpenoids.
The levels of gossypol, hemigossypolone, and heliocides H1-H4 were measured in the seed and six non-seed tissues obtained from cotton plants grown at the TAMU Field Laboratory (Sommerville, TX) during the 2009–2015 growing seasons. Analytes were measured by high performance liquid chromatography and results are reported as μg/mg dry weight of tissue (mean ± SE; n=3 in 2009, 2010, 2011 and 2012; n=4 in 2013, 2014 and 2015).

		2009			2010			2011			
		Coker 312	**66-49B**	**66-81**	**Coker 312**	**66-49B**	**66-81**	**Coker 312**	**66-49B**	**66-81**	**66-250**
Bracts	**G**	0.08 (±0.008)	0.06 (±0.003)	0.11 (±0.011)	0.11 (±0.01)	0.10 (±0.03)	0.12 (±0.02)	0.08 (±0.00)	0.06 (±0.00)	0.16 (±0.02)	0.11 (±0.00)
	HGQ	0.15 (±0.02)	0.10 (±0.012)	0.10 (±0.014)	0.17 (±0.04)	0.12 (±0.04)	0.11 (±0.03)	0.25 (±0.04)	0.24 (±0.02)	0.32 (±0.08)	0.38 (±0.02)
	H1-H4	1.25 (±0.05)	0.89 (±0.06)	1.05 (±0.10)	1.78 (±0.32)	1.59 (±0.46)	1.44 (±0.32)	1.51 (±0.25)	1.07 (±0.59)	1.75 (±0.47)	1.82 (±0.09)
Floral buds	**G**	5.65 (±0.42)	5.04 (±0.39)	6.41 (±0.91)	3.15 (±0.28)	3.14 (±0.43)	3.47 (±0.19)	3.09 (±0.10)	3.17 (±0.05)	4.01 (±0.08)	3.93 (±0.07)
	HGQ	1.11 (±0.14)	0.96 (±0.11)	0.88 (±0.23)	0.45 (±0.09)	0.36 (±0.06)	0.40 (±0.07)	0.51 (±0.06)	0.61 (±0.09)	0.66 (±0.02)	0.87 (±0.04)
	H1-H4	2.39 (±0.09)	1.99 (±0.22)	1.90 (±0.40)	1.47 (±0.32)	1.46 (±0.19)	0.90 (±0.03)	1.89 (±0.13)	2.07 (±0.19)	2.30 (±0.13)	2.40 (±0.12)
Terminal part of axillary branch	**G**	1.04 (±0.30)	0.99 (±0.26)	0.82 (±0.03)	1.02 (±0.03)	0.98 (±0.24)	1.15 (±0.12)	0.92 (±0.14)	1.15 (±0.08)	1.32 (±0.31)	1.17 (±0.19)
	HGQ	1.62 (±0.16)	1.22 (±0.19)	1.15 (±0.17)	1.24 (±0.06)	0.98 (±0.05)	1.12 (±0.09)	2.22 (±0.21)	1.89 (±0.19)	1.96 (±0.27)	2.27 (±0.12)
	H1-H4	2.84 (±0.27)	1.77 (±0.23)	2.43 (±0.40)	1.64 (±0.14)	1.74 (±0.25)	1.94 (±0.17)	3.19 (±0.43)	2.70 (±0.11)	2.91 (±0.21)	3.67 (±0.16)
Leaves	**G**	0.34 (±0.08)	0.19 (±0.02)	0.48 (±0.13)	0.80 (±0.01)	0.43 (±0.04)	0.97 (±0.04)	0.65 (±0.09)	0.62 (±0.04)	1.12 (±0.12)	0.63 (±0.01)
	HGQ	1.66 (±0.49)	0.78 (±0.06)	1.37 (±0.37)	2.25 (±0.23)	1.25 (±0.09)	2.09 (±0.08)	4.04 (±0.40)	3.73 (±0.30)	4.38 (±0.18)	3.57 (±0.15)
	H1-H4	2.68 (±0.36)	1.77 (±0.23)	2.46 (±0.76)	1.81 (±0.38)	0.91 (±0.07)	1.14 (±0.09)	2.94 (±0.25)	2.77 (±0.59)	3.51 (±0.47)	2.57 (±0.11)
2-day bolls	**G**	1.69 (±0.09)	1.37 (±0.08)	1.88 (±0.07)	2.02 (±0.24)	1.22 (±0.05)	1.47 (±0.06)	1.06 (±0.06)	0.86 (±0.02)	1.04 (±0.06)	0.89 (±0.01)
	HGQ	7.29 (±0.05)	6.43 (±0.23)	6.58 (±0.41)	4.29 (±0.28)	3.69 (±0.24)	3.05 (±0.05)	4.95 (±0.41)	4.57 (±0.26)	3.98 (±0.25)	4.32 (±0.03)
	H1-H4	11.03 (±0.14)	9.05 (±0.54)	7.64 (±0.39)	11.27 (±3.58)	9.96 (±1.88)	5.81 (±1.31)	8.66 (±0.73)	8.74 (±0.54)	5.51 (±0.60)	7.62 (±0.38)
Petals	**G**	5.70 (±0.15)	6.28 (±0.22)	6.25 (±0.25)	4.77 (±0.26)	5.92 (±0.13)	5.97 (±0.05)	3.60 (±0.19)	3.78 (±0.30)	4.15 (±0.18)	4.22 (±0.14)
Seed kernel	**G**	6.46 (±0.65)	0.15 (±0.01)	0.31 (±0.03)	6.35 (±0.40)	0.14 (±0.02)	0.21 (±0.02)	7.48 (±0.05)	0.14 (±0.00)	0.38 (±0.00)	0.45 (±0.00)

G: Gossypol; HGQ: Hemigossypolone; H1-H4: Heliocides. Note that the predominant terpenoid in the seed and flower petal glands is gossypol.

Table A-3, continued. Levels of gossypol and related terpenoids.
The levels of gossypol, hemigossypolone, and heliocides H1-H4 were measured in the seed and six non-seed tissues obtained from cotton plants grown at the TAMU Field Laboratory (Sommerville, TX) during the 2009–2015 growing seasons. Analytes were measured by high performance liquid chromatography and results are reported as μg/mg dry weight of tissue (mean ± SE; n=3 in 2009, 2010, 2011 and 2012; n=4 in 2013, 2014 and 2015).

		2012				2013			
		Coker 312	**66-274**	**66-316**	**66-317**	**Coker 312**	**66-103**	**66-163C**	**66-193B**
Bracts	**G**	0.11 (±0.01)	0.17 (±0.01)	0.08 (±0.01)	0.15 (±0.03)	0.09 (±0.02)	0.09 (±0.01)	0.11 (±0.02)	0.11 (±0.01)
	HGQ	0.29 (±0.04)	0.60 (±0.05)	0.22 (±0.03)	0.59 (±0.18)	0.37 (±0.07)	0.25 (±0.03)	0.36 (±0.05)	0.32 (±0.03)
	H1-H4	2.37 (±0.15)	2.2 (±0.06)	1.57 (±0.10)	1.96 (±0.33)	1.49 (±0.15)	1.98 (±0.25)	1.93 (±0.13)	1.89 (±0.17)
Floral buds	**G**	2.07 (±0.25)	1.73 (±0.22)	2.07 (±0.23)	1.53 (±0.19)	2.99 (±0.12)	3.53 (±0.15)	2.51 (±0.08)	3.76 (±0.19)
	HGQ	1.02 (±0.07)	1.06 (±0.11)	1.11 (±0.14)	1.01 (±0.12)	0.89 (±0.11)	0.59 (±0.05)	0.63 (±0.07)	0.75 (±0.04)
	H1-H4	3.3 (±0.24)	2.55 (±0.30)	3.11 (±0.23)	2.07 (±0.31)	2.25 (±0.13)	2.20 (±0.14)	2.09 (±0.14)	2.18 (±0.20)
Terminal part of axillary branch	**G**	0.51 (±0.06)	0.77 (±0.01)	1.07 (±0.10)	0.97 (±0.07)	0.44 (±0.01)	0.51 (±0.05)	0.54 (±0.07)	0.45 (±0.01)
	HGQ	1.31 (±0.12)	1.52 (±0.04)	2.51 (±0.23)	2.14 (±0.11)	1.34 (±0.07)	1.46 (±0.10)	1.73 (±0.13)	1.39 (±0.04)
	H1-H4	2.12 (±0.18)	2.66 (±0.11)	3.43 (±0.24)	3.05 (±0.20)	2.08 (±0.08)	3.35 (±0.14)	3.30 (±0.24)	2.73 (±0.21)
Leaves	**G**	0.95 (±0.09)	1.01 (±0.10)	1.17 (±0.17)	0.93 (±0.06)	0.51 (±0.04)	0.54 (±0.08)	0.45 (±0.06)	0.47 (±0.05)
	HGQ	5.38 (±0.39)	4.48 (±0.32)	4.77 (±0.33)	4.11 (±0.12)	2.40 (±0.10)	2.42 (±0.28)	2.10 (±0.23)	2.17 (±0.13)
	H1-H4	5.67 (±0.50)	3.32 (±0.34)	3.85 (±0.80)	2.66 (±0.44)	1.53 (±0.09)	2.02 (±0.29)	1.52 (±0.14)	1.52 (±0.09)
2-day bolls	**G**	0.90 (±0.08)	0.84 (±0.05)	0.98 (±0.07)	0.89 (±0.07)	1.07 (±0.03)	0.79 (±0.04)	1.36 (±0.07)	0.88 (±0.06)
	HGQ	4.30 (±0.18)	3.69 (±0.24)	4.70 (±0.25)	4.98 (±0.26)	4.64 (±0.20)	3.69 (±0.14)	5.21 (±0.35)	4.09 (±0.18)
	H1-H4	8.4 (±0.30)	6.46 (±0.37)	9.73 (±0.48)	7.29 (±0.46)	9.03 (±0.52)	8.69 (±0.58)	13.51 (±1.12)	8.09 (±0.90)
Petals	**G**	2.96 (±0.28)	3.00 (±0.06)	4.00 (±0.18)	3.78 (±0.11)	2.75 (±0.16)	3.72 (±0.25)	3.11 (±0.06)	3.45 (±0.23)
Seed kernel	**G**	8.12 (±0.02)	0.16 (±0.00)	0.33 (±0.00)	0.10 (±0.00)	9.23 (±0.32)	0.75 (±0.09)	0.31 (±0.00)	0.61 (±0.00)

G: Gossypol; HGQ: Hemigossypolone; H1-H4: Heliocides. Note that the predominant terpenoid in the seed and flower petal glands is gossypol.

Table A-3, continued. Levels of gossypol and related terpenoids.
The levels of gossypol, hemigossypolone, and heliocides H1-H4 were measured in the seed and six non-seed tissues obtained from cotton plants grown at the TAMU Field Laboratory (Sommerville, TX) during the 2009–2015 growing seasons. Analytes were measured by high performance liquid chromatography and results are reported as μg/mg dry weight of tissue (mean ± SE; n=3 in 2009, 2010, 2011 and 2012; n=4 in 2013, 2014 and 2015).

		2014		2015		
		Coker 312	**66-239**	**Coker 312**	**66-49B**	**66-274**
Bracts	**G**	0.09 (±0.00)	0.12 (±0.01)	0.09 (±0.01)	0.03 (±0.00)	0.08 (±0.00)
	HGQ	0.24 (±0.01)	0.31 (±0.03)	0.13 (±0.03)	0.04 (±0.00)	0.17 (±0.01)
	H1-H4	1.44 (±0.06)	1.75 (±0.11)	1.31 (±0.25)	0.64 (±0.08)	1.17 (±0.07)
Floral buds	**G**	2.91 (±0.15)	2.78 (±0.10)	4.32 (±0.34)	2.77 (±0.22)	2.56 (±0.12)
	HGQ	0.62 (±0.04)	0.56 (±0.02)	0.50 (0.05)	0.48 (±0.03)	0.37 (±0.01)
	H1-H4	1.8 (±0.17)	1.91 (±0.06)	1.45 (±0.06)	1.34 (±0.13)	1.22 (±0.07)
Terminal part of axillary branch	**G**	0.46 (±001)	0.49 (±0.04)	0.85 (±0.03)	0.60 (±0.02)	0.72 (±0.05)
	HGQ	1.00 (±005)	0.95 (±009)	1.93 (±0.05)	1.38 (±0.11)	1.78 (±0.18)
	H1-H4	1.40 (±0.06)	2.01 (±0.39)	3.17 (±0.18)	2.33 (±0.12)	2.66 (±0.22)
Leaves	**G**	0.38 (±0.01)	0.52 (±0.03)	0.757 (±0.02)	0.73 (±0.04)	0.84 (±0.06)
	HGQ	2.04 (±0.07)	2.19 (±0.19)	2.315 (±0.4)	3.01 (±0.34)	3.74 (±0.22)
	H1-H4	0.95 (±0.09)	1.01 (±012)	1.42 (±0.28)	2.12 (±0.29)	1.83 (±0.1)
2-day bolls	**G**	0.90 (±0.02)	1.16 (±0.10)	1.34 (±0.09)	0.74 (±0.01)	0.82 (±0.06)
	HGQ	1.92 (±0.03)	2.11 (±(0.20)	4.77 (±0.24)	4.11 (±0.08)	3.87 (±0.20)
	H1-H4	7.04 (±0.35)	8.23 (±0.66)	9.69 (±0.4)	8.5 (±0.28)	6.7 (±0.25)
Petals	**G**	3.76 (±0.19)	3.23 (±0.09)	4.5 (±0.25)	4.1 (±0.21)	3.8 (±0.17)
Seed kernel	**G**	9.76 (±0.53)	0.53 (±0.05)	11.2 (±1.12)	0.31 (±0.04)	0.32 (±0.04)

G: Gossypol; HGQ: Hemigossypolone; H1-H4: Heliocides. Note that the predominant terpenoid in the seed and flower petal glands is gossypol.

Appendix B.

Materials and Methods for Molecular Characterization of TAM66274

Molecular analyses were performed to characterize the integrated T-DNA in TAM66274 (T6 generation). The analyses included the following:

A. Southern blot hybridizations were performed to investigate the presence or absence of the vector backbone sequences that are outside of the T-DNA borders, and to characterize the integration pattern of the T-DNA in the genome of TAM66274. Southern blot analysis was also used to demonstrate the stability of the inserted T-DNA in three different breeding generations.
B. Sequence of the genomic DNA directly adjacent to the T-DNA borders, was determined by High-Efficiency Thermal Asymmetric InterLaced Polymerase Chain Reaction (HE-TAIL PCR).
C. The integrity and the genomic organization of the T-DNA insert of TAM66274 were determined by overlap PCR amplification, followed by sequencing of the amplified products.
D. Analysis of the genomic DNA flanking the T-DNA insert in TAM66274 showed that the T-DNA integration occurred in an intron of a putative *α-hydrolase* gene. However, qRT-PCR analysis showed that there was no impact on mRNA expression from this gene in TAM66274 compared to expression of the same gene in non-transgenic cv. Coker 312.

Materials and methods used for the above molecular characterizations of TAM66274 are described in this Appendix B.

A. Southern Blot Analysis

Plant Materials.

TAM66274 cottonseeds from four generations (T1, T2 and T3 for generational stability, T6 generation for molecular characterization) were planted in the greenhouse [Texas A&M University (TAMU), College Station, TX]. After three weeks of growth, true leaves were harvested from each plant for genomic DNA isolation that was used for various molecular analyses. Non-transgenic cv. Coker 312 served as a control.

Reference Materials.

pART27-LCT66 plasmid DNA digested with the restriction enzyme *Eco*RI or *Kpn*I [New England Biolabs (NEB), Ipswich, MA] was added to the *Eco*RI or *Kpn*I digested genomic DNA of the non-transgenic cv. Coker 312 at a ratio approximately equivalent to one copy of the transgene per cotton genome (genome size ~2.2×10^9 bp; Li et al. 2015) and used as a positive control for the Southern hybridizations. *Eco*RI or *Kpn*I digested non-transgenic cv. Coker 312 genomic DNA was used as a negative control for the Southern hybridizations. 1 kb Plus DNA molecular weight marker (Invitrogen, Thermo Fisher Scientific) served as the size standard for agarose gel electrophoresis and Southern blot analysis.

Sample Collection and Genomic DNA Extraction.

Cotton genomic DNA was isolated using the Plant Isolate DNA Extraction Kit (Alfa Aesar, Catalog #J67858) following manufacturer's instructions. Approximately 100 mg of newly opened leaf tissue from a greenhouse-grown cotton plant was frozen in liquid nitrogen and ground with mortar and pestle in 1 ml of the Plant Isolate buffer mixed with 0.5 µl RNase A. The sample was then transferred to a 1.5 ml microcentrifuge tube, incubated at 65ºC for 30 minutes, and then centrifuged at 16000 *x g* for 5 minutes. Approximately 600 µl of supernatant was transferred to a different 1.5 ml microcentrifuge tube. To this, 60 µl of the same buffer and 600 µl of chloroform were added. The mixture was shaken vigorously and then centrifuged again at 16000 *x g* for 5 minutes. The upper, aqueous phase was carefully removed and transferred to a new 1.5 ml microcentrifuge tube. DNA was precipitated by adding an equal volume of isopropanol and mixing the suspension gently by inverting the tube 20 times and then leaving the tube on a stand for 5 minutes at room temperature. The tube was centrifuged at 16000 *x g* for 20 minutes to collect the precipitated DNA as a pellet. The pellet was washed with 70% ethanol, air-dried, and dissolved in water. The DNA was quantitated using NanoDrop® ND-1000 (NanoDrop, Thermo Fisher Scientific) spectrophotometer and the samples were stored at -20ºC until use.

DNA Digestion and Electrophoretic Separation of the DNA Fragments.

Genomic DNA isolated from TAM66274 and non-transgenic cv. Coker 312 leaf tissue was digested with either *Eco*RI or *Kpn*I restriction enzymes by combining 12 µg of genomic DNA with 5 units of the restriction enzyme per µg of DNA in the corresponding reaction buffer, 1mM spermidine, and 10 µg bovine serum albumen (BSA) in a 100 µl reaction volume. The digestion was carried out overnight at 37ºC. The restriction enzyme was inactivated by heating the digested samples at 65ºC for 20 minutes. Digested genomic DNA from non-transgenic cv. Coker 312 plants spiked with the pART27-LCT66 plasmid DNA (approximately one copy equivalent of the cotton genome), also digested with the same enzyme, served as a positive control. Non-transgenic cv. Coker 312 genomic DNA, digested with the same enzyme, was used as a negative control. DNA from the digested samples was reprecipitated with 1/10 volume of 3M sodium acetate (pH 5.2) and an equal volume of isopropanol, washed with 70% ethanol and dissolved in 25 µl water before loading on the gel. The digested DNA samples of TAM66274, the positive and negative controls along with molecular size markers were electrophoresed through a 1% agarose gel in 0.5X tris/borate/EDTA (TBE) buffer at 22 volts for 18-22 hours to achieve fragment separation. The gel was stained with ethidium bromide and photographed using an AlphaImager® Mini UV transilluminator (ProteinSimple, San Jose, CA) with a UV fluorescent ruler next to the molecular weight marker in order to determine the size of the hybridizing bands in a later step.

Southern Hybridization.

DNA fragments in the agarose gel were depurinated, denatured, neutralized *in situ*, and transferred to a nylon membrane (Amersham Hybond-N+, GE Healthcare Life Sciences) in 10X saline sodium citrate (SSC) buffer using capillary action (Sambrook and Russell, 2001). After the transfer to the membrane, the DNA was fixed to the membrane by crosslinking in a GS Gene Linker® UV chamber (Bio-Rad, Hercules, CA) at 150 mJoule. The membrane was prehybridized at 65°C for 4 hours in 25 ml buffer containing 6.25 ml of 20X SSC, 0.625 ml of 20% sodium dodecyl sulfate (SDS), 2.5 ml of 50X Denhardt's solution, and 1 mg denatured herring sperm DNA (Invitrogen, Catalog #15634-017).

Probe template DNA containing sequences from plasmid pART27-LCT66 was prepared by PCR amplification using standard protocol. The sequence of the primers used for probe preparations are shown in Table B-1. Approximately 50 ng of denatured probe template was radiolabeled with [α-^{32}P] deoxycytidine triphosphate (dCTP; PerkinElmer, Waltham, MA) using RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The radiolabelled probe was mixed with 25 ml hybridization buffer containing 0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA, and used to hybridize the blot at 65°C for 16 hours. The blot was washed once with 50 ml wash I buffer containing 2X SSC and 0.1% SDS solution for 25 minutes at 65 °C, followed by two washes each with wash II buffer containing 0.5X SSC and 0.1% SDS solution at 65 °C for 10 minutes. The pattern of the hybridizing bands was visualized by exposing the blot to the X-ray film (Amersham Hyperfilm™ MP, GE Healthcare Life Sciences). Developed films were aligned with the previously photographed gel for size estimation of hybridizing bands. Films were scanned and electronic images were generated.

Table B-1. Primers used to make probes for Southern blot analyses.

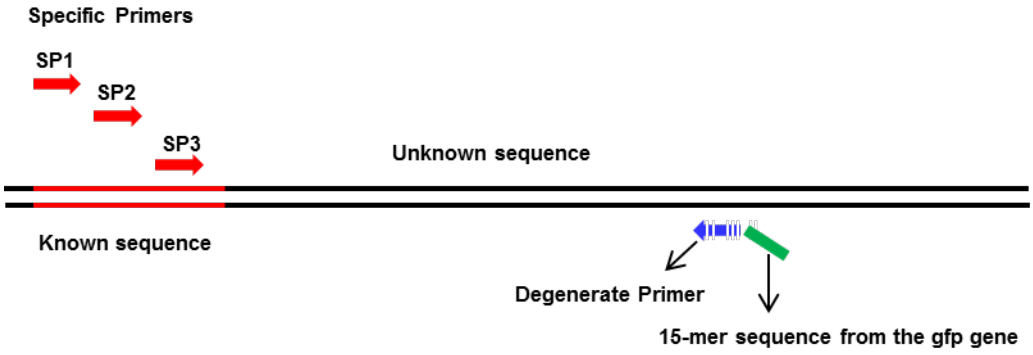
Primer	Primer sequence 5' to 3'
Probe 1F*	GCCGTTACTAGTGATATCCC
Probe 1R	AGTACGTGCTCGCTCGATGCG
Probe 3F	GTGATTGGTAACTTCAGTTCCAGC
Probe 3R	TCGCTATAATGACCCCGAAG
Probe 4F	GGTCATTATAGCGATTTTTTCGG
Probe 4R	TCATGCAGCTCCACCGATTTTGAGAA
Probe 5F	AATCGGTGGAGCTGCATGA
Probe 5R	ATAGTTCCTCGCGTGTCGAT
Probe 6F	ACGCGAGGAACTATGACGAC
Probe 6R	CGAGCGATACTGAGCGAAG
Probe 7F	CAGTATCGCTCGGGACGCA
Probe 7R	CCGGCTGAGAAAGCCCAGTAAG
Probe 8F	TTTCTCAGCCGGGATGGCGCTAA
Probe 8R	GGATCTAGGTGAAGATCCTTTTTG
Probe 9F	GATCTTCACCTAGATCC
Probe 9R	GTGTCGGCTTGAATGAATTG
Probe 10F	ACGCCTAACAATTCATTCAAGC
Probe 10R	CATCGCAAGTACGAGGCTTA
Probe 11F	GCGATCTGTTGAAGGTGGTT
Probe 11R	GCGAAAACGCCTGATTTTAC
Probe 12F	AAACTCGCGTAAAATCAGGCG
Probe 12R	CTAAGAGAAAAGAGCGTTTA
Probe 13F	CTGCTGAGCCTCGACATGTTGT
Probe 13R	CTGCTTTAATGAGATATGCGAGA
Probe 14F	CACATCCCTTCGATTCCGATTAC
Probe 14R	TCCACAATAGAAGCCATGGCTATC
Probe 15F	TATTCATGTTCGACTAATTC
Probe 15R	CAACATAGTAATGTAAAAAAATATGACA
Probe 16F	GATTACGATAAGCTCTGTATT
Probe 16R	TCATCCTATTTAGAAATCCAAG

*F and R refer to forward and reverse DNA strands

B. High-Efficiency Thermal Asymmetric Interlaced (HE-TAIL) PCR

High Efficiency Thermal Asymmetric Interlaced PCR (HE-TAIL PCR) method proposed by Tan and Singh (2011) is an improved version of the original TAIL-PCR method (Liu and Chen, 2007) and was chosen for the LB and RB T-DNA flanking sequence analysis of TAM66274. Although effective for the isolation of unknown genomic sequences flanking the T-DNA borders from several species, TAIL-PCR is limited by the high frequency amplification of undesired smaller non-target sequences from large genomes such as cotton. This limitation is overcome in HE-TAIL PCR by the inclusion of arbitrary degenerate (AD) primers that have an additional, unique 15-mer sequence corresponding to the green fluorescent protein (GFP) gene at their 5'ends (Figure B-1). The GFP sequence is used as a primer in the subsequent (primary, secondary and tertiary) PCR reactions following the pre-amplification step in order to favor amplification of target-specific and longer PCR products and discriminate smaller nontarget sequences from the target sequence. The sequences of the arbitrary degenerate (AD) primers used for the HE-TAIL PCR in the current analyses are the same as described by Tan and Singh (2011). Location and sequence of the LB T-DNA specific (New *nos*-1, 2, 3) and RB T-DNA specific primers (New RB-1, 2, 3) are provided in Figure B-2 and B-3, respectively. Details of the sequence-specific primers used for the LB flanking region analysis (New *nos*-1, 2, 3), RB flanking region analysis (New RB-1, 2, 3), the degenerate (QTLAD1(1-4), and the *gfp* primer (QTAC-1) are provided in Table B-2.

Figure B-1. Principle of the HE-TAIL PCR method.



HE-TAIL PCR was performed in four separate runs using the genomic DNA from TAM66274. These PCR runs include pre-amplification, primary amplification, secondary amplification and tertiary amplification steps, and were conducted on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) using the conditions described by Tan and Singh (2011). Phusion® High Fidelity DNA Polymerase (NEB, Ipswich, MA) and Phusion® 5X reaction buffer were used to perform the PCR reactions. A detailed account of PCR conditions and thermal cycling program are presented in Tables B-3 – B-5.

Figure B-2. HE-TAIL PCR primers used to determine the LB T-DNA flanking sequence.

Figure B-3. HE-TAIL PCR primers used to determine the RB T-DNA flanking sequence.

Gel Electrophoresis and Sequencing.

Amplicons from the HE-TAIL PCR reactions were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized as described previously under Southern blots. The primary reaction amplicons were not typically analyzed as this reaction produces only a very small amount of the specific amplicon, not yet visible on the gel. In general, the specific amplicons start to emerge only at the secondary reactions. Prominent bands from secondary and tertiary reactions were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced. All sequencing analyses were performed by the Laboratory for Genome Technology (TAMU, College Station, TX) using the BigDye® Terminator chemistry (Applied Biosystems, Foster City, CA). Sequencing results obtained were aligned to obtain a consensus sequence using Sequencher® 4.8 (Gene Codes, Ann Arbor, MI) and SnapGene® 3.2.1 (GSL Biotech) software.

Table B-2. Primers used for HE-TAIL PCR.

Primer	Name	Sequence (5' – 3')	Length (bp)	Average Tm (^{o}C)	Average GC (%)	Degeneracy	Reference
Degenerate primers	QTLAD1-1	TAGCGGCTGAAGCACCTGCAGGCVNVNNNGGAA	33	66	63	2304	Tan and Singh, 2011
	QTLAD1-2	TAGCGGCTGAAGCACCTGCAGGCBNBNNNGGTT	33	66	63	2304	Tan and Singh, 2011
	QTLAD1-3	TAGCGGCTGAAGCACCTGCAGGCVVNVNNNCCAA	34	66	63	6912	Tan and Singh, 2011
	QTLAD1-4	TAGCGGCTGAAGCACCTGCAGGCBDNBNNNCGGT	34	67	67	6912	Tan and Singh, 2011
Primer specific to the *gfp* tag of degenerate primers	QTAC-1	TAGCGGCTGAAGCAC	15	45	60	NA	Tan and Singh, 2011
Primers specific to T-DNA insert sequences	New *nos*-1	GGAGTGCGTCGAAGCAGATCGTTC	24	64	58	NA	Current work
	New *nos*-2	TGAATCCTGTTGCCGGTCTTGCGATG	26	69	54	NA	Current work
	New *nos*-3	GATAAATTATCGCGCGCGGTGTCATCTATG	30	67	47	NA	Current work
	New RB-1	GTCGATATGGGAGAGCTCCCAACGCG	26	68	62	NA	Current work
	New RB-2	TCCACACAACATACGAGCCGGAAGC	25	65	56	NA	Current work
	New RB-3	AGAGGCGGTTTGCGTATTGGGGCTG	25	68	60	NA	Current work

* Underlined sequence is the *gfp* tag attached to the 5' end of the degenerate primers.

Table B-3. HE-TAIL PCR conditions - RB-flanking sequence determination.

Components	*Pre-Amplification* 1st Run	*Primary Amplification* 2nd Run	*Secondary Amplification* 3rd Run	*Tertiary Amplification* 4th Run
Template DNA	50 ng template DNA	1 µl of 40-fold dilution of 1st Run	1 µl of 5-fold dilution of 2nd Run	1 µl of 500-fold dilution of 3rd Run
5 X HF Buffer	5 µl	5 µl	5 µl	5 µl
10 mM dNTPs	1 µl (400 µM)	1 µl (400 µM)	1 µl (400 µM)	1 µl (400 µM)
10 µM Forward Primer	1 µl (0.4 µM) QTLAD1-1, 1-2, 1-3, 1-4	1 µl (0.4 µM) QTAC-1	1 µl (0.4 µM) QTAC-1	1 µl (0.4 µM) QTAC-1
10 µM Reverse Primer	1 µl (0.4 µM) New RB-1	1 µl (0.4 µM) New RB-1	1 µl (0.4 µM) New RB-2	1 µl (0.4 µM) New RB-3
Phusion DNA Polymerase	0.25 µl	0.25 µl	0.25 µl	0.25 µl
Water	To 25 µl	To 25 µl	To 25 µl	To 25 µl

The numbers in parenthesis are final concentration in 25 µl of reaction mixture.
dNTP. Deoxyribonucleotide triphosphate

Table B-4. HE-TAIL PCR conditions - LB-flanking sequence determination.

Components	*Pre-Amplification* 1st Run	*Primary Amplification* 2nd Run	*Secondary Amplification* 3rd Run	*Tertiary Amplification* 4th Run
Template DNA	50 ng template DNA	1 µl of 40-fold dilution of 1st Run	1 µl of 5-fold dilution of 2nd Run	1 µl of 500-fold dilution of 3rd Run
5 X HF Buffer	5 µl	5 µl	5 µl	5 µl
10 mM dNTPs	1 µl (400 µM)	1 µl (400 µM)	1 µl (400 µM)	1 µl (400 µM)
10 µM Forward Primer	1 µl (0.4 µM) New *nos*-1	1 µl (0.4 µM) New *nos*-1	1 µl (0.4 µM) New *nos*-2	1 µl (0.4 µM) New *nos*-3
10 µM Reverse Primer	1 µl (0.4 µM) QTLAD1-1, 1-2, 1-3, 1-4	1 µl (0.4 µM) QTAC-1	1 µl (0.4 µM) QTAC-1	1 µl (0.4 µM) QTAC-1
Phusion DNA Polymerase	0.25 µl	0.25 µl	0.25 µl	0.25 µl
Water	To 25 µl	To 25 µl	To 25 µl	To 25 µl

The numbers in parenthesis are final concentration in 25 µl of reaction mixture.

Table B-5. HE-TAIL PCR program (Tan and Singh, 2011).

Step	**Pre-amplification**		Step	**Primary TAIL-PCR**		Step	**Secondary TAIL-PCR**		Step	**Tertiary TAIL-PCR**	
	Temp. (°C)	Time		Temp. (°C)	Time		Temp. (°C)	Time		Temp. (°C)	Time
1	93	2:00	1	94	0:20	1	94	0:20	1	94	0:20
2	95	1:00	2	65	1:00	2	68	1:00	2	94	0:20
3	94	0:30	3	72	3:00	3	72	3:00	3	56	1:00
4	60	1:00	4	Go to Step 1	1 time	4	94	0:20	4	72	3:00
5	72	3:00	5	94	0:20	5	50	1:00	5	Go to Step 2	35 times
6	Go to Step 3	10 times	6	68	1:00	6	72	3:00	6	72	5:00
7	94	0:30	7	72	3:00	7	Go to Step 1	13 times	7	4	∞
8	25	2:00	8	94	0:20	8	72	5:00			
9	72	3:00	9	50	1:00	9	4	∞			
10	94	0:20	10	72	3:00						
11	58	1:00	11	Go to Step 5	13 times						
12	72	3:00	12	72	5:00						
13	Go to Step 11	25 times	13	4	∞						
14	72	5:00									
15	4	∞									

Figure B-4. Genomic flanking sequence on the right and left border of the integrated T-DNA in TAM66274, and deleted cotton genomic DNA at the site of T-DNA integration

The 1035 bp underlined sequence is the genomic sequence flanking the right border of the T-DNA. The 1152 bp sequence in regular type is genomic sequence flanking the left border of the T-DNA in TAM66274. The 44 bp sequence in bold type and grey highlight between the right and left border flanking genomic DNA sequences was the cotton genomic DNA which was deleted when the T-DNA was integrated in the genome.

GTGCAGCTATGTTTGCTCCAATGGTCAATCCTTATGATTCACTGATGAATAGGGGAGAAAGATAT
GGAATCTGGGAAAAGTGGACTCGGAAAAGGAAATTTATGTATTTTTTGGCTCGAAGATTTCCTAA
ATTTCTATCTTACTTCTACCGGCAAAGCTTCCTCTCTGGAAAGCATGGTCAGATTGATCAATGGCT
AGCATTGACACTGGGAAGAAGGGTGAGTGATTTTCTACACTATGAGCTCTCTAAGGTTCCGGAAT
CCAGTTAAGTACAGTGCATAATATGCAATTTTTTGTTTCCAAGGCACTCTACGGTCTTAAATGTGG
AAATGCATAATATTCTAAGCGAGTTACTAGATTTTTGCAGCATTTCACTTGTCAAGCATCGAATCT
AAATTCATATATTGTCTTTACAGGATAGAGCTTTGATAGAAGACCCTATCTATGAAGAATTCTGG
CAAAGGGATGTCGAAGAATCAATCCGACAAGGAAATGCAAAACCTTTTGTGGAGGAAGCTGTAT
TGCAAGTTTCTAATTGGGGATTCAGCCTTGCAGACCTCAAATTACAGAAGAAACAGAGAGGAAA
AGGAATCCTAAATTTGATCAAGTTTTTTCTTAGTGGCTCTGAGGAAGAATATACTGGTTTTCTTGG
TCCAATACACATATGGCAGGTATAATTTCATCCTATGTTGCTGTGACTCTTCCATTTTCTTGAACT
ACTCGTATCTTTCACTTGTGTCCAACACATATCTAGACATATGATCCTTCAAAGACCCTCCAATTA
CATGGAAAAACTTGTTAAAGAAAAAGAACATACCTATGTTGGAATGGACCTGTATCTGGTACTCA
AACTCACCTGAGTAACATAGATTCCATCCTTATTCATACATTGCCGCTGCATTAAACTGTTCTGCT
ACACTTTTTCCTGTTCATTGAAGCATTACGGATTTACTGATCTATTGTTTTCTAGTGTAATATGTGA
TAGCTGAGAAGTTCTTTGCTTTAGAGGCTTCCATTTTACTTTTGTTTTTGTGG**TTAATTTGTTGCC**
AACTATTATCATATTTAACTTGCTTTTTACATTTTGGATTCTTGCAGTCACGTTAATATAATTTC
TTGGAACTACATTTTTTCCAAAACCTATTTGCTCAATTTGGTAACAAAGAAGCCTCCTTGTACTAA
TAATAAAAATAAAAAAAGGCTAGCTTTCTGGTATTGCTTAAACATGAAATGTCTAACCCATAGAG
CACTTGATAGATGCTTAGTACATCAAACTTTCTTTTCGGAAGAAAGTACCATGGCACTAAGTTAC
TGCGACACTTCATTTTTCTTGAAGAACCCCTTTTCAACTTCTATGTCCAGCCCATAGGTATAACCT
CCATAGACCCACATGATATGATATATGGAAAAACTTAGAAAAGCTTGAATATACCCATGTCAAA
ACCTGAGTCCTAGTAACAAAGCCTTGGTATATAAGATCATCAATGAAACAACATTTGGTTTTGAT
TCCCAAGATATGAACTTTTAATCTAAAATCGTACGAGTATTAGTTTGCTTGCAACGTATAAACTAT
GGTTTTATTTTGCAATTTGAGAGCAGAACAAGACATGGATTTCTATTCCCAAGGAAATTTTAGTTG
AACCTTCTTTCTTTTTAATTTTATTTGCTAAATTTTTTGGTGTTATACAGGGGATGGATGATAAAGT
AGTCCCACCTTCAATGACTGATTTCGTTCATAGGGTTCTGCCAAGTGCTGCAGTTCATAAACTCCC
ATATGAGGGTCATTTTACATATTTATATTTCTGTGATGAATGCCATAGACAGATATTTACCACACT
TTTTGGAACCCCACAAGGCCCTCTCCCTGTCAACAATACCATAGAAGTGGAACAAACACCATTGG
ATGATATACAAGTGCAGGAAGATGCTTCAACTCAGGATGATTTTAAGACAGACTGAGATATCGA
AGTTTTCTACAATTAGGTTTGAGTTTTGACATGTAATGTAAGGTTGGTTGTATATATAGCATAGGT
TTATTCTATCACTTGTGATTAGAAAAGTTGAATAAAATTTTCTCATATATTTATGTGGCAATGGAA
TGGAGATTTGAGAAACATTTTGAAGTTGTTGGCTGGCTACAATGGAAATAAACATAATCAAGGA
AAAGGTGATGACTTGTTCTGATTTGTTGTTAGCTGCTTTCAATTTAATCTTGAGACAATAGTTTTTT
TACA

C. DNA Sequence Analyses to Confirm the Integrity of the Insert in TAM66274 Genome

Overlapping PCR products were generated that span several segments of the insert and adjacent 5′ and 3′ flanking genome sequences in TAM66274. Each PCR amplicon was sequenced to determine the nucleotide sequence of the T-DNA insert in TAM66274, as well as that of the genomic DNA flanking the 5′ and 3′ ends of the insert. The PCR analysis to obtain the overlapping products that span the T-DNA insert were conducted using ~100 ng of genomic DNA template in a 50 µl reaction volume. Along with the test sample, pART27-LCT66 plasmid DNA was used as a positive control. PCR reaction without any template DNA and genomic DNA from the non-transgenic cv. Coker 312 served as negative controls. The reaction volume contained 10 µl of 5X Phusion® reaction buffer and, a final concentration of 0.4 µM of each primer, 0.4 mM of dNTPs, and 0.4 units of Phusion® High Fidelity DNA Polymerase (NEB, Ipswich, MA). The sequence of primers used for the overlap PCR reactions is presented in Table B-6. The PCR amplification was performed under the following cycling conditions: 1 cycle at 98ºC for 30 seconds, 35 cycles at 95ºC for 30 seconds, 64ºC for 30 seconds, and 72ºC for 1 minute, followed by final extension at 72ºC for 10 minutes. The PCR products were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify whether the products were of expected sizes. The bands representing the amplicons were excised from the gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR product was sequenced using the same forward and reverse primers that were used for PCR amplification. All sequencing was performed by the Laboratory for Genome Technology (TAMU, College Station, TX) using the BigDye® Terminator chemistry (Applied Biosystems, Foster City, CA). The PCR amplifications and sequencing of the amplified products were performed twice. Thus, at least four sequencing results were obtained for each segment of the T-DNA examined (and the LB and RB flanking sequence).

Sequencher® 4.8 and SnapGene® 3.2.1 software was used to generate a consensus sequence by compiling sequencing results obtained from multiple sequencing reactions performed on the overlapping PCR products (Table B-7). This consensus sequence was aligned to the pART27-LCT66 sequence to determine the integrity and organization of the integrated DNA and the 5′ and 3′ insert-to-flank DNA junctions in TAM66274.

Table B-6. Primers used for overlap PCR and sequencing of amplicons.

Primer	Primer sequence 5' to 3'
66274 flank-RB-691F (1F*)	GGAATGGACCTGTATCTGGTACTCA
66insert (*ocs*)-581R (1R)	TTTGCGACAACATGTCGAGGC
66insert (*ocs*)-521F (2F)	CACTAGTAAGCTAGCTTGCATGCC
66insert (*ocs*)-1250R (2R)	GAGATATGCGAGACGCCTATGATCG
66insert (*ocs*)-1159F (3F)	GGATCTGAGCTACACATGCTC
66insert (*pdk*)-2120R (3R)	GCTAATATAACAAAGCGCAAGATC
66insert (*pdk*)-1950F (4F)	GACATGATCTATCATGTTACCTTG
66insert (*pdk*)-2524R (4R)	GACAAGTGATGTGTAAGACGAAGAAG
66insert (*pdk*)-2441F (5F)	CATCTTACATGTTCGATCAAATTC
66insert (AGP)-3507R (5R)	GGCATCTCGATATCTACCCACCAC
66insert (AGP)-3373F (6F)	GAAGGTGGAGCTGTGGAAGGTG
66insert- 4721R (6R)	CAATTTCCATTCGCCATTCAGGC
66insert (T7)-4519F (7F)	CGGGCCCAATTCGCCCTATAGT
66insert (*nptII*)-5773R (7R)	TCGCTTGGTCGGTCATTTCGA
66insert (*nptII*)-5690F (8F)	CGATTCGCAGCGCATCGCCTT
LB flank of 66274 (8R)	TGTACTAAGCATCTATCAAGTGCTCTATGG

*F and R refer to forward and reverse DNA strands

Table B-7. DNA Sequences of eight overlap amplicons covering the entire T-DNA and flanking sequences in TAM66274. Genomic nucleotide sequences flanking the T-DNA insert in TAM66274 cotton are shown in bold and underlined text. The overlapping sequences of the amplicons are shown in bold and italics.

Product No.	Sequence
Product A	**CTGGTACTCAAACTCACCTGAGTAACATAGATTCCATCCTTATTCATACATTGCCGC TGCATTAAACTGTTCTGCTACACTTTTTCCTGTTCATTGAAGCATTACGGATTTACTG ATCTATTGTTTTCTAGTGTAATATGTGATAGCTGAGAAGTTCTTTGCTTTAGAGGCTT CCATTTTACTTTTGTTTTTGTGG**ACTGATAGTTTAAACTGAAGGCGGGAAACGACAATC TGATCATGAGCGGAGAATTAAGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACA AGCCGTTTTACGTTTGGAACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCCCAAT ACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTT TCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTA GGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGA TAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTATTTAGGTGACAC TATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATCGACCTGCAGG CGGCCGCACTAGTAAGCTAG***CTTGCATGCCTGCAGGTCCTGCTGAGCCTC***	Product B	***CTTGCATGCCTGCAGGTCCTGCTGAGCCTC***GACATGTTGTCGCAAAATTCGCCCTGGACCC GCCCAACGATTTGTCGTCACTGTCAAGGTTTGACCTGCACTTCATTTGGGGCCCACATACA CCAAAAAAATGCTGCATAATTCTCGGGGCAGCAAGTCGGTTACCCGGCCGCCGTGCTGGA CCGGGTTGAATGGTGCCCGTAACTTTCGGTAGAGCGGACGGCCAATACTCAACTTCAAGG AATCTCACCCATGCGCGCCGGCGGGGAACCGGAGTTCCCTTCAGTGAGCGTTATTAGTTC GCCGCTCGGTGTGTCGTAGATACTAGCCCCTGGGGCACTTTTGAAATTTGAATAAGATTT ATGTAATCAGTCTTTTAGGTTTGACCGGTTCTGCCGCTTTTTTTAAAATTGGATTTGTAAT AATAAAACGCAATTGTTTGTTATTGTGGCGCTCTATCATAGATGTCGCTATAAACCTATTC AGCACAATATATTGTTTTCATTTTAATATTGTACATATAAGTAGTAGGGTACAATCAGTAA ATTGAACGGAGAATATTATTCATAAAAATACGATAGTAACGGGTGATATATTCATTAGAA TGAACCGAAACCGGCGGTAAGGATCTGAGCTACACATGCTCAGGTTTTTTACAACGTGCA CAACAGAAT***TGAAAGCAAATATCATGCGATCATA***
Product C	***TTGAAAGCAAATATCATGCGATCATA***GGCGTCTCGCATATCTCATTAAAGCAGGACTCTAG TCGAGATGCCGAGAACGACCTCTACACCACATCCCTTCGATTCCGATTACTCCGAGAGCA TGGATTCAATGTTTCATGCGACGTATTCAACAAGTTTAAAGACGAGCAAGGGAATTTCAA GTCATCCGTGACAAGCGATGTTCGAGGATTGTTGGAACTTTACCAAGCTTCCTATTTGAG GGTTCATGGGGAAGATATATTGGATGAAGCAATTTCTTTCACCACCAACCATTTAAGCCT TGCAGTAGCATCTTTGGACTATCCGTTATCCGAAGAGGTTTCACATGCTTTGAAACAATC AATTCGAAGAGGCTTGCCAAGGGTTGAGGCAAGACACTATCTTTCAGTATACCAAGATAT TGAGTCCCATAATAAGGTTTTGTTGGAGTTTGCTAAGATCGATTTCAACATGGTACAACTT TTGCATAGGAAAGAGCTAAGTGAGATTTCTAGGTGGTGGAAGGATTTAGACTTTCAAAGA AAGTTGCCATACGCAAGAGATAGAGTGGTTGAAGGCTATTTTTGGATCTCAGGAGTGTAC TTTGAGCCCCAATATTCTCTTGGTAGAAAGATGTTGACAAAAGTGATAGCCATGGCTTCT ATTGTGGAGGATCCAAGCTTATCGATTTCGAACCCAGCTTCCCAACTGTAATCAATCCAA ATGTAAGATCAATGATAACACAATGACATGATCTATCAT***GTTACCTTGTTTATTCATGTTC GACTAATTCATTTAATTAATAGTCAATCCATTTAGAAGTTAATAAAACTACAAGTATTATTTA GAAATTAATAAGAATGTTGATTGAAAAATAATACTATATAAAATTGA***

Table B-7, continued. DNA sequences of eight overlap amplicons covering the entire T-DNA and flanking sequences in TAM66274. Genomic nucleotide sequences flanking the T-DNA insert in TAM66274 cotton are shown in bold and underlined text. The overlapping sequences of the amplicons are shown in bold and italics.

Product D	***GTTACCTTGTTTATTCATGTTCGACTAATTCATTTAATTAATAGTCAATCCATTTAGAAGTTA ATAAAACTACAAGTATTATTTAGAAATTAATAAGAATGTTGATTGAAAAATAATACTATATAA AATTGA***TAGATCTTGCGCTTTGTTATATTAGCATTAGATTATGTTTTGTTACATTAGATTAC TGTTTCTATTAGTTTGATATTATTTGTTACTTTAGCTTGTTATTTAATATTTTGTTTATTGAT AAATTACAAGCAGATTGGAATTTCTAACAAAATATTTATTAACTTTTAAACTAAAATATTT AGTAATGGTATAGATATTTAATTATATAATAAACTATTAATCATAAAAAAATATTATTTTA ATTTATTTATTCTTATTTTTACTATAGTATTTTATCATTGATATTTAATTCATCAAACCAGC TAGAATTACTATTATGATTAAAACAAATATTAATGCTAGTATATCATCTTACATGTTCGAT CAAATTCATTAAAAATAATATACTTAC***TCTCAACTTTTATCTTCTTCGT***
Product E	***TCTCAACTTTTATCTTCTTCGT***CTTACACATCACTTGTCATATTTTTTTACATTACTATGTTG TTTATGTAAACAATATATTTATAAATTATTTTTTCACAATTATAACAACTATATTATTATA ATCATACTAATTAACATCACTTAACTATTTTATACTAAAAGGAAAAAAGAAAATAATTAT TTCCTTACCAAGCTGGGGTACCGAATTCGGATCCTCCACAATAGAAGCCATGGCTATCAC TTTTGTCAACATCTTTCTACCAAGAGAATATTGGGGCTCAAAGTACACTCCTGAGATCCA AAAATAGCCTTCAACCACTCTATCTCTTGCGTATGGCAACTTTCTTTGAAAGTCTAAATCC TTCCACCACCTAGAAATCTCACTTAGCTCTTTCCTATGCAAAAGTTGTACCATGTTGAAAT CGATCTTAGCAAACTCCAACAAAACCTTATTATGGGACTCAATATCTTGGTATACTGAAA GATAGTGTCTTGCCTCAACCCTTGGCAAGCCTCTTCGAATTGATTGTTTCAAAGCATGTGA AACCTCTTCGGATAACGGATAGTCCAAAGATGCTACTGCAAGGCTTAAATGGTTGGTGGT GAAAGAAATTGCTTCATCCAATATATCTTCCCCATGAACCCTCAAATAGGAAGCTTGGTA AAGTTCCAACAATCCTCGAACATCGCTTGTCACGGATGACTTGAAATTCCCTTGCTCGTCT TTAAACTTGTTGAATACGTCGCATGAAACATTGAATCCATGCTCTCGGAGTAATCGGAAT CGAAGGGATGTGGTGTAGAGGTCGTTCTCGGCATCTCGAGCGGCCGCCAGTGTGATGGAT ATCTGCAGAATTCGGCTTGGGACGCGTATCGATTACGATAAGCTCTGTATTTTGTTACTGT GTGATGGTAATAGCAAAGAGTGGTAATGTAT***TTATAGAAGGTGGAGCTGTGGAAGGTGAT ATTTTTGCATGCAAATCTTCATCAACGTGTTGAAGACATTGACATGCAAGATGACGAGTGTG CAAATTAAAGAAGACGAAATATTGTCTCTTTTTTTTTTTTTTGTATAGTGGTGG***
Product F	***TTATAGAAGGTGGAGCTGTGGAAGGTGATATTTTTGCATGCAAATCTTCATCAACGTGTTGA AGACATTGACATGCAAGATGACGAGTGTGCAAATTAAAGAAGACGAAATATTGTCTCTTTTT TTTTTTTTTGTATAGTGGTGG***GTAGATATCGAGATGCCACATCCCTTCACATGGTACTAG CAAGGTTCGGACAAACTTTGTTGCAGGGGGGTTCATGGTTGCATCTGTAACTGGAAGGGG CGAAATGATGATGCTTTAACAGCAGAAAGATGATGGACCGTGTTGTGTTGTATGTGAACT CAGTTGAATTCAAAGAGTGTTGAAACTGGGAAGGGTTTTAAAGTGAGACAGAGATGTCC CGATTCACTGAGTTAAGGGTTGAGTTGATAGAGGACAAGTCAAGTGTACACATGTTGCTG TGCATGGTGATGATCTATGAGTTGCAGGAGATATGAACAAATTCAGATATGTATACTTTT GGTATCCTGTACGTTTGATGCTCATACAAATTAGTCCTTTCAAAGTTTGAGGTATTTTTAT TCTTTTTCAATAATATTATCTAAGTATTACATATTATATCATTATATAAATTTATATAATAA GAATGGAAAATAAAATGTTTCACTAAAAACGCTTAAAAGTAAGGATTTGGATTCAATATA GATAATAGTATATAAGTTATACAGTCCAATCTAACATAAGGTGCCACGTATTAAGAAATA TGGTAATTTATTTTTTCATAAATTTTAAATTAATTATACTATTTATTAATAATTTTATATAA TCCTAACAATATATTATACTATGTTAGTTTATTAAAAACAAACAAGTAGGCGAGGGGCTA GGGCCATGACTCTTTAATTTTAGGGTAATCTATAAAAATAGTCATTTTTGTTTGCCTCAGG TTATATTTTAATCATTTATGTTTGAAATGTTACACTTTAGTCACTTTTGTTATTATTTTGTT ACAAAGTGATCACTCTACCGTTAAGCTCCGTTATCTCTCTAACGATAATCCTACATGGCA GTCCAACTAAATTTTAGGTGTCAACTTGGATTTCTAAATAGGATGAAAATAGCTGCAGGC ATG

Table B-7, continued. DNA sequences of eight overlap amplicons covering the entire T-DNA and flanking sequences in TAM66274. Genomic nucleotide sequences flanking the T-DNA insert in TAM66274 cotton are shown in bold and underlined text. The overlapping sequences of the amplicons are shown in bold and italics.

Product F, cont.	CAAGCTTAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGATATCCCGCGGCCATG GCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTC ACTGGCCGTCGTTTTACAACG***TCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGC CTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCC CTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGAAATTG***
Product G	***TCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCG CCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCT GAATGGCGAATGGAAATTG***TAAGCGTTAATGGGTTTCTGGAGTTTAATGAGCTAAGCACA TACGTCAGAAACCATTATTGCGCGTTCAAAAGTCGCCTAAGGTCACTATCAGCTAGCAAA TATTTCTTGTCAAAAATGCTCCACTGACGTTCCATAAATTCCCCTCGGTATCCAATTAGAG TCTCATATTCACTCTCAATCCAAATAATCTGCAATGGCAATTACCTTATCCGCAACTTCTT TACCTATTTCCGCCCGGATCCGGGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCG GCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAG CGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGC AGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTG CTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCA GGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATG CGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGC ATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGA AGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCG ACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAA ATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGG ACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCT TCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCC***TTCT TGACGAGTTCTTCTGAG***
Product H	***TTCTTGACGAGTTCTTCTGAG***CGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCC CAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGG AATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTT CTTCGCCCACCCCGATCCAACACTTACGTTTGCAACGTCCAAGAGCAAATAGACCACGAA CGCCGGAAGGTTGCCGCAGCGTGTGGATTGCGTCTCAATTCTCTCTTGCAGGAATGCAAT GATGAATATGATACTGACTATGAAACTTTGAGGGAATACTGCCTAGCACCGTCACCTCAT AACGTGCATCATGCATGCCCTGACAACATGGAACATCGCTATTTTTCTGAAGAATTATGC TCGTTGGAGGATGTCGCGGCAATTGCAGCTATTGCCAAAATCGAAATACCCCTCACGCAT GCATTCATCAATATTATTCATGCGGGGAAAGGCAAGATTAATCCAACTGGCAAATCATCC AGCGTGATTGGTAACTTCAGTTCCAGCGACTTGATTCGTTTTGGTGCTACCCACGTTTTCA ATAAGGACGAGATGGTGGAGTAAAGAAGGAGTGCGTCGAAGCAGATCGTTCAAACATTT GGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATT TCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGA TGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATA TAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGAATT AATTCAGTACATTAAAAACGTCCGCAATGTGTTATTAAGTTGTCTAAGCGTCAATTTGTTT ACA**<u>TTTTGGATTCTTGCAGTCACGTTAATATAATTTCTTGGAACTACATTTTTTCCAA AACCTATTTGCTCAATTTGGTAACAAAGAAGCCTCCTTGTACTAATAATAAAAATAA AAAAAGGCTAGCTT</u>**

D. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cotyledon, hypocotyl and root tissues of three-day-old seedlings of TAM66274 and non-transgenic cv. Coker 312 using the Spectrum Plant Total RNA Kit (Sigma, St. Louis, MO). Isolated RNA was subjected to DNase treatment using RNase free DNase Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized from various RNA samples using 1 µg of total RNA and a Taqman® Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following manufacturer's recommendations. Quantitative reverse transcription PCR (qRT-PCR) analysis was performed to determine the level of mRNA expression of the *α-hydrolase* gene in various tissues. The technical variability of the PCR reaction was standardized by inclusion of a template normalization step using constitutively expressed reference gene, *Gh histone 3A* (Accession AF024716). Samples were run in duplicate on each plate using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) following manufacturer recommendations. Primer sequences are shown in Table B-8. The qRT-PCR results were analyzed using CFX Manager software (Bio-Rad, Hercules, CA) and reported as relative α-hydrolase values (mean ± SE, n=4).

Table B-8. Primers used for *α-hydrolase* gene expression.

Primer	Primer sequence 5' to 3'
AH qPCR F1	ACTCTCCGTCTTCCTTCTTGC
AH qPCR R1	TCCGCACTAGGAGGATGTATG
Histone3-F	TCGTGAAATTGCCCAGGACT
Histone3-R	GCGCAAAGGTTGGTGTCTTC

AH = *α-hydrolase;* Histone3 = *Gossypium hirsutum histone3A.*

Appendix C.

Gel-based, Event-specific Polymerase Chain Reaction Method for Detection of TAM66274 Cottonseed and Certificates of Analysis for Test and Control Seed Used in Safety Assessment Studies

Texas A&M University (TAMU) has developed cotton event TAM66274 that exhibits ultra-low levels of the antinutrient gossypol in the cottonseed, referred to as ultra-low gossypol cottonseed (ULGCS). The phenotype was achieved by introducing plasmid pART27-LCT66 into cottonseed variety Coker 312 by *Agrobacterium tumefaciens*-mediated transformation, and the phenotype is attributable to RNAi-mediated suppression of *δ-cadinene synthase* genes that encode a key enzyme in gossypol biosynthesis. The RNAi construct interferes with expression of δ-cadinene synthase in the seed, while leaving gossypol levels unchanged in other plant tissues (Sunilkumar et al., 2006; Rathore et al., 2012; Palle et al., 2013).

A gel-based, event-specific polymerase chain reaction (PCR) method was developed to detect TAM66274 cottonseed deoxyribonucleic acid (DNA). This method used oligonucleotide primers to amplify a 733 base pair (bp) DNA fragment that spans the right border junction between the cotton genome and the T-DNA in TAM66274. Oligonucleotide primers were also developed to detect cottonseed DNA of TAM6649B, another ULGCS event developed by TAMU, which was used in this study to demonstrate the specificity of the detection method for individual cotton events. This PCR method was used to verify the identity of seeds planted in field studies to assess phenotypic, agronomic, and environmental characteristics of TAM66274 and to verify the identity of seed harvested from field studies and used in composition analysis, gossypol analysis, mycotoxin analysis, seed germination/dormancy, and fiber quality studies of TAM66274. Certificates of analysis for test and control seed used in these safety studies are included in this appendix.

Principle of the Method

For specific detection of TAM66274 genomic DNA, a unique fragment that spans the insert-to-plant genome junction at the right border (RB) region in TAM66274 cottonseed is amplified using two primers. The forward primer binding site is located in the cotton genomic sequence of TAM66274 and the reverse primer binding site is located in the T-DNA insert (Figure C-1).

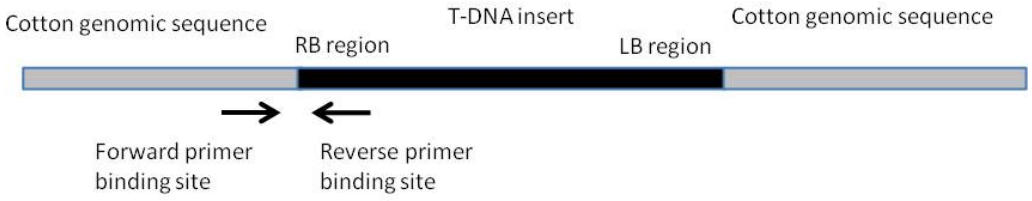


Figure C-1. Location of the event-specific PCR primer binding sites.

Reagents and Equipment

All materials used (e.g., vials, containers, and pipette tips) were suitable for PCR and molecular biology applications. Table C-1 contains a list of equipment and materials needed to perform the PCR method. Materials were deoxyribonuclease-free, DNA-free, sterile, and unable to absorb protein or DNA. To avoid contamination, materials for use in this method were stored separately from materials used in other laboratory procedures, benches and pipettes were regularly cleaned with 70% ethanol, filter tips were used with all pipettes, and disposable gloves were used and changed often. An electronic, repeat pipette was used to reduce sample-to-sample variability and to reduce the time needed to set up the reactions. Genomic DNA was extracted from plants using a cetyltrimethyl ammonium bromide (CTAB)-based extraction method described below and quantitated using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). Table C-2 contains a list of reagents and solutions needed to perform the PCR method.

DNA Extraction

Cottonseed DNA was isolated using a published protocol (Paterson et al., 1993) with some modifications. DNA was isolated from cottonseeds by manually removing the seed coat using a razor blade and transferring the seed kernel to a 2-ml microcentrifuge tube containing 900 µl DNA extraction buffer containing 2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA pH 8.0, 1.4 M sodium chloride, 2% polyvinylpyrrolidone (PVP) and 2.5 µl/ml β-mercaptoethanol. Seed samples were then homogenized using a TissueLyser II (QIAGEN®). The samples were then incubated at 65ºC for 5 minutes. Equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) were then added to each tube and mixed gently. The tubes were centrifuged at 13,000 rpm for 15 minutes. The upper aqueous phase was transferred to a sterile 1.5-ml microcentrifuge tube and equal volumes of chloroform:isoamyl alcohol (24:1) were added to each tube and mixed by inversion. The tubes were then centrifuged at 13,000 rpm for 15 minutes. The upper aqueous phase was transferred to a sterile 1.5-ml microcentrifuge tube. An equal volume of isopropanol was added to each tube and mixed by inversion. The tubes were centrifuged at 13,000 rpm for 15 minutes. The precipitated DNA pellet at the bottom of each tube was washed with 1 ml of 70% ethanol. The tubes were then centrifuged at 10,000 rpm for 5 minutes, the ethanol was removed with a pipette, and the DNA pellet was air-dried before dissolving it in 250 µl of sterile double-distilled water.

Table C-1. Equipment and materials.

Equipment and materials	Specification
TissueLyser II	Quiagen Catalog Number 85300
Veriti 96 well Thermal Cycler	Invitrogen Catalog Number 4375786
PCR tube cap strip	Fisherbrand, Fisher Scientific Catalog Number 07-200-259
Pipettes with adjustable volume	Pipetman, Gilson 1 to 10 µl; 2 to 20 µl; 20 to 200 µl; 100 to 1000 µl.
Aerosol filter pipet tips	VWR International Catalog Number 16466.006
Microcentrifuge tubes 1.5 ml	VWR International Catalog Number 20170-038

Table C-2. Reagents, buffers and solutions.

Reagents, buffers and solutions	Specification
Taq polymerase	Invitrogen Catalog Number 18067-017 or equivalent
dNTPs	Invitrogen Catalog Number 18427-088 or equivalent
Nuclease-free water	Invitrogen Catalog Number 10977-015 or equivalent
1 kb DNA Ladder	Invitrogen Catalog Number 10787-026

Primers and Amplicons – TAM66274

For the specific detection of TAM66274 genomic DNA, two primers (Table C-3) were used to amplify a 733 bp fragment that spans the insert-to-plant genome junction at the RB region. The forward primer (274 flank-RB-691F) binding site is located within the TAM66274 plant genome, and the reverse primer (OCS-581R) binding site is located within the T-DNA insert in TAM66274.

Table C-3. Primers used with TAM66274 event-specific PCR method

Primer name	Length (bp)	Primer sequence 5' to 3'
274 flank-RB-691F	25	GGAATGGACCTGTATCTGGTACTCA
OCS-581R	21	TTTGCGACAACATGTCGAGGC

Primers and Amplicons – TAM6649B

TAM6649B, another ULGCS event developed by TAMU, was used as a control in this study to demonstrate the specificity of the detection method for individual cotton events. For the specific detection of TAM6649B genomic DNA, two primers (Table C-4) were used to amplify a 697 bp fragment that spans the insert-to-plant genome junction at the RB region. The forward primer (150 bp RB flank of 49BF) binding site is located within the TAM6649B plant genome and the reverse primer (OCS-581R) binding site is located within the TAM6649B insert.

Table C-4. Primers used with TAM6649B event-specific PCR method.

Primer name	Length (bp)	Primer sequence 5' to 3'
150 bp RB flank of 49BF	24	CGTACGCAAAATACATTTGGAGT
OCS-581R	21	TTTGCGACAACATGTCGAGGC

Master Mix

All reagents were thawed, as necessary, and thoroughly mixed before each use. A master mix that contained all components of the PCR reaction except the DNA (Table C-5) was prepared in sufficient quantities before the reactions were performed.

Table C-5. Master mix components for the gel-based, event-specific PCR method.

Components	Volume per reaction (µl)[a]	Final concentration
10X Taq reaction buffer	2.5	1x
10 mM dNTPs	1	400 µM
Forward Primer, 10 µM	1	0.4 µM
OCS-851R Primer, 10 µM	1	0.4 µM
Template DNA	1	100 ng
Taq DNA Polymerase	0.25	1.25 units/25 µl PCR
Nuclease-free water	to 25 µl	Not applicable

[a] Total PCR reaction volume is 25 µl (1 µl template DNA plus 24 µl master mix)

PCR Method Controls

The following controls were used for this method:

1. Negative control 1: genomic DNA from non-transgenic cv. Coker 312 cottonseed
2. Negative control 2: genomic DNA from transgenic cottonseed not expected to provide a PCR amplicon of the target primer (e.g. the PCR primers used for detection of event TAM66274 were tested on genomic DNA from event TAM6649B to show specificity of the primers for event TAM66274, and vice versa)

Cycling Parameters

The method was performed with the cycling parameters shown in Table C-6.

Table C-6. Cycling parameters.

Cycle	Step	Temperature (°C)	Time (seconds)	Number of cycles
A	1	95°C	300	1
B	1	95°C	30	30
	2	64°C	30	
	3	72°C	55	
C	1	72°C	420	1
D	1	4°C	Hold	Not applicable

Step-by-Step Instructions for Performing the Method.

1. At room temperature, prepare a master mix of all reagents, including event-specific primers, except the template DNA.
2. Thoroughly mix the solution.
3. Aliquot the appropriate amount of master mix into individual tubes (i.e. total volume of PCR reaction minus the DNA volume to be added).
4. Add DNA samples and controls in the following order:
   1 µl genomic DNA (100 ng/µl) from non-transgenic cv. Coker 312 cottonseed – 4 tubes
   1 µl genomic DNA (100 ng/µl) from ULGCS TAM6649B – 4 tubes
   1 µl of genomic DNA (100 ng/µl) from ULGCS TAM66274 – 4 tubes
5. Cap the PCR tubes.
6. Centrifuge the tubes for approximately 20 seconds.
7. Perform PCR using the cycling parameters in Table C-6.
8. Following completion of the PCR, maintain the PCR products at 4°C until further analysis.
9. Load the molecular weight marker and 10 µl of each PCR reaction onto a 1% agarose gel in 0.5X tris-borate-EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide and electrophorese at 100 volts for 30 to 40 minutes.
10. Capture the image under ultraviolet light.

Analytical Results

The event-specific PCR primers designed for TAM66274 were highly specific for the corresponding cotton event (Figure C-2). The gel-based PCR results showed that TAM66274-specific PCR primers amplified only DNA from TAM66274 template DNA, but not from TAM6649B DNA, and generated the expected 733 bp amplicon.

TAM6649B, another ULGCS event developed by TAMU, was used as a control in this study to demonstrate the specificity of the detection method for individual cotton events. TAM6649B-specific primers amplified only DNA from TAM6649B template DNA, but not from TAM66274 DNA, and generated the expected 697 bp amplicon. DNA obtained from non-transgenic cv. Coker 312 seeds did not generate any PCR amplicons from primers designed for either TAM66274 or TAM6649B and served as a negative control for the event-specific PCR. Similar PCR results were obtained when genomic DNA was extracted from leaf tissue (data not shown).

Certificates of analysis are presented in this appendix for test and control seed planted in field studies during 2014 and 2015 (Figures C-3 and C-4), as well as for test and control seed harvested in 2014 and 2015 for gossypol analysis, fiber quality, composition analysis, seed germination/dormancy, and mycotoxin analysis [2015 only] (Figures C-5 to C-8).

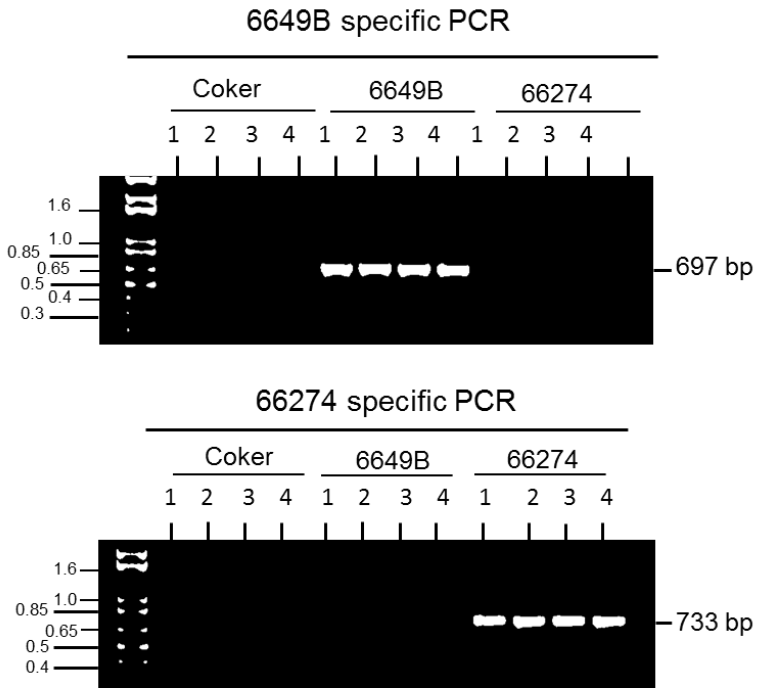


Figure C-2. Agarose gel image of the PCR products generated by amplification with TAM66274 and TAM6649B event-specific primers. Approximately 100 ng of genomic DNA obtained from non-transgenic cv. Coker 312, TAM6649B and TAM66274 seed kernels were PCR amplified with either TAM6649B-specific PCR primers (upper panel) or TAM66274-specific PCR primers (lower panel). PCR was performed on four biological replicates (Lanes 1-4) for each entry. Lane 0 (unmarked): 1 kb DNA ladder; Lanes 1-4 (Coker): Negative control (genomic DNA from non-transgenic cv. Coker 312); Lanes 1-4 (6649B): Genomic DNA from TAM6649B; Lanes 1-4 (66274): Genomic DNA from TAM66274.

Certificate of Analysis

Seed used to plant ULGCS events TAM66274 and TAM6649B, and non-transgenic cv. Coker 312 in 2014 field trials to evaluate phenotypic, agronomic and ecological plant characteristics

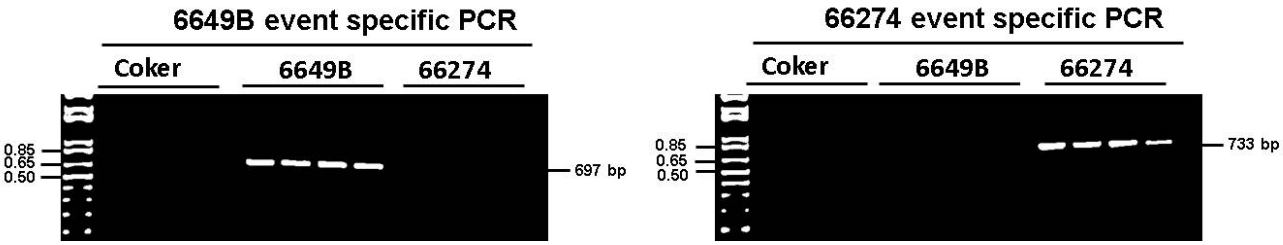


Figure C-3. Event-specific PCR of test and control seeds used for 2014 multi-location field trials. TAM6649B specific PCR (left panel) and TAM66274 specific PCR (right panel) analyses were performed on genomic DNA isolated from individual seed kernels of non-transgenic cv. Coker 312, and ULGCS events, TAM6649B and TAM66274. Seeds were obtained from plants grown in a greenhouse at TAMU in 2013. Four seeds of each genotype were randomly selected and tested by event-specific PCR. PCR analysis produced bands of the expected size for TAM6649B (697 bp) and TAM66274 (733 bp), which confirms the identity of the T-DNA introduced into non-transgenic cv. Coker 312.

Certificate of Analysis

Seed used to plant ULGCS events TAM66274 and TAM6649B, and non-transgenic cv. Coker 312 in 2015 field trials to evaluate phenotypic, agronomic and ecological plant characteristics

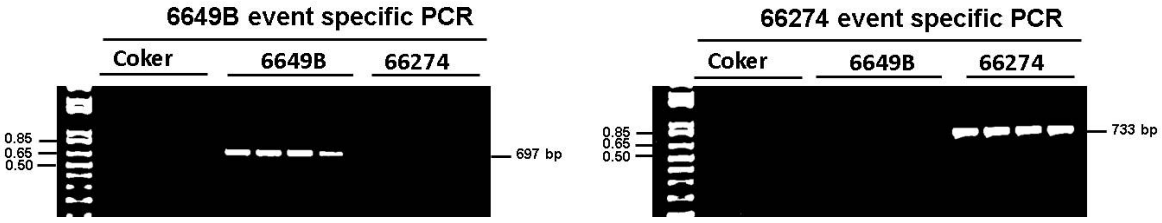


Figure C-4. Event-specific PCR of test and control seeds used for 2015 multi-location field trials. TAM6649B specific PCR (left panel) and TAM66274 specific PCR (right panel) analyses were performed on genomic DNA isolated from individual seed kernels of non-transgenic cv. Coker 312, and ULGCS events, TAM6649B and TAM66274. Seeds were obtained from plants grown in a greenhouse at TAMU in 2014. PCR analysis produced bands of the expected size for TAM6649B (697 bp) and TAM66274 (733 bp), which confirms the identity of the T-DNA introduced into non-transgenic cv. Coker 312.

Certificate of Analysis

Seed of ULGCS events TAM66274 and TAM6649B, and non-transgenic cv. Coker 312 obtained from 25-boll samples from 2014 field trials used for HPLC-based gossypol analysis (TAMU) and fiber quality analysis

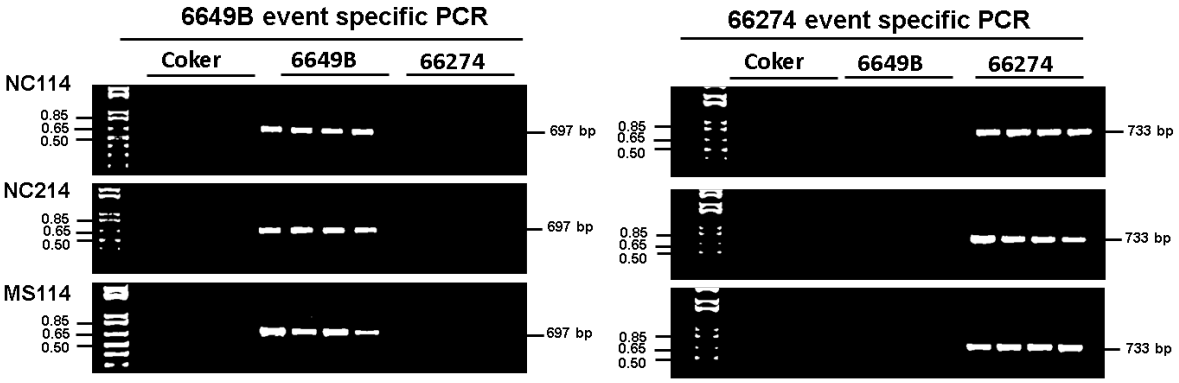


Figure C-5. Event-specific PCR of test and control seeds of 25-boll samples used for HPLC-based gossypol analysis (TAMU) and fiber quality analysis. TAM6649B specific PCR (left panel) and TAM66274 specific PCR (right panel) analyses were performed on genomic DNA isolated from individual seed kernels of non-transgenic cv. Coker 312, and ULGCS events, TAM6649B and TAM66274, grown in three locations in 2014 [North Carolina site 1 (NC114), North Carolina site 2 (NC214) and Mississippi site 1 (MS114)]. Each lane in the gel image represents a single seed from each of the four replicated plots per treatment per location. PCR analysis produced bands of the expected size for TAM6649B (697 bp) and TAM66274 (733 bp), which confirms the identity of the T-DNA introduced into non-transgenic cv. Coker 312.

Certificate of Analysis

Seed of ULGCS events TAM66274 and TAM6649B, and non-transgenic cv. Coker 312 obtained from 2014 field trials used for composition analysis (Covance) and germination tests (TAMU)

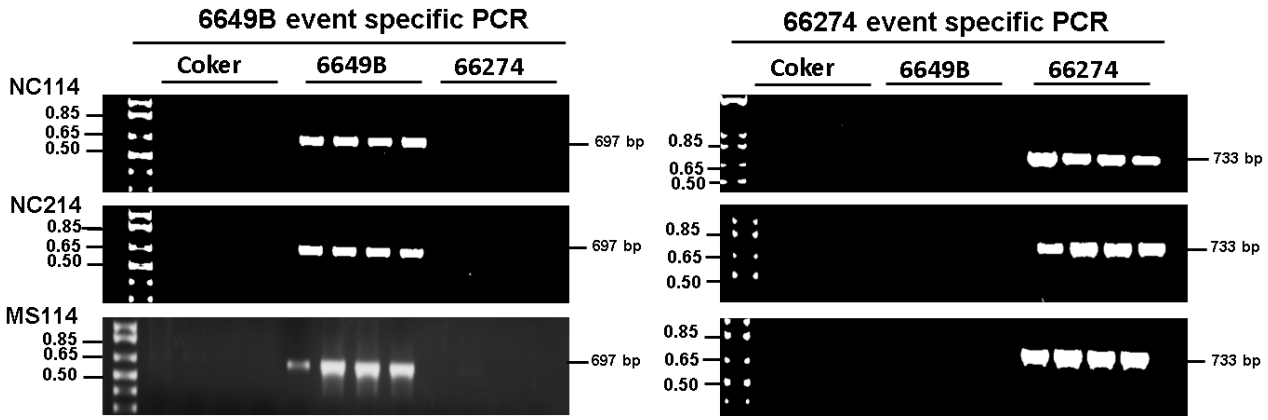


Figure C-6. Event-specific PCR of test and control seeds used for composition analysis (Covance) and seed germination analysis (TAMU). TAM6649B specific PCR (left panel) and TAM66274 specific PCR (right panel) analyses were performed on genomic DNA isolated from individual seed kernels of non-transgenic cv. Coker 312, and ULGCS events, TAM6649B and TAM66274, grown in three locations in 2014 [North Carolina site 1 (NC114), North Carolina site 2 (NC214) and Mississippi site 1 (MS114)]. Each lane in the gel image represents a single seed from each of the four replicated plots per treatment per location. PCR analysis produced bands of the expected size for TAM6649B (697 bp) and TAM66274 (733 bp), which confirms the identity of the T-DNA introduced into non-transgenic cv. Coker 312.

Certificate of Analysis

Seed of ULGCS events TAM66274 and TAM6649B, and non-transgenic cv. Coker 312 obtained from 25-boll samples from 2015 field trials used for HPLC-based gossypol analysis (TAMU) and fiber quality analysis

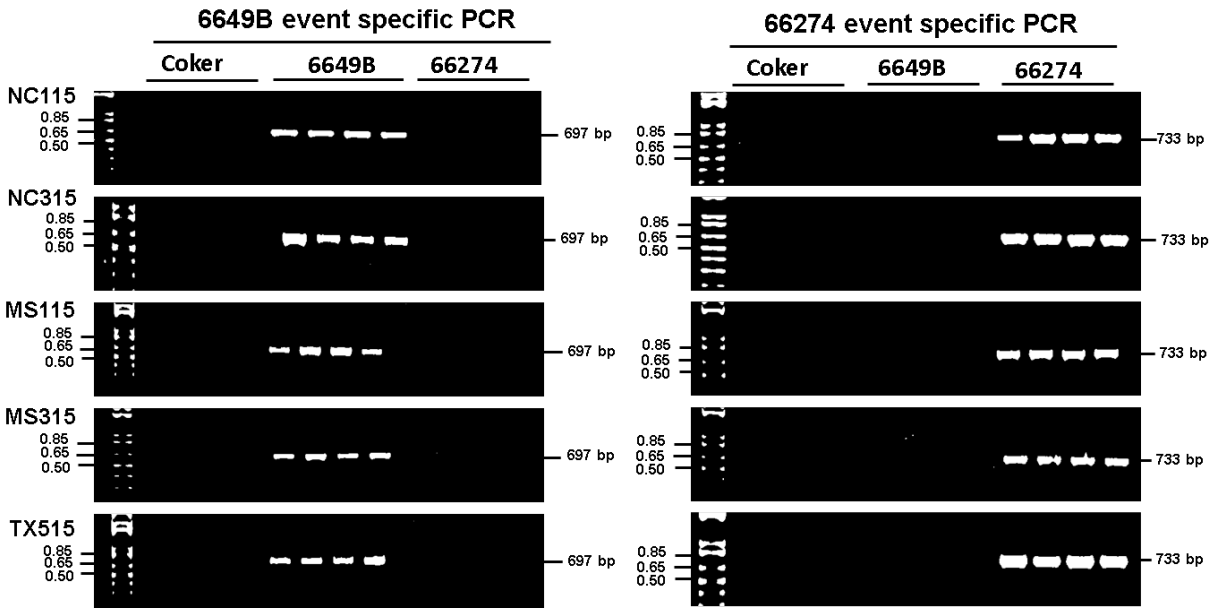


Figure C-7. Event-specific PCR of test and control seeds obtained from 25-boll samples used for HPLC-based gossypol analysis (TAMU) and fiber quality analysis. TAM6649B specific PCR (left panel) and TAM66274 specific PCR (right panel) analyses were performed on genomic DNA isolated from individual seed kernels of non-transgenic cv. Coker 312, and ULGCS events, TAM6649B and TAM66274, grown in five locations in 2015 [North Carolina site 1 (NC115), North Carolina site 3 (NC315), Mississippi site 1 (MS115), Mississippi site 3 (MS315), and Texas site 5 (TX515)]. Each lane in the gel image represents a single seed from each of the four replicated plots per treatment per location. PCR analysis produced bands of the expected size for TAM6649B (697 bp) and TAM66274 (733 bp), which confirms the identity of the T-DNA introduced into non-transgenic cv. Coker 312.

Certificate of Analysis

Seed of ULGCS events TAM66274 and TAM6649B, and non-transgenic cv. Coker 312 obtained from 2015 field trials used for composition analysis (Covance), germination tests (TAMU) and mycotoxin analysis (Romer)

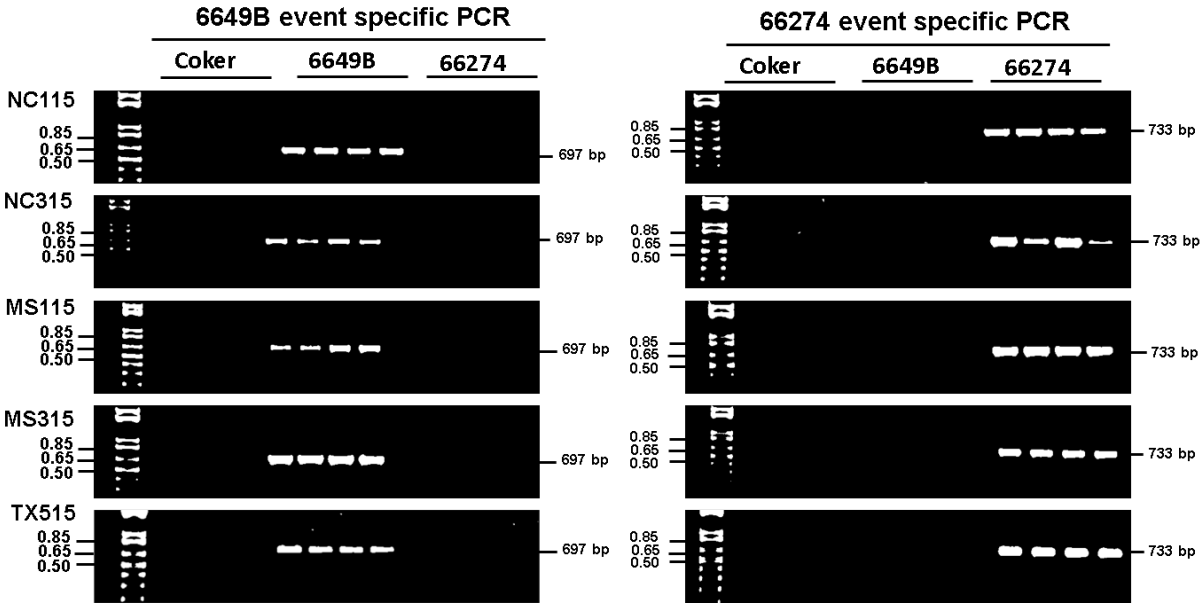


Figure C-8. Event-specific PCR of test and control seeds used for composition analysis (Covance), seed germination analysis (TAMU) and mycotoxin analysis (Romer). TAM6649B specific PCR (left panel) and TAM66274 specific PCR (right panel) analyses were performed on genomic DNA isolated from individual seed kernels of non-transgenic cv. Coker 312, and ULGCS events, TAM6649B and TAM66274, grown in five locations in 2015 [North Carolina site 1 (NC115), North Carolina site 3 (NC315), Mississippi site 1 (MS115), Mississippi site 3 (MS315), and Texas site 5 (TX515)]. Each lane in the gel image represents a single seed from each of the four replicated plots per treatment per location. PCR analysis produced bands of the expected size for TAM6649B (697 bp) and TAM66274 (733 bp), which confirms the identity of the T-DNA introduced into non-transgenic cv. Coker 312.

Appendix D.

A. Methods to Measure Levels of *dCS* Transcripts in Tissues of TAM66274 and Non-Transgenic cv. Coker 312, and Bioinformatics Analysis of Potential Non-Target Effects of *dCS* RNAi

B. Methods to Measure Levels of the NPTII Variant Protein in Tissues of TAM66274 and Non-Transgenic cv. Coker 312

C. Results of Bioinformatics Analyses of Amino Acid Sequences Encoded by Putative Open Reading Frames (ORFs) in the T-DNA and Flanking Genomic DNA of TAM66274 Compared to Amino Acid Sequences of Known Allergens and Toxins

The T-DNA insert in the genome of TAM66274 contains two gene cassettes. First, the RNAi cassette which silences *δ-cadinene synthase* (*dCS*) genes that encode δ-cadinene synthase (dCS), a key enzyme involved in gossypol biosynthesis, and results in ultra-low gossypol levels in TAM66274 cottonseed. The second gene cassette introduced in TAM66274 contains the selectable marker *nptII* variant gene, which was used to select transformed cotton cells and therefore used to generate TAM66274. This gene encodes the enzyme neomycin phosphotransferase type II (NPTII), which confers resistance to the antibiotics neomycin and other related aminoglycosides.

In addition to the intended expression of the *dCS* RNAi transcripts and the NPTII variant protein in TAM66274, an assessment of both intended and potential unintended open reading frames (ORFs), created by the inserted DNA and contiguous plant genomic DNA in TAM66274, was conducted. This assessment included a comprehensive bioinformatic analysis to investigate similarity between putative amino acid sequences, encoded by ORFs within the TAM66274 cotton T-DNA insert and genomic flanking sequences, and amino acid sequences of known allergens and toxins.

Appendix D presents the following:

A) Materials and methods for measurement of transcript levels of the *dCS* genes in cottonseed and other plant tissues of TAM66274 compared to levels in the non-transgenic cv. Coker 312, and bioinformatics analysis of potential non-target effects of *dCS* RNAi.
B) Materials and methods for measurement of levels of the NPTII variant protein in cottonseed and other plant tissues of TAM66274 compared to levels in non-transgenic cv. Coker 312.
C) Results of bioinformatics analyses of the amino acid sequences encoded by both intended and unintended ORFs in the T-DNA and flanking genomic DNA of TAM66274 compared to amino acid sequences of known allergens and toxins.

A. Materials and Methods to Measure Transcript Levels of the *dCS* Genes in TAM66274 and Non-transgenic cv. Coker 312, and Bioinformatics Analysis of Potential Non-target Effects of *dCS* RNAi

In TAM66274, the seed-specific reduction of the dCS enzyme results from the suppression of *dCS* gene expression, mediated by RNAi. Therefore, measurements of transcript expression of the endogenous *dCS* gene in TAM66274 were made by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and compared to levels in the non-transgenic cv. Coker 312. Measurement of transcript levels of the target gene is a well-recognized method for

measuring suppression of endogenous enzymes by RNAi (Liu et al., 2002; Ogita et al., 2004; Flores et al., 2008). The *dCS* transcript levels were measured in seed embryos and tissues of TAM66274 collected from plants grown in a 2015 TAMU field trial.

Production of Test and Control Plants.

Cotton plants for production of test and control tissues for analysis of levels of *dCS* transcripts were grown in a replicated-plot field trial at Texas A&M Field Laboratory (Sommerville, TX) during the 2015 growing season. A description of the field design and agronomic practices used to maintain the plots throughout the growing season are described in Table D-1.

Test and Control Substances.

The test substance in this study was RNA extracted from developing embryos at 31 days post anthesis (dpa) and from root, leaf, bract, floral bud, and axillary bud tissues harvested from individual plants of TAM66274. The control substance in this study was RNA extracted from developing embryos at 31 dpa and from root, leaf, bract, floral bud, and axillary bud tissues harvested from individual plants of non-transgenic cv. Coker 312. Initial characterization of the test and control substances was by documentation of the seed pedigree from the study director. Primary characterization was then conducted during the study by qRT-PCR analyses described herein.

Samples for Analysis of Expression of dCS Transcripts.

Approximately 2 g of leaf, bract, floral bud, and axillary bud tissues were collected from each of four replicated plots of 10-week old test and control plants grown at the Texas A&M Field Laboratory. Tissue samples were harvested from three plants from each plot and constituted a replicate. Root samples were obtained by carefully pulling 45-day old plants from the soil, carefully removing most of the soil from the roots. Root samples were harvested from three plants from each plot and constituted a replicate. Developing embryos were collected at 31 dpa from unopened bolls harvested from three plants from each plot and constituted a replicate. All tissues were frozen in liquid nitrogen immediately after harvesting and stored at -80°C until used for RNA extraction.

Sample Analysis: RNA Extraction and qRT-PCR Amplification.

Quantitation of *dCS* transcript expression in TAM66274 and non-transgenic cv. Coker 312 plant tissues was performed using a CFX96 Touch™ Real-Time PCR Detection System (Biorad, Hercules CA). qRT-PCR was selected as the method of choice to quantify *dCS* transcripts in TAM66274. qRT-PCR has become one of the most widely used methods for detection and/or comparison of gene expression levels. qRT-PCR is attractive because it requires template cDNA synthesized from minute quantities of RNA and uses fluorescent reporter molecules to monitor the amplification products during each cycle of the PCR reaction. qRT-PCR combines nucleic acid amplification and detection steps into one homogeneous assay with a large dynamic range of

detection and high sensitivity. Unlike Northern hybridizations, the need for post-handling of RNA is eliminated in qRT-PCR, thus making it a more accurate method of choice for comparing gene expression levels. qRT-PCR differs from classical PCR by the measurement of the amplified PCR product at each cycle throughout the PCR reaction. In practice, a video camera records in real-time the light emitted by the fluorochrome incorporated into the newly synthesized PCR product.

Total RNA was extracted from test and control substance tissue samples using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, MO). Isolated RNA was subjected to DNase treatment using RNase-free DNase Kit (Qiagen, Valencia, CA). Total RNA was isolated from four biological replicates of embryos (31 dpa) and three biological replicates of other non-seed tissues (axillary bud, bract, floral bud, leaf and root). Complementary DNA (cDNA) was synthesized from these RNA samples using 1 µg of total RNA and a Taqman® Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following manufacturer's recommendations. qRT-PCR analysis was performed to determine the level of *dCS* transcripts in various tissues of test plants compared to their respective control plants. The technical variability of the PCR reaction was standardized by inclusion of a template normalization step using a constitutively expressed reference gene, *Gh histone 3A* (GenBank Accession AF024716). Samples were run in duplicate for embryo cDNA and triplicate for the cDNA from the rest of the non-seed tissues on a 384-well plate using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) following manufacturer's instructions. Primer sequences are presented in Table D-2, and quantification cycle (Cq) values for expression of *dCS* transcripts in non-seed tissues and in embryos in Tables D-3 and D-4, respectively. The qRT-PCR results were analyzed using the Bio-Rad CFX Manager™ software (Bio-Rad, Hercules, CA) and reported as relative *dCS* values [mean ± SE, n=8 (for embryos) or n=9 (for the rest of the samples)].

Table D-1. Field site characteristics for production of test and control plant materials. TAM66274 and non-transgenic cv. Coker 312 were grown in a replicated plot field trial at the Texas A&M University Field Laboratory during the summer of 2015. Plant tissue samples were collected at the appropriate growth stage, frozen in liquid nitrogen and were transported to laboratory facilities for analysis.

Trial Information	
Site code	TAMU0001
County, State	Burleson, TX
Principal Investigators	Devendra Pandeya, PhD
	Sreenath Palle, PhD
USDA permit	15-048-109n
GPS coordinates	30.54246, -096.43451
Crop Description	
Planting date	05/01/2015
Planting method	Hand planted
Seeding rate	0.67 seed/ft
Depth	0.5 inch
Row spacing	36 inches
Spacing in row	18 inches
Seed bed	Rows
Soil moisture	Adequate
Sample dates	6/15/2015; 7/16/2015; 8/23/2015
Site Design	
Plot width	3 feet
Plot length	15 feet
Plot area	45 sq feet
Replications	4
Study design	RCB*
Soil Description	
% Sand	11%
% Silt	30%
% Clay	59%
Texture	Clay loam
Soil type	Belk
Fert level	Good
Drainage	Good

RCB. Randomized Complete Block.

Table D-2. Primers for quantification of expression of *dCS* transcripts in TAM66274 and non-transgenic cv. Coker 312.

Primer name	Sequence (5'-3')	Product size (bp)	Gene
qPCR-dCS-F1	TTACCTTGTGGAGGCCAGATG	90	*dCS*
qPCR-dCS-R1	GCATAACCACAAGTTGGCAAT		
Histone3-F	TCGTGAAATTGCCCAGGACT	120	*Gh histone 3A*
Histone3-R	GCGCAAAGGTTGGTGTCTTC		

Table D-3. Quantification cycle (Cq) values for expression of *dCS* transcripts in non-seed tissues of TAM66274 and non-transgenic cv. Coker 312.

Tissue	Sample	*dCS* Cq	Histone Cq
Axillary bud - TAM66274	TAM66274 - Ax Bud	26.78	20.47
	TAM66274 - Ax Bud	27.06	20.57
	TAM66274 - Ax Bud	26.85	20.63
	TAM66274 - Ax Bud	26.97	20.50
	TAM66274 - Ax Bud	26.96	20.51
	TAM66274 - Ax Bud	26.93	20.26
	TAM66274 - Ax Bud	25.11	18.73
	TAM66274 - Ax Bud	25.33	18.87
	TAM66274 - Ax Bud	25.17	18.81
Bract - TAM66274	TAM66274 - Bract	27.57	17.47
	TAM66274 - Bract	27.48	17.57
	TAM66274 - Bract	27.41	17.33
	TAM66274 - Bract	28.14	16.99
	TAM66274 - Bract	27.91	17.21
	TAM66274 - Bract	27.60	17.11
	TAM66274 - Bract	26.67	16.23
	TAM66274 - Bract	26.82	16.48
	TAM66274 - Bract	26.42	16.18

Table D-3, continued. Quantification cycle (Cq) values for expression of *dCS* transcripts in non-seed tissues of TAM66274 and non-transgenic cv. Coker 312.

Tissue	Sample	*dCS* Cq	Histone Cq
Floral bud - TAM66274	TAM66274 - Fl Bud	29.57	23.61
	TAM66274 - Fl Bud	29.71	22.49
	TAM66274 - Fl Bud	29.01	22.85
	TAM66274 - Fl Bud	29.35	22.73
	TAM66274 - Fl Bud	29.57	23.13
	TAM66274 - Fl Bud	29.38	23.08
	TAM66274 - Fl Bud	27.14	20.89
	TAM66274 - Fl Bud	27.19	21.11
	TAM66274 - Fl Bud	27.25	20.78
Leaf - TAM66274	TAM66274 - Leaf	27.52	17.57
	TAM66274 - Leaf	27.71	18.04
	TAM66274 - Leaf	27.60	17.76
	TAM66274 - Leaf	28.14	17.20
	TAM66274 - Leaf	28.18	17.47
	TAM66274 - Leaf	28.28	17.61
	TAM66274 - Leaf	27.13	17.31
	TAM66274 - Leaf	27.42	17.45
	TAM66274 - Leaf	27.21	17.38
Root - TAM66274	TAM66274 - Root	25.82	20.44
	TAM66274 - Root	25.45	20.12
	TAM66274 - Root	25.41	20.00
	TAM66274 - Root	25.67	20.28
	TAM66274 - Root	25.66	20.36
	TAM66274 - Root	25.22	20.42
	TAM66274 - Root	26.13	21.02
	TAM66274 - Root	26.41	20.88
	TAM66274 - Root	26.11	20.91
Axillary bud – Coker 312	Coker 312 - Ax Bud	33.92	26.68
	Coker 312 - Ax Bud	33.44	26.75
	Coker 312 - Ax Bud	33.65	26.67
	Coker 312 - Ax Bud	33.43	26.69
	Coker 312 - Ax Bud	34.16	26.85
	Coker 312 - Ax Bud	33.43	26.55
	Coker 312 - Ax Bud	33.13	26.31
	Coker 312 - Ax Bud	33.17	26.82
	Coker 312 - Ax Bud	33.24	26.72

Table D-3, continued. Quantification cycle (Cq) values for expression of *dCS* transcripts in non-seed tissues of TAM66274 and non-transgenic cv. Coker 312.

Tissue	Sample	*dCS* Cq	Histone Cq
Bract – Coker 312	Coker 312 - Bract	27.82	17.14
	Coker 312 - Bract	27.57	17.31
	Coker 312 - Bract	27.53	17.15
	Coker 312 - Bract	27.56	17.60
	Coker 312 - Bract	27.94	17.79
	Coker 312 - Bract	27.38	17.68
	Coker 312 - Bract	27.23	17.12
	Coker 312 - Bract	27.32	17.23
	Coker 312 - Bract	27.62	17.31
Floral bud – Coker 312	Coker 312 - Fl Bud	32.88	25.82
	Coker 312 - Fl Bud	32.72	25.32
	Coker 312 - Fl Bud	32.41	25.84
	Coker 312 - Fl Bud	24.42	18.13
	Coker 312 - Fl Bud	24.75	18.41
	Coker 312 - Fl Bud	24.44	18.34
	Coker 312 - Fl Bud	24.67	18.24
	Coker 312 - Fl Bud	24.19	18.08
	Coker 312 - Fl Bud	24.78	18.51
Leaf-Coker 312	Coker 312 - Leaf	27.78	17.13
	Coker 312 - Leaf	27.92	17.49
	Coker 312 - Leaf	27.69	17.27
	Coker 312 - Leaf	27.77	17.55
	Coker 312 - Leaf	27.87	17.95
	Coker 312 - Leaf	27.87	17.70
	Coker 312 - Leaf	27.51	17.22
	Coker 312 - Leaf	27.45	17.31
	Coker 312 - Leaf	27.39	17.12
Root-Coker 312	Coker 312- Root	27.25	21.57
	Coker 312- Root	27.36	21.63
	Coker 312- Root	27.08	21.59
	Coker 312- Root	24.87	19.27
	Coker 312- Root	24.85	19.30
	Coker 312- Root	24.74	19.39
	Coker 312- Root	24.36	19.24
	Coker 312- Root	24.28	18.97
	Coker 312- Root	24.48	19.01

Table D-4. Quantification Cycle (Cq) values for expression of *dCS* transcripts in 31 dpa embryos.

	Samples	Cq dCS	Cq Histone
Embryo-31 dpa	TAM66274	23.76	18.44
	TAM66274	23.31	18.41
	TAM66274	23.19	18.47
	TAM66274	23.03	18.28
	TAM66274	23.45	18.49
	TAM66274	22.95	18.24
	TAM66274	23.99	18.82
	TAM66274	23.60	18.44
	Coker 312	21.31	18.82
	Coker 312	21.06	18.68
	Coker 312	21.22	18.43
	Coker 312	20.65	18.56
	Coker 312	20.60	19.11
	Coker 312	21.09	19.11
	Coker 312	20.18	18.84
	Coker 312	20.39	18.33

Statistical Analysis.
Statistical analysis was performed using Microsoft® Excel® (Version 14.6.8, 2011). The mean, standard error, and range of values were reported for each tissue type.

Bioinformatics Analysis of Potential Non-target Effects of dCS RNAi.
The *dCS* RNAi cassette in TAM66274 was designed to specifically silence the endogenous *dCS* genes in cottonseed, but not have any effect on *dCS* genes in other parts of the plant. The *dCS* RNAi cassette contains a 604 bp long internal sequence (Trigger A) of the *dCS* gene from cotton and a reverse complement of the Trigger A sequence (Trigger B). Expression of this cassette in TAM66274 results in the formation of a dsRNA transcript containing a fragment of the *dCS* genes in cotton, and it is recognition and processing of this dsRNA by the cotton plant's RNAi machinery which results in suppression of expression of the dCS protein in cottonseed.

To determine any potential non-target effects of *dCS* RNAi, the 604 bp *dCS* gene sequence (Figure D-1) from the RNAi hairpin construct (Trigger A or B) was used to identify regions of similarity to sequences in the National Center for Biotechnology Information (NCBI) databases. The sequence was queried against human, cow, pig, chicken, fish, shrimp, dog and cat expressed sequence tags (ESTs) in the NCBI database using the BLASTN algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn).

Figure D-1. The 604 bp *dCS* trigger DNA sequence.

```
  1 ATGCCGAGAACGACCTCTACACCACATCCCTTCGATTCCGATTACTCCGAGAGCATGGAT
 61 TCAATGTTTCATGCGACGTATTCAACAAGTTTAAAGACGAGCAAGGGAATTTCAAGTCAT
121 CCGTGACAAGCGATGTTCGAGGATTGTTGGAACTTTACCAAGCTTCCTATTTGAGGGTTC
181 ATGGGGAAGATATATTGGATGAAGCAATTTCTTTCACCACCAACCATTTAAGCCTTGCAG
241 TAGCATCTTTGGACTATCCGTTATCCGAAGAGGTTTCACATGCTTTGAAACAATCAATTC
301 GAAGAGGCTTGCCAAGGGTTGAGGCAAGACACTATCTTTCAGTATACCAAGATATTGAGT
361 CCCATAATAAGGTTTTGTTGGAGTTTGCTAAGATCGATTTCAACATGGTACAACTTTTGC
421 ATAGGAAAGAGCTAAGTGAGATTTCTAGGTGGTGGAAGGATTTAGACTTTCAAAGAAAGT
481 TGCCATACGCAAGAGATAGAGTGGTTGAAGGCTATTTTTGGATCTCAGGAGTGTACTTTG
541 AGCCCCAATATTCTCTTGGTAGAAAGATGTTGACAAAAGTGATAGCCATGGCTTCTATTG
601 TGGA
```

The BLASTN search was performed using four criteria.

1. Optimized for *highly similar* sequences (megablast) (>95% identity)
2. Optimized for *more dissimilar* sequences (discontiguous megablast)
3. Optimized for *somewhat similar* sequences (BLASTN) (at least 7 base match)
4. Optimized for *similar* sequences (BLASTN) (20 base contiguous match)

BLASTN Search Results and Conclusion.

No similarity was found between the *dCS* trigger sequence and any ESTs from human, cow, pig, chicken, fish, shrimp, dog and cat in the NCBI database at the *highly similar* level (>95%). No similarity was found with any of the ESTs from human, cow, pig, chicken, fish, shrimp, dog and cat in the NCBI database by discontiguous megablast search. Similarity was found to several ESTs from human, cow, pig, chicken, fish, shrimp, dog and cat in the NCBI database at the *somewhat similar* level (at least 7 base match). This level of similarity over such a short stretch may not be sufficient to trigger any non-target effects. No similarity with any of the ESTs from human, cow, pig, chicken, fish, shrimp, dog and cat in the NCBI database was found in the 20 base contiguous stretch.

Based on the bioinformatic analysis results, we foresee no likelihood of any non-target effects on humans and animals that are likely to consume the cottonseed or cottonseed products derived from TAM66274.

B. Materials and Methods to Measure Levels of the NPTII Variant Protein in TAM66274 and Non-transgenic cv. Coker 312

Production of Test and Control Plants.

The TAM66274 and non-transgenic cv. Coker 312 cotton plants used for analysis of NPTII variant protein levels in different tissues were the same plants described above in part A of this Appendix, which were used for determination of *dCS* transcript levels.

Test and Control Substances.

The test substance in this study was protein extracted from leaf, root, seed and pollen harvested from individual plants of TAM66274. The control substance in this study was protein extracted from leaf, root, seed and pollen harvested from individual plants of non-transgenic cv. Coker 312. Initial characterization of the test and control substances was by documentation of the seed pedigree from the study director. Primary characterization was then conducted during the study by NPTII analyses described herein.

Samples for NPTII Expression Analysis.

Approximately 5 g of tissue of each type (leaf, root and seed) and approximately 500 mg pollen was collected from four replicated plots of test and control plants grown at the Texas A&M Field Laboratory during the 2015 growing season. Leaf and root samples were harvested from 45 day old plants. Root sampling was as described above for roots analyzed for *dCS* transcript levels. The pollen sample was collected from the plants at the flowering stage (70-80 day old plants). Fully matured seed samples were used after harvesting plants at the end of the trial. Therefore, a total of 32 samples were harvested from the field (2 entries X 4 tissue types X 4 replicates). All tissues were frozen in liquid nitrogen immediately after harvesting and stored at -80°C until use. Samples were lyophilized (FreeZone Model 7948040; Labconco, Kansas City, MO) and ground to a fine powder using an agate mortar and pestle (Fisher Scientific, Catalog No. 12950C) before protein extraction and analysis.

Sample Analysis: Protein Extraction and Quantification.

Total protein extraction and quantitative ELISA was performed using the Agdia PathoScreen® Kit (Catalog No. PSP 73000; Agdia, Elkhart, IN). Approximately 10 mg freeze-dried tissue powder was mixed with 200 µl 1X protein extraction buffer 1 (PEB1) in a 1.5 ml Eppendorf tube. The tube was vortexed for 5 minutes and centrifuged at 14,000 rpm for 15 minutes. The supernatant was collected and used for the NPTII ELISA. The NPTII protein standard (Catalog No. LST 73000; Agdia, Elkhart, IN) was diluted according to manufacturer's instructions. NPTII protein standard or test and control substance sample extract was added at 100 µl per well to the NPTII antibody-coated microplates and incubated for 2 hours at room temperature in a humid box. The wells were washed seven times with 1X phosphate buffered saline with Tween 20 (PBST). Anti-NPTII and peroxidase enzyme conjugate diluted with enzyme conjugate diluent (1:100) was added at 100 µl per well and incubated for 2 hours at room temperature in a humid

box. The wells were again rinsed seven times with 1X PBST. The plates were developed by adding 100 µl, per well, of horseradish peroxidase substrate tetramethylbenzidene (TMB) solution. The optical density (OD) value was measured at 650 nanometers (nm) using a Victor™ X3 Multilabel Plate Reader (PerkinElmer, Waltham, MA). Quantification of the NPTII variant protein in each tissue type was accomplished by interpolation from an NPTII standard curve that ranged from 0.5-18 ng/ml. This conversion utilized protein standard curve, sample dilution factor and tissue-to-buffer ratio. According to the manufacturer, the assay limit of detection (LOD) is 1.25 ng/ml in PEB1 extraction buffer. Results were reported as ng NPTII variant protein per gram dry weight tissue sample (mean ± SE, n=4). The standard curve for NPTII quantitation is presented in Table D-5 and Figure D-2, and optical density values for NPTII variant protein expression analyses in different tissues of TAM66274 and non-transgenic cv. Coker 312 are presented in Table D-6.

Table D-5. Optical density of NPTII protein concentrations in serial dilutions of the NPTII protein standard.

NPTII protein concentration (ng/ml)	OD (650 nm)
18	0.783
9	0.418
4.5	0.220
2.25	0.144
1.125	0.101
0.571	0.089
0.0	0.078

Figure D-2. NPTII standard curve.
Starting with 18 ng/ml of NPTII protein (Agdia Inc., Catalog No. LST7300), serial dilutions were made and used to generate the standard curve. Tissue samples were analyzed according to the manufacturer's protocol.

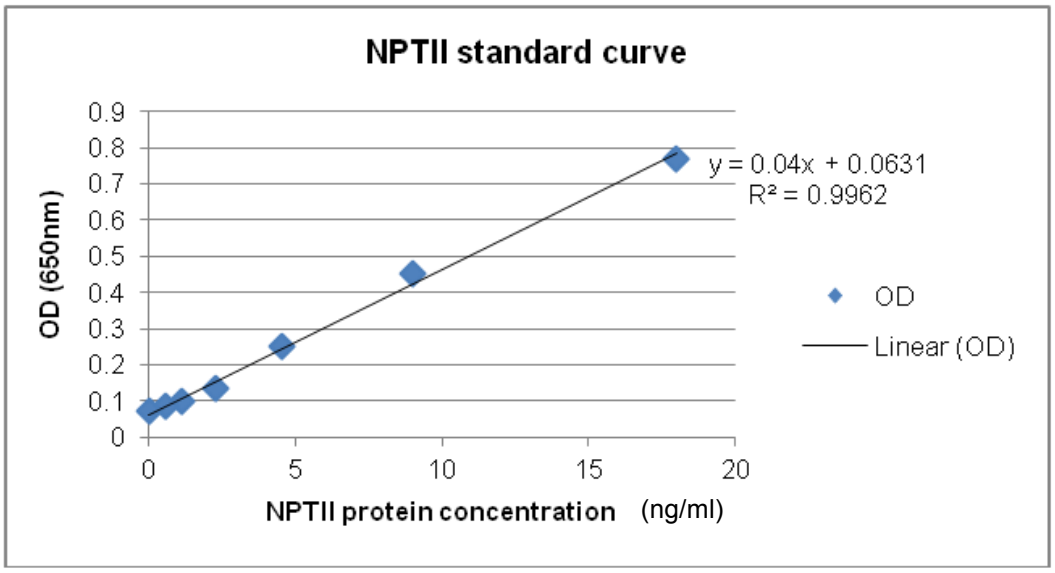


Table D-6. Optical density of NPTII variant protein samples from tissues of TAM66274 and non-transgenic cv. Coker 312.

OD (650 nm) values for Leaf samples	
Coker312-1	0.063
Coker312-2	0.064
Coker312-3	0.065
Coker312-4	0.063
TAM66274-1	0.356
TAM66274-2	0.499
TAM66274-3	0.647
TAM66274-4	0.777

OD (650 nm) values for Pollen samples	
Coker312-1	0.094
Coker312-2	0.093
Coker312-3	0.096
Coker312-4	0.092
TAM66274-1	0.093
TAM66274-2	0.086
TAM66274-3	0.089
TAM66274-4	0.078

OD (650 nm) values for Root samples	
Coker312-1	0.064
Coker312-2	0.065
Coker312-3	0.064
Coker312-4	0.065
TAM66274-1	0.210
TAM66274-2	0.175
TAM66274-3	0.164
TAM66274-4	0.172

OD (650 nm) values for Seed Kernel samples	
Coker312-1	0.094
Coker312-2	0.096
Coker312-3	0.088
Coker312-4	0.094
TAM66274-1	0.209
TAM66274-2	0.243
TAM66274-3	0.207
TAM66274-4	0.167

Statistical Analysis.

Statistical analysis was performed using Microsoft® Excel® (Version 14.6.8, 2011). The mean, standard error, and range of values were reported for each tissue type.

C. Bioinformatics Analyses of Amino Acid Sequences of Intended and Unintended ORFs in the T-DNA and Flanking Genomic DNA of TAM66274 Compared to Amino Acid Sequences of Known Allergens and Toxins

As detailed in Section 5 of the petition, a total of 33 putative ORFs were identified in the T-DNA and genomic flanking sequences of TAM66274 cotton, and there were only six that encoded proteins or peptides greater than 80 amino acids. Putative ORFs were tabulated and subjected to various search strategies for homology to allergens and toxins. Two protein databases were queried for homology of putative ORFs to known allergens: Food and Allergy Research and Resource Program (FARRP) AllergenOnline and the NCBI Entrez protein database. The two-database search strategy was used to ensure queries of the most current and comprehensive databases of known allergens. The NCBI Entrez protein database was queried for homology of putative ORFs to known toxins. Results of these different searches are presented below.

AllergenOnline.

The AllergenOnline search routine employs three comparative bioinformatics approaches:

1. Search for full-length alignments by FASTA (referred to as "Full FASTA")
2. Search for 80 amino acid alignments by FASTA (80mer sliding window search with FASTA)
3. Search for 8 amino acid alignments by FASTA (8mer exact match search with FASTA)

All three approaches were used to query the putative translated ORFs in TAM66274 cotton against the database.

Full FASTA. A total of 33 putative ORFs were identified in the T-DNA and genomic flanking sequences of TAM66274 cotton, and there were only six that encoded proteins or peptides greater than 80 amino acids. Therefore, the full FASTA search was only conducted on the amino acid sequences encoded by these six ORFs. The full FASTA search was conducted using the AllergenOnline database on October 20, 2016. Results of these analyses are presented in Table D-7 below.

80mer sliding window search with FASTA. A FASTA search of every possible 80 amino acid segment of the six putative translated ORFs (described above) was conducted using the AllergenOnline database on October 20, 2016. Results of these analyses are presented in Table D-8 below.

8mer exact match search with FASTA. A FASTA search of every possible 8 amino acid segment of the 33 putative translated ORFs was conducted using the AllergenOnline database on October 20, 2016. Results of these analyses are presented in Table D-9 below.

Table D-7. Full FASTA search results of six putative translated ORFs in TAM66274 cotton. A full FASTA search of the AllergenOnline database (version 16, January 29, 2016) of six putative translated ORFs equal to or greater than 80 amino acids in length in TAM66274 cotton was conducted on October 20, 2016.

TAM66274-1F (101 aa)

```
MTMITPSYLG  DTIEYSSYAS  NALGALPYRP  AGGRTSKLAC  MPAGPAEPRH  VVAKFALDPP
NDLSSLSRFD  LHFIWGPHTP  KKCCIILGAA  SRLPGRRAGPG

No sequences with E() < 1.000000
```

TAM66274-5F (80 aa)

```
MVQLLHRKEL  SEISRWWKDL  DFQRKLPYAR  DRVVEGYFWI  SGVYFEPQYS  LGRKMLTKVI
AMASIVEDPS  LSISNPASQL

The best scores are:                                      opt  z-sc E(1956)  %_id  %_sim alen
gi|303387468|gid|1856|lipid binding protein (Felis catu ( 228) 65 112.2   0.38  0.355  0.645   31
gi|262272877|gid|1593|allergen Bla g 3 isoform 2 precur ( 657) 70 110.5   0.47  0.400  0.600   40
gi|262272875|gid|1593|allergen Bla g 3 isoform 1 precur ( 657) 70 110.5   0.47  0.400  0.600   40
gi|549179|gid|200|Venom allergen 2 precursor (Venom all ( 138) 58 105.4   0.9   0.303  0.515   66
gi|2833325|gid|214|Allergen Cr-PI precursor (Allergen P ( 685) 67 105.2   0.93  0.375  0.600   40
gi|289721058|gid|214|Per a 3 allergen (Periplaneta amer ( 685) 67 105.2   0.93  0.375  0.600   40

>>gi|303387468|gid|1856|lipid binding protein (Felis catus) (Felis catus)     (228 aa)
initn:  54 init1:  54 opt:  65  Z-score: 112.2  bits: 26.5 E(): 0.38
Smith-Waterman score: 65; 35.5% identity (64.5% similar) in 31 aa overlap (18-47:135-165)

                                    10        20        30        40         50
TAM662                          MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWI-SGVYFEPQYSLGRKML
                                       ::.: . .: ... :.:    :: :: .. :
gi|303 DFKGIDLRMPLAFSIQIKFPALNPYIFHVRTDMKVQLYLEKDVDNRYQLTFGHCRIVPETVWIQSGNFITPMKNFIVENI
          100       110       120       130       140       150       160       170

         60        70        80
TAM662 TKVIAMASIVEDPSLSISNPASQL
gi|303 ERALGNVIIHNFGAKMCPFINSWLYNLNPQVTNQLISLLLQHGTYQATVEIPAK
          180       190       200       210       220

>>gi|262272877|gid|1593|allergen Bla g 3 isoform 2 precursor (Blattella germ  (657 aa)
initn:  53 init1:  53 opt:  70  Z-score: 110.5  bits: 27.7 E(): 0.47
Smith-Waterman score: 70; 40.0% identity (60.0% similar) in 40 aa overlap (6-41:77-116)

                                             10        20         30        40
TAM662                                  MVQLLHRKE---LSEISRWWKDLD-FQRKLPYARDRVVEGYFWIS
                                           ::..   : :.  . .: : : .   .::::: ::.:  :
gi|262 YDIEANINNYKNPRVVKNFMALYKKDPVKRGEPFSTYYIKHREQAIMLFELFYYANDYDTFYKTACWARDRVNEGMFLYS
         40        50        60        70        80        90       100       110

             50        60        70        80
TAM662 GVYFEPQYSLGRKMLTKVIAMASIVEDPSLSISNPASQL
gi|262 FNIAIMHREDMQDIVVPAFYEIYPFLFVENDVIQKAYDYKMKESGHLNEPHTHVIPVNFTLRNQEQLLSYFTEDVFLNAF
        120       130       140       150       160       170       180       190

>>gi|262272875|gid|1593|allergen Bla g 3 isoform 1 precursor (Blattella germ  (657 aa)
initn:  53 init1:  53 opt:  70  Z-score: 110.5  bits: 27.7 E(): 0.47
Smith-Waterman score: 70; 40.0% identity (60.0% similar) in 40 aa overlap (6-41:77-116)

                                             10        20         30        40
TAM662                                  MVQLLHRKE---LSEISRWWKDLD-FQRKLPYARDRVVEGYFWIS
                                           ::..   : :.  . .: : : .   .::::: ::.:  :
gi|262 YDIEANINNYKNPRVVKNFMALYKKDPVKRGEPFSTYYIKHREQAIMLFELFYYANDYDTFYKTACWARDRVNEGMFLYS
         40        50        60        70        80        90       100       110

             50        60        70        80
TAM662 GVYFEPQYSLGRKMLTKVIAMASIVEDPSLSISNPASQL
```

Table D-7, continued. Full FASTA search results of six putative translated ORFs in TAM66274 cotton.

```
gi|262 FNIAIMHREDMQDIVIPAFYEIYPFLFVENDVIQKAYDYKMKESGHLNEPHTHVIPVNFTLRNQEQLLSYFTEDVFLNAF
        120       130       140       150       160       170       180       190

>>gi|549179|gid|200|Venom allergen 2 precursor (Venom allergen II) (Allergen  (138 aa)
initn:  64 init1:  41 opt:  58  Z-score: 105.4  bits: 24.5 E():  0.9
Smith-Waterman score: 58; 30.3% identity (51.5% similar) in 66 aa overlap (7-68:28-93)

                                    10        20        30        40        50
TAM662                          MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWISGVYFEPQYSLGR----KM
                                     ::...:  :       :   : ::  : .  .   ::: .:. .. .    ::
gi|549 MKSFVLATCLLGFAQIIYADNKELKIIRKDVAECLRTLPKCGNQPDDPLARVDVWHCAMAKRGVYDNPDPAVIKERSMKM
              10        20        30        40        50        60        70        80

          60        70        80
TAM662 LTKVIAMASIVEDPSLSISNPASQL
       ::.:.  . ::.
gi|549 CTKIITDPANVENCKKVASRCVDRETQGPKSNRQKAVNIIGCALRAGVAETTVLARKK
              90       100       110       120       130

>>gi|2833325|gid|214|Allergen Cr-PI precursor (Allergen Per a 3) (Periplanet  (685 aa)
initn:  54 init1:  54 opt:  67  Z-score: 105.2  bits: 26.8 E(): 0.93
Smith-Waterman score: 67; 37.5% identity (60.0% similar) in 40 aa overlap (6-41:102-141)

                                                 10        20         30        40
TAM662                                       MVQLLHRKE---LSEISRWWKDLD-FQRKLPYARDRVVEGYFWIS
                                                 ::..   : ..  . .: : : .   .::::: ::.:  :
gi|283 MTSKQTSATTVPPSGEAVHGVLQEGHARPRGEPFSVNYEKHREQAIMLYDLLYFANDYDTFYKTACWARDRVNEGMFMYS
             70        80        90       100       110       120       130       140

             50        60        70        80
TAM662 GVYFEPQYSLGRKMLTKVIAMASIVEDPSLSISNPASQL
gi|283 FSIAVFHRDDMQGVMLPPPYEVYPYLFVDHDVIHMAQKYWMKNAGSGEHHSHVIPVNFTLRTQDHLLAYFTSDVNLNAFN
             150       160       170       180       190       200       210       220


>>gi|289721058|gid|214|Per a 3 allergen (Periplaneta americana) (Periplaneta  (685 aa)
initn:  54 init1:  54 opt:  67  Z-score: 105.2  bits: 26.8 E(): 0.93
Smith-Waterman score: 67; 37.5% identity (60.0% similar) in 40 aa overlap (6-41:102-141)

                                                 10        20         30        40
TAM662                                       MVQLLHRKE---LSEISRWWKDLD-FQRKLPYARDRVVEGYFWIS
                                                 ::..   : ..  . .: : : .   .::::: ::.:  :
gi|289 MTSKQTSATTVPPSGEAVHGVLQEGHARPRGEPFSVNYEKHREQAIMLYDLLYFANDYDTFYKTACWARDRVNEGMFMYS
             70        80        90       100       110       120       130       140

             50        60        70        80
TAM662 GVYFEPQYSLGRKMLTKVIAMASIVEDPSLSISNPASQL
gi|289 FSIAVFHRDDMQGVMLPPPYEVYPYLFVDHDVIHMAQKYWMKNAGSGEHHSHVIPVNFTLRTQDHLLAYFTSDVNLNAFN
             150       160       170       180       190       200       210       22
```

TAM66274-12F (273 aa)

```
MAITLSATSL  PISARIRAGS  PAAWVERLFG  YDWAQQTIGC  SDAAVFRLSA  QGRPVLFVKT
DLSGALNELQ  DEAARLSWLA  TTGVPCAAVL  DVVTEAGRDW  LLLGEVPGQD  LLSSHLAPAE
KVSIMADAMR  RLHTLDPATC  PFDHQAKHRI  ERARTRMEAG  LVDQDDLDEE  HQGLAPAELF
ARLKARMPDG  EDLVVTHGDA  CLPNIMVENG  RFSGFIDCGR  LGVADRYQDI  ALATRDIAEE
LGGEWADRFL  VLYGIAAPDS  QRIAFYRLLD  EFF
```

```
No sequences with E() < 1.000000
```

Table D-7, continued. Full FASTA search results of six putative translated ORFs in TAM66274 cotton.

TAM66274-14F (128 aa)

```
MNCRTRQRGY  RGWPRRAFLA  QLCSTLSLKR  EGTGCYWAKC  RGRISCHLTL  LLPRKYPSWL
MQCGGCIRLI  RLPAHSTTKR  NIASSEHVLG  WKPVLSIRMI  WTKSIRGSRQ  PNCSPGSRRA
CPTARISS

The best scores are:                                      opt  z-sc  E(1956)   %id  %sim
alen
gi|75009997|gid|1734|RecName: Full=Venom protease; AltN ( 243) 77 106.6     0.77 0.364 0.606
33
>>>TAM66274-14F, 128 aa vs fasta/version16.fasta library

>>gi|75009997|gid|1734|RecName: Full=Venom protease; AltName: Allergen=Bom p  (243 aa)
initn:  43 init1:  43 opt:  77  Z-score: 106.6  bits: 26.2 E(): 0.77
Smith-Waterman score: 77; 36.4% identity (60.6% similar) in 33 aa overlap (53-82:26-56)

             20        30        40        50        60        70            80
TAM662 WPRRAFLAQLCSTLSLKREGTGCYWAKCRGRISCHLTLLLPRKYPSWLMQCGGCIRLIR---LPAHSTTKRNIASSEHVL
                                           :.: : :  .::: .:. :     :: . .:..
gi|750               VVGGKPAKLGAWPWMVALGFHNYRQPKKSPEW—KCGGSLRISRHVLTAAHCAIHRSLYVVRIAD
                              10        20        30          40        50        60

      90       100       110       120
TAM662 GWKPVLSIRMIWTKSIRGSRQPNCSPGSRRACPTARISS
gi|750 LNLKRDDDGAHPIQMGIESKLIHPDYVYSEHHDDIAILKLEKDVSFSEYIRPICLPIEESLRNNNFIGYNPFVAGWGRLR
            70        80        90       100       110       120       130       140
```

TAM66274-3R (178 aa)

```
MAGWASLGRS  FRTPESRSEE  LVKKAIEGDA  LRIGSGDTVK  HEEAVSPFAA  KLFSNITGSQ
RYVLIAVRHT  QPATVDESRK  AAIFHHDIRQ  AGIAMGHDEI  LAVGHARLEP  GEQFGWREPL
MLFVQIILID  KTGFHPSTCS  LDAMFRLVVE  WAGSRIKRMQ  PPHCISHDGY  FLGRSKVR

The best scores are:                                      opt  z-sc E(1956)  %_id %_sim alen
gi|741844|gid|109|major allergen Par j I (Parietaria ju ( 143) 77 115.1    0.26 0.237 0.500   80

>>gi|741844|gid|109|major allergen Par j I (Parietaria judaica)              (143 aa)
initn:  66 init1:  66 opt:  77  Z-score: 115.1  bits: 27.5 E(): 0.26
Smith-Waterman score: 77; 23.8% identity (50.0% similar) in 80 aa overlap (49-123:50-127)

       10        20        30        40        50             60        70        80
TAM662 RSFRTPESRSEELVKKAIEGDALRIGSGDTVKHEEAVSPFAAKLFSNITGS-----QRYVLIAVRHTQPATVDESRKAAI
                                           : : .:.: :.      ..  :.  .  :  :. . :..
gi|741 PFVQGKEKEPSKGCCSGAKRLDGETKTGPQRVHACECIQTAMKTYSDIDGKLVSEVPKHCGIVDSKLPPIDVNMDCKTVG
      10        20        30        40        50        60        70        80

            90       100       110       120       130       140       150       160
TAM662 FHHDIRQAGIAMGHDEILAVGHARLEPGEQFGWREPLMLFVQIILIDKTGFHPSTCSLDAMFRLVVEWAGSRIKRMQPPH
             :  ... :  .  .: .: .:  . :::.: . :
gi|741 VVPRQPQLPVSLRHGPV--TGPSRSRPPTKHGWRDPRLEFRPPHRKKPNPAFSTLG
      90       100         110       120       130       140
```

TAM66274-9R (89 aa)

```
MVQLLHRKEL  SEISRWWKDL  DFQRKLPYAR  DRVVEGYFWI  SGVYFEPQYS  LGRKMLTKVI
AMASIVEDPN  SVPQLGKEII  IFFFPFSIK

The best scores are:                                      opt  z-sc E(1956) %_id %_sim alen
gi|303387468|gid|1856|lipid binding protein (Felis catu ( 228) 65 109.8   0.51 0.355 0.645   31
gi|6136162|gid|200|Venom allergen 2 (Venom allergen II) ( 119) 61 109.4   0.54 0.260 0.519   77
gi|262272875|gid|1593|allergen Bla g 3 isoform 1 precur ( 657) 70 108.1   0.64 0.400 0.600   40
gi|262272877|gid|1593|allergen Bla g 3 isoform 2 precur ( 657) 70 108.1   0.64 0.400 0.600   40
```

Table D-7, continued. Full FASTA search results of six putative translated ORFs in TAM66274 cotton.

```
gi|549179|gid|200|Venom allergen 2 precursor (Venom all ( 138) 59 104.6      1 0.300 0.529   70

>>gi|303387468|gid|1856|lipid binding protein (Felis catus) (Felis catus)     (228 aa)
initn:  54 init1:  54 opt:  65  Z-score: 109.8  bits: 26.2 E(): 0.51
Smith-Waterman score: 65; 35.5% identity (64.5% similar) in 31 aa overlap (18-47:135-165)

                                     10        20        30        40         50
TAM662                          MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWI-SGVYFEPQYSLGRKML
                                          ::.: . .: ... :.:    :: :: .. :
gi|303 DFKGIDLRMPLAFSIQIKFPALNPYIFHVRTDMKVQLYLEKDVDNRYQLTFGHCRIVPETVWIQSGNFITPMKNFIVENI
         100       110       120       130       140       150       160       170

         60        70        80
TAM662 TKVIAMASIVEDPNSVPQLGKEIIIFFFPFSIK

gi|303 ERALGNVIIHNFGAKMCPFINSWLYNLNPQVTNQLISLLLQHGTYQATVEIPAK
         180       190       200       210       220

>>gi|6136162|gid|200|Venom allergen 2 (Venom allergen II) (Allergen Sol r 2)  (119 aa)
initn:  37 init1:  37 opt:  61  Z-score: 109.4  bits: 25.2 E(): 0.54
Smith-Waterman score: 61; 26.0% identity (51.9% similar) in 77 aa overlap (3-79:5-79)

                10        20        30        40        50        60        70
TAM662   MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWISGVYFEPQYSLGRKMLTKVIAMASIVEDPNSVPQLGKE
           : . ::...: .:       :   : ::  : .  .   ::: .:. .. ..  .:.    .:. :: .: .  :
gi|613 DIEAQRVLRKDIAECARTLPKCVNQPDDPLARVDVWHCAMSKRGVYDNPDPAVVKEKNSKMCP--KIITDPADVENCKKV
             10        20        30        40        50        60          70

        80
TAM662 IIIFFFPFSIK
       .
gi|613 VSRCVDRETQRPRSNRQKAINITGCILRAGVVEATVLAREK
       80        90       100       110

>>gi|262272875|gid|1593|allergen Bla g 3 isoform 1 precursor (Blattella germ  (657 aa)
initn:  53 init1:  53 opt:  70  Z-score: 108.1  bits: 27.4 E(): 0.64
Smith-Waterman score: 70; 40.0% identity (60.0% similar) in 40 aa overlap (6-41:77-116)

                                              10        20         30        40
TAM662                                   MVQLLHRKE---LSEISRWWKDLD-FQRKLPYARDRVVEGYFWIS
                                              ::..   : :.  . .: : : .   .:::::: ::.:  :
gi|262 YDIEANINNYKNPRVVKNFMALYKKDPVKRGEPFSTYYIKHREQAIMLFELFYYANDYDTFYKTACWARDRVNEGMFLYS
         40        50        60        70        80        90        100       110

              50        60        70        80
TAM662 GVYFEPQYSLGRKMLTKVIAMASIVEDPNSVPQLGKEIIIFFFPFSIK
gi|262 FNIAIMHREDMQDIVIPAFYEIYPFLFVENDVIQKAYDYKMKESGHLNEPHTHVIPVNFTLRNQEQLLSYFTEDVFLNAF
        120       130       140       150       160       170       180       190

>>gi|262272877|gid|1593|allergen Bla g 3 isoform 2 precursor (Blattella germ  (657 aa)
initn:  53 init1:  53 opt:  70  Z-score: 108.1  bits: 27.4 E(): 0.64
Smith-Waterman score: 70; 40.0% identity (60.0% similar) in 40 aa overlap (6-41:77-116)

                                              10        20         30        40
TAM662                                   MVQLLHRKE---LSEISRWWKDLD-FQRKLPYARDRVVEGYFWIS
                                              ::..   : :.  . .: : : .   .:::::: ::.:  :
gi|262 YDIEANINNYKNPRVVKNFMALYKKDPVKRGEPFSTYYIKHREQAIMLFELFYYANDYDTFYKTACWARDRVNEGMFLYS
         40        50        60        70        80        90        100       110

              50        60        70        80
TAM662 GVYFEPQYSLGRKMLTKVIAMASIVEDPNSVPQLGKEIIIFFFPFSIK

gi|262 FNIAIMHREDMQDIVVPAFYEIYPFLFVENDVIQKAYDYKMKESGHLNEPHTHVIPVNFTLRNQEQLLSYFTEDVFLNAF
        120       130       140       150       160       170       180       190
```

Table D-7, continued. Full FASTA search results of six putative translated ORFs in TAM66274 cotton.

```
>>gi|549179|gid|200|Venom allergen 2 precursor (Venom allergen II) (Allergen  (138 aa)
initn:  60 init1:  38 opt:  59  Z-score: 104.6  bits: 24.5 E():    1
Smith-Waterman score: 59; 30.0% identity (52.9% similar) in 70 aa overlap (7-72:28-97)

                                    10        20        30        40        50
TAM662                         MVQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWISGVYFEPQYSLGR----KM
                                     ::...:  :       :   : ::  : .  .   ::: .:. .. .    ::
gi|549 MKSFVLATCLLGFAQIIYADNKELKIIRKDVAECLRTLPKCGNQPDDPLARVDVWHCAMAKRGVYDNPDPAVIKERSMKM
               10        20        30        40        50        60        70        80

          60        70        80
TAM662 LTKVIAMASIVEDPNSVPQLGKEIIIFFFPFSIK
        ::.:.  . ::. ..:
gi|549 CTKIITDPANVENCKKVASRCVDRETQGPKSNRQKAVNIIGCALRAGVAETTVLARKK
               90       100       110       120       130
```

Table D-8. FASTA 80mer sliding window search results of six putative translated ORFs in TAM66274 cotton. A FASTA 80mer sliding window search of the AllergenOnline database (version 16, January 29, 2016) of six putative translated ORFs equal to or greater than 80 amino acids in length in TAM66274 cotton was conducted on October 20, 2016.

1. TAM66274-1F (101 aa)

```
MTMITPSYLG  DTIEYSSYAS  NALGALPYRP  AGGRTSKLAC  MPAGPAEPRH  VVAKFALDPP
NDLSSLSRFD  LHFIWGPHTP  KKCCIILGAA  SRLPGRRAGPG
Number of 80 mers:      22
Number of Sequences with hits:      0
No Matches of Greater than 35% Identity Found
```

2. TAM66274-5F (80 aa)

```
MVQLLHRKEL  SEISRWWKDL  DFQRKLPYAR  DRVVEGYFWI  SGVYFEPQYS  LGRKMLTKVI
AMASIVEDPS  LSISNPASQL
Number of 80 mers:      1
Number of Sequences with hits:      0
No Matches of Greater than 35% Identity Found
```

3. TAM66274-12F (273 aa)

```
MAITLSATSL  PISARIRAGS  PAAWVERLFG  YDWAQQTIGC  SDAAVFRLSA  QGRPVLFVKT
DLSGALNELQ  DEAARLSWLA  TTGVPCAAVL  DVVTEAGRDW  LLLGEVPGQD  LLSSHLAPAE
KVSIMADAMR  RLHTLDPATC  PFDHQAKHRI  ERARTRMEAG  LVDQDDLDEE  HQGLAPAELF
ARLKARMPDG  EDLVVTHGDA  CLPNIMVENG  RFSGFIDCGR  LGVADRYQDI  ALATRDIAEE
LGGEWADRFL  VLYGIAAPDS  QRIAFYRLLD  EFF
Number of 80 mers:      194
Number of Sequences with hits:      0
No Matches of Greater than 35% Identity Found
```

4. TAM66274-14F (128 aa)

```
MNCRTRQRGY  RGWPRRAFLA  QLCSTLSLKR  EGTGCYWAKC  RGRISCHLTL  LLPRKYPSWL
MQCGGCIRLI  RLPAHSTTKR  NIASSEHVLG  WKPVLSIRMI  WTKSIRGSRQ  PNCSPGSRRA
CPTARISS
Number of 80 mers:      49
Number of Sequences with hits:      0
No Matches of Greater than 35% Identity Found
```

5. TAM66274-3R (178 aa)

```
MAGWASLGRS  FRTPESRSEE  LVKKAIEGDA  LRIGSGDTVK  HEEAVSPFAA  KLFSNITGSQ
RYVLIAVRHT  QPATVDESRK  AAIFHHDIRQ  AGIAMGHDEI  LAVGHARLEP  GEQFGWREPL
MLFVQIILID  KTGFHPSTCS  LDAMFRLVVE  WAGSRIKRMQ  PPHCISHDGY  FLGRSKVR
Number of 80 mers:      99
Number of Sequences with hits:      0
No Matches of Greater than 35% Identity Found
```

6. TAM66274-9R (89 aa)

```
MVQLLHRKEL  SEISRWWKDL  DFQRKLPYAR  DRVVEGYFWI  SGVYFEPQYS  LGRKMLTKVI
AMASIVEDPN  SVPQLGKEII  IFFFPFSIK
Number of 80 mers:      10
Number of Sequences with hits:      0
No Matches of Greater than 35% Identity Found
```

Table D-9. FASTA 8mer exact match search results of 33 putative translated ORFs in TAM66274 cotton. A FASTA search of the AllergenOnline database (version 16, January 29, 2016) for exact match (100% identity) of 8 amino acid segments of 33 putative translated ORFs in TAM66274 cotton was conducted on October 20, 2016.

1. TAM66274-1F (101 aa)

```
MTMITPSYLG DTIEYSSYAS NALGALPYRP AGGRTSKLAC MPAGPAEPRH VVAKFALDPP NDLSSLSRFD
LHFIWGPHTP KKCCIILGAA SRLPGRRAGP G
Number of 8mers = 94
No sequences found with an exact 8mer match
```

2. TAM66274-2F (35 aa)

```
MLSQNSPWTR PTICRHCQGL TCTSFGAHIH QKNAA
Number of 8mers = 28
No sequences found with an exact 8mer match
```

3. TAM66274-3F (35 aa)

```
MVPVTFGRAD GQYSTSRNLT HARRRGTGVP FSERY
Number of 8mers = 28
No sequences found with an exact 8mer match
```

4. TAM66274-4F (41 aa)

```
MPRTTSTPHP FDSDYSESMD SMFHATYSTS LKTSKGISSH P
Number of 8mers = 34
No sequences found with an exact 8mer match
```

5. TAM66274-5F (80 aa)

```
MVQLLHRKEL  SEISRWWKDL  DFQRKLPYAR  DRVVEGYFWI  SGVYFEPQYS  LGRKMLTKVI
AMASIVEDPS  LSISNPASQL
Number of 8mers = 73
No sequences found with an exact 8mer match
```

6. TAM66274-6F (67 aa)

```
MLVYHLTCSI KFIKNNILTL NFYLLRLTHH LSYFFTLLCC LCKQYIYKLF FHNYNNYIII IILINIT
Number of 8mers = 60
No sequences found with an exact 8mer match
```

7. TAM66274-7F (36 aa)

```
MVGGERNCFI QYIFPMNPQI GSLVKFQQSS NIACHG
Number of 8mers = 29
No sequences found with an exact 8mer match
```

8. TAM66274-8F (30 aa)

```
MDICRIRLGT RIDYDKLCIL LLCDGNSKEW
Number of 8mers = 23
No sequences found with an exact 8mer match
```

9. TAM66274-9F (65 aa)

```
MQIFINVLKT LTCKMTSVQI KEDEILSLFF FFCIVVGRYR DATSLHMVLA RFGQTLLQGG SWLHL
Number of 8mers = 58
No sequences found with an exact 8mer match
```

Table D-9, continued. FASTA 8mer exact match search results of 33 putative translated ORFs in TAM66274 cotton.

10. TAM66274-10F (33 aa)

```
MSRFTELRVE LIEDKSSVHM LLCMVMIYEL QEI
Number of 8mers = 26
No sequences found with an exact 8mer match
```

11. TAM66274-11F (45 aa)

```
MEIVSVNGFL EFNELSTYVR NHYCAFKSRL RSLSASKYFL SKMLH
Number of 8mers = 38
No sequences found with an exact 8mer match
```

12. TAM66274-12F (273 aa)

```
MAITLSATSL  PISARIRAGS  PAAWVERLFG  YDWAQQTIGC  SDAAVFRLSA  QGRPVLFVKT
DLSGALNELQ  DEAARLSWLA  TTGVPCAAVL  DVVTEAGRDW  LLLGEVPGQD  LLSSHLAPAE
KVSIMADAMR  RLHTLDPATC  PFDHQAKHRI  ERARTRMEAG  LVDQDDLDEE  HQGLAPAELF
ARLKARMPDG  EDLVVTHGDA  CLPNIMVENG  RFSGFIDCGR  LGVADRYQDI  ALATRDIAEE
LGGEWADRFL  VLYGIAAPDS  QRIAFYRLLD  EFF
Number of 8mers = 266
No sequences found with an exact 8mer match
```

13. TAM66274-13F (35 aa)

```
MTGHNRQSAA LMPPCSGCQR RGARFFLSRP TCPVP
Number of 8mers = 28
No sequences found with an exact 8mer match
```

14. TAM66274-14F (128 aa)

```
MNCRTRQRGY RGWPRRAFLA QLCSTLSLKR EGTGCYWAKC RGRISCHLTL LLPRKYPSWL MQCGGCIRLI
RLPAHSTTKR NIASSEHVLG WKPVLSIRMI WTKSIRGSRQ PNCSPGSRRA CPTARISS
Number of 8mers = 121
No sequences found with an exact 8mer match
```

15. TAM66274-15F (33 aa)

```
MAMPACRISW WKMAAFLDSS TVAGWVWRTA IRT
Number of 8mers = 26
No sequences found with an exact 8mer match
```

16. TAM66274-16F (63 aa)

```
MTDQATPNLP SRDFDSTAAF YERLGFGIVF RDAGWMILQR GDLMLEFFAH PDPTLTFATS KSK
Number of 8mers = 56
No sequences found with an exact 8mer match
```

17. TAM66274-17F (58 aa)

```
MHALTTWNIA IFLKNYARWR MSRQLQLLPK SKYPSRMHSS ILFMRGKARL IQLANHPA
Number of 8mers = 51
No sequences found with an exact 8mer match
```

18. TAM66274-18F (78 aa)

```
MEHRYFSEEL CSLEDVAAIA AIAKIEIPLT HAFINIIHAG KGKINPTGKS SSVIGNFSSS DLIRFGATHV
FNKDEMVE
Number of 8mers = 71
No sequences found with an exact 8mer match
```

Table D-9, continued. FASTA 8mer exact match search results of 33 putative translated ORFs in TAM66274 cotton.

19. TAM66274-19F (34 aa)

```
MLVGGCRGNC SYCQNRNTPH ACIHQYYSCG ERQD
Number of 8mers = 27
No sequences found with an exact 8mer match
```

20. TAM66274-1R (38 aa)

```
MIIIARPATG FNLKKLYCQM FERSASTHSF FTPPSRPY
Number of 8mers = 31
No sequences found with an exact 8mer match
```

21. TAM66274-2R (33 aa)

```
MHDARYEVTV LGSIPSKFHS QYHIHHCIPA RENY
Number of 8mers = 26
No sequences found with an exact 8mer match
```

22. TAM66274-3R (178 aa)

```
MAGWASLGRS FRTPESRSEE LVKKAIEGDA LRIGSGDTVK HEEAVSPFAA KLFSNITGSQ RYVLIAVRHT
QPATVDESRK AAIFHHDIRQ AGIAMGHDEI LAVGHARLEP GEQFGWREPL MLFVQIILID KTGFHPSTCS
LDAMFRLVVE WAGSRIKRMQ PPHCISHDGY FLGRSKVR
Number of 8mers = 171
No sequences found with an exact 8mer match
```

23. TAM66274-4R (44 aa)

```
MNPEKRPFST MIFGKQASPW VTTRSSPSGM RALSLANSSA GASP
Number of 8mers = 37
No sequences found with an exact 8mer match
```

24. TAM66274-5R (35 aa)

```
MRCFAWWSNG QVAGSSVCSR RIASAMMDTF SAGAR
Number of 8mers = 28
No sequences found with an exact 8mer match
```

25. TAM66274-6R (46 aa)

```
MSIKRTGYQK YTYLNLFISP ATHRSSPCTA TCVHLTCPLS TQPLTQ
Number of 8mers = 39
No sequences found with an exact 8mer match
```

26. TAM66274-7R (41 aa)

```
MNPPATKFVR TLLVPCEGMW HLDIYPPLYK KKKKETIFRL L
Number of 8mers = 34
No sequences found with an exact 8mer match
```

27. TAM66274-8R (41 aa)

```
MPRTTSTPHP FDSDYSESMD SMFHATYSTS LKTSKGISSH P
Number of 8mers = 34
No sequences found with an exact 8mer match
```

Table D-9, continued. FASTA 8mer exact match search results of 33 putative translated ORFs in TAM66274 cotton.

28. TAM66274-9R (89 aa)

```
MVQLLHRKEL SEISRWWKDL DFQRKLPYAR DRVVEGYFWI SGVYFEPQYS LGRKMLTKVI AMASIVEDPN
SVPQLGKEII IFFFPFSIK
Number of 8mers = 82
No sequences found with an exact 8mer match
```

29. TAM66274-10R (36 aa)

```
MVGGERNCFI QYIFPMNPQI GSLVKFQQSS NIACHG
Number of 8mers = 29
No sequences found with an exact 8mer match
```

30. TAM66274-11R (48 aa)

```
MRDAYDRMIF AFNSVVHVVK NLSMCSSDPY RRFRFILMNI SPVTIVFL
Number of 8mers = 41
No sequences found with an exact 8mer match
```

31. TAM66274-12R (33 aa)

```
MIERHNNKQL RFIITNPILK KAAEPVKPKR LIT
Number of 8mers = 26
No sequences found with an exact 8mer match
```

32. TAM66274-13R (67 aa)

```
MQHFFGVCGP QMKCRSNLDS DDKSLGGSRA NFATTCRGSA GPAGMQASLL VRPPAGRYGR APNALDA
Number of 8mers = 60
No sequences found with an exact 8mer match
```

33. TAM66274-14R (42 aa)

```
MSELTHINCV ALTARFPVGK PVVPAALMNR PTRGERRFAY WG
Number of 8mers = 35
No sequences found with an exact 8mer match
```

Entrez Protein Database.

A BLASTP search was used to compare the amino acid sequence of the six putative translated ORFs with more than 80 aa residues against the entire Entrez protein database. The results of BLASTP searches were tabulated into a list of sequences with identities over at least 80 aa of the query sequence to known allergens, and results are presented in Table D-10.

A BLASTP search was also conducted to compare the amino acid sequence of all 33 putative translated ORFs against the Entrez protein database for protein toxins. The results of BLASTP searches were tabulated into a list of aligned sequences from best to least similar, *E*-value and a percent identity of the overlapping alignment, and the best alignment between the query sequence and aligned protein for further evaluation. Results are presented in Table D-11.

Table D-10. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton. A BLASTP search of the Entrez protein database of 6 putative translated ORFs in TAM66274 cotton was conducted on October 19, 2016. The search was restricted to putative translated ORFs of 80 or more amino acids in length and database sequences identified as "allergen."

1. TAM66274-1F (101 aa)

```
MTMITPSYLG DTIEYSSYAS NALGALPYRP AGGRTSKLAC MPAGPAEPRH VVAKFALDPP NDLSSLSRFD
LHFIWGPHTP KKCCIILGAA SRLPGRRAGP G

                                                                  Score     E
Sequences producing significant alignments:                       (Bits)  Value
XP_014612495.1  PREDICTED: MD-2-related lipid-recognition prot...  29.3    0.27

ALIGNMENTS

>XP_014612495.1 PREDICTED: MD-2-related lipid-recognition protein-like (Polistes canadenisis)
Length=162

Score = 29.3 bits (64),  Expect = 0.27, Method: Compositional matrix adjust.
Identities = 16/83 (19%), Positives = 35/83 (42%), Gaps = 3/83 (4%)

Query  5    TPSYLGDTIEYSSYASNALGALPYRPAGGRTSKLACMPAGPAEPRHVVAKFALDPPNDLS  64
            T ++ GD +E  +Y ++A+  LP+    G    +   PA P + +    +  +
Sbjct  77   TLNFSGDKLETRAYWASAVADLPFIGMSGDACTMTACPAVPGQKQTYNVQLFISKK---F  133
Query  65   SLSRFDLHFIWGPHTPKKCCIIL  87
             +  +DL +       ++CC +
Sbjct  134  PIRMYDLKWKMWNEQEQECCFMF  156
```

2. TAM66274-5F (80 aa)

```
MVQLLHRKEL  SEISRWWKDL  DFQRKLPYAR  DRVVEGYFWI  SGVYFEPQYS  LGRKMLTKVI
AMASIVEDPS  LSISNPASQL

No significant similarity found.
```

Table D-10, continued. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton.

3. TAM66274-12F (273 aa)

```
MAITLSATSL  PISARIRAGS  PAAWVERLFG  YDWAQQTIGC  SDAAVFRLSA  QGRPVLFVKT
DLSGALNELQ  DEAARLSWLA  TTGVPCAAVL  DVVTEAGRDW  LLLGEVPGQD  LLSSHLAPAE
KVSIMADAMR  RLHTLDPATC  PFDHQAKHRI  ERARTRMEAG  LVDQDDLDEE  HQGLAPAELF
ARLKARMPDG  EDLVVTHGDA  CLPNIMVENG  RFSGFIDCGR  LGVADRYQDI  ALATRDIAEE
LGGEWADRFL  VLYGIAAPDS  QRIAFYRLLD  EFF
```

Sequences producing significant alignments:	Score (Bits)	E Value
gi|1036768828|ref|XP_017087060.1| PREDICTED: venom allergen 5...	33.5	0.10
gi|1036768520|ref|XP_017040791.1| PREDICTED: venom allergen 5...	32.7	0.17
gi|1037043392|ref|XP_017125940.1| PREDICTED: venom allergen 5...	32.3	0.19
gi|1037063026|ref|XP_016990735.1| PREDICTED: venom allergen 5...	32.3	0.21
gi|1036915151|ref|XP_017072698.1| PREDICTED: venom allergen 5...	32.3	0.21
gi|194754086|ref|XP_001959328.1| uncharacterized protein Dana...	32.3	0.21
gi|1036794411|ref|XP_016953055.1| PREDICTED: venom allergen 5...	32.3	0.21
gi|1036050781|ref|XP_016928162.1| PREDICTED: venom allergen 5...	32.3	0.22
gi|195346421|ref|XP_002039756.1| GM15725 (Drosophila sechellia)	32.3	0.23
gi|567967163|gb|ETK92347.1| hypothetical protein L915_04276 (...	31.6	0.25
gi|1036830438|ref|XP_017022249.1| PREDICTED: venom allergen 5...	32.0	0.26
gi|195585418|ref|XP_002082478.1| uncharacterized protein Dsim...	32.0	0.26
gi|970633761|gb|KUF79289.1| hypothetical protein AM587_100114...	32.0	0.27
gi|24656989|ref|NP_611582.1| uncharacterized protein Dmel_CG1...	32.0	0.27
gi|1036987849|ref|XP_017007825.1| PREDICTED: venom allergen 5...	31.6	0.35
gi|195486506|ref|XP_002091541.1| uncharacterized protein Dyak...	31.6	0.35
gi|970633753|gb|KUF79281.1| SCP extracellular protein (Phytop...	31.2	0.51
gi|194881898|ref|XP_001975050.1| uncharacterized protein Dere...	31.2	0.57
gi|968087096|ref|XP_002061399.2| uncharacterized protein Dwil...	30.8	0.65
gi|1060207003|ref|XP_017836482.1| PREDICTED: venom allergen 3...	30.8	0.65
gi|970648792|gb|KUF88238.1| hypothetical protein AM588_100017...	30.8	0.68
gi|924557510|gb|ALC42783.1| CG17974 (Drosophila busckii)	30.8	0.78
gi|195025732|ref|XP_001986115.1| GH21184 (Drosophila grimshawi)	30.4	0.91
gi|567995603|gb|ETL45735.1| hypothetical protein L916_04236 (...	30.4	0.92
gi|567995605|gb|ETL45737.1| hypothetical protein L916_04235 (...	30.0	0.99

ALIGNMENTS

>gi|1036768828|ref|XP_017087060.1| PREDICTED: venom allergen 5 (Drosophila bipectinata)
Length=259

Score = 33.5 bits (75), Expect = 0.10, Method: Compositional matrix adjust.
Identities = 27/88 (31%), Positives = 38/88 (43%), Gaps = 18/88 (20%)

```
Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER---  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  74   RNFLALGKVPG-------YYPATRMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYRYAN  126

Query  153  ------ARTRMEAGLVDQDDLDEEHQGL  174
                  A  R  +  V+   L EE  GL
Sbjct  127  SGQNLCAVWRPRSPYVNVTSLVEECVGL  154
```

>gi|1036768520|ref|XP_017040791.1| PREDICTED: venom allergen 5 (Drosophila ficusphila)
Length=262

Score = 32.7 bits (73), Expect = 0.17, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

```
Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  126
```

Table D-10, continued. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton.

```
>gi|1037043392|ref|XP_017125940.1| PREDICTED: venom allergen 5 (Drosophila elegans)
Length=264

Score = 32.3 bits (72),  Expect = 0.19, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  79   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  128


>gi|1037063026|ref|XP_016990735.1| PREDICTED: venom allergen 5 (Drosophila rhopaloa)
Length=262

Score = 32.3 bits (72),  Expect = 0.21, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  126


>gi|1036915151|ref|XP_017072698.1| PREDICTED: venom allergen 5 (Drosophila eugracilis)
Length=258

Score = 32.3 bits (72),  Expect = 0.21, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  73   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  122


>gi|194754086|ref|XP_001959328.1| uncharacterized protein Dana_GF12099 (Drosophila ananassae)
gi|190620626|gb|EDV36150.1| uncharacterized protein Dana_GF12099 (Drosophila ananassae)
Length=263

Score = 32.3 bits (72),  Expect = 0.21, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  78   RNFLALGKVPG-------YYPATRMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  127


>gi|1036794411|ref|XP_016953055.1| PREDICTED: venom allergen 5 (Drosophila biarmipes)
Length=262

Score = 32.3 bits (72),  Expect = 0.21, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  126


>gi|1036050781|ref|XP_016928162.1| PREDICTED: venom allergen 5 (Drosophila suzukii)
Length=262

Score = 32.3 bits (72),  Expect = 0.22, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  126
```

Table D-10, continued. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton.

```
>gi|195346421|ref|XP_002039756.1| GM15725 (Drosophila sechellia)
 gi|194135105|gb|EDW56621.1| GM15725 (Drosophila sechellia)
Length=258

Score = 32.3 bits (72),  Expect = 0.23, Method: Compositional matrix adjust.
Identities = 36/126 (29%), Positives = 50/126 (40%), Gaps = 28/126 (22%)

Query  44   AVFRLSAQGRPVLFVKTDLSGALNELQDEAARLSWLATTGV------PCAAVLDVVTEAG  97
            AVF+L+ Q    L +  D S    +L     R     TTG       P A  +DV
Sbjct  8    AVFQLTFQ----LILAKDYSWCDPDLCGNGVRHIACRTTGNFHRRCQPDAVQVDVSRHKA  63

Query  98   ---------RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQA  146
                     R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH
Sbjct  64   DFLHAHNKRRNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNTRTCKLDHDD  116

Query  147  KHRIER  152
             H   R
Sbjct  117  CHNTYR  122


>gi|567967163|gb|ETK92347.1| hypothetical protein L915_04276 (Phytophthora parasitica)
Length=160

Score = 31.6 bits (70),  Expect = 0.25, Method: Compositional matrix adjust.
Identities = 15/50 (30%), Positives = 25/50 (50%), Gaps = 0/50 (0%)

Query  51  QGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDW  100
           QG P L +   L  A     D+ A+ +++A  G   + +   +TEAG +W
Sbjct  43  QGVPALCMNKKLQAAAQRHSDDMAKNNYMAHDGADGSTMSQRITEAGYEW  92


>gi|1036830438|ref|XP_017022249.1| PREDICTED: venom allergen 5 (Drosophila kikkawai)
Length=262

Score = 32.0 bits (71),  Expect = 0.26, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNTRTCKLDHDDCHNTYR  126


>gi|195585418|ref|XP_002082478.1| uncharacterized protein Dsimw501_GD25202 (Drosophila simulans)
gi|194194487|gb|EDX08063.1| GD25202 (Drosophila simulans)
gi|900893985|gb|KMY95530.1| uncharacterized protein Dsimw501_GD25202 (Drosophila simulans)
Length=258

Score = 32.0 bits (71),  Expect = 0.26, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  73   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNTRTCKLDHDDCHNTYR  122


>gi|970633761|gb|KUF79289.1| hypothetical protein AM587_10011430 (Phytophthora nicotianae)
Length=236

Score = 32.0 bits (71),  Expect = 0.27, Method: Compositional matrix adjust.
Identities = 36/151 (24%), Positives = 61/151 (40%), Gaps = 18/151 (12%)

Query  49   SAQGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGE--V  106
            +A G P L     L  A     D+ A   ++  TG    +V + +T +G DW  + E
Sbjct  52   AAYGLPALCTNKKLQAAAQGHSDDQAANDYMDHTGTDGTSVSERITRSGYDWSAVAENVA  111

Query  107  PGQDLLSSHL-----APAEKVSIMADAMRRLHTLDPATCPFDHQAKHRIERARTRMEAGL  161
             GQ  + S +     +P  + +I+ D     +T+    C + H A    +   T+ + G
Sbjct  112  AGQPDVDSVMENWMNSPGHRENILGD-----YTM--FGCAYAHNAGTTYQHYWTQ-DFGT  163
```

Table D-10, continued. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton.

```
Query  162  VDQDDLDEEHQGLA---PAELFARLKARMPD  189
            D ++ D E   +    P E F    A  P+
Sbjct  164  GDAEECDGEETPIVIVDPPEAFTDPVAEQPE  194
```

>gi|24656989|ref|NP_611582.1| uncharacterized protein Dmel_CG17974 (Drosophila melanogaster)
gi|21645218|gb|AAF46718.2| uncharacterized protein Dmel_CG17974 (Drosophila melanogaster)
gi|223718734|gb|ACN22204.1| MIP05446p (Drosophila melanogaster)
Length=259

```
Score = 32.0 bits (71),  Expect = 0.27, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  74   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNTRTCKLDHDDCHNTYR  123
```

>gi|1036987849|ref|XP_017007825.1| PREDICTED: venom allergen 5 (Drosophila takahashii)
Length=262

```
Score = 31.6 bits (70),  Expect = 0.35, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 27/57 (47%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGRVPG-------YYPAARMATMVWDDELQYLSMLNSRTCKLDHDDCHNTYR  126
```

>gi|195486506|ref|XP_002091541.1| uncharacterized protein Dyak_GE13718 (Drosophila yakuba)
gi|194177642|gb|EDW91253.1| uncharacterized protein Dyak_GE13718 (Drosophila yakuba)
Length=262

```
Score = 31.6 bits (70),  Expect = 0.35, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 28/57 (49%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG+VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  77   RNFLALGKVPG-------YYPAARMATMVWDDELQYLSMLNTRTCKLDHDDCHNTFR  126
```

>gi|970633753|gb|KUF79281.1| SCP extracellular protein (Phytophthora nicotianae)
Length=239

```
Score = 31.2 bits (69),  Expect = 0.51, Method: Compositional matrix adjust.
Identities = 15/50 (30%), Positives = 25/50 (50%), Gaps = 0/50 (0%)

Query  51  QGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDW  100
           QG P L +   L  A     D+ A+ +++A  G   + +   +TEAG +W
Sbjct  43  QGVPALCMNKKLQAAAQRHSDDMAKNNYMAHDGADGSTMSQRITEAGYEW  92
```

>gi|194881898|ref|XP_001975050.1| uncharacterized protein Dere_GG20780 (Drosophila erecta)
gi|190658237|gb|EDV55450.1| uncharacterized protein Dere_GG20780 (Drosophila erecta)
Length=261

```
Score = 31.2 bits (69),  Expect = 0.57, Method: Compositional matrix adjust.
Identities = 19/57 (33%), Positives = 27/57 (47%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R++L LG VPG         PA +++ M   D ++ L  L+  TC  DH   H   R
Sbjct  76   RNFLALGRVPG-------YYPAARMATMVWDDELQYLSMLNTRTCKLDHDDCHNTFR  125
```

>gi|968087096|ref|XP_002061399.2| uncharacterized protein Dwil_GK20746 (Drosophila willistoni)
gi|946580156|gb|EDW72385.2| uncharacterized protein Dwil_GK20746 (Drosophila willistoni)
Length=264

Table D-10, continued. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton.

```
Score = 30.8 bits (68),  Expect = 0.65, Method: Compositional matrix adjust.
Identities = 20/60 (33%), Positives = 28/60 (47%), Gaps = 9/60 (15%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIERART  155
            R++L LG+VPG         PA +++ M   D +  L  L+  TC  DH   H   R  T
Sbjct  79   RNFLALGKVPG-------YYPAARMATMVWDDELEYLSRLNTRTCVLDHDDCHNTYRFAT  131


>gi|1060207003|ref|XP_017836482.1| PREDICTED: venom allergen 3 (Drosophila busckii)
Length=260

Score = 30.8 bits (68),  Expect = 0.65, Method: Compositional matrix adjust.
Identities = 18/57 (32%), Positives = 30/57 (53%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R+++ LG++PG         PA +++ M   D ++ L +L+  TC  DH A H   R
Sbjct  75   RNFVALGKLPG-------YYPAARMTTMMWDDELQYLASLNVRTCKLDHDACHNSYR  124


>gi|970648792|gb|KUF88238.1| hypothetical protein AM588_10001748 (Phytophthora nicotianae)
 Length=238

Score = 30.8 bits (68),  Expect = 0.68, Method: Compositional matrix adjust.
Identities = 15/50 (30%), Positives = 25/50 (50%), Gaps = 0/50 (0%)

Query  51  QGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDW  100
           QG P L +   L  A     D+ A+ +++A  G   + +   +TEAG +W
Sbjct  43  QGVPALCMNKKLQAAAQRHSDDMAKNNYMAHDGADGSTMSQRITEAGYEW  92


>gi|924557510|gb|ALC42783.1| CG17974 (Drosophila busckii)
Length=283

Score = 30.8 bits (68),  Expect = 0.78, Method: Compositional matrix adjust.
Identities = 18/57 (32%), Positives = 30/57 (53%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R+++ LG++PG         PA +++ M   D ++ L +L+  TC  DH A H   R
Sbjct  98   RNFVALGKLPG-------YYPAARMTTMMWDDELQYLASLNVRTCKLDHDACHNSYR  147


>gi|195025732|ref|XP_001986115.1| GH21184 (Drosophila grimshawi)
 gi|193902115|gb|EDW00982.1| GH21184 (Drosophila grimshawi)
 Length=264

Score = 30.4 bits (67),  Expect = 0.91, Method: Compositional matrix adjust.
Identities = 17/57 (30%), Positives = 29/57 (51%), Gaps = 9/57 (16%)

Query  98   RDWLLLGEVPGQDLLSSHLAPAEKVSIMA--DAMRRLHTLDPATCPFDHQAKHRIER  152
            R+++ LG++PG         PA +++ M   D ++ L +L+  TC  DH   H   R
Sbjct  79   RNFIALGKLPG-------YYPAARMTTMVWDDELQYLSSLNVRTCILDHDDCHNTYR  128


>gi|567995603|gb|ETL45735.1| hypothetical protein L916_04236, partial (Phytophthora parasitica)
Length=358

Score = 30.4 bits (67),  Expect = 0.92, Method: Compositional matrix adjust.
Identities = 17/57 (30%), Positives = 26/57 (46%), Gaps = 0/57 (0%)

Query  49   SAQGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGE  105
            +A G PVL     L  A     D+ A   ++  TG    +V + +T +G DW  + E
Sbjct  99   AAYGLPVLCTNKKLQAAAQGHSDDQAANDYMDHTGTDGTSVSERITRSGYDWSAVAE  155


>gi|567995605|gb|ETL45737.1| hypothetical protein L916_04235, partial (Phytophthora parasitica)
gi|570957313|gb|ETP22292.1| hypothetical protein F441_04368, partial (Phytophthora parasitica
CJ01A1)
```

Table D-10, continued. Protein allergen BLASTP search results of six putative translated ORFs in TAM66274 cotton.

```
Length=232

Score = 30.0 bits (66),  Expect = 0.99, Method: Compositional matrix adjust.
Identities = 15/50 (30%), Positives = 25/50 (50%), Gaps = 0/50 (0%)

Query  51  QGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDW  100
           QG P L +   L  A     D+ A+ +++A  G   + +   +TEAG +W
Sbjct  43  QGVPALCMNKKLQAAAQRHSDDMAKNNYMAHDGADGSTMSQRITEAGYEW  92
```

4. TAM66274-14F (128 aa)

MNCRTRQRGY RGWPRRAFLA QLCSTLSLKR EGTGCYWAKC RGRISCHLTL LLPRKYPSWL MQCGGCIRLI RLPAHSTTKR NIASSEHVLG WKPVLSIRMI WTKSIRGSRQ PNCSPGSRRA CPTARISS

No significant similarity found.

5. TAM66274-3R (178 aa)

MAGWASLGRS FRTPESRSEE LVKKAIEGDA LRIGSGDTVK HEEAVSPFAA KLFSNITGSQ RYVLIAVRHT QPATVDESRK AAIFHHDIRQ AGIAMGHDEI LAVGHARLEP GEQFGWREPL MLFVQIILID KTGFHPSTCS LDAMFRLVVE WAGSRIKRMQ PPHCISHDGY FLGRSKVR

Sequences producing significant alignments:

```
ALIGNMENTS
>KPM05668.1 Sar s 27 allergen (serpin-like protein 4) (Sarcoptes scabiei)
Length=419

Score = 30.4 bits (67),  Expect = 0.58, Method: Compositional matrix adjust.
Identities = 17/71 (24%), Positives = 30/71 (42%), Gaps = 2/71 (3%)

Query  55   NITGSQRYVLIAVRHTQPATVDESRKAAIFHHDIRQAGIAMGHDEILAVGHARLEPGEQF  114
            N+   ++  L  +   +   VD+ R AAI   DI + G        +      L+P   F
Sbjct  335  NVFDRKKADLSGINDQEQVIVDDIRHAAIM--DINEEGTEAAASTYVGFVKMSLQPSTVF  392

Query  115  GWREPLMLFVQ  125
             +  P +LF++
Sbjct  393  NFNRPFILFIR  403
```

6. TAM66274-9R (89 aa)

MVQLLHRKEL SEISRWWKDL DFQRKLPYAR DRVVEGYFWI SGVYFEPQYS LGRKMLTKVI AMASIVEDPN SVPQLGKEII IFFFPFSIK

No significant similarity found.

Table D-11. Protein toxin BLASTP search results of 33 putative translated ORFs in TAM66274 cotton. A BLASTP search of the Entrez protein database of 33 putative translated ORFs in TAM66274 cottonseed was conducted on October 22, 2016. The search was restricted to database sequences identified as a "toxin."

1. TAM66274-1F (101 aa)

MTMITPSYLG DTIEYSSYAS NALGALPYRP AGGRTSKLAC MPAGPAEPRH VVAKFALDPP NDLSSLSRFD LHFIWGPHTP KKCCIILGAA SRLPGRRAGP G

```
Sequences producing significant alignments:                      (Bits)  Value
AAS77687.2  LacZ-alpha (Shuttle vector pLPV111)                   55.1    8e-10

ALIGNMENTS
>AAS77687.2 LacZ-alpha (Shuttle vector pLPV111)
Length=121

 Score = 55.1 bits (131),  Expect = 8e-10, Method: Compositional matrix adjust.
 Identities = 26/26 (100%), Positives = 26/26 (100%), Gaps = 0/26 (0%)

Query  1   MTMITPSYLGDTIEYSSYASNALGAL  26
           MTMITPSYLGDTIEYSSYASNALGAL
Sbjct  1   MTMITPSYLGDTIEYSSYASNALGAL  26
```

2. TAM66274-2F (35aa)

MLSQNSPWTR PTICRHCQGL TCTSFGAHIH QKNAA

No significant similarity found.

3. TAM66274-3F (35aa)

MVPVTFGRAD GQYSTSRNLT HARRRGTGVP FSERY

No significant similarity found.

4. TAM66274-4F (41 aa)

MPRTTSTPHP FDSDYSESMD SMFHATYSTS LKTSKGISSH P

No significant similarity found.

5. TAM66274-5F (80 aa)

MVQLLHRKEL SEISRWWKDL DFQRKLPYAR DRVVEGYFWI SGVYFEPQYS LGRKMLTKVI AMASIVEDPS LSISNPASQL

```
Sequences producing significant alignments:                      (Bits)  Value
BAM29049.1  geraniol synthase (Citrus jambhiri)                   61.2    2e-11

ALIGNMENTS
>BAM29049.1 geraniol synthase (Citrus jambhiri)
Length=612

Score = 61.2 bits (147),  Expect = 2e-11, Method: Compositional matrix adjust.
Identities = 24/67 (36%), Positives = 45/67 (67%), Gaps = 0/67 (0%)

Query  2    VQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWISGVYFEPQYSLGRKMLTKVIA  61
            +Q ++++EL +IS WWK+     KL +ARD +V  + W  G+  EPQ++  R+++T  IA
Sbjct  277  LQAIYQEELKDISGWWKETGLGEKLSFARDSLVASFLWSMGIGSEPQFAYCRRIVTIAIA  336

Query  62   MASIVED  68
            + ++++D
Sbjct  337  LITVIDD  343
```

Table D-11, continued. Protein toxin BLASTP search results of 33 putative translated ORFs in TAM66274 cotton.

6. TAM66274-6F (67aa)

```
MLVYHLTCSI KFIKNNILTL NFYLLRLTHH LSYFFTLLCC LCKQYIYKLF FHNYNNYIII IILINIT
No significant similarity found.
```

7. TAM66274-7F (36aa)

```
MVGGERNCFI QYIFPMNPQI GSLVKFQQSS NIACHG
No significant similarity found.
```

8. TAM66274-8F (30aa)

```
MDICRIRLGT RIDYDKLCIL LLCDGNSKEW
No significant similarity found.
```

9. TAM66274-9F (65aa)

```
MQIFINVLKT LTCKMTSVQI KEDEILSLFF FFCIVVGRYR DATSLHMVLA RFGQTLLQGG SWLHL
No significant similarity found.
```

10. TAM66274-10F (33aa)

```
MSRFTELRVE LIEDKSSVHM LLCMVMIYEL QEI
No significant similarity found.
```

11. TAM66274-11F (45aa)

```
MEIVSVNGFL EFNELSTYVR NHYCAFKSRL RSLSASKYFL SKMLH
No significant similarity found.
```

12. TAM66274-12F (273 aa, NPTII variant)

```
MAITLSATSL PISARIRAGS PAAWVERLFG YDWAQQTIGC SDAAVFRLSA QGRPVLFVKT DLSGALNELQ
DEAARLSWLA TTGVPCAAVL DVVTEAGRDW LLLGEVPGQD LLSSHLAPAE KVSIMADAMR RLHTLDPATC
PFDHQAKHRI ERARTRMEAG LVDQDDLDEE HQGLAPAELF ARLKARMPDG EDLVVTHGDA CLPNIMVENG
RFSGFIDCGR LGVADRYQDI ALATRDIAEE LGGEWADRFL VLYGIAAPDS QRIAFYRLLD EFF
```

Sequences producing significant alignments:		(Bits)	Value
BAL46488.1	neomycin-kanamycin phosphotransferase (Ti-curing v...	521	0.0
BAS53447.1	neomycin resistance protein (Gene-trapping transpo...	520	0.0
AAR17784.1	neomycin phosphotransferase II (Cloning vector pZG...	520	0.0
BAG12832.1	beta-geo-lessCpG (Exchangeable gene trap vector pU...	522	4e-176
EZQ43232.1	aminoglycoside phosphotransferase (Escherichia col...	159	2e-46
ALL88228.1	aminoglycoside phosphotransferase (Escherichia coli)	159	2e-46
KLG86115.1	aminoglycoside phosphotransferase (Escherichia coli)	159	3e-46
EZE14596.1	aminoglycoside phosphotransferase (Escherichia col...	159	3e-46
EIL24887.1	aminoglycoside 3'-phosphotransferase (Escherichia ...	159	4e-46
YP_004172623.1	Aph-3 (Enterococcus faecium)	117	2e-30
AGR48366.1	aminoglycoside 3'-phosphotransferase (Escherichia ...	117	2e-30
YP_003937710.1	streptomycin 3''-kinase (Escherichia coli)	105	7e-26
KJW21141.1	aminoglycoside phosphotransferase (Escherichia coli)	105	8e-26
EJE84154.1	hypothetical protein ECO9455_10069 (Escherichia co...	89.7	1e-20
EIL23135.1	hypothetical protein ECO9545_07778 (Escherichia co...	90.1	1e-20
EZA13907.1	hypothetical protein BW75_15400 (Escherichia coli ...	53.1	3e-08

13. TAM66274-13F (35 aa)

```
MTGHNRQSAA LMPPCSGCQR RGARFFLSRP TCPVP
No significant similarity found.
```

Table D-11, continued. Protein toxin BLASTP search results of 33 putative translated ORFs in TAM66274 cotton.

14. TAM66274-14F (128 aa)

MNCRTRQRGY RGWPRRAFLA QLCSTLSLKR EGTGCYWAKC RGRISCHLTL LLPRKYPSWL MQCGGCIRLI
RLPAHSTTKR NIASSEHVLG WKPVLSIRMI WTKSIRGSRQ PNCSPGSRRA CPTARISS

No significant similarity found.

15. TAM66274-15F (33 aa)

MAMPACRISW WKMAAFLDSS TVAGWVWRTA IRT
No significant similarity found.

16. TAM66274-16F (63 aa)

MTDQATPNLP SRDFDSTAAF YERLGFGIVF RDAGWMILQR GDLMLEFFAH PDPTLTFATS KSK
No significant similarity found.

17. TAM66274-17F (58 aa)

MHALTTWNIA IFLKNYARWR MSRQLQLLPK SKYPSRMHSS ILFMRGKARL IQLANHPA
No significant similarity found.

18. TAM66274-18F (78 aa)

MEHRYFSEEL CSLEDVAAIA AIAKIEIPLT HAFINIIHAG KGKINPTGKS SSVIGNFSSS DLIRFGATHV
FNKDEMVE
No significant similarity found.

19. TAM66274-19F (34 aa)

MLVGGCRGNC SYCQNRNTPH ACIHQYYSCG ERQD
No significant similarity found.

20. TAM66274-1R (38 aa)

MIIIARPATG FNLKKLYCQM FERSASTHSF FTPPSRPY
No significant similarity found.

21. TAM66274-2R (33 aa)

MHDARYEVTV LGSIPSKFHS QYHIHHCIPA REN
No significant similarity found.

22. TAM66274-3R (178 aa)

MAGWASLGRS FRTPESRSEE LVKKAIEGDA LRIGSGDTVK HEEAVSPFAA KLFSNITGSQ RYVLIAVRHT
QPATVDESRK AAIFHHDIRQ AGIAMGHDEI LAVGHARLEP GEQFGWREPL MLFVQIILID KTGFHPSTCS
LDAMFRLVVE WAGSRIKRMQ PPHCISHDGY FLGRSKVR
No significant similarity found.

23. TAM66274-4R (44 aa)

MNPEKRPFST MIFGKQASPW VTTRSSPSGM RALSLANSSA GASP
No significant similarity found.

24. TAM66274-5R (35 aa)

MRCFAWWSNG QVAGSSVCSR RIASAMMDTF SAGAR
No significant similarity found.

Table D-11, continued. Protein toxin BLASTP search results of 33 putative translated ORFs in TAM66274 cotton.

25. TAM66274-6R (46 aa)

MSIKRTGYQK YTYLNLFISP ATHRSSPCTA TCVHLTCPLS TQPLTQ
No significant similarity found.

26. TAM66274-7R (41 aa)

MNPPATKFVR TLLVPCEGMW HLDIYPPLYK KKKKETIFRL L
No significant similarity found.

27. TAM66274-8R (41 aa)

MPRTTSTPHP FDSDYSESMD SMFHATYSTS LKTSKGISSH P
No significant similarity found.

28. TAM66274-9R (89 aa)

MVQLLHRKEL SEISRWWKDL DFQRKLPYAR DRVVEGYFWI SGVYFEPQYS LGRKMLTKVI AMASIVEDPN
SVPQLGKEII IFFFPFSIK

```
Sequences producing significant alignments:                      (Bits)  Value
BAM29049.1  geraniol synthase (Citrus jambhiri)                   60.8    3e-11

ALIGNMENTS
>BAM29049.1 geraniol synthase (Citrus jambhiri)
Length=612

Score = 60.8 bits (146),  Expect = 3e-11, Method: Compositional matrix adjust.
Identities = 27/81 (33%), Positives = 50/81 (62%), Gaps = 0/81 (0%)

Query  2    VQLLHRKELSEISRWWKDLDFQRKLPYARDRVVEGYFWISGVYFEPQYSLGRKMLTKVIA  61
            +Q ++++EL +IS WWK+     KL +ARD +V  + W  G+  EPQ++  R+++T  IA
Sbjct  277  LQAIYQEELKDISGWWKETGLGEKLSFARDSLVASFLWSMGIGSEPQFAYCRRIVTIAIA  336

Query  62   MASIVEDPNSVPQLGKEIIIF  82
            + ++++D   V     E+ +F
Sbjct  337  LITVIDDIYDVYGTLDELELF  357
```

29. TAM66274-10R (36 aa)

MVGGERNCFI QYIFPMNPQI GSLVKFQQSS NIACHG
No significant similarity found.

30. TAM66274-11R (48 aa)

MRDAYDRMIF AFNSVVHVVK NLSMCSSDPY RRFRFILMNI SPVTIVFL
No significant similarity found.

31. TAM66274-12R (33 aa)

MIERHNNKQL RFIITNPILK KAAEPVKPKR LIT
No significant similarity found.

32. TAM66274-13R (67 aa)

MQHFFGVCGP QMKCRSNLDS DDKSLGGSRA NFATTCRGSA GPAGMQASLL VRPPAGRYGR APNALDA
No significant similarity found.

33. TAM66274-14R (42 aa)

MSELTHINCV ALTARFPVGK PVVPAALMNR PTRGERRFAY WG
No significant similarity found.

Appendix E.

Methods and Results of Cottonseed Composition Analyses

Texas A&M University (TAMU) developed cotton event TAM66274 that exhibits ultra-low levels of the antinutrient gossypol in the cottonseed by introducing plasmid pART27-LCT66 into non-transgenic cotton variety Coker 312 by *Agrobacterium tumefaciens*-mediated transformation. An important component of the safety and product assessment of TAM66274 is the comparison of seed gossypol levels to safety and legal standards established for the intended food and feed uses. Provided that seed gossypol levels do not exceed the legal standards for their intended uses, TAM66274 may be legally used in food and feed products. Another important component of the safety and product assessment of TAM66274 is the comparison of the nutrient and antinutrient levels in the cottonseed both to the parental variety, non-transgenic cv. Coker 312, and to published values for other commercial cotton varieties with a history of safe use in food and feed products. Compositional equivalence confirms the appropriateness of this cotton event for use in food and feed products.

Therefore, the purpose of these studies was to analyze and compare the gossypol composition of cottonseed of TAM66274 to cottonseed gossypol levels established for safety and legal standards for intended food and feed uses. Also, to compare the nutrient and antinutrient levels in cottonseed of TAM66274 to levels in cottonseed of non-transgenic cv. Coker 312 and to published values for other commercial cotton varieties. Cottonseed for these compositional analyses were produced in field trials in multi-locations in the U.S. in 2014 and 2015. Further, cottonseed of TAM66274 and non-transgenic cv. Coker 312 harvested from 2015 field trials were analyzed for mycotoxins. Details of the production and subsequent processing of the cottonseed used in these analyses are presented below, as well as the methods used for analyses and the results for each treatment and field site of production.

Cottonseed Source.

The cottonseed of TAM66274 and non-transgenic cv. Coker 312 was produced from plants grown in replicated field trials at three locations in the U.S. during the summer of 2014, and five U.S. locations in 2015. The locations in 2014 were one site in Washington County, Mississippi, and two independent sites in Perquimans County, North Carolina. In 2015, the locations were two independent sites in Perquimans County, North Carolina, two independent sites in Washington County, Mississippi, and one site in Tom Green County, Texas. Field sites were selected as representative of major cotton-growing regions in the United States. Characterization of the seed of TAM66274 and non-transgenic cv. Coker 312 used for planting was based on the documentation of the seed pedigree from the study director and by gel-based, event-specific PCR. The plants were grown under standard agronomic practices in a complete randomized block design with four replicate blocks per location. Details of the field trials and agronomic practices for plant growth and production of cottonseed of each treatment are described in Section 7 of the submission and in Appendix F.

Sample Collection, Handling, Identification, Preparation and Storage.
Seed cotton samples were harvested from replicated plots at each field site, ginned by Cotton Incorporated (Cary, NC), acid delinted by TAMU laboratory personnel, and shipped to Covance Laboratories (Madison, WI) for nutrient and antinutrient analyses under Good Laboratory Practices (GLP). In addition, samples harvested from 2015 field trials were shipped to Romer Laboratories (Union, MO) for mycotoxin analyses. Also, some seed cotton was picked separately (25-boll samples) and used for total gossypol analysis at TAMU. There were some differences in sampling for the different analyses (by Covance Laboratories, TAMU and Romer Laboratories) and these are shown below.

For cottonseed nutrient and antinutrient analyses conducted by Covance Laboratories, the seed cotton produced in both 2014 and 2015 field trials was hand harvested from the two middle rows of each replicate plot of each treatment at plant maturity (approximately 10 lb of seed cotton per replicate plot). Seed cotton samples from each replicate plot were individually labeled and packed, and shipped to Cotton Incorporated for ginning. Seed cotton samples were ginned with a saw-gin to separate lint and fuzzy seed. Fuzzy seed samples were individually labeled and packed, and shipped to TAMU for further processing. For samples collected from 2014 field trials, 40 g of cottonseed from each replicate sample of fuzzy seed was pooled by treatment and by location before acid delinting. Therefore, a total of 160 g of cottonseed per treatment per location was used for acid delinting. The pooled samples were thoroughly mixed during acid delinting. After acid delinting, 100 g of cottonseed was subsampled from each 160 g pooled sample. Test and control substances were individually labeled and packed, and shipped to Covance Laboratories for the composition analysis phase of this study. For samples collected from 2015 field trials, approximately 250 g of fuzzy seed from each replicate sample was acid delinted separately. After acid delinting, 28 g of cottonseed was subsampled from each replicate sample, and the subsamples were thoroughly mixed by treatment and by field location. Test and control pooled cottonseed samples (approximately 110 g) were individually labeled and packed, and shipped to Covance Laboratories for the composition analysis phase of this study. For samples collected in both 2014 and 2015 studies, identity of each sample was confirmed by TAMU by chain of custody records and by PCR analysis (Appendix C). Cotton samples were ginned, stored, and shipped at ambient temperatures.

The sampling and processing of samples for total gossypol analysis by TAMU was as follows. From the same middle two rows of each replicate plot from which the 10 lb of seed cotton were harvested (described above), 25-boll samples were collected from each replicate plot of each treatment at each location. Each 25-boll sample was processed separately and were not pooled by treatment at each location. Seeds were separated from the lint on a roller gin from each 25-boll sample. Twenty seeds were subsampled from each replicate, roller-ginned 25-boll sample. The seed coat was removed manually using a razor blade. Kernels from each subsample were ground to a fine powder separately using an agate mortar and pestle in the dark at room temperature.

Each powdered seed sample was processed and analyzed by HPLC within a day of grinding, or stored at -80°C until analysis. Identity of each sample was confirmed by TAMU by chain of custody records and by PCR analysis (Appendix C).

For cottonseed mycotoxin analyses conducted by Romer Laboratories, the seed cotton was harvested from 2015 field trials, shipped to Cotton Incorporated for ginning, and the fuzzy seed shipped to Texas A&M, as described above for samples analyzed by Covance Laboratories. At TAMU, the fuzzy seed for mycotoxin analyses were processed as follows. A sample of approximately 150 g of cottonseed from each replicate of fuzzy seed were pooled by treatment and by location before acid delinting. Approximately, 600 g of cottonseed per treatment per location was acid delinted. The pooled samples were thoroughly mixed during acid delinting. After acid delinting, seed samples, each weighing 500 g, of TAM66274 and non-transgenic cv. Coker 312 were individually labeled and packed, and shipped to Romer Laboratories for mycotoxin analysis. The identity of each sample was confirmed by TAMU by chain of custody records and by PCR analysis (Appendix C). Cotton samples were ginned, stored, and shipped at ambient temperatures.

Analytical Methods.
The nutrient and antinutrient composition of test and control substances was analyzed by Covance Laboratories, Inc. In addition to the compositional analyses conducted by Covance Laboratories, Inc., total gossypol levels in the cottonseed from 25-boll samples were also analyzed by TAMU. All analytical details are described below.

Upon receipt of the samples at Covance Laboratories, Inc., the test and control samples were stored in a freezer set to maintain -20 ± 10ºC except during sample preparation and analysis. The cottonseed samples were prepared with liquid nitrogen and ground into a homogenized powder using a Waring blender. Equipment was cleaned in between each use with a liquid nitrogen rinse. Results were recorded on a fresh weight (FW) basis and adjusted for moisture content and recorded on a dry weight (DW) basis. Statistical analysis was conducted using the dry weight data.

Moisture. The samples were dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture (AOAC, 2012a and b). The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100% on a fresh weight basis.

Protein. The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The

percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOCS, 2011a). The results are reported on a dry weight basis. The limit of quantitation was calculated as 0.100% on a fresh weight basis.

Fat by Soxhlet Extraction. The samples were weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the samples to remove the fat. The extract was then evaporated, dried, and weighed (AOAC, 2012c and d). The limit of quantitation was calculated as 0.100% on a fresh weight basis.

Ash. All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash (AOAC, 2012e). The limit of quantitation was calculated as 0.100% on a fresh weight basis.

Carbohydrate. The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

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

The limit of quantitation was calculated as 0.100% on a fresh weight basis (USDA, 1973).

Calories. Calories were calculated using the Atwater factors with the fresh weight-derived data and the following equation:

$$\text{Calories (Kcal/100g)} = (4 \times \%\ \text{protein}) + (9 \times \%\ \text{fat}) + (4 \times \%\ \text{carbohydrates})$$

The limit of quantitation was calculated as 2.00 Kcalories/100 g on a fresh weight basis (USDA, 1975).

Crude Fiber. Crude fiber was quantitated as the loss on ignition of dried residue remaining after digestion of the sample with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions under specific conditions (AOAC, 2012f). The results were reported on a dry weight basis. The limit of quantitation was 0.100%.

Total Dietary Fiber. Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each of the samples to precipitate the soluble fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the samples was calculated using protein and ash values and the weighed residue

fractions (AOAC, 2012g). The results are reported on a dry weight basis. The limit of quantitation was calculated as 1.00% on a fresh weight basis.

Acid Detergent Fiber. Sample aliquots were weighed into pre-weighed ANKOM filter bags (ANKOM, 2013a). Samples were placed in an ANKOM Fiber analyzer and treated with an acid detergent solution containing sulfuric acid with cetyl trimethylammonium bromide then filtered to remove proteins, starches, simple sugars, pectins, and ash. Fats and pigments were removed via an acetone wash leaving cellulose and lignin fractions. Due to the high fat content of the samples, an additional 12-hour acetone soak was conducted with no agitation. The remaining residue was the acid detergent fiber and was determined gravimetrically. The results are reported on a dry weight basis. The limit of quantitation was calculated as 1.00% on a fresh weight basis.

Neutral Detergent Fiber. Sample aliquots were weighed into pre-weighed filter bags. Samples were placed in an ANKOM Fiber analyzer (ANKOM, 2013b) and treated with a neutral detergent solution containing EDTA then filtered to remove proteins, simple sugars, pectins, and ash. Fats and pigments were removed via an acetone wash leaving hemicellulose, cellulose, and lignin fractions. Due to the high fat content of the samples, an additional 12-hour acetone soak was conducted with no agitation. Starches were removed with a heat stable alpha soak. The remaining residue was the neutral detergent fiber and was determined gravimetrically. The results are reported on a dry weight basis. The limit of quantitation was calculated as 1.00% on a fresh weight basis.

Amino Acid Composition. Levels of the following amino acids were determined in test and control samples:

- Total alanine
- Total arginine
- Total aspartic acid (including asparagine)
- Total cystine (including cysteine)
- Total glutamic acid (including glutamine)
- Total glycine
- Total histidine
- Total isoleucine
- Total leucine
- Total lysine
- Total methionine
- Total phenylalanine
- Total proline
- Total serine
- Total threonine
- Total tryptophan
- Total tyrosine
- Total valine

The samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-118ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine were converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours. The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-

phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection (AOAC, 2012h; Barkholt and Jensen, 1989; Henderson and Brooks, 2010; Henderson, et al., 2000; Schuster, 1988). The results are reported on a dry weight basis. The limit of quantitation was calculated as 0.1 mg/g on a fresh weight basis.

The reference standards used for the amino acid analyses of samples collected from 2014 field studies were as follows:

Reference Standards:

Analyte	**Manufacturer**	**Lot No.**	**Purity** (%)
L-Alanine	Sigma-Aldrich	060M1776V	>99
L-Arginine Monohydrochloride	Sigma-Aldrich	SLBF3348V	100
L-Aspartic Acid	Sigma-Aldrich	091M0201V	100
L-Cystine	Sigma-Aldrich	SLBB9524V	100
L-Glutamic Acid	Sigma-Aldrich	060M01711V	100
Glycine	Sigma-Aldrich	059K0040V	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	110M00481V	100
L-Isoleucine	Sigma-Aldrich	090M00842V	100
L-Leucine	Sigma-Aldrich	110M00492V	100
L-Lysine Monohydrochloride	Sigma-Aldrich	051M0016V	100
L-Methionine	Sigma-Aldrich	SLBF3077V	100
L-Phenylalanine	Sigma-Aldrich	SLBF2036V	100
L-Proline	Sigma-Aldrich	SLBF1872V	100
L-Serine	Sigma-Aldrich	098K0161V	99
L-Threonine	Sigma-Aldrich	081M01921V	99
L-Tryptophan	Sigma-Aldrich	SLBK2108V	100
L-Tyrosine	Sigma-Aldrich	BCBG4812V	100
L-Valine	Sigma-Aldrich	SLBF7406V	100

The reference standards used for the amino acid analyses of samples collected from 2015 field studies were as follows:

Reference Standards:

Analyte	**Manufacturer**	**Lot No.**	**Purity** (%)
L-Alanine	Sigma-Aldrich	051M1830V	>99
L-Arginine Monohydrochloride	Sigma-Aldrich	SLBH7875V	100
L-Aspartic Acid	Sigma-Aldrich	SLBB8906V	100
L-Cystine	Sigma-Aldrich	WXBB4439V	100
L-Glutamic Acid	Sigma-Aldrich	SLBL8200V	99
Glycine	Sigma-Aldrich	SLBK0853V	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	SLBG5999V	100
L-Isoleucine	Sigma-Aldrich	SLBK1375V	100
L-Leucine	Sigma-Aldrich	SLBL0518V	99
L-Lysine Monohydrochloride	Sigma-Aldrich	051M0016V	100
L-Methionine	Sigma-Aldrich	SLBL7822V	100
L-Phenylalanine	Sigma-Aldrich	SLBL5637V	100

Analyte	Manufacturer	Lot No.	Purity (%)
L-Proline	Sigma-Aldrich	SLBL4766V	100
L-Serine	Sigma-Aldrich	SLBK9059V	100
L-Threonine	Sigma-Aldrich	SLBK2255V	100
L-Tryptophan	Sigma-Aldrich	SLBL3563V	100
L-Tyrosine	Sigma-Aldrich	BCBK5272V	99.7
L-Valine	Sigma-Aldrich	SLBK2573V	100

Fatty Acid Profile. Levels of the following fatty acids were measured in test and control samples:

8:0 Caprylic
10:0 Capric
12:0 Lauric
14:0 Myristic
14:1 Myristoleic
15:0 Pentadecanoic
15:1 Pentadecenoic
16:0 Palmitic
16:1 Palmitoleic
17:0 Heptadecanoic
17:1 Heptadecenoic
18:0 Stearic
18:1 Oleic
18:2 Linoleic
18:3 Gamma linolenic
18:3 Linolenic
18:4 Octadecatetraenoic
20:0 Arachidic
20:1 Eicosenoic
20:2 Eicosadienoic
20:3 Eicosatrienoic
20:4 Arachidonic
20:5 Eicosapentaenoic
22:0 Behenic
22:1 Erucic
22:5 Docosapentaenoic
22:6 Docosahexaenoic

Levels of the cyclopropenoid fatty acids, malvalic, sterculic and dihydrosterculic were also determined.

After addition of tritridecanoin (C13:0 triglyceride) as an internal standard, a weighed aliquot of the lipid was transesterified with 0.5 N sodium methoxide in methanol at ambient temperature. The resulting methyl esters of the fatty acids were extracted with heptane and analyzed by gas chromatography (GC) using external standards of known concentration for quantitation. For analysis of samples collected from 2014 field studies, methods AOCS (2009a) and Christie (2013) were followed. For analysis of samples collected from 2015 field studies, in addition to the methods described in the above references, methods described by Mitchell et al. (2015) and Park and Rhee (1988) were incorporated in the analytical procedures. Because standards were not available for malvalic and sterculic methyl ester, dihydrosterculic methyl ester was used for quantitation of the cyclopropenoid fatty acids. The limit of quantitation for all fatty acids in cottonseed was calculated as 0.0110% on a fresh weight basis.

The reference standards used for the fatty acid analyses of samples collected from 2014 field studies were as follows:

Manufacturer	Lot No.	Component	JA14-Z		O1-X	
			Weight (%)	Purity (%)	Weight (%)	Purity (%)
Nu-Chek Prep GLC Reference Standard Covance 1 Covance 2	JA14-Z 01-X	Methyl Octanoate	3.0	99.7	1.25	99.7
		Methyl Decanoate	3.25	99.7	1.25	99.7
		Methyl Laurate	3.25	99.8	1.25	99.8
		Methyl Myristate	3.25	99.8	1.25	99.8
		Methyl Myristoleate	1.0	99.5	1.25	99.5
		Methyl Pentadecanoate	1.0	99.6	1.25	99.6
		Methyl Pentadecenoate	1.0	99.5	1.25	99.4
		Methyl Palmitate	10.0	99.8	15.75	99.8
		Methyl Palmitoleate	3.0	99.7	1.25	99.7
		Methyl Heptadecanoate	1.0	99.6	1.25	99.6
		Methyl 10-Heptadecenoate	1.0	99.5	1.25	99.5
		Methyl Stearate	7.0	99.8	14.00	99.8
		Methyl Oleate	10.0	99.8	15.75	99.8
		Methyl Linoleate	10.0	99.8	15.75	99.8
		Methyl Gamma Linolenate	1.0	99.4	1.25	99.5
		Methyl Linolenate	3.0	99.5	1.25	99.6
		Methyl Arachidate	2.0	99.8	1.25	99.8
		Methyl 11-Eicosenoate	2.0	99.6	1.25	99.6
		Methyl 11-14 Eicosadienoate	1.0	99.5	1.25	99.5
		Methyl 11-14-17 Eicosatrienoate	1.0	99.5	1.25	99.5
		Methyl Arachidonate	1.0	99.5	1.25	99.4
		Methyl Eicosapentaenoate	5.0	99.4	1.25	99.4
		Methyl Behenate	1.0	99.8	1.25	99.8
		Methyl Erucate	1.0	99.6	1.25	99.7
		Methyl Docosapentaenoate	5.0	99.4	1.25	99.4
		Methyl Docosahexaenoate	5.0	99.6	1.25	99.6

Manufacturer	Component	Lot No.	Purity (%)
Matreya LLC	Methyl Dihydrosterculate	23608	98.47

The reference standards used for the fatty acid analyses of samples collected from 2015 field studies were as follows:

Manufacturer	Lot No.	Component	N25-Z		O1-X	
			Weight (%)	Purity (%)	Weight (%)	Purity (%)
Nu-Chek Prep GLC Reference Standard Covance 1	N25-Z O1-X	Methyl Octanoate	3.0	99.8	1.25	99.7
		Methyl Decanoate	3.25	99.7	1.25	99.7
		Methyl Laurate	3.25	99.8	1.25	99.8
		Methyl Myristate	3.25	99.8	1.25	99.8
		Methyl Myristoleate	1.0	99.5	1.25	99.5

Manufacturer	Lot No.	Component	N25-Z		O1-X	
Covance 2		Methyl Pentadecanoate	1.0	99.6	1.25	99.6
		Methyl Pentadecenoate	1.0	99.5	1.25	99.4
		Methyl Palmitate	10.0	99.8	15.75	99.8
		Methyl Palmitoleate	3.0	99.7	1.25	99.7
		Methyl Heptadecanoate	1.0	99.6	1.25	99.6
		Methyl 10-Heptadecenoate	1.0	99.5	1.25	99.5
		Methyl Stearate	7.0	99.8	14.00	99.8
		Methyl Oleate	10.0	99.8	15.75	99.8
		Methyl Linoleate	10.0	99.8	15.75	99.8
		Methyl Gamma Linolenate	1.0	99.5	1.25	99.5
		Methyl Linolenate	3.0	99.5	1.25	99.6
		Methyl Arachidate	2.0	99.8	1.25	99.8
		Methyl 11-Eicosenoate	2.0	99.6	1.25	99.6
		Methyl 11-14 Eicosadienoate	1.0	99.5	1.25	99.5
		Methyl 11-14-17 Eicosatrienoate	1.0	99.5	1.25	99.5
		Methyl Arachidonate	1.0	99.5	1.25	99.4
		Methyl Eicosapentaenoate	5.0	99.4	1.25	99.4
		Methyl Behenate	1.0	99.8	1.25	99.8
		Methyl Erucate	1.0	99.7	1.25	99.7
		Methyl Docosapentaenoate	5.0	99.4	1.25	99.4
		Methyl Docosahexaenoate	5.0	99.6	1.25	99.6

Manufacturer	Component	Lot No.	Purity (%)
Matreya LLC	Methyl Dihydrosterculate	23608	98.47

Minerals: ICP Emission Spectrometry. Test and control cottonseed samples were analyzed for the following minerals: calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc. The samples were dried, precharred, and ashed overnight in a muffle furnace set to maintain 500°C. The ashed samples were re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown samples, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions (AOAC, 2012i; j; k). The results are reported on a dry weight basis.

The reference standards used for the mineral analyses of samples collected from 2014 field studies were as follows:

Inorganic Ventures Reference Standards:

Mineral	Lot No.	Certified Value (µg/ml)
Calcium	J2-MEB576142, J2-MEB576144	200.0, 1000
Copper	J2-MEB576142, J2-MEB576143MCA	2.000, 10.00
Iron	J2-MEB576142, J2-MEB576145	10.00, 50.00
Magnesium	J2-MEB576142, J2-MEB576143MCA	50.00, 250.0
Manganese	J2-MEB576142, J2-MEB576143MCA	2.000, 10.00
Phosphorus	J2-MEB576142, K2-MEB576144	200.0, 1000
Potassium	J2-MEB576142, K2-MEB576144	200.0, 1000
Sodium	J2-MEB576142, K2-MEB576144	200.0, 1000
Zinc	J2-MEB576142, J2-MEB576143MCA	10.00, 49.99

The reference standards used for the mineral analyses of samples collected from 2015 field studies were as follows:

Inorganic Ventures Reference Standards:

Mineral	Lot No.	Certified Value (µg/ml)
Calcium	J2-MEB610130, K2-MEB637070	200.0, 1000
Copper	J2-MEB610130, J2-MEB576143	2.001, 10.00
Iron	J2-MEB610130, J2-MEB576145	10.00, 50.00
Magnesium	J2-MEB610130, J2-MEB576143	50.00, 250.0
Manganese	J2-MEB610130, J2-MEB576143	2.000, 10.00
Phosphorus	J2-MEB610130, K2-MEB637070	200.0, 1000
Potassium	J2-MEB610130, K2-MEB637070	200.0, 1000
Sodium	J2-MEB610130, K2-MEB637070	200.0, 1000
Zinc	J2-MEB610130, J2-MEB576143	10.00, 49.99

The following limits of quantitation for each mineral were calculated on a fresh weight basis:

Mineral	Limit of Quantitation (ppm)
Calcium	20.0
Copper	0.500
Iron	2.00
Magnesium	20.0
Manganese	0.300
Phosphorus	20.0
Potassium	100
Sodium	100
Zinc	0.400

Alpha-Tocopherol. The samples were saponified with potassium hydroxide to break down any fat and release the alpha-tocopherol. The saponified mixture was extracted with diethyl ether.

The oil samples were diluted with hexane. Sample extracts were then quantitated by ultra or high-performance liquid chromatography using fluorescence detection (Speek et al., 1985; Cort et al., 1983; McMurray et al., 1980). The results are reported on a dry weight basis. The limit of quantitation was calculated as 0.500 mg/100 g on a fresh weight basis.

The reference standards used for the alpha-tocopherol analyses of samples collected from 2014 field studies were as follows:

Reference Standards:

Manufacturer	**Analyte**	**Lot No.**	**Purity** (%)
USP	Alpha Tocopherol	O0K291	98.5
Sigma-Aldrich	(+)-delta-Tocopherol	SLBG1716V	93
Sigma-Aldrich	D-gamma-Tocopherol	SLBL8950V	98

The reference standards used for the alpha-tocopherol analyses of samples collected from 2015 field studies were as follows:

Reference Standards:

Manufacturer	**Analyte**	**Lot No.**	**Purity** (%)
USP	Alpha Tocopherol	O0K291	98.5
Sigma-Aldrich	(+)-delta-Tocopherol	SLBG1716V	93
ACROS	D-gamma-Tocopherol	A0083534	99.3

Note: The alpha-tocopherol standard is part of a mixed standard which also includes beta, delta, and gamma isomers. The reference standard material for those isomers may contain small amounts of alpha tocopherol.

Phytic Acid. The samples were extracted using hydrochloric acid and sonication, purified using a silica-based anion exchange column, concentrated and injected onto a high-performance liquid chromatography (HPLC) system with a refractive index detector (Lehrfeld, 1989 and 1994). The results are reported on a dry weight basis. The limit of quantitation was calculated as 0.125% on a fresh weight basis.

The reference standard used for phytic acid analyses of samples collected from 2014 and 2015 field studies was as follows:

Reference Standard:

Manufacturer	**Analyte**	**Lot No.**	**Purity** (%)
Sigma-Aldrich	Phytic Acid Sodium Salt Hydrate	BCBK8062V	82.187

Total Gossypol, Free Gossypol and Gossypol Isomers.
The different forms of gossypol in the cottonseed were measured by several different methods and different laboratories. Total gossypol was measured by three different methods and two separate laboratories. Covance Laboratories measured total gossypol and free gossypol by the aniline method, as well as by an HPLC method. Gossypol isomers were measured by HPLC and total gossypol concentration calculated as the sum of the concentrations of the two isomers, all described below. In addition, TAMU measured total gossypol in the kernels of the cottonseed by an HPLC method, described in detail below.

Total Gossypol Measured by the Aniline Method (Covance Laboratories). Total gossypol defines gossypol and gossypol derivatives, both free and bound, in cottonseed products that are capable of reacting with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol under the conditions of the method. Gossypol, gossypol analogs, and gossypol derivatives having an available aldehyde moiety were measured by the method AOCS (2011b). The results are reported on a dry weight basis. The limit of quantitation was calculated as 0.00200% on a fresh weight basis.

The reference standard used for total gossypol analyses of samples collected from 2014 and 2015 field studies, as measured by the aniline method, was as follows:

Reference Standard:

Manufacturer	**Analyte**	**Lot No.**	**Purity** (%)
Sigma-Aldrich	Gossypol	024M4030V	98.90

Total Gossypol Measured by the HPLC Method (TAMU). Gossypol analyses were performed on cottonseed samples using HPLC following the methods described by Stipanovic et al. (1988) and Benson et al. (2001). Briefly, a 500 mg of sample was mixed with 20 ml of solvent consisting of ethanol:ether:water:glacial acetic acid (59:17:24:0.2) in a flask. The sample was then agitated for an hour on a shaker at 200 rpm in the dark under N_2 atmosphere, to facilitate extraction of gossypol. The suspension was then transferred to a 50 ml centrifuge tube and the extraction solvent was brought up to 50 ml before centrifugation at 2800 *x g* for 15 min. A 50 µl fraction of the supernatant was analyzed on a LC-1200 (Agilent Technology) High Pressure Liquid Chromatograph, equipped with a diode array detector for compound spectral identification, multichannel integrator, and autoinjector using a 4.6 mm x 25 cm Scientific Glass Engineering (SGE) ProteCol-GP-C18-125 (5µm) column maintained at 30ºC. The mobile phase was 26% water with 0.1% phosphoric acid and 74% SGE-#3B organic mix of acetonitrile:ethanol:isopropyl alcohol:dimethylformamide:methanol:ethyl acetate:phosphoric acid (32.3:26.7:19.4:8.2:7.4:6.08:0.1). The flowrate was 1.25 ml/min and the runtime was 15 min.

The chromatogram signal was obtained at 272 nm (band width = 20 nm) and spectra were stored over 240-400 nm. The injection volume of the samples was 50 µl. The limit of detection of the method was calculated to be 14.8 µg/g cottonseed on a dry weight basis.

The standard curve was created using standards prepared from purified gossypol (99.1%).

Free Gossypol (Aniline Method/Covance Laboratories). The samples were extracted with an aqueous acetone solution and filtered. Duplicate aliquots were made and the active aliquot was reacted with aniline with heat applied in a water bath. Active and inactive aliquots were brought to volume with an aqueous isopropyl alcohol solution and read on a spectrophotometer at 440 nm. The absorbance difference was then compared to a linear curve calculated from standards that were aliquoted, reacted, and read in the same fashion as the samples (AOCS, 2009b). The results are reported on a dry weight basis. The limit of quantitation was calculated as 0.00200% on a fresh weight basis.

The reference standard used for free gossypol analyses of samples collected from 2014 and 2015 field studies was as follows:

Reference Standard:

Manufacturer	**Analyte**	**Lot No.**	**Purity** (%)
Sigma-Aldrich	Gossypol	024M4030V	98.90

Gossypol Isomers (HPLC Method/Covance Laboratories). The samples were treated with a complexing reagent (2 ml D-alaninol, 10 ml acetic acid, 88 ml dimethylformamide) to produce (+)- and (−)-gossypol-aminopropanol. The samples were mixed with the complexing reagent and heated at 100ºC for 30 min. The mixture of sample and complexing reagent were diluted with the mobile phase of acetonitrile and 10 mM potassium phosphate (78:22), and filtered. The samples were further diluted with the mobile phase and analyzed using HPLC with ultraviolet (UV) detection. The HPLC analysis used a Prodigy™ 5µ ODS3 100Å column (3.2 mm x 150 mm) [Phenomenex, CA], the mobile phase is described above, the flowrate was 1.00 ml/min, the chromatogram signal was obtained at 254 nm and the injection volume of the samples was 16 µl (AOCS, 2011c). The results are reported on a dry weight basis. The limit of quantitation for total gossypol isomers was calculated as 170 µg/g on a fresh weight basis. The limit of quantitation for the (+)- and (–)- gossypol- aminopropanol isomers was calculated at 50.0 µg/g on a fresh weight basis.

The reference standard used for analysis of gossypol isomers in samples collected from 2014 and 2015 field studies was as follows:

Manufacturer	**Analyte**	**Lot No.**	**Purity** (%)
Sigma-Aldrich	Gossypol-acetic acid	014M4065V	98.99

Mycotoxins.
The mycotoxins analyzed in test and control cottonseed were aflatoxins B1, B2, G1 and G2, as well as deoxynivalenol, acetyldeoxynivalenol and zearalenone.

Upon receipt at Romer Laboratories, the cottonseed samples were stored at ambient room temperatures before sample preparation and analysis. The cottonseed samples were ground using a Waring® blender to crack open the seed and expose the kernel. A 25 g portion of the cracked cottonseed was weighed into an 18 oz Whirl-Pak® bag. Pre-extraction matrix spiked samples were prepared by weighing out aliquots of cottonseed. One aliquot was spiked with predetermined amounts of aflatoxin B1, B2, G1, G2, deoxynivalenol and zearalenone; another aliquot was weighed as-is, which served as an unspiked control. Extraction solvent consisting of 100 ml of acetonitrile:deionized water (84:16 v/v) was added to the pre-extraction and control samples and shaken for a minimum of 90 minutes on a gyratory shaker. The spiked matrix and control solutions were filtered using Whatman® filter paper and the filtrates were collected for analysis using the methods described below.

Aflatoxins B1, B2, G1 and G2. The sample filtrates were purified using a MycoSep® 228 AflaPat column. The purified samples were collected, mixed with aflatoxin mobile phase carrier solution, and injected onto a high-performance liquid chromatography (HPLC) system with a fluorescence detector (AOAC, 2000). Concentrations of the different aflatoxins in test and control cottonseed samples were determined from standard curves of freshly prepared reference standards. The results are reported on a fresh weight (FW) basis. The limits of detection for the different aflatoxins were calculated as B1 (0.7 ppb), B2 (0.9 ppb), G1 (0.7 ppb) and G2 (0.8 ppb) on a fresh weight basis.

Deoxynivalenol and Acetyldeoxynivalenol. The sample filtrates were concentrated using a heated water bath under vacuum, reconstituted with LC-MS/MS solution, and injected onto a liquid chromatography/tandem mass spectroscopy (LC-MS/MS) system (Sulyok et al., 2006). Concentrations of deoxynivalenol and acetyldeoxynivalenol in test and control cottonseed samples were determined from standard curves of freshly prepared reference standards. The results are reported on a fresh weight (FW) basis. The limits of detection were calculated as deoxynivalenol (0.6 ppb) and acetyldeoxynivalenol (0.8 ppb) on a fresh weight basis.

Zearalenone. The sample filtrates were acidified and purified using a MycoSep® 226 AflaZon column. The purified samples were concentrated using a heated water bath under vacuum, mixed with mobile phase carrier solution, and injected onto a high-performance liquid chromatography (HPLC) system with a fluorescence detector (Silva and Vargas, 2001). Concentrations of zearalenone in test and control cottonseed samples were determined from standard curves of a freshly prepared reference standard. The results are reported on a fresh weight (FW) basis. The limit of detection was calculated as 43.1 ppb on a fresh weight basis.

Statistical Analysis.

In statistics, a *numerical* variable is one that can be assigned a numerical value, either a discrete (whole) number or continuous (non-integer) numbers. In the case of TAM66274 data, 66 compositional characteristics (i.e., nutrients, anti-nutrients) were measured on a continuous scale.

For the purpose of statistical analysis of TAM66274 compositional data, a normal distribution was assumed in the underlying population from which sample data were drawn. Sample means, standard error of the mean (S.E.M.), and mean square error (MSE) were calculated and evaluated using a parametric statistical method, analysis of variance (ANOVA) to compare treatment means. Statistical analysis was performed using JMP software (Version 9, 2011) (SAS Institute, Cary NC, USA). The same statistical model was used for all variables (i.e., all data was treated as continuous). Orthogonal contrasts were calculated comparing the two genotypes (TAM66274, non-transgenic cv. Coker 312). All compositional variables used the same randomized complete block statistical model. Location samples were an equal representation from each of the four replications. Only a single composite sample of the four replications was analyzed, and it represents a single estimate for each location. Accordingly, statistical analysis of variability with each field location was not possible. Entry (genotype) differences were analyzed across locations with entry as a fixed variable and locations a random variable using maximum likelihood REML. Entries (genotypes) were declared different if $P > F$ was ≤ 0.05. The least significant difference between genotypes was calculated by Fisher's Protected Least Significant Difference (LSD) (0.05). All variables were also analyzed across all sites (locations) by a mixed-design model using residual (or restricted) maximum likelihood (REML) with entry and site as random effects and replication as a fixed effect. Significant differences were declared in the same manner as individual sites using $P > F$ of $\alpha = 0.05$ for contrasts between test and control substances.

For the mycotoxin analyses, more than 80% of the observed values for each analyte in this study were less than the assay limit of detection (LOD). In order to proceed with the statistical analysis of any component in this study, at least 50% of the observed values for that analyte needed to be greater than the assay LOD. Due to these statistical constraints, statistical analysis of the data was not performed.

Analytical Results.

Table E-1. Cottonseed proximate composition.
Proximate composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Moisture is expressed on a fresh weight (FW) basis, and the other proximates on a dry weight (DW) basis.

		Analytes					
		Moisture (% FW)	**Protein (% DW)**	**Total Fat (% DW)**	**Ash (% DW)**	**Carbohydrates (% DW)**	**Calories (Kcal/100 g DW)**
Sites	**Treatments**	**2014 Studies**					
NC114	TAM66274	7.92	27.4	22.5	3.87	46.3	497
	Coker 312	7.80	26.7	23.6	3.71	46.0	503
NC214	TAM66274	7.50	28.1	21.4	3.62	46.8	493
	Coker 312	7.59	29.5	21.9	3.73	44.8	495
MS114	TAM66274	6.99	26.9	23.5	4.16	45.5	501
	Coker 312	7.02	27.1	23.4	4.25	45.3	500
Sites	**Treatments**	**2015 Studies**					
NC115	TAM66274	8.49	27.5	22.6	4.28	45.6	496
	Coker 312	8.30	27.5	23.8	4.42	44.3	502
NC315	TAM66274	8.59	28.6	21.9	4.13	45.4	493
	Coker 312	8.25	30.0	23.4	4.04	42.5	501
MS115	TAM66274	8.43	25.8	22.2	4.40	47.6	493
	Coker 312	7.77	26.8	23.7	4.43	45.1	501
MS315	TAM66274	7.95	29.1	21.3	4.32	45.3	489
	Coker 312	7.52	30.8	22.0	4.41	42.7	493
TX515	TAM66274	7.93	29.2	20.5	4.07	46.2	486
	Coker 312	7.69	30.0	22.3	3.98	43.7	496

Table E-2. Cottonseed fiber composition.
Fiber composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fiber levels are expressed on a percent dry weight (DW) basis.

		Analytes			
		Crude Fiber (% DW)	**Total Dietary Fiber (% DW)**	**Acid Detergent Fiber (% DW)**	**Neutral Detergent Fiber (% DW)**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	21.2	45.7	27.9	38.1
	Coker 312	20.8	43.3	25.8	35.9
NC214	TAM66274	22.1	43.5	29.0	37.4
	Coker 312	19.5	42.2	27.2	35.9
MS114	TAM66274	20.9	43.9	26.7	34.5
	Coker 312	20.2	40.3	25.8	32.9
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	21.2	41.2	28.4	38.1
	Coker 312	21.0	40.5	27.0	34.6
NC315	TAM66274	21.7	42.5	28.9	37.7
	Coker 312	19.3	37.5	25.6	31.8
MS115	TAM66274	21.8	44.2	28.9	38.2
	Coker 312	20.1	39.0	26.3	32.2
MS315	TAM66274	18.9	39.1	26.6	34.7
	Coker 312	17.6	38.5	24.6	30.6
TX515	TAM66274	19.7	41.1	29.1	36.2
	Coker 312	19.9	37.4	26.4	32.1

Table E-3. Cottonseed amino acid composition.
Amino acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Amino acid levels are expressed on a dry weight (DW) basis.

		Analytes				
		Alanine (mg/g DW)	**Arginine (mg/g DW)**	**Aspartic Acid (mg/g DW)**	**Cystine (mg/g DW)**	**Glutamic Acid (mg/g DW)**
Sites	**Treatments**	**2014 Studies**				
NC114	TAM66274	10.3	29.2	23.7	4.55	50.2
	Coker 312	10.3	30.4	24.0	4.45	51.1
NC214	TAM66274	11.2	32.3	25.9	5.01	55.0
	Coker 312	10.7	32.4	25.2	4.71	53.6
MS114	TAM66274	10.7	29.8	24.4	4.90	51.8
	Coker 312	10.0	28.8	22.8	4.42	48.6
Sites	**Treatments**	**2015 Studies**				
NC115	TAM66274	11.7	30.8	24.5	4.43	49.8
	Coker 312	10.6	30.9	23.7	4.40	49.4
NC315	TAM66274	11.5	32.5	25.5	4.59	52.1
	Coker 312	11.5	34.6	27.0	4.87	55.6
MS115	TAM66274	10.3	29.1	23.1	4.29	47.9
	Coker 312	10.6	30.6	24.8	4.80	51.6
MS315	TAM66274	11.0	32.6	25.9	4.86	52.9
	Coker 312	12.4	37.6	28.6	5.34	59.1
TX515	TAM66274	10.8	33.1	26.0	4.95	54.6
	Coker 312	11.0	32.3	25.4	5.02	54.1

Table E-3, continued. Cottonseed amino acid composition.
Amino acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Amino acid levels are expressed on a dry weight (DW) basis.

		Analytes				
		Glycine (mg/g DW)	**Histidine (mg/g DW)**	**Isoleucine (mg/g DW)**	**Leucine (mg/g DW)**	**Lysine (mg/g DW)**
Sites	**Treatments**	**2014 Studies**				
NC114	TAM66274	11.0	7.46	8.62	15.5	11.2
	Coker 312	11.0	7.49	8.66	15.4	11.3
NC214	TAM66274	11.8	8.02	9.29	16.5	11.8
	Coker 312	11.3	7.68	8.90	15.9	11.3
MS114	TAM66274	11.2	7.52	8.97	15.7	11.2
	Coker 312	10.5	6.69	8.26	14.6	10.2
Sites	**Treatments**	**2015 Studies**				
NC115	TAM66274	11.2	7.39	8.76	15.7	11.8
	Coker 312	10.7	7.28	8.59	15.3	11.4
NC315	TAM66274	11.2	7.67	9.26	16.1	11.8
	Coker 312	11.9	8.07	9.63	17.0	12.5
MS115	TAM66274	10.5	6.94	8.22	14.6	11.0
	Coker 312	11.0	7.25	8.64	15.5	11.6
MS315	TAM66274	11.4	7.55	9.07	16.1	12.0
	Coker 312	12.4	8.46	9.95	17.5	13.0
TX515	TAM66274	11.3	7.30	9.08	16.1	12.1
	Coker 312	11.1	7.42	8.90	15.9	11.7

Table E-3, continued. Cottonseed amino acid composition.
Amino acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Amino acid levels are expressed on a dry weight (DW) basis.

		Analytes			
		Methionine (mg/g DW)	**Phenylalanine (mg/g DW)**	**Proline (mg/g DW)**	**Serine (mg/g DW)**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	3.97	14.6	9.88	11.3
	Coker 312	4.20	14.6	9.77	11.3
NC214	TAM66274	4.19	15.8	10.6	12.2
	Coker 312	4.27	15.1	10.2	11.8
MS114	TAM66274	4.33	14.5	9.95	11.7
	Coker 312	3.83	13.8	9.25	10.8
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	4.26	14.5	10.2	11.7
	Coker 312	4.11	14.4	9.86	11.4
NC315	TAM66274	4.13	15.2	11.5	12.1
	Coker 312	4.41	15.9	10.9	12.6
MS115	TAM66274	3.95	13.5	9.49	11.4
	Coker 312	3.94	14.4	10.0	11.8
MS315	TAM66274	4.18	15.1	10.7	12.2
	Coker 312	4.47	16.8	11.8	13.3
TX515	TAM66274	4.29	15.1	10.6	12.5
	Coker 312	3.91	14.9	10.2	12.2

Table E-3, continued. Cottonseed amino acid composition.
Amino acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Amino acid levels are expressed on a dry weight (DW) basis.

		Analytes			
		Threonine (mg/g DW)	**Tryptophan (mg/g DW)**	**Tyrosine (mg/g DW)**	**Valine (mg/g DW)**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	8.62	3.63	8.26	12.1
	Coker 312	8.59	3.60	8.18	12.1
NC214	TAM66274	9.29	3.69	8.92	13.1
	Coker 312	8.80	3.50	8.53	12.3
MS114	TAM66274	8.91	3.38	8.44	12.1
	Coker 312	8.16	3.67	7.77	11.4
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	9.04	3.69	8.40	11.8
	Coker 312	8.64	3.89	8.26	11.6
NC315	TAM66274	9.04	3.51	8.68	12.3
	Coker 312	9.47	4.07	9.16	12.8
MS115	TAM66274	8.35	3.82	7.92	11.2
	Coker 312	8.79	3.83	8.36	11.7
MS315	TAM66274	9.11	3.78	8.63	12.1
	Coker 312	9.81	3.96	9.51	13.5
TX515	TAM66274	9.19	4.02	8.90	12.1
	Coker 312	8.73	4.30	8.62	12.0

Table E-4. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)			
		8:0 Caprylic (%)	10:0 Capric (%)	12:0 Lauric (%)	14:0 Myristic (%)
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	<LOQ	<LOQ	<LOQ	0.531
	Coker 312	<LOQ	<LOQ	<LOQ	0.682
NC214	TAM66274	<LOQ	<LOQ	<LOQ	0.555
	Coker 312	<LOQ	<LOQ	<LOQ	0.727
MS114	TAM66274	<LOQ	<LOQ	<LOQ	0.537
	Coker 312	<LOQ	<LOQ	<LOQ	0.692
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	<LOQ	<LOQ	<LOQ	0.539
	Coker 312	<LOQ	<LOQ	<LOQ	0.781
NC315	TAM66274	<LOQ	<LOQ	<LOQ	0.556
	Coker 312	<LOQ	<LOQ	<LOQ	0.772
MS115	TAM66274	<LOQ	<LOQ	<LOQ	0.633
	Coker 312	<LOQ	<LOQ	<LOQ	0.835
MS315	TAM66274	<LOQ	<LOQ	<LOQ	0.688
	Coker 312	<LOQ	<LOQ	<LOQ	0.946
TX515	TAM66274	<LOQ	<LOQ	<LOQ	0.839
	Coker 312	<LOQ	<LOQ	<LOQ	1.150

LOQ. Limit of Quantification

Table E-4, continued. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)			
		14:1 Myristoleic (%)	**15:0 Pentadecanoic (%)**	**15:1 Pentadecenoic (%)**	**16:0 Palmitic (%)**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	<LOQ	<LOQ	<LOQ	20.9
	Coker 312	<LOQ	<LOQ	<LOQ	22.3
NC214	TAM66274	<LOQ	<LOQ	<LOQ	20.7
	Coker 312	<LOQ	<LOQ	<LOQ	22.6
MS114	TAM66274	<LOQ	<LOQ	<LOQ	21.4
	Coker 312	<LOQ	<LOQ	<LOQ	22.9
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	<LOQ	<LOQ	<LOQ	21.0
	Coker 312	<LOQ	<LOQ	<LOQ	23.9
NC315	TAM66274	<LOQ	<LOQ	<LOQ	21.0
	Coker 312	<LOQ	<LOQ	<LOQ	23.3
MS115	TAM66274	<LOQ	<LOQ	<LOQ	22.2
	Coker 312	<LOQ	<LOQ	<LOQ	24.9
MS315	TAM66274	<LOQ	<LOQ	<LOQ	22.8
	Coker 312	<LOQ	<LOQ	<LOQ	25.4
TX515	TAM66274	<LOQ	<LOQ	<LOQ	24.9
	Coker 312	<LOQ	<LOQ	<LOQ	28.0

LOQ. Limit of Quantification

Table E-4, continued. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)			
		16:1 Palmitoleic (%)	**17:0 Heptadecanoic (%)**	**17:1 Heptadecenoic (%)**	**18:0 Stearic (%)**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	0.454	0.0811	<LOQ	2.10
	Coker 312	0.455	0.0861	<LOQ	2.22
NC214	TAM66274	0.455	0.0856	<LOQ	2.00
	Coker 312	0.464	0.0834	<LOQ	2.13
MS114	TAM66274	0.469	0.0836	<LOQ	2.12
	Coker 312	0.467	0.0832	<LOQ	2.23
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	0.467	0.0886	<LOQ	2.20
	Coker 312	0.503	0.0887	<LOQ	2.27
NC315	TAM66274	0.469	0.0919	<LOQ	2.13
	Coker 312	0.520	0.0816	<LOQ	2.16
MS115	TAM66274	0.482	0.0881	<LOQ	2.30
	Coker 312	0.531	0.0778	<LOQ	2.26
MS315	TAM66274	0.497	0.0888	<LOQ	2.26
	Coker 312	0.564	0.0895	<LOQ	2.29
TX515	TAM66274	0.559	0.0856	<LOQ	2.30
	Coker 312	0.619	0.0850	<LOQ	2.38

LOQ. Limit of Quantification

Table E-4, continued. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)			
		18:1 Oleic (%)	**18:2 Linoleic (%)**	**18:3 γ-Linolenic (%)**	**18:3 Linolenic (%)**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	14.0	60.5	<LOQ	0.167
	Coker 312	13.8	58.9	<LOQ	0.150
NC214	TAM66274	14.3	60.5	<LOQ	0.172
	Coker 312	14.1	58.3	<LOQ	0.152
MS114	TAM66274	13.8	60.3	<LOQ	0.165
	Coker 312	13.3	58.8	<LOQ	0.148
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	13.8	60.4	<LOQ	0.202
	Coker 312	13.3	57.5	<LOQ	0.165
NC315	TAM66274	14.4	59.9	<LOQ	0.201
	Coker 312	13.7	57.9	<LOQ	0.166
MS115	TAM66274	14.8	58.2	<LOQ	0.162
	Coker 312	13.7	56.2	<LOQ	0.141
MS315	TAM66274	14.6	57.7	<LOQ	0.189
	Coker 312	14.0	55.2	<LOQ	0.153
TX515	TAM66274	16.2	53.9	<LOQ	0.137
	Coker 312	15.6	51.0	<LOQ	0.123

LOQ. Limit of Quantification

Table E-4, continued. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)			
		18:4 Octadecatetraenoic (%)	**20:0 Arachidic (%)**	**20:1 Eicosenoic (%)**	**20:2 Eicosadienoic (%)**
Site	**Treatments**	**2014 Studies**			
NC114	TAM66274	<LOQ	0.225	0.0724	<LOQ
	Coker 312	<LOQ	0.255	0.0643	<LOQ
NC214	TAM66274	<LOQ	0.223	0.0700	<LOQ
	Coker 312	<LOQ	0.254	0.0669	<LOQ
MS114	TAM66274	<LOQ	0.235	0.0694	<LOQ
	Coker 312	<LOQ	0.259	0.0608	<LOQ
Site	**Treatments**	**2015 Studies**			
NC115	TAM66274	<LOQ	0.247	<LOQ	<LOQ
	Coker 312	<LOQ	0.278	0.0675	<LOQ
NC315	TAM66274	<LOQ	0.251	0.0769	<LOQ
	Coker 312	<LOQ	0.259	<LOQ	<LOQ
MS115	TAM66274	<LOQ	0.289	<LOQ	<LOQ
	Coker 312	<LOQ	0.280	0.0567	<LOQ
MS315	TAM66274	<LOQ	0.280	0.0670	<LOQ
	Coker 312	<LOQ	0.301	<LOQ	<LOQ
TX515	TAM66274	<LOQ	0.298	<LOQ	<LOQ
	Coker 312	<LOQ	0.331	<LOQ	<LOQ

LOQ. Limit of Quantification

Table E-4, continued. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)			
		20:3 Eicosatrienoic (%)	**20:4 Arachidonic (%)**	**20:5 Eicosapentaenoic (%)**	**22:0 Behenic (%)**
Site	**Treatments**	**2014 Studies**			
NC114	TAM66274	<LOQ	<LOQ	<LOQ	0.117
	Coker 312	<LOQ	<LOQ	<LOQ	0.125
NC214	TAM66274	<LOQ	<LOQ	<LOQ	0.121
	Coker 312	<LOQ	<LOQ	<LOQ	0.133
MS114	TAM66274	<LOQ	<LOQ	<LOQ	0.112
	Coker 312	<LOQ	<LOQ	<LOQ	0.127
Site	**Treatments**	**2015 Studies**			
NC115	TAM66274	<LOQ	<LOQ	<LOQ	0.093
	Coker 312	<LOQ	<LOQ	<LOQ	0.109
NC315	TAM66274	<LOQ	<LOQ	<LOQ	0.095
	Coker 312	<LOQ	<LOQ	<LOQ	0.141
MS115	TAM66274	<LOQ	<LOQ	<LOQ	0.121
	Coker 312	<LOQ	<LOQ	<LOQ	0.137
MS315	TAM66274	<LOQ	<LOQ	<LOQ	0.151
	Coker 312	<LOQ	<LOQ	<LOQ	0.156
TX515	TAM66274	<LOQ	<LOQ	<LOQ	0.154
	Coker 312	<LOQ	<LOQ	<LOQ	0.164

LOQ. Limit of Quantification

Table E-4, continued. Cottonseed fatty acid composition.
Fatty acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Fatty acid levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)		
		22:1 Erucic (%)	**22:5 Docosapentaenoic (%)**	**22:6 Docosahexaenoic (%)**
Site	**Treatments**	**2014 Studies**		
NC114	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
NC214	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
MS114	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
Site	**Treatments**	**2015 Studies**		
NC115	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
NC315	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
MS115	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
MS315	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ
TX515	TAM66274	<LOQ	<LOQ	<LOQ
	Coker 312	<LOQ	<LOQ	<LOQ

LOQ. Limit of Quantification

Table E-5. Cottonseed mineral composition.
Mineral composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Mineral levels are expressed as parts per million (ppm) on a dry weight basis.

		Analytes				
		Copper (ppm)	**Iron (ppm)**	**Manganese (ppm)**	**Zinc (ppm)**	**Calcium (ppm)**
Site	**Treatments**	**2014 Studies**				
NC114	TAM66274	9.21	45.2	11.5	40.0	1090
	Coker 312	8.57	43.2	10.8	38.6	1050
NC214	TAM66274	9.55	42.7	13.1	47.4	1050
	Coker 312	8.54	42.2	12.7	47.9	1090
MS114	TAM66274	8.84	49.8	14.0	39.4	999
	Coker 312	8.97	51.9	14.1	40.3	1030
Site	**Treatments**	**2015 Studies**				
NC115	TAM66274	9.06	47.8	11.6	37.3	1240
	Coker 312	8.83	46.7	11.7	39.2	1210
NC315	TAM66274	5.93	44.9	14.4	48.1	1120
	Coker 312	5.15	46.3	12.9	57.4	984
MS115	TAM66274	9.42	54.6	14.9	43.0	1770
	Coker 312	9.48	52.6	14.1	45.5	1630
MS315	TAM66274	9.69	52.1	13.8	42.0	1440
	Coker 312	9.88	53.7	13.2	50.4	1380
TX515	TAM66274	11.90	51.9	18.2	53.0	2160
	Coker 312	11.50	50.7	16.0	53.9	1980

Table E-5, continued. Cottonseed mineral composition.
Mineral composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Mineral levels are expressed as parts per million (ppm) on a dry weight basis.

		Analytes			
		Magnesium (ppm)	**Phosphorus (ppm)**	**Potassium (ppm)**	**Sodium (ppm)**
Site	**Treatments**	**2014 Studies**			
NC114	TAM66274	3870	6220	11400	873
	Coker 312	3920	6050	10700	996
NC214	TAM66274	3630	5600	10900	923
	Coker 312	3850	5450	10500	1110
MS114	TAM66274	4030	7110	11500	824
	Coker 312	4390	7570	11200	796
Site	**Treatments**	**2015 Studies**			
NC115	TAM66274	3830	6390	11700	902
	Coker 312	4110	6320	12200	1100
NC315	TAM66274	3660	5650	11400	917
	Coker 312	3770	5630	11400	878
MS115	TAM66274	4080	7150	10500	881
	Coker 312	4360	7440	10800	1050
MS315	TAM66274	3820	6820	11100	800
	Coker 312	4070	6920	11100	805
TX515	TAM66274	3690	6440	10000	888
	Coker 312	3590	5890	9890	793

Table E-6. Cottonseed alpha-tocopherol composition.
Alpha tocopherol composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Alpha tocopherol levels are expressed as mg/100 g dry weight (DW).

		Analyte
		Alpha-tocopherol (mg/100 g DW)
Sites	**Treatments**	**2014 Studies**
NC114	TAM66274	10.6
	Coker 312	12.7
NC214	TAM66274	10.3
	Coker 312	12.0
MS114	TAM66274	12.3
	Coker 312	15.4
Site	**Treatments**	**2015 Studies**
NC115	TAM66274	14.2
	Coker 312	17.1
NC315	TAM66274	13.4
	Coker 312	15.6
MS115	TAM66274	15.8
	Coker 312	18.4
MS315	TAM66274	15.3
	Coker 312	18.2
TX515	TAM66274	15.4
	Coker 312	16.3

Table E-7. Cottonseed phytic acid composition.
Phytic acid composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Phytic acid levels are expressed as a percent on a dry weight (DW) basis.

		Analyte
		Phytic Acid (% DW)
Sites	**Treatments**	**2014 Studies**
NC114	TAM66274	1.74
	Coker 312	1.70
NC214	TAM66274	1.51
	Coker 312	1.49
MS114	TAM66274	2.01
	Coker 312	2.19
Sites	**Treatments**	**2015 Studies**
NC115	TAM66274	1.73
	Coker 312	1.72
NC315	TAM66274	1.49
	Coker 312	1.51
MS115	TAM66274	1.88
	Coker 312	1.93
MS315	TAM66274	1.88
	Coker 312	1.90
TX515	TAM66274	1.75
	Coker 312	1.59

Table E-8. Cottonseed cyclopropenoid fatty acid (CPFA) composition.
CPFA composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. CPFA levels are expressed as a percent of total fatty acids.

		Analytes (% of total fatty acids)		
		Malvalic Acid (%)	**Sterculic Acid (%)**	**Dihydrosterculic Acid (%)**
Sites	**Treatments**	**2014 Studies**		
NC114	TAM66274	0.486	0.232	0.114
	Coker 312	0.574	0.265	0.168
NC214	TAM66274	0.467	0.232	0.124
	Coker 312	0.567	0.266	0.163
MS114	TAM66274	0.472	0.217	0.116
	Coker 312	0.543	0.246	0.159
Sites	**Treatments**	**2015 Studies**		
NC115	TAM66274	0.530	0.254	0.130
	Coker 312	0.569	0.275	0.181
NC315	TAM66274	0.504	0.260	0.106
	Coker 312	0.562	0.294	0.178
MS115	TAM66274	0.432	0.194	0.114
	Coker 312	0.484	0.236	0.183
MS315	TAM66274	0.373	0.179	0.114
	Coker 312	0.455	0.230	0.175
TX515	TAM66274	0.329	0.171	0.113
	Coker 312	0.294	0.176	0.153

Table E-9. Cottonseed total and free gossypol composition measured by the aniline method. Total and free gossypol composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Total gossypol was measured by Covance Laboratories using the aniline method, as described above in the Analytical Methods section of this Appendix. Gossypol levels are expressed as a percent on a dry weight basis.

		Analytes	
		Total Gossypol (%) (by aniline)	**Free Gossypol (%) (by aniline)**
Sites	**Treatments**	**2014 Studies**	
NC114	TAM66274	0.0429	0.0286
	Coker 312	0.9710	0.7890
NC214	TAM66274	0.0404	0.0272
	Coker 312	0.9300	0.7630
MS114	TAM66274	0.0502	0.0332
	Coker 312	0.9880	0.7800
Sites	**Treatments**	**2015 Studies**	
NC115	TAM66274	0.0496	0.0276
	Coker 312	1.0000	0.8780
NC315	TAM66274	0.0508	0.0288
	Coker 312	0.9840	0.8440
MS115	TAM66274	0.0388	0.0209
	Coker 312	1.0400	0.9050
MS315	TAM66274	0.0355	0.0239
	Coker 312	0.8980	0.8220
TX515	TAM66274	0.0346	0.0276
	Coker 312	0.7810	0.7010

Table E-10. Cottonseed total gossypol composition measured by HPLC.
Total gossypol composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Total gossypol was measured in seeds from 25-boll samples by TAMU using an HPLC method, as described above in the Analytical Methods section of this Appendix. Each replicate sample of each treatment at each field site was analyzed separately and data are presented for each replicate sample. Gossypol levels are expressed as a percent on a dry weight basis.

		Total Gossypol (%) (by HPLC)			
		Replicate 1	**Replicate 2**	**Replicate 3**	**Replicate 4**
Sites	**Treatments**	**2014 Studies**			
NC114	TAM66274	0.0281	0.0320	0.0380	0.0403
	Coker 312	1.1106	1.2221	1.0998	1.1361
NC214	TAM66274	0.0326	0.0351	0.0302	0.0333
	Coker 312	0.8808	0.9403	0.9685	1.0072
MS114	TAM66274	0.0448	0.0415	0.0482	0.0428
	Coker 312	1.0212	0.9532	1.0126	0.9670
Sites	**Treatments**	**2015 Studies**			
NC115	TAM66274	0.0475	0.0273	0.0322	0.029
	Coker 312	1.1809	1.0174	1.2814	1.1626
NC315	TAM66274	0.0457	0.0234	0.0395	0.0248
	Coker 312	0.8478	0.8752	0.9546	0.9646
MS115	TAM66274	0.0270	0.0285	0.0262	0.0258
	Coker 312	1.2537	1.2251	1.1752	1.1067
MS315	TAM66274	0.0318	0.0315	0.0276	0.0310
	Coker 312	0.8842	1.0024	1.1026	0.8290
TX515	TAM66274	0.0459	0.0219	0.0198	0.0187
	Coker 312	0.7314	0.8381	0.8746	0.7828

Table E-11. Cottonseed (+)- and (−)-gossypol isomers and total gossypol composition.
Gossypol isomers and total gossypol composition of cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). The four replicate samples of each treatment at each field site were pooled and analyzed as a single sample. Levels of the gossypol isomers were measured by Covance Laboratories using an HPLC method, as described above in the Analytical Methods section of this Appendix. Total gossypol content of the cottonseed was calculated as the sum of the content of the (+)- and (−)-gossypol isomers. Gossypol levels are expressed on a dry weight (DW) basis.

		Analytes		
		(+)-gossypol (µg/g DW)	(−)-gossypol (µg/g DW)	Total gossypol (µg/g DW)
Sites	**Treatments**	**2014 Studies**		
NC114	TAM66274	144	104	248
	Coker 312	4010	2920	6930
NC214	TAM66274	141	104	245
	Coker 312	3800	2670	6470
MS114	TAM66274	158	118	276
	Coker 312	3870	2870	6740
Sites	**Treatments**	**2015 Studies**		
NC115	TAM66274	179	132	311
	Coker 312	4520	3090	7610
NC315	TAM66274	192	146	338
	Coker 312	4110	2730	6840
MS115	TAM66274	122	97.6	220
	Coker 312	4600	3000	7600
MS315	TAM66274	135	105	240
	Coker 312	3920	2600	6520
TX515	TAM66274	173	132	305
	Coker 312	3870	2220	6090

Table E-12. Mycotoxin levels.
Mycotoxin levels in cottonseed from TAM66274 and control non-transgenic cv. Coker 312. Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Four replicate samples of each treatment at each field site were pooled and analyzed as a single sample.

		Analytes						
		Aflatoxin B1 (ppb)	**Aflatoxin B2 (ppb)**	**Aflatoxin G1 (ppb)**	**Aflatoxin G2 (ppb)**	**DON (ppm)**	**ADON (ppm)**	**ZEA (ppb)**
	LOD	0.7	0.9	0.7	0.8	0.6	0.8	43.1
Sites	**Treatments**							
NC115	TAM66274	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
	Coker 312	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
NC315	TAM66274	<0.7	<0.9	<0.7	<0.8	1.6	<0.8	<43.1
	Coker 312	<0.7	<0.9	<0.7	<0.8	0.8	<0.8	<43.1
MS115	TAM66274	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
	Coker 312	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
MS315	TAM66274	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
	Coker 312	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
TX515	TAM66274	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1
	Coker 312	<0.7	<0.9	<0.7	<0.8	<0.6	<0.8	<43.1

LOD. Limits of detection
DON. Deoxynivalenol
ADON. Acetyldeoxynivalenol
ZEA. Zearalenone

Appendix F.

Materials and Methods of Phenotypic, Agronomic and Ecological Characteristics of TAM66274

The environmental safety of ULGCS event TAM66274 was assessed by evaluating the phenotypic, agronomic and ecological characteristics of TAM66274 relative to non-transgenic control cv. Coker 312. The evaluations included:

A) Seed germination and dormancy characteristics conducted under controlled environment conditions; and
B) Field evaluations of phenotypic, agronomic and ecological characteristics of TAM66274, conducted in U.S. cotton growing regions in 2014 and 2015.

This appendix presents information on production and processing of seed cotton for seed germination studies, as well as the germination methods and statistical methods used to analyze the data. This appendix also presents details of field evaluations of phenotypic, agronomic and ecological characteristics of TAM66274 including field site characteristics, field trial management practices, methods of data collection and data analysis, weather data for each field site, as well as results of evaluations of TAM66274 for individual field sites.

A. Seed Germination and Dormancy Characteristics

Cottonseed Source.

TAM66274 and non-transgenic control cv. Coker 312 were grown in three U.S. locations in 2014 (MS114, Washington County, MS; NC114, Perquimans County, NC; NC214, Perquimans County, NC) and five U.S. locations in 2015 (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX). Field sites were selected as representative of major cotton-growing regions in the United States. Characterization of the seed of TAM66274 and non-transgenic cv. Coker 312 used for planting was based on the documentation of the seed pedigree from the study director and by gel-based, event-specific PCR (Appendix C). The plants were grown under standard agronomic practices in a complete randomized block design with four replicate blocks per location. Details of the field trials and agronomic practices for plant growth and production of cottonseed of each treatment are described in section B of this appendix.

Sample Collection, Handling, Identification, Preparation and Storage.

Seed cotton was hand-harvested from replicated plots of each treatment at each location for a total of 64 samples. Samples were individually packed and shipped to Cotton Incorporated (Cary, NC), where samples were ginned to separate lint and fuzzy seed. Ginned samples were labeled and shipped to Texas A&M University (TAMU, College Station, TX) for processing and analysis. Fuzzy seed samples were individually packed and stored under ambient conditions until analyzed. Fuzzy seed samples were acid delinted by Texas A&M laboratory personnel. Acid delinting was performed using sulfuric acid (97%). Seeds were placed in a plastic beaker with a perforated bottom and partially submerged in an acid bath. The contents were stirred until all lint

was removed from the cottonseed. The beaker was removed from the acid bath and the cottonseeds were rinsed with tap water for approximately 3 minutes. The remaining acid was neutralized by partially submerging the beaker in a lime suspension. Treated cottonseeds were rinsed with tap water, spread on a wire mesh tray, and dried at 49°C for 8 hours. Acid delinted seed of each treatment were thoroughly mixed and one hundred seed of each replicate of each treatment (TAM66274 and non-transgenic cv. Coker 312) were subsampled and stored at ambient temperatures until ready for the laboratory phase of this study.

Germination Assays.

Warm and cool germination assays were conducted on TAM66274 and non-transgenic cv. Coker 312 seed using the following methods adapted from the AOSA Seed Vigor Testing Handbook (AOSA, 2009).

Warm Germination Method. One hundred acid-delinted seeds for each replicate of TAM66274 and non-transgenic cv. Coker 312 (four replicates of each treatment from each of eight field sites) were evenly distributed on two water saturated sheets of non-toxic germination paper. These sheets were then loosely rolled and placed upright in a five-liter plastic beaker with 500 ml water. Each beaker was covered with a perforated plastic bag to maintain humidity and placed in a 30°C incubator. Water was added to the beaker, as needed, to maintain the moisture for the duration of the experiment. On day four, TAM66274 and non-transgenic cv. Coker 312 seed were observed for germination and evaluated in accordance with the AOSA Seedling Evaluation guidelines (AOSA, 2009). For warm germination, normal seedlings were defined as seedlings having a combined hypocotyl and root length of 4 cm (1-10/16 in) or longer. All other germinated seedlings that did not meet these criteria were classified as abnormal, and grouped with the non-germinated cottonseeds.

Cool Germination Method. One hundred acid-delinted seeds for each replicate of TAM66274 and non-transgenic cv. Coker 312 (four replicates of each treatment from each of eight field sites) were evenly distributed on two water saturated sheets of non-toxic germination paper. These sheets were then loosely rolled and placed upright in a five-liter plastic beaker with 500 ml water. Prior to use, all towels had been kept at 18°C for at least 16 hours. Each beaker was covered with a perforated plastic bag to maintain humidity and kept at 18°C for seven days in the dark. The germination of TAM66274 and non-transgenic cv. Coker 312 seed was recorded on day seven. For cool germination, normal seedlings were defined as seedlings having a combined hypocotyl and root length of 4 cm (1-10/16 inch) or longer (AOSA, 2009). All other germinated seedlings that did not meet these criteria were classified as abnormal, and grouped with the non-germinated cottonseeds.

Results were reported as percent normal germinated seed for TAM66274 and non-transgenic cv. Coker 312 at warm and cool temperatures.

% normal germinated seed = [number of normal germinated seed ÷ number of seed assayed] x 100

Statistical Analysis.

In statistics, a *categorical* variable is a variable that can take on one of a limited, and usually fixed, number of possible values, assigning each observation to a particular group or nominal category on the basis of some qualitative property. Survey data by ethnicity or blood type are examples of categorical variables. Statistical analysis of categorical data is ordinarily conducted by determining an over treatment distribution of data, then using Chi-Square analysis to determine if the distribution of individual categorical data differs from the mean of all data. In the case of TAM66274 phenotypic, agronomic, and ecological data, six characteristics (i.e., plant vigor, plant lodging, boll type, disease incidence, insect damage and rodent damage) were assigned a nominal rating on a scale of one to nine where each numerical rating was defined in the test protocol. The ratings were assumed to be scaled proportionally (i.e., the difference between ratings of, for example, one and three are equal to the difference between ratings of seven and nine). Accordingly, calculated means that are not discrete (whole numbers) can be treated the same as numerical (continuous) values. In contrast, a *numerical* variable is one that can be assigned a numerical value, either a discrete (whole) number or continuous (non-integer) numbers. In the case of TAM66274 data, 22 phenotypic, agronomic, and ecological characteristics (i.e., warm germination, cool germination, seedling emergence, stand counts, plant height, days to bloom, total seeds per boll, seed index, line percent, lint yield, seed yield, fiber micronaire, fiber elongation, fiber strength, fiber length, short fiber content, fiber uniformity, total nodes, height to node ratio, total bolls, number of first position bolls, number of second position bolls, final stand count) were measured on a numerical scale.

For the purpose of statistical analysis of TAM66274 field data, a normal distribution was assumed in the underlying population from which sample data were drawn. Sample means, standard error of the mean (S.E.M.), and mean square error (MSE) were calculated and evaluated using a parametric statistical method, analysis of variance (ANOVA) to compare treatment means. Statistical analysis was performed using JMP software (Version 9, 2011) (SAS Institute, Cary NC). The same statistical model was used for all variables (i.e., all data was treated as continuous). Orthogonal contrasts were calculated comparing the two genotypes (TAM66274, non-transgenic cv. Coker 312). Analysis within a single site used a randomized complete block design with four replications and two entries (genotypes). Both replication and genotype were fixed effects. Standard Least Squares was the ANOVA method selected. Within a single site, genotypes were declared different if $P > F$ was ≤ 0.05. The least significant difference between genotypes was calculated by Fisher's Protected Least Significant Difference (LSD) (0.05). All variables were also analyzed across all sites (locations) by a mixed-design model using residual (or restricted) maximum likelihood (REML) with entry and site as random effects and replication

as a fixed effect. Significant differences were declared in the same manner as individual sites using $P > F$ of $\alpha = 0.05$ for contrasts between test and control substances.

B. Field Evaluations of Phenotypic, Agronomic, and Ecological Characteristics of TAM66274

The environmental safety of TAM66274 was assessed by evaluating the phenotypic, agronomic and ecological characteristics of TAM66274 relative to non-transgenic cv. Coker 312. Phenotypic, agronomic and ecological data were collected by field study personnel at three field sites in 2014, one in Mississippi (Washington County, designated as MS114 in the Tables), and two separate field trials in North Carolina (Perquimans County, designated as NC114 and NC214 in the Tables)[1] and at five field sites in 2015, two separate field sites were located in North Carolina (Perquimans County, designated as NC115 and NC315 in the Tables), two in Mississippi (Washington County, designated MS115 and MS315 in the Tables), and one in Texas (Tom Green County, designated as TX515 in the Tables). Field sites were selected as representative of major cotton-growing regions of the United States. Each field trial study was designed as a randomized complete block (RCB) with four replications per treatment.

Plant phenotypic, agronomic and ecological characteristics encompassed six general categories: 1) seedling emergence and stand count; 2) vegetative growth (plant vigor, height and lodging); 3) reproductive development (days to bloom, seeds per boll, seed index [g/100 seed], lint percent, lint yield and seed yield); 4) fiber quality (micronaire, elongation, strength, length, short fiber content and uniformity); 5) plant mapping (total nodes, height to node ratio, total bolls, number of first and second position bolls and boll type); and 6) plant susceptibility to diseases and insect pests, as well as to rodents.

This section of the appendix presents details of field site characteristics, field trial management practices, methods of data collection and data statistical analysis, weather data for each field site, as well as results of field evaluations of phenotypic, agronomic and ecological characteristics of TAM66274 compared to non-transgenic cv. Coker 312 at individual field sites.

Test Materials.

The test substance in this study was seed from genetically engineered cottonseed event TAM66274. For 2014 field studies, the test substance was T5 generation seed of TAM66274, which were homozygous for the low seed-gossypol trait imparted by RNAi construct pART27-

[1] A total of six field trials with TAM66274 and control Coker 312 were planted in 2014. However, two field sites (TX114, Hale County, TX; TX214, Hale County, TX) were terminated early due to weather damage that rendered the field sites impractical to continue. Termination of TX114 and TX214 occurred nine and 14 weeks after planting, respectively. No plant material was harvested from either field site. One field site (MS314, Washington County, MS) was terminated early due to loss of reproductive isolation after inadvertent destruction of border rows. Termination of MS314 occurred seven weeks after planting. No plant material was harvested from this field site.

LCT66. The test substance was grown in a contained greenhouse at TAMU between June - November, 2013 and treated at TAMU prior to delivery of seeds to field study personnel. For 2015 field studies, the test substance was T6 generation seed of TAM66274, which were homozygous for the low seed-gossypol trait. The test substance was grown in a contained greenhouse at TAMU between May - October in 2014 and treated at TAMU prior to delivery of seeds to field study personnel. Initial characterization of the test substance seed for both 2014 and 2015 field studies was by documentation of the seed pedigree from the study director. Primary characterization was conducted during the 2014 and 2015 field studies by a gel-based, event-specific polymerase chain reaction (PCR) method, and details of the method and results are presented in Appendix C.

Control Materials.

The control substance in both 2014 and 2015 field studies was seed of non-transgenic cv. Coker 312, a genetic background identical to the test substance, but which does not contain any T-DNA genetic elements of plasmid pART27-LCT66. For 2014 field studies, the control substance was grown in a contained greenhouse at TAMU between June - November, 2013 and treated at TAMU prior to delivery of seeds to field study personnel. For 2015 field studies, the control substance was grown in a contained greenhouse at TAMU between May - October, 2014 and treated at TAMU prior to delivery to field study personnel. Initial characterization of the control substance seed was verified by documentation of the seed pedigree from the study director. Primary characterization was conducted during the 2014 and 2015 field studies by a gel-based, event-specific polymerase chain reaction (PCR) method, and results are presented in Appendix C.

Phenotypic, Agronomic and Ecological Characteristics.

Forty measurements were made at six in-season stages and at harvest comparing the phenotype, agronomic, and ecological characteristics of TAM66274 to non-transgenic cv. Coker 312, in both 2014 and 2015 field studies. Characteristics were evaluated in replicated plots by field study personnel who were qualified by training and experience with production and evaluation of cotton varieties. Field study personnel recorded raw data in field notebooks. The phenotypic, agronomic and ecological characteristics evaluated in the field studies are presented in Table 7-3 of the petition.

Test System.

In 2014, the test system consisted of three field sites in cotton-growing regions of the United States. One field site was located in Washington County, Mississippi (designated as MS114 in the Tables); and two separate sites in Perquimans County, North Carolina (designated as NC114 and NC214 in the Tables). Characteristics of the three field sites in the test system are described in Table F-1. In 2015, the test system consisted of five field sites in cotton-growing regions of the United States. Two field sites were located in North Carolina (Perquimans County,

designated as NC115 and NC315 in the Tables), two in Mississippi (Washington County, designated MS115 and MS315 in the Tables), and one in Texas (Tom Green County, designated as TX515 in the Tables). Characteristics of the five field sites in the test system are described in Table F-2. Field sites were selected as representative of major cotton-growing regions of the United States. The field study trials were designed as a randomized complete block with four replications per treatment. Test and control substances were planted in 4-row plots (30 - 40 row-ft). Plots were planted at a rate of 420 - 800 seed per plot (3.5 - 5 seed/ft) with row spacing of 38 - 40 inches (48,000 - 65,500 seed/acre).

Table F-1. Location and characteristics of the 2014 field sites.
The test system consisted of three field sites in 2014 representative of major cotton-growing regions of the United States[2]. One field site was located in Washington County, Mississippi (MS114); and two in Perquimans County, North Carolina (NC114 and NC214).

2014 Field Site Information			
Site code	MS114	NC114	NC214
Field scientist	Tyler Horn	Matt Winslow	Matt Winslow
Address	Stoneville R&D 103 Research Road Greenville, MS 38701	Tidewater Agronomics 313 Turnpike Rd Belvidere, NC 27919	Tidewater Agronomics 313 Turnpike Rd Belvidere, NC 27919
County	Washington	Perquimans	Perquimans
Field ID	South Farm Section C5-6	RF1	NG2
GPS Coords	33.31041, -091.12577 33.31081, -091.12546 33.31111, -091.12623 33.31069, -091.12648	36.3300, -076.4849 36.3296, -076.4851 36.3295, -076.4839 36.3291, -076.4843	36.3205, -076.4707 36.3202, -076.4701 36.3196, -076.4706 36.3199, -076.4711
Status	Harvested	Harvested	Harvested
USDA notification	14-057-103n	14-057-103n	14-057-103n

[2] A total of six field trials with TAM66274 and control Coker 312 were planted in 2014. However, two field sites (TX114, Hale County, TX; TX214, Hale County, TX) were terminated early due to weather damage that rendered the field sites impractical to continue. Termination of TX114 and TX214 occurred nine and 14 weeks after planting, respectively. No plant material was harvested from either field site. One field site (MS314, Washington County, MS) was terminated early due to loss of reproductive isolation after inadvertent destruction of border rows. Termination of MS314 occurred seven weeks after planting. No plant material was harvested from this field site.

Table F-1, continued. Location and characteristics of the 2014 field sites.
The test system consisted of three field sites in 2014 representative of major cotton-growing regions of the United States. One field site was located in Washington County, Mississippi (MS114); and two in Perquimans County, North Carolina (NC114 and NC214).

2014 Planting and Harvest Description			
Site code	MS114	NC114	NC214
Planting date	05/24/2014	05/19/2014	05/20/2014
Planting method	4-row cone planter	Air planter	Air planter
Seeding rate	4 seed/ft	3.5 seed/ft	3.5 seed/ft
Depth	0.5 inch	0.67 inch	0.67 inch
Row spacing	38 inches	38 inches	38 inches
Spacing in row	3 inches	3.5 inches	3.5 inches
Seed bed	Rows	Rows	Rows
Soil moisture	Adequate	Good	Good
Harvest date	11/03/2014	10/21/2014	10/21/2014
Harvest width	6.33 feet	6.34 feet	6.34 feet
Border rows	ST4946	PHY499, DP0912	PHY499, DP0912
Site Design			
Plot width	12.67 feet	12.67 feet	12.67 feet
Plot length	40 feet	30 feet	30 feet
Plot area	506.8 sq feet	380 sq feet	380 sq feet
Replications	4	4	4
Study design	RCB*	RCB	RCB
Soil Description			
% Sand	30	88	81
% Silt	57	8	14
% Clay	12	4	5
Texture	Silt loam	Loamy sand	Sandy loam
Soil type	Commerce	Dragston	Arapahoe
Fert level	Good	Good	Good
Drainage	Good	Good	Good

*RCB. Randomized complete block

Table F-2. Location and characteristics of the 2015 field sites.
The test system in 2015 consisted of five field sites representative of major cotton-growing regions of the United States. Field sites were located in three states: Washington County, Mississippi; Perquimans County, North Carolina; and Tom Green County, Texas.

2015 Field Site Information

Site code	MS115	MS315	NC115	NC315	TX515
Field scientist	Tyler Horn	Tyler Horn	Matt Winslow	Matt Winslow	Brandon Ripple
Cooperator	Stoneville R&D 103 Research Rd Greenville, MS 38701	Stoneville R&D 103 Research Rd Greenville, MS 38701	Tidewater Agronomics 313 Turnpike Rd Belvidere, NC 27919	Tidewater Agronomics 313 Turnpike Rd Belvidere, NC 27919	Ripple Ag Research 8346 Ripple Rd San Angelo, TX 76904
County/State	Washington MS	Washington MS	Perquimans NC	Perquimans NC	Tom Green TX
Field ID	South Farm Section C5-6	North Farm Section A1	RF1	NG6	Ripple Ag Blk C
GPS Coords	33.31041, -091.12577 33.31081, -091.12546 33.31111, -091.12623 33.31069, -091.12648	33.44464, -090.99328 33.44463, -090.99300 33.44387, -090.99348 33.44390, -090.99303	36.3300, -076.4849 36.3296, -076.4851 36.3295, -076.4839 36.3291, -076.4843	36.3205, -076.4707 36.3202, -076.4701 36.3196, -076.4706 36.3199, -076.4711	31.37110, -100.27251 31.37092, -100.27174 31.37059, -100.27186 31.37075, -100.27257
Status	Harvested	Harvested	Harvested	Harvested	Harvested
USDA notification	15-054-101n	15-054-101n	15-054-101n	15-054-101n	15-054-101n

Table F-2, continued. Location and characteristics of the 2015 field sites.
The test system consisted of five field sites in 2015 representative of major cotton-growing regions of the United States. Field sites were located in three states: Washington County, Mississippi; Perquimans County, North Carolina; and Tom Green County, Texas. All five field sites were successfully harvested.

2015 Planting and Harvest Description					
Site code	MS115	MS315	NC115	NC315	TX515
Planting date	06/04/2015	06/08/2015	05/20/2015	05/21/2015	06/19/2015
Planting method	4-row cone planter	4-row cone planter	4-row cone planter	4-row cone planter	4-row cone planter
Seeding rate	4 seed/ft	4 seed/ft	4 seed/ft	4 seed/ft	3.5 seed/ft
Depth	0.5 inch	0.5 inch	1.0 inch	1.0 inch	1.5 inches
Row spacing	38 inches	38 inches	38 inches	38 inches	39 inches
Spacing in row	3 inches	4 inches	3 inches	3 inches	3.5 inches
Seed bed	Rows	Rows	Rows	Rows	Rows
Soil moisture	Adequate	Adequate	Good	Good	Good
Harvest date	11/14/2015	11/04/2015	10/26/2015	10/27/2015	12/05/2015
Harvest width	6.33 feet	6.33 feet	6.34 feet	6.34 feet	6.5 feet
Border rows	ST4946	ST4744	PHY499, DP1137	PHY499, DP1137	ST4946
Field Trial Design					
Plot width	12.67 feet	12.67 feet	12.67 feet	12.67 feet	13 feet
Plot length	30 feet	30 feet	30 feet	30 feet	30 feet
Plot area	380 sq feet	380 sq feet	380 sq feet	380 sq feet	390 sq feet
Replications	4	4	4	4	4
Study design	RCB*	RCB	RCB	RCB	RCB
Soil Description					
% Sand	30	31	80	70	17
% Silt	57	60	12	18	36
% Clay	12	7	8	12	47
Texture	Silt loam	Silt loam	Loamy sand	Sandy loam	Clay loam
Soil type	Commerce	Dundee	Dragston	Portsmouth	Angelo
Fert level	Good	Good	Good	Good	Good
Drainage	Good	Good	Good	Good	Good

*RCB. Randomized complete block

Pre-plant Seed Treatment.
The test and control substances were treated with a commercial seed treatment before packaging and shipment to field study personnel. The same pre-plant seed treatments and rates of application were used in both 2014 and 2015 field studies, and the components of the seed treatments are listed in Table F-3.

Maintenance of Field Plots.
Crop maintenance practices typical of cotton production for the region were uniformly applied to all plots of test and control substances. Crop maintenance practices at field sites were recorded by field study personnel in field notebooks and are listed in Table F-4 (2014 field studies) and Table F-5 (2015 field studies). Rainfall and temperatures at field sites in 2014 and 2015 were recorded by field study personnel in field notebooks and are summarized in Table F-6 (2014 field studies) and Table F-7 (2015 field studies).

Disease Susceptibility and Insect Damage Measurements.
Assessments of disease susceptibility and insect damage of TAM66274 and non-transgenic control cv. Coker 312 cotton were made throughout the growing seasons in both 2014 and 2015 field trials. The diseases and insects observed at each assessment are listed in Table F-8 (2014 field trials) and Table F-9 (2015 field trials).

Sample Collection.
Samples were collected by field study personnel from each replicated plot for processing and laboratory studies. Twenty-five bolls (seed and lint only, no burs) were collected by field study personnel from impartially selected, healthy, representative plants in the two middle rows of replicated plots of test and control substances (one 25-boll sample per treatment). After collecting the 25-boll samples, seed cotton samples (6-10 lb) were collected by field study personnel from the two middle rows of each replicated plot of test and control substances (8 total plot samples at each field site). Boll samples were individually packed and shipped at ambient temperatures to TAMU (College Station TX) for processing and analysis. Replicated plot seed cotton samples were individually packed and shipped at ambient temperatures to Cotton Incorporated (Cary NC) for ginning.

Fiber Analysis.
Boll samples were ginned with a roller-gin (Porter Morrison & Son Laboratory Cotton Gin) to separate lint and fuzzy seed. Lint samples were individually packed and shipped at ambient temperatures to Cotton Incorporated (Cary NC) for fiber analysis by High Volume Instrumentation (Uster Technologies, Knoxville TN) according to manufacturer's directions. Fiber measurements included micronaire, length, uniformity, strength, short fiber content and elongation. Seed samples were individually packed and stored at TAMU at ambient temperatures for gossypol analysis at TAMU, and for shipment to Covance Laboratories, Inc. for compositional analyses (refer to Section 6 of the petition and to Appendix E for details).

Statistical Analysis.

Statistical analysis was performed using JMP software (Version 9, 2011) (SAS Institute, Cary NC). The same statistical model was used for all variables. Analysis within a single site used a randomized complete block design with four replications and two entries (genotypes). Both replication and genotype were fixed effects. Within a single site, genotypes were declared different if P > F was ≤ 0.05. The actual probability of difference (α) is listed in tables as "Significance Level." The least significant difference between genotypes was calculated by Fischer's Protected LSD (0.05). Additionally, test substances were directly compared to the control substance using orthogonal analysis. The P > F values for orthogonal contrasts are listed in the same row as the statement of the two entries being contrasted.

All variables were also analyzed across all sites (locations) by a mixed-design model using residual (or restricted) maximum likelihood (REML) with entry and site as random effects and replication as a fixed effect. Significant differences were declared in the same manner as individual sites using P > F of α = 0.05 for orthogonal contrasts between test and control substances. P > F for the interaction of site by genotype is given in tables for each variable. The actual probability of difference (α) was considered significant if ≤ 0.05.

Table F-3. Pre-plant seed treatment.
A commercial seed treatment (Bayer CropScience, Lubbock TX) was applied to the test and control substances before planting the 2014 and 2015 field trials. Composition of the seed treatment is described in the Table. Applications were made by TAMU laboratory personnel prior to packaging and shipment to field study personnel. The seed treatment was applied using a Batch Lab Seed Treater (Seedburro) in 900 g batches according to supplier directions at the rate of 31.2 ml per 1.2 kg of acid-delinted cottonseed.

Component	Quantity	Active Ingredient	Function
Aeris	233.2 ml	Thiodicarb, imidicloprid	AERIS seed-applied insecticide provides protection of cotton seedlings against injury by early season thrips, aphids, lygus, fleahoppers, cutworms and reniform and root knot nematodes.
Vortex 450 FL	85.4 ml	Ipconazole	VORTEX is a systemic broad-spectrum fungicide seed dressing for protection against soilborne and seedborne disease caused by *Rhizoctonia*, *Fusarium*, and *Phomopsis* and provides activity against weakly pathogenic fungi such as *Mucor*, *Rhizopus*, *Aspergillus*, *Penicillium*, *Alternaria*, and *Cladosporium*.
Spera 240 FS	17.3 ml	Myclobutanil	SPERA seed treatment fungicide is recommended to protect against sore shin (*Rhizoctonia solani*) and black root rot (*Thielaviopsis basicola*), which impairs good cottonseed germination and seedling development.
Allegiance FL	7.54 ml	Metalaxyl	ALLEGIANCE FL seed treatment fungicide is a systemic fungicide seed dressing specifically for control of systemic downy mildews, *Pythium*, and *Phytophthora* spp.
Evergol	3.2 ml	Penflufen	EVERGOL seed treatment fungicide is for protection against seed rot and damping-off caused by *Rhizoctonia solani*.
Pro-ized blue colorant	10.42 ml		Colorant applied to discolor treated seed
ColorCoat white	10.02 ml		Colorant applied to discolor treated seed
Seed Gloss 661	60.14 ml		Liquid seed conditioner colorant gloss
Calcium carbonate	57.34 ml		Seed conditioner
Total	484.6 ml		

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Table F-4. Crop maintenance history for 2014 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2014 field locations.

Site Code MS114
Location Washington County, MS

Tillage and Cultural Practices

Date	Practice	Depth
1/23/14	Disked	
1/31/14	Hipped	
5/20/14	Seed bed preparation	
11/4/14	Disked	
11/11/14	Disked	
11/12/14	Sub-soiled	
11/13/14	Disked	
11/14/14	Hipped	

Fertilizer

Date	Formulation	Rate/Acre
6/19/14	Liquid N-sol (32%)	28 gal

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
5/20/14	Cotoran	1 qt	Pre-emerge weeds	Fluometuron
5/20/14	Dual	1 pt	Pre-emerge weeds	Metalochlor
5/20/14	Staple	1.7 fl oz	Pre-emerge weeds	Pryithiobac sodium
5/20/14	Prowl	1 pt	Pre-emerge weeds	Pendimethalin
6/17/14	Bidrin	3.2 fl oz	Thrips	Dicrotophos
6/25/14	Dual	1.25 pt	Post-emerge weed	Metalochlor
6/25/14	Orthene	0.75 lb	Lygus	Acephate
7/03/14	Orthene	0.75 lb	Lygus	Acephate
7/03/14	Mepiquat	10 fl oz	Growth regulator	Mepiquat chloride
7/14/14	MSMA	2.4 pt	Post-emerge weeds	Methanearsonate
7/14/14	Diuron	1 pt	Post-emerge weeds	Diuron
7/15/14	Bidrin	8 fl oz	Tarnished plant bug	Dicrotophos
7/15/14	Baythiroid	2 fl oz	Tarnished plant bug	β-cyfluthrin
7/15/14	Diamond	6 fl oz	Tarnished plant bug	Novaluron
7/15/14	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
7/25/14	Transform	1.5 oz	Plant bugs, aphids	Sulfoxaflor
7/25/14	Karate	2 fl oz	Lygus	Lambda-cyhalothrin
7/25/14	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
8/07/14	Besiege	9 fl oz	Stinkbug, lygus	Lambda-cyhalothrin, chlorantraniliprole
8/09/14	Diamond	6 fl oz	Plant bugs, stinkbug	Novaluron
8/09/14	Orthene	1 lb	Lygus	Acephate
8/09/14	Karate	2 fl oz	Lygus	Lambda-cyhalothrin
8/09/14	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
8/14/14	Besiege	9 fl oz	Cotton bollworm	Lambda-cyhalothrin, chlorantraniliprole
8/14/14	Coragen	7 fl oz	Cotton bollworm	Chlorantraniliprole

Table F-4, continued. Crop maintenance history for 2014 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2014 field locations.

Site Code MS114
Location Washington County, MS

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
8/22/14	Endigo	6 fl oz	Cotton bollworm, Tarnished plant bugs, stinkbug	Lambda-cyhalothrin, thiamethoxam
8/22/14	Transform	1.5 fl oz	Plant bugs, aphids	Sulfoxaflor
8/22/14	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
10/01/14	Dropp	2 fl oz	Defoliant	Thidiazuron
10/01/14	Def	8 fl oz	Defoliant	Tributyl phosphorotrithioate
10/01/14	Prep	1 qt	Growth regulator	Ethephon
10/10/14	Def	1 pt	Defoliant	Tributyl phosphorotrithioate

Table F-4, continued. Crop maintenance history for 2014 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2014 field locations.

Site Code NC114
Location Perquimans County, NC

Tillage and Cultural Practices

Date	Practice	Depth
11/28/13	Disked, cultivated, rowed	6 inch

Fertilizer

Date	Formulation	Rate/Acre
3/28/14	10-15-25	300 lb
6/26/14	17-0-12-19	350 lb
7/12/14	Boron	1 qt
7/30/14	Boron	1 qt

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
4/10/14	Roundup Weather Max	32 oz	Burndown weeds	Glyphosate
4/10/14	Clarity	6 oz	Burndown weeds	Dicamba, diglycolamine salt
4/10/14	Valor	1.75 oz	Burndown weeds	Flumioxazin
5/21/14	Prowl H2O	1.5 pt	Pre-emerge weeds	Pendimethalin
5/21/14	Cotoran	1.5 pt	Pre-emerge weeds	Fluometuron
5/21/14	Roundup Power Max	32 oz	Pre-emerge weeds	Glyphosate
6/04/14	Brawl	1.25 pt	Early-post weeds	Metalochlor
6/04/14	Acephate	12 oz	Thrips	Acephate
6/11/14	Radiant	3 oz	Thrips	Spinetoram
6/14/14	Arrow	10 oz	Grass	Clethodim
6/17/14	Pyrimax	3 oz	Mid-post weeds	Pyrithiobac sodium
7/08/14	Suprend	1.25 lb	Lay-by weeds	Prometryn, trifloxysulfuon sodium
7/08/14	Suprend	1 qt	Lay-by weeds	Prometryn, trifloxysulfuon sodium
7/09/14	Brigadier	6.5 oz	Lygus	Imidicloprid, bifenthrin
7/09/14	Transform	1.5 oz	Lygus	Sulfoxaflor
7/09/14	Mepichlor	12 oz	Growth regulator	Mepiquat chloride
7/12/14	Transform	1.75 oz	Lygus	Sulfoxaflor
7/12/14	Mepiquat	16 oz	Growth regulator	Mepiquat chloride
7/30/14	Sky Raider	6.4 oz	Lygus	Bifenthrin, imidicloprid
7/30/14	Mepiquat	24 oz	Growth regulator	Mepiquat chloride
8/06/14	Belt	2.5 oz	Cotton bollworm	Flubendiamide
8/13/14	Belt	2 oz	Cotton bollworm	Flubendiamide
8/13/14	Bifenthrin	6 oz	Lygus	Bifenthrin
8/13/14	Acephate	8 oz	Lygus	Acephate
8/21/14	Belt	2 oz	Cotton bollworm	Flubendiamide
8/21/14	Karate Z	2.56 oz	Lygus	Lambda cyhalothrin
8/21/14	Centric	2.5 oz	Lygus	Thiamethoxam
10/02/14	Super Boll	1 pt	Conditioner	Ethephon
10/07/14	Super Boll	1 pt	Final defol	Ethephon
10/07/14	Finish	1 pt	Final defol	Ethephon, cyclanilide
10/07/14	Resource	5 oz	Final defol	Flumiclorac pentyl
10/07/14	Free Fall	4 oz	Final defol	Thidiazuron

Table F-4, continued. Crop maintenance history for 2014 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2014 field locations.

Site Code NC214
Location Perquimans County, NC

Tillage and Cultural Practices

Date	Practice	Depth
4/03/14	Disked and cultivated	6 inch
4/04/14	Rowed and rolled	
6/20/14	Hand chopped weeds	

Fertilizer

Date	Formulation	Rate/Acre
3/28/14	Dolomitic lime	1 ton
3/28/14	10-15-25	300 lb
6/26/14	17-0-12-19	290 lb
7/12/14	Boron	1 qt
7/30/14	Boron	1 qt

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
5/22/14	Warrant	2.5 pt	Pre-emerge weeds	Acetochlor
5/22/14	Cotoran	1.75 pt	Pre-emerge weeds	Fluometuron
5/22/14	Roundup Power Max	24 oz	Pre-emerge weeds	Glyphosate
6/04/14	Brawl	1.25 pt	Early-post weeds	Metalochlor
6/04/14	Acephate	12 oz	Thrips	Acephate
6/11/14	Radiant	3 oz	Thrips	Spinetoram
6/17/14	Pyrimax	3 oz	Mid-post weeds	Pyrithiobac sodium
6/24/14	Brigadier	6.5 oz	Lygus	Imidicloprid, bifenthrin
6/25/14	Arrow	12 oz	Grass	Clethodim
7/08/14	Suprend	1.25 lb	Lay-by weeds	Prometryn, trifloxysulfuon sodium
7/08/14	Suprend	1 qt	Lay-by weeds	Prometryn, trifloxysulfuon sodium
7/09/14	Brigadier	6.5 oz	Lygus	Imidicloprid, bifenthrin
7/09/14	Transform	1.5 oz	Lygus	Sulfoxaflor
7/09/14	Mepichlor	12 oz	Growth regulator	Mepiquat chloride
7/12/14	Transform	1.75 oz	Lygus	Sulfoxaflor
7/12/14	Mepiquat	16 oz	Growth regulator	Mepiquat chloride
7/14/14	Sky Raider	6.5 oz	Lygus	Bifenthrin, imidicloprid
7/14/14	Acephate	11 oz	Lygus	Acephate
7/23/14	Sky Raider	6.4 oz	Lygus	Bifenthrin, imidicloprid
7/23/14	Transform	1.5 oz	Lygus	Sulfoxaflor
7/23/14	Mepiquat	20 oz	Growth regulator	Mepiquat chloride
7/30/14	Sky Raider	6.4 oz	Lygus	Bifenthrin, imidicloprid
7/30/14	Mepiquat	24 oz	Growth regulator	Mepiquat chloride
8/06/14	Belt	2.5 oz	Cotton bollworm	Flubendiamide
8/13/14	Belt	2 oz	Cotton bollworm	Flubendiamide
8/13/14	Bifenthrin	6 oz	Lygus	Bifenthrin
8/13/14	Acephate	8 oz	Lygus	Acephate

Table F-4, continued. Crop maintenance history for 2014 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2014 field locations.

Site Code NC214
Location Perquimans County, NC

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
8/13/14	Envoke	0.1 oz	Morning glory	Trifloxysulfuron sodium
8/21/14	Belt	2 oz	Cotton bollworm	Flubendiamide
8/21/14	Karate Z	2.56 oz	Lygus	Lambda cyhalothrin
8/21/14	Centric	2.5 oz	Lygus	Thiamethoxam
10/02/14	Super Boll	1 pt	Conditioner	Ethephon
10/07/14	Super Boll	1 pt	Final defol	Ethephon
10/07/14	Finish	1 pt	Final defol	Ethephon, cyclanilide
10/07/14	Folex	1 pt	Final defol	Tributyl phosphorotrithioate
10/07/14	Free Fall	4 oz	Final defol	Thidiazuron

Table F-5. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code MS115
Location Washington County, MS

Tillage and Cultural Practices

Date	Practice	Depth
6/4/15	Seed bed preparation (do-all)	
12/10/15	Disked	

Fertilizer

Date	Formulation	Rate/Acre
6/25/15	Liquid N-sol (32%)	21 gal

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
2/10/15	Roundup PowerMax	1 qt	Pre-plant burndown	Glyphosate
2/10/15	Firstshot	0.5 oz	Pre-plant burndown	Thifensulfuron-methyl, Tribenuron-methyl
4/22/15	Roundup	1 pt	Pre-emerge weeds	Glyphosate
4/22/15	Liberty	29 fl oz	Pre-emerge weeds	Glufosinate
6/04/15	Gramoxone	3 pt	Post-emerge weeds	Paraquat
6/04/15	Cotoran	1 pt	Post-emerge weeds	Fluometuron
6/04/15	Dual	1.25 pt	Pre-emerge weeds	Metalochlor
6/04/15	Staple	1.7 fl oz	Pre-emerge weeds	Pryithiobac sodium
6/18/15	Bidrin	4 fl oz	Tarnished plant bug	Dicrotophos
6/29/15	TriMax	2 fl oz	Post-emerge weeds	Triclopyr butoxy ethyl ester
6/29/15	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
7/11/15	Transform	1.5 oz	Plant bugs, aphids	Sulfoxaflor
7/11/15	Diamond	6 fl oz	Tarnished plant bug	Novaluron
7/15/15	Karate	2 fl oz	Lygus	Lambda-cyhalothrin
7/15/14	Bidrin	8 fl oz	Tarnished plant bug	Dicrotophos
7/15/15	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
7/15/15	Belt	3 fl oz	Armyworm	Flubendiamide
7/17/15	Linuron	1 pt	Post-emerge weeds	Linuron
7/17/15	MSMA	2.7 pt	Post-emerge weeds	Methanearsonate
7/22/15	Tundra	6.4 fl oz	Thrips	Bifenthrin
7/22/15	Transform	1.5 oz	Plant bugs, aphids	Sulfoxaflor
7/22/15	Diamond	6 fl oz	Tarnished plant but	Novaluron
7/22/15	Mepiquat	1 pt	Growth regulator	Mepiquat chloride
7/22/15	Tracer	3 fl oz	Armyworm	Spinosad
7/30/15	Prevathon	14 fl oz	Cotton bollworm	Chlorantraniliprole
7/30/15	Bidrin	8 fl oz	Tarnished plant bug	Dicrotophos
8/03/15	Tundra	6.4 fl oz	Thrips	Bifenthrin
8/03/15	Orthene	1 lb	Lygus	Acephate
8/03/15	Mepiquat	1 pt	Tarnished plant bug	Dicrotophos
8/13/15	Abamectin	2.5 fl oz	Spider mites, thrips	Abamectin
8/13/15	Mepiquat	1 pt	Tarnished plant bug	Dicrotophos
8/13/15	Endigo	6 fl oz	Cotton bollworm, Tarnished plant bugs, stinkbug	Lambda-cyhalothrin, thiamethoxam

Table F-5. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code MS115
Location Washington County, MS

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
8/25/15	Prevathon	14 fl oz	Cotton bollworm	Chlorantraniliprole
8/25/15	Reveal	6.4 fl oz	Spider mites, thrips	Bifenthrin
8/25/15	Abamectin	2.5 fl oz	Spider mites, thrips	Abamectin
8/25/15	Orthene	1 lb	Lygus	Acephate
8/25/15	Mepiquat	1 pt	Tarnished plant bug	Dicrotophos
10/09/15	Dropp	2.4 fl oz	Defoliant	Thidiazuron
10/09/15	Def	6 fl oz	Defoliant	Tributyl phosphorotrithioate
10/09/15	Prep	42 fl oz	Defoliant	Ethephon

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code MS315
Location Washington County, MS

Tillage and Cultural Practices

Date	Practice	Depth
6/8/15	Seed bed preparation	
6/18/15	Plowed middles	
6/18/15	Irrigation, furrow/rill	2 inch
7/9/15	Plowed middles	
7/21/15	Irrigation, furrow/rill	2 inch
8/3/15	Irrigation, furrow/rill	2 inch
8/8/15	Irrigation, furrow/rill	2 inch
8/28/15	Irrigation, furrow/rill	2 inch
11/13/15	Disked	

Fertilizer

Date	Formulation	Rate/Acre
6/24/15	Liquid N-sol (32%)	21 gal

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
2/11/15	Roundup PowerMax	1 qt	Pre-emerge weeds	Glyphosate
2/11/15	Firstshot	0.5 oz	Pre-plant burndown	Thifensulfuron-methyl, Tribenuron-methyl
5/01/15	Gramoxone	3 pt	Post-emerge weeds	Paraquat
6/03/15	Gramoxone	3 pt	Post-emerge weeds	Paraquat
6/08/15	Gramoxone	3 pt	Post-emerge weeds	Paraquat
6/04/15	Cotoran 4L	1 qt	Post-emerge weeds	Fluometuron
6/08/15	Staple LX	1.7 fl oz	Pre-emerge weeds	Pryithiobac sodium
6/04/15	Dual Magnum EC	21.28 fl oz	Pre-emerge weeds	Metalochlor
6/18/15	Bidrin 8 EC	4 fl oz	Tarnished plant bug	Dicrotophos
6/26/15	Select 2 EC	8 fl oz	Post-emerge weeds	Clethodim
7/09/15	Dual Magnum EC	21.28 fl oz	Pre-emerge weeds	Metalochlor
7/11/15	Transform WG	1.5 oz	Plant bugs, aphids	Sulfoxaflor
7/11/15	Diamond 0.83 EC	6 fl oz	Tarnished plant bug	Novaluron
7/16/15	Compact L	16 fl oz	Growth regulator	Mepiquat chloride
7/16/15	Karate w/Zeon	2 fl oz	Lygus	Lambda-cyhalothrin
7/16/15	Bidrin 8 EC	8 fl oz	Tarnished plant bug	Dicrotophos
7/16/15	Belt SC	3 fl oz	Armyworm	Flubendiamide
7/24/15	Transform WG	1.5 oz	Plant bugs, aphids	Sulfoxaflor
7/24/15	Diamond 0.83 EC	6 fl oz	Tarnished plant bug	Novaluron
7/24/15	Tundra EC	6.4 fl oz	Thrips	Bifenthrin
7/24/15	Tracer	3 fl oz	Armyworm	Spinosad
7/24/15	Compact L	10 fl oz	Growth regulator	Mepiquat chloride
7/29/15	Direx 4L	16 fl oz	Post-emerge weeds	Diuron
7/29/15	MSMA 6 Plus	43 fl oz	Post-emerge weeds	Methanearsonate
8/06/15	Prevathon	14 fl oz	Cotton bollworm	Chlorantraniliprole
8/06/15	Bidrin 8 EC	8 fl oz	Tarnished plant bug	Dicrotophos

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code MS315
Location Washington County, MS

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
8/06/15	Compact L	16 fl oz	Growth regulator	Mepiquat chloride
8/06/15	Tundra EC	6.4 fl oz	Thrips	Bifenthrin
8/12/15	Agri-Mek SC	2.5 fl oz	Spider mites	Abamectin
8/12/15	Compact L	16 fl oz	Growth regulator	Mepiquat chloride
8/12/15	Endigo ZC	6 fl oz	Cotton bollworm, tarnished plant bugs, stinkbug	Lambda-cyhalothrin, thiamethoxam
8/26/15	Prevathon	14 fl oz	Cotton bollworm	Chlorantraniliprole
8/26/15	Orthene	1 lb	Lygus	Acephate
8/26/15	Compact L	16 fl oz	Growth regulator	Mepiquat chloride
8/26/15	Agri-Mek SC	2.5 fl oz	Spider mites	Abamectin
8/26/15	Intruder	2.3 oz	Whitefly	Acetamiprid
10/08/15	Dropp SC	2.4 fl oz	Defoliant	Thidiazuron
10/08/15	Folex	6 fl oz	Defoliant	Tributyl phosphorotrithioate
10/08/15	Boll'd	47 fl oz	Defoliant	Ethephon

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code NC115
Location Perquimans County, NC

Tillage and Cultural Practices

Date	Practice	Depth
6/17/15	Hand weed plot alleys	
7/27/15	Irrigation	0.5 inch
7/30/15	Irrigation	0.5 inch
8/21/15	Irrigation	0.75 inch
10/27/15	Harvest plots	
10/27/15	Mow stubble, trial destruct	

Fertilizer

Date	Formulation	Rate/Acre
4/09/15	10-15-24-8	394 lb
6/30/15	15-0-15	450 lb
7/29/15	Solubor (20.5% boron)	1 lb

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
3/25/15	Roundup PowerMax	32 fl oz	Burndown weeds	Glyphosate
3/25/15	Valor	2 oz	Burndown weeds	Flumioxazin
3/25/15	LI 700		Burndown weeds	Adjuvant
5/08/15	Roundup PowerMax	32 fl oz	Burndown weeds	Glyphosate
5/21/15	Prowl H2O	1.5 pt	Pre-emerge weeds	Pendimethalin
5/21/15	Cotoran	1.5 pt	Pre-emerge weeds	Fluometuron
6/03/15	Dual II Magnum	1.5 pt	Early-post weeds	Metalochlor
6/03/15	Orthene	8 fl oz	Thrips	Acephate
6/17/15	Volunteer	12 oz	Grass	Clethodim
6/17/15	Crop oil concentrate		Grass	Adjuvant
6/18/15	Staple	3.2 fl oz	Mid-post weeds	Pyrithiobac sodium
7/01/15	Bifenthrin	6.4 oz	Lygus	Bifenthrin
7/01/15	Mepex	12 oz	Growth regulator	Mepiquat chloride
7/08/14	Bifenthrin	6.4 oz	Lygus	Bifenthrin
7/08/15	Acephate	8 oz	Lygus	Acephate
7/08/15	Mepstar	12 oz	Growth regulator	Mepiquat chloride
7/08/15	Volunteer	12 oz	Grass	Clethodim
7/08/15	Crop oil concentrate		Grass	Adjuvant
7/09/15	Suprend	1 lb	Lay-by weeds	Prometryn, trifloxysulfuon sodium
7/09/15	MSMA	1 qt	Lay-by weeds	Methanearsonate
7/22/15	Sky Raider	6.4 oz	Lygus	Bifenthrin, imidicloprid
7/22/15	Transform	1.5 oz	Aphids	Sulfoxaflor
7/22/15	Mepstar	16 oz	Growth regulator	Mepiquat chloride
7/22/15	Belt	2 oz	Cotton bollworm	Flubendiamide
7/29/15	Belt	1 oz	Cotton bollworm	Flubendiamide
8/10/15	Besiege	8 oz	Lygus	Lambda-cyhalothrin, chlorantraniliprole

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code NC115
Location Perquimans County, NC

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
8/10/15	Karate Z	1 oz	Lygus	Lambda-cyhalothrin
9/23/15	Resource	4 oz	Final defol	Flumiclorac pentyl
9/23/15	Free Fall	6 oz	Final defol	Thidiazuron
9/23/15	Finish	1 qt	Final defol	Ethephon
9/23/15	Prep	1 pt	Final defol	Ethephon
9/23/15	80/20		Final defol	Surfactant

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code NC315
Location Perquimans County, NC

Tillage and Cultural Practices

Date	Practice	Depth
6/05/15	Hand weed plot alleys	
6/24/15	Hand weed plots	
7/09/15	Hand weed plot alleys	

Fertilizer

Date	Formulation	Rate/Acre
4/09/15	10-15-24-8	394 lb
6/30/15	15-0-15	300 lb
7/29/15	Solubor (20.5% boron)	1 lb

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
4/06/15	Roundup PowerMax	32 fl oz	Burndown weeds	Glyphosate
4/06/15	Valor	2 oz	Burndown weeds	Flumioxazin
4/06/15	Backdrop	2 gal	Burndown weeds	Adjuvant
4/06/15	LI 700		Burndown weeds	Adjuvant
5/14/15	Liberty	40 oz	Early-post weeds	Glufosinate
5/14/15	80/20		Early-post weeds	Surfactants
5/21/15	Cotoran	3 pt	Pre-emerge weeds	Fluometuron
5/21/14	Roundup Power Max	24 fl oz	Pre-emerge weeds	Glyphosate
5/21/15	Warrant	2 pt	Pre-emerge weeds	Acetochlor
5/21/15	Faststrike		Pre-emerge weeds	Adjuvant
6/03/15	Dual II Magnum	1.5 pt	Early-post weeds	Metalochlor
6/03/15	Orthene	8 fl oz	Thrips	Acephate
6/08/15	Select	8 fl oz	Post-emerge weeds	Clethodim
6/08/15	Acephate	12 oz	Thrips	Acephate
7/01/15	Bifenthrin	6.4 oz	Lygus	Bifenthrin
7/02/15	Mepex	12 oz	Growth regulator	Mepiquat chloride
7/02/15	Bifenthrin	6.4 oz	Lygus	Bifenthrin
7/08/15	Bifenthrin	6.4 oz	Lygus	Bifenthrin
7/08/15	Acephate	8 oz	Lygus	Acephate
7/08/15	Mepstar	12 oz	Growth regulator	Mepiquat chloride
7/13/15	Sky Raider	6.4 oz	Lygus	Bifenthrin, imidicloprid
7/13/15	Pix WG	16 oz	Growth regulator	Mepiquat chloride
7/22/15	Sky Raider	6.4 oz	Lygus	Bifenthrin, imidicloprid
7/22/15	Transform	1.5 oz	Aphids	Sulfoxaflor
7/22/15	Mepstar	16 oz	Growth regulator	Mepiquat chloride
7/22/15	Belt	2 oz	Cotton bollworm	Flubendiamide
7/29/15	Belt	1 oz	Cotton bollworm	Flubendiamide
8/03/15	Bifenthrin	6.4 oz	Lygus	Bifenthrin
8/03/15	Belt	1 oz	Cotton bollworm	Flubendiamide
8/03/15	Transform	1 oz	Plant bugs, aphids	Sulfoxaflor
8/03/15	Siltrate	10 gm	Plant bugs, aphids	Adjuvant

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code NC315
Location Perquimans County, NC

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
8/10/15	Besiege	8 oz	Lygus	Lambda-cyhalothrin, chlorantraniliprole
8/10/15	Karate Z	1 oz	Lygus	Lambda cyhalothrin
8/20/15	Bifenthrin	6.4 oz	Lygus	Bifenthrin
8/20/15	Belt	2 oz	Cotton bollworm	Flubendiamide
8/20/15	Approach Prima	6 oz	Leaf spot	Picoxystrobin, cyproconazole
10/08/15	Resource	6 oz	Final defol	Flumiclorac pentyl
10/08/15	Free Fall	6 oz	Final defol	Thidiazuron
10/08/15	Prep	1.5 pt	Final defol	Ethephon
10/08/15	Finish	1.5 pt	Final defol	Ethephon, cyclanilide
10/08/15	Methylated soybean oil	1 pt / 100 gal	Final defol	Adjuvant

Table F-5, continued. Crop maintenance history for 2015 field sites.
Crop maintenance practices consistent with commercial cotton production practices were uniformly applied to all plots of test and control substances for the 2015 field locations.

Site Code TX515
Location Tom Green County, TX

Tillage and Cultural Practices

Date	Practice	Depth
5/28/15	Disc pre-emerge herbicide	
7/15/15	Hand weed plots	
7/15/15	Cultivated trial site (sweep)	
7/17/15	Furrow irrigation	2 inches
7/28/15	Furrow irrigation	2 inches
8/08/15	Furrow irrigation	2 inches
12/22/15	Shred trial site	

Fertilizer

Date	Formulation	Rate/Acre
5/28/15	Fertilizer 17-17-0-5	20 gal

Agro-Chemicals

Date	Treatment	Rate/Acre	Target Pest	Active Ingredient
5/28/15	Trifluralin HF	1.33 pt	Pre-emerge weeds	Trifluralin
6/19/15	Topguard Terra	8 fl oz	Root rot (plots)	Flutriafol
6/20/15	Topguard Terra	8 fl o	Root rot (borders)	Flutriafol
11/12/15	Ginstar EC	6.5 oz	Final defol	Thidiazuron, diuron
11/12/15	Finish 6 Pro	16 oz	Final defol	Ethephon, cyclanilide
11/12/15	Induce	3.2 fl oz	Final defol	Adjuvant

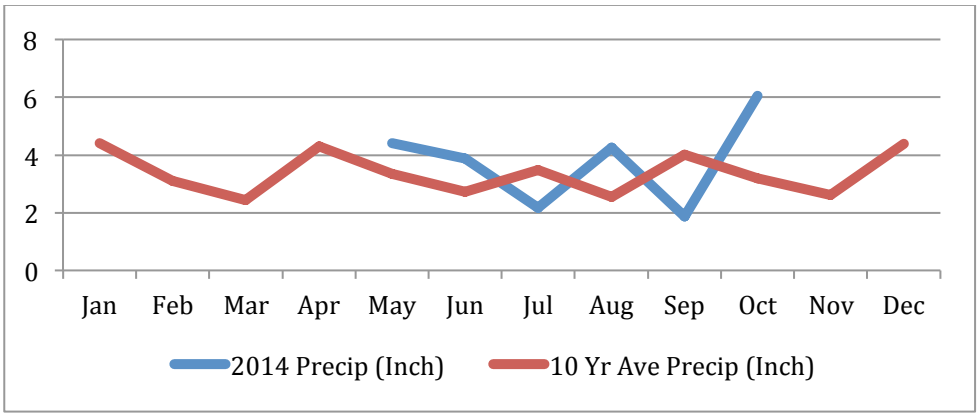
Table F-6. Weather data for 2014 field sites.
Temperature and precipitation data were collected at three field sites during the 2014 growing season (MS115, Washington County MS; NC114, Perquimans County, NC; and NC214, Perquimans County, NC) and are presented below. Data is compared to the 15 year averages to evaluate plant responses to abiotic stresses during the 2014 growing season.

Site Code MS114
Location Washington County, MS

2014 Temp (°F)	*	*	*	*	74	81	79	80	77	67	*	*
10 Yr Ave Temp (°F)	46	57	58	66	74	81	83	83	77	66	55	46
2014 Precip (inch)	*	*	*	*	4.4	3.9	2.2	4.3	1.9	6.1	*	*
10 Yr Ave Precip (inch)	4.4	3.1	2.4	4.3	3.4	2.7	3.5	2.6	4.0	3.2	2.6	4.4
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

(*) data reported for experimental phase only

2014 Precipitation vs. 10 Year Average Precipitation



2014 Mean Air Temperature vs. 10 Year Average Air Temperature

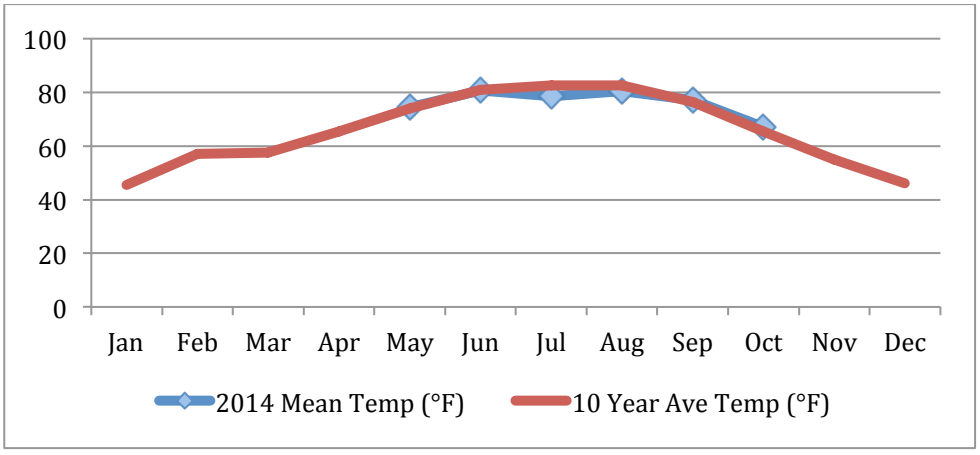


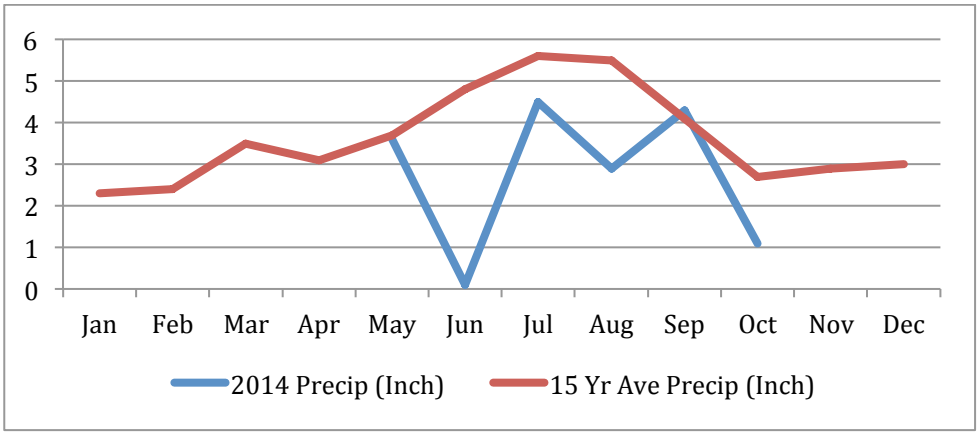
Table F-6, continued. Weather data for 2014 field sites.
Temperature and precipitation data were collected at three field sites during the 2014 growing season (MS115, Washington County MS; NC114, Perquimans County, NC; and NC214, Perquimans County, NC) and are presented below. Data is compared to the 15 year averages to evaluate plant responses to abiotic stresses during the 2014 growing season.

Site Code NC114, NC214
Location Perquimans County, NC

2014 Temp (°F)	*	*	*	*	70	75	77	76	74	65	*	*
15 Yr Ave Temp (°F)	42	45	51	60	68	75	80	79	73	62	53	45
2014 Precip (inch)	*	*	*	*	3.6	0.1	4.5	2.9	4.3	1.1	*	*
15 Yr Ave Precip (inch)	2.3	2.4	3.5	3.1	3.7	4.8	5.6	5.5	4.1	2.7	2.9	3.0
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

(*) data reported for experimental phase only

2014 Precipitation vs. 15 Year Average Precipitation



2014 Mean Air Temperature vs. 15 Year Average Air Temperature

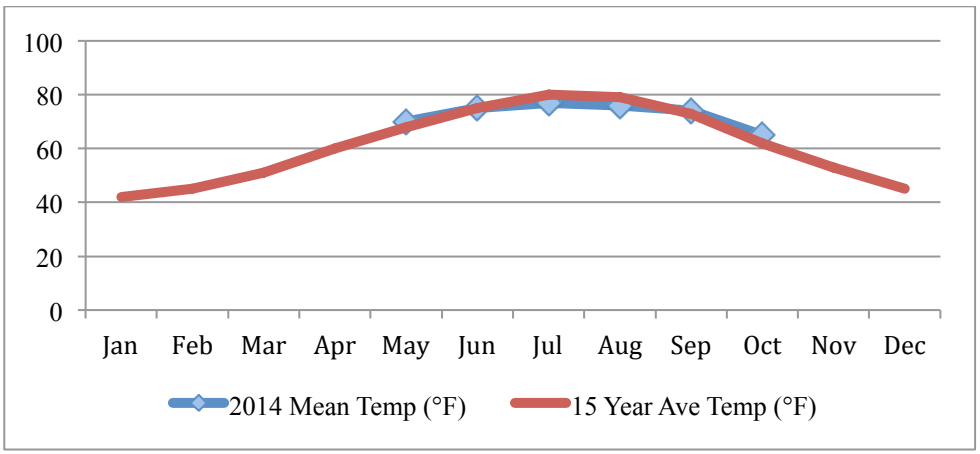


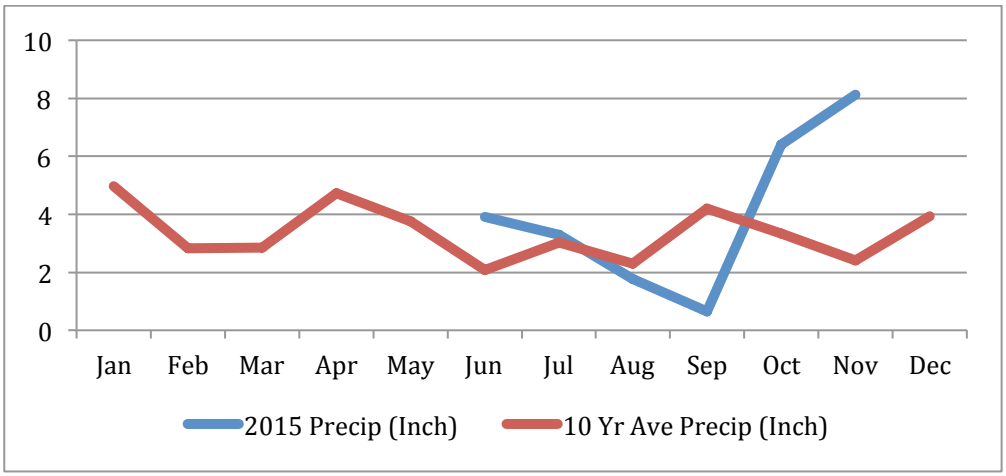
Table F-7. Weather data for 2015 field sites.
Temperature and precipitation data were collected at five field sites during the 2015 growing season (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) and are presented below. Data is compared to 10 year averages to evaluate plant response to abiotic stresses during the 2015 growing season.

Site Code MS115
Location Washington County, MS

2015 Temp (°F)	*	*	*	*	*	80	84	82	78	68	54	*
10 Yr Ave Temp (°F)	45	48	57	66	74	81	82	83	77	65	54	47
2015 Precip (inch)	*	*	*	*	*	3.9	3.3	1.8	0.7	6.4	8.1	*
10 Yr Ave Precip (inch)	5.0	2.8	2.8	4.7	3.8	2.1	3.0	2.3	4.2	3.3	2.4	3.9
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

(*) data reported for experimental phase only

2015 Precipitation vs. 10 Year Average Precipitation



2015 Mean Air Temperature vs. 10 Year Average Air Temperature

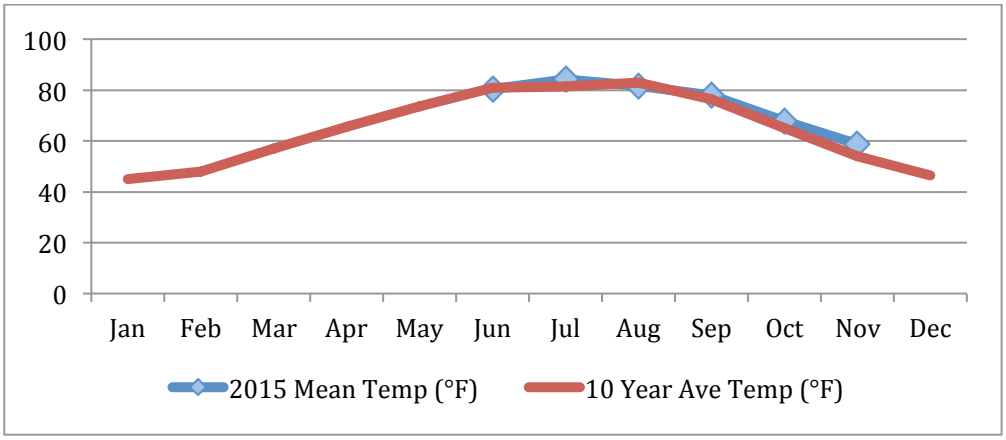


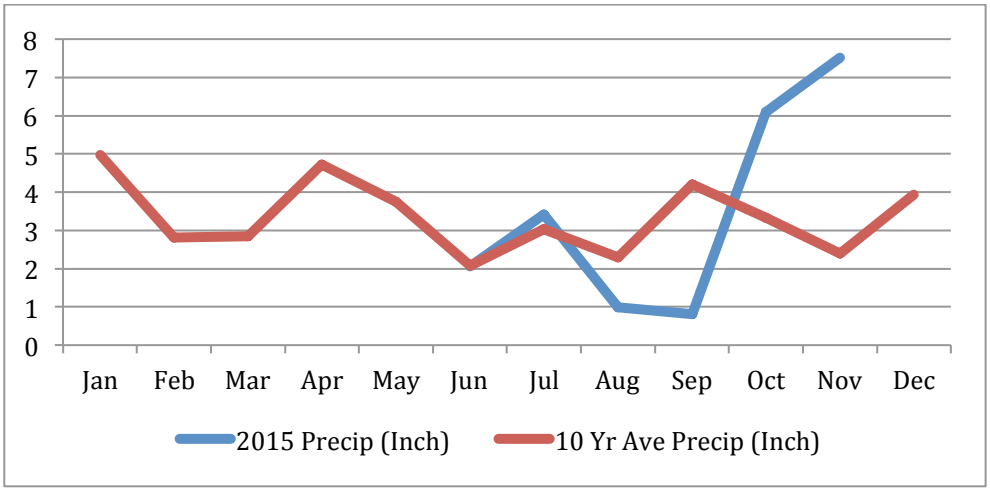
Table F-7, continued. Weather data for 2015 field sites.
Temperature and precipitation data were collected at five field sites during the 2015 growing season (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) and are presented below. Data is compared to 10 year averages to evaluate plant response to abiotic stresses during the 2015 growing season.

Site Code MS315
Location Washington County, MS

2015 Temp (°F)	*	*	*	*	*	80	84	82	78	68	54	*
10 Yr Ave Temp (°F)	45	48	57	66	74	81	82	83	77	65	54	47
2015 Precip (inch)	*	*	*	*	*	3.9	3.3	1.8	0.7	6.4	8.1	*
10 Yr Ave Precip (inch)	5.0	2.8	2.8	4.7	3.8	2.1	3.0	2.3	4.2	3.3	2.4	3.9
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

(*) data reported for experimental phase only

2015 Precipitation vs. 15 Year Average Precipitation



2015 Mean Air Temperature vs. 15 Year Average Air Temperature

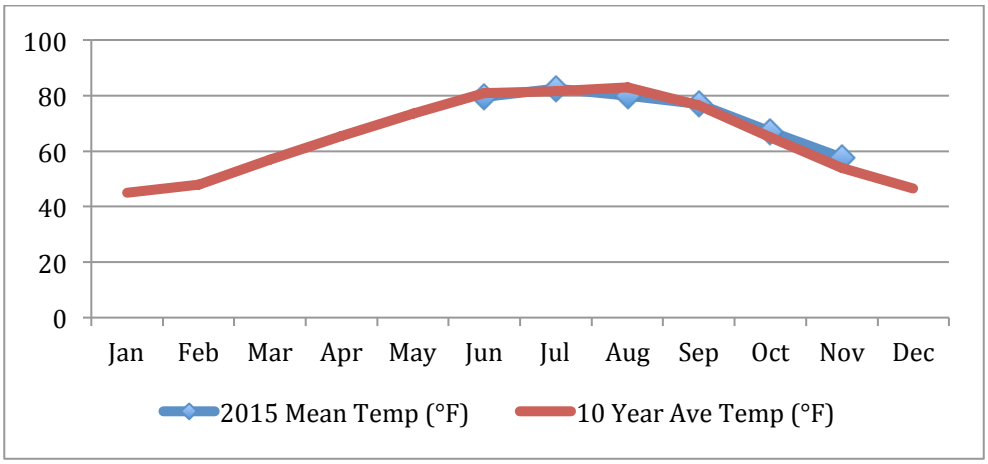


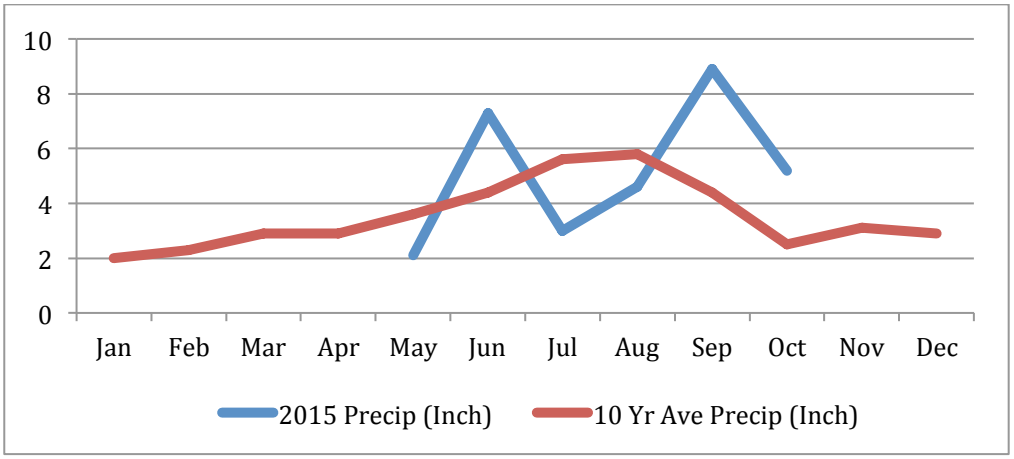
Table F-7, continued. Weather data for 2015 field sites.
Temperature and precipitation data were collected at five field sites during the 2015 growing season (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) and are presented below. Data is compared to 10 year averages to evaluate plant response to abiotic stresses during the 2015 growing season.

Site Code NC115, NC315
Location Perquimans County, NC

2015 Temp (°F)	*	*	*	*	69	78	79	86	76	63	*	*
10 Yr Ave Temp (°F)	43	44	50	60	68	77	80	79	73	63	53	47
2015 Precip (inch)	*	*	*	*	2.1	7.3	3.0	4.6	8.9	5.2	*	*
10 Yr Ave Precip (inch)	2.0	2.3	2.9	2.9	3.6	4.4	5.6	5.8	4.4	2.5	3.1	2.9
Month	Jan	Feb	Mar	Apr	Mau	Jun	Jul	Aug	Sep	Oct	Nov	Dec

(*) data reported for experimental phase only

2015 Precipitation vs. 15 Year Average Precipitation



2015 Mean Air Temperature vs. 15 Year Average Air Temperature

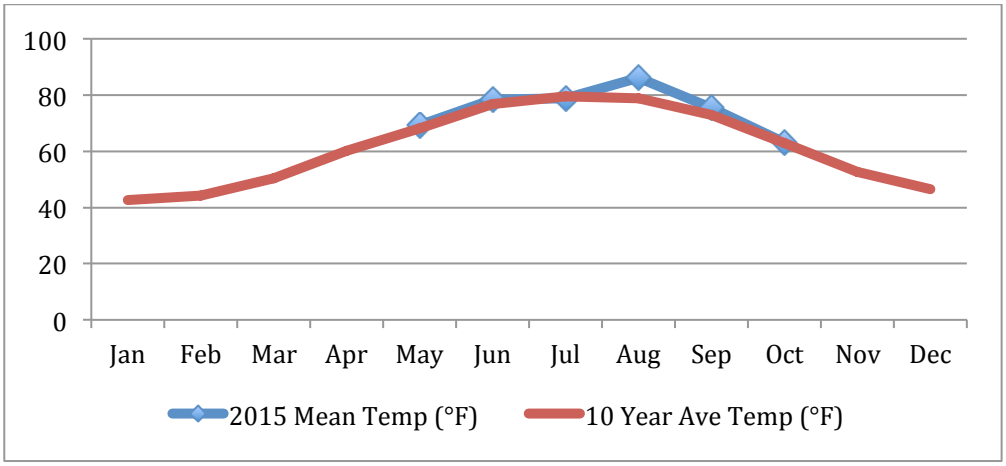


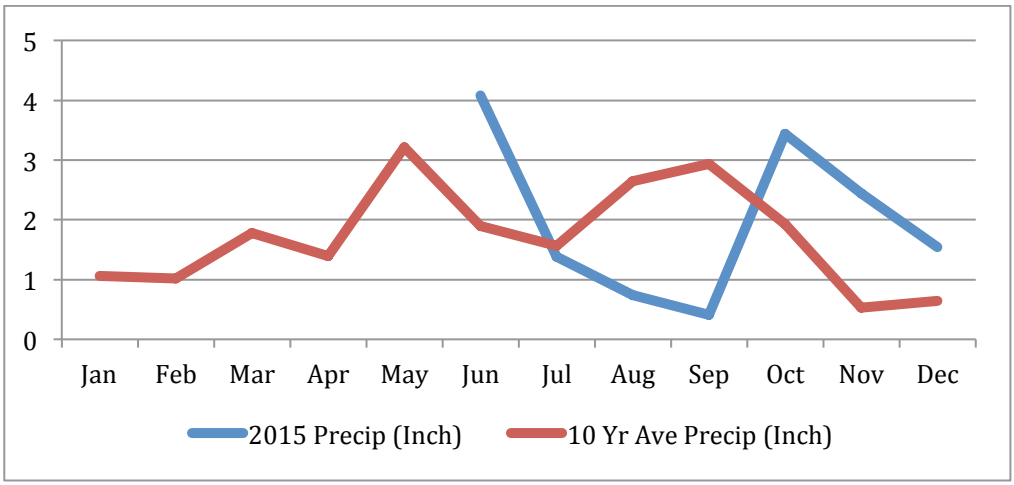
Table F-7, continued. Weather data for 2015 field sites.
Temperature and precipitation data were collected at five field sites during the 2015 growing season (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) and are presented below. Data is compared to 10 year averages to evaluate plant response to abiotic stresses during the 2015 growing season.

Site Code TX515
Location Tom Green County, TX

2015 Temp (°F)	*	*	*	*	*	81	85	100	81	70	57	65
10 Yr Ave Temp (°F)	47	51	60	68	75	83	84	84	77	67	57	48
2015 Precip (inch)	*	*	*	*	*	4.1	1.4	0.7	0.4	3.4	2.4	1.5
10 Yr Ave Precip (inch)	1.1	1.0	1.8	1.4	3.2	1.9	1.6	2.7	2.9	1.9	0.5	0.6
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

(*) data reported for experimental phase only

2015 Precipitation vs. 15 Year Average Precipitation



2015 Mean Air Temperature vs. 15 Year Average Air Temperature

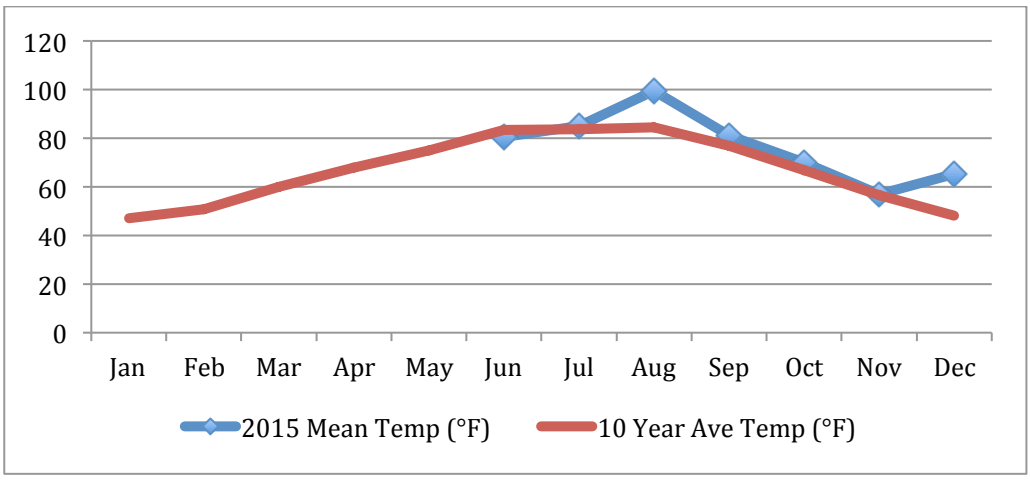


Table F-8. Diseases and insects observed in 2014 field trials.
Diseases and insects observed at three field sites in 2014 (MS114, Washington County, MS; NC114, Perquimans County, NC; NC214, Perquimans County, NC) are presented below. Field study personnel applied crop protection chemicals to all plots to maximize yield.

Site Code MS114
Location Washington County, MS

Date	Crop Stage	Diseases Present	Insects Present
05/28/2014	7 DAP	*	*
06/03/2014	14 DAP	None observed	Thrips
06/20/2014	28 DAP	None observed	Thrips
07/16/2014	56 DAP / Bloom	None observed	Tarnished plant bug
08/15/2014	84 DAP	Leaf spot	Cotton bollworm
09/15/2014	112 DAP	Leaf spot	Cotton bollworm, tarnished plant bug, stinkbug
10/20/2014	Harvest	*	*

Site Code NC114
Location Perquimans County, NC

Date	Crop Stage	Diseases Present	Insects Present
05/29/2014	7 DAP	*	*
06/04/2014	14 DAP	None observed	None observed
06/17/2014	28 DAP	None observed	Thrips
07/17/2014	56 DAP / Bloom	None observed	Spider mites, lygus
08/15/2014	84 DAP	Leaf spot	Lygus, stinkbug
09/15/2014	112 DAP	Leaf spot, boll rot	Stinkbug
10/20/2014	Harvest	*	*

Site Code NC214
Location Perquimans County, NC

Date	Crop Stage	Diseases Present	Insects Present
05/29/2014	7 DAP	*	*
06/04/2014	14 DAP	None observed	None observed
06/17/2014	28 DAP	None observed	Thrips
07/17/2014	56 DAP / Bloom	None observed	None observed
08/15/2014	84 DAP	None observed	None observed
09/15/2014	112 DAP	Leaf spot, boll rot	Stinkbug
10/20/2014	Harvest	*	*

(*) Observation for disease or insects not performed at this time point
DAP, Days after planting.

Table F-9. Diseases and insects observed in 2015 field trials.
Diseases and insects observed by field study personnel at five field sites in 2015 (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) are presented below. Field study personnel applied crop protection chemicals to all plots to maximize yield.

Site Code: MS115
Location Washington County, MS

Date	Crop Stage	Diseases Present	Insects Present
	7 DAP	*	*
06/18/15	14 DAP	None observed	Thrips
07/06/15	28 DAP	None observed	Thrips
07/30/15	56 DAP / Bloom	None observed	Cotton bollworm, tarnished plant bugs
08/27/15	84 DAP	None observed	Spider mite
09/24/15	112 DAP	None observed	Cotton bollworm
	Harvest	*	*

Site Code MS315
Location Washington County, MS

Date	Crop Stage	Diseases Present	Insects Present
	7 DAP	*	*
06/22/15	14 DAP	None observed	Tarnished plant bugs
07/09/15	28 DAP	None observed	Tarnished plant bugs, aphids
08/03/15	56 DAP / Bloom	None observed	Cotton bollworm, plant bugs, thrips
09/02/15	84 DAP	None observed	Cotton bollworm, lygus, spider mites
09/30/15	112 DAP	None observed	Cotton bollworm, lygus, spider mites
	Harvest	*	*

(*) Observation for disease or insects not performed at this time point.
DAP, Days after planting.

Table F-9, continued. Diseases and insects observed in 2015 field trials.
Diseases and insects observed by field study personnel at five field sites in 2015 (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) are presented below. Field study personnel applied crop protection chemicals to all plots to maximize yield.

Site Code NC115
Location Perquimans County, NC

Date	Crop Stage	Diseases Present	Insects Present
	7 DAP	*	*
06/04/15	14 DAP	None observed	Thrips
06/17/15	28 DAP	None observed	None observed
07/14/15	56 DAP / Bloom	None observed	Lygus, stinkbug, spider mite
08/13/15	84 DAP	Leaf spot	Lygus, stinkbug, spider mite
09/11/15	112 DAP	Leaf spot	Stinkbug
	Harvest	*	*

Site Code NC315
Location Perquimans County, NC

Date	Crop Stage	Diseases Present	Insects Present
	7 DAP	*	*
06/04/15	14 DAP	None observed	None observed
06/17/15	28 DAP	None observed	None observed
07/14/15	56 DAP / Bloom	None observed	Lygus, stinkbug
08/13/15	84 DAP	Leaf spot	Lygus, spider mite, stinkbug
09/17/15	112 DAP	Leaf spot	Stinkbug
	Harvest	*	*

(*) Observation for disease or insects not performed at this time point.
DAP, Days after planting.

Table F-9, continued. Diseases and insects observed in 2015 field trials.
Diseases and insects observed by field study personnel at five field sites in 2015 (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX) are presented below. Field study personnel applied crop protection chemicals to all plots to maximize yield.

Site Code TX515
Location Tom Green County, TX

Date	Crop Stage	Diseases Present	Insects Present
	7 DAP	*	*
07/03/15	14 DAP	None observed	None observed
07/17/15	28 DAP	None observed	Aphids
08/13/15	56 DAP / Bloom	None observed	Aphids
09/10/15	84 DAP	None observed	None observed
10/09/15	112 DAP	None observed	None observed
	Harvest	*	*

(*) Observation for disease or insects not performed at this time point.
DAP, Days after planting.

Results of Phenotypic, Agronomic and Ecological Characterizations for Each Field Site in 2014 and 2015.

The ULGCS event TAM66274 and non-transgenic cv. Coker 312 were planted in three U.S. field sites in 2014 and five U.S. field sites in 2015. Phenotypic, agronomic and ecological data were collected by field study personnel at the three field sites in 2014 (MS114, Washington County, MS; NC114, Perquimans County, NC; NC214, Perquimans County, NC) and the five field sites in 2015 (MS115, Washington County, MS; MS315, Washington County, MS; NC115, Perquimans County, NC; NC315, Perquimans County, NC; TX515, Tom Green County, TX). All plants in the field trials were grown to maturity and trials successfully completed.

The field trials were monitored from stand establishment through harvest by agronomists experienced in cotton production and research. Forty phenotypic, agronomic and ecological characteristics were evaluated comparing TAM66274 to non-transgenic cv. Coker 312. Data collection encompassed six general categories: 1) seedling emergence and plant stand; 2) vegetative growth; 3) reproductive development; 4) fiber quality; 5) plant mapping; and 6) plant susceptibility to diseases and insects, and rodent damage. The collected data was subjected to statistical analyses collected for the treatments at each field site to detect significant differences between TAM66274 and non-transgenic cv. Coker 312 ($P<0.05$), and these results are presented below in Tables F-10 – F-18.

Table F-10. Plant stand.
Comparison of plant stand of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Plant stand values for each treatment are presented as the means of four replicated plots of each treatment at each field site.

		Plant Stand		
		7 DAP	**14 DAP**	**Harvest**
Site	**Treatments**	**2014 Studies** (Means)		
NC114	Coker 312	90.5	89.8	87.3
	TAM66274	93.5	90.8	90.3
NC214	Coker 312	90.1	89.5	85.9
	TAM66274	88.1	84.6	84.3
MS114	Coker 312	108.1	109.5	107.5
	TAM66274	108.9	108.7	107.5
Site	**Treatments**	**2015 Studies** (Means)		
NC115	Coker 312	97.6	97.0	87.1
	TAM66274	101.5	100.9	87.9
NC315	Coker 312	90.4	94.9	81.5
	TAM66274	94.5 †	93.3	81.8
MS115	Coker 312	101.8	109.4	105.0
	TAM66274	105.6	107.8	113.4 †
MS315	Coker 312	82.3	95.4	90.8
	TAM66274	78.3	87.5	83.6
TX515	Coker 312	74.4	74.9	74.4
	TAM66274	71.9	71.5	72.1

* Stand count was the number of emerged plants in two rows, standardized to 30 ft rows.
† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-11. Plant vigor.
Comparison of plant vigor of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Plant vigor values are presented as the means of four replicated plots of each treatment at each field site.

		Plant Vigor*		
		28 DAP (1-9 scale)	**56 DAP** (1-9 scale)	**84 DAP** (1-9 scale)
Site	**Treatments**	**2014 Studies** (Means)		
NC114	Coker 312	8.0	7.0	7.8
	TAM66274	8.0	7.0	7.5
NC214	Coker 312	7.0	7.0	7.5
	TAM66274	7.0	7.0	7.5
MS114	Coker 312	8.3	9.0	9.0
	TAM66274	8.3	9.0	9.0
Site	**Treatments**	**2015 Studies** (Means)		
NC115	Coker 312	7.0	7.0	7.0
	TAM66274	6.0 †	6.9	7.0
NC315	Coker 312	6.8	7.0	7.0
	TAM66274	6.0 †	7.0	7.0
MS115	Coker 312	8.0	9.0	9.0
	TAM66274	8.0	9.0	9.0
MS315	Coker 312	7.5	8.0	7.8
	TAM66274	6.9	8.0	7.8
TX515	Coker 312	7.0	7.6	7.6
	TAM66274	7.0	7.9	7.9

* Plant vigor was rated on a 1-9 scale: 1 = short plants with small leaves, 9 = tall plants with robust leaves.
† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-12. Plant height and lodging.
Comparison of plant height and lodging of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Values are presented as the means of four replicated plots of each treatment at each field site.

		Plant Height*				**Lodging**
		28 DAP (inches)	**56 DAP** (inches)	**84 DAP** (inches)	**Harvest** (inches)	**Harvest** (1-9 scale)
Site	**Treatment**	**2014 Studies** (Means)				
NC114	Coker 312	4.9	28.1	35.9	35.5	2.3
	TAM66274	4.8	28.0	35.3	35.0	2.0
NC214	Coker 312	4.4	27.6	34.3	34.0	1.3
	TAM66274	4.1	26.7	34.2	34.0	1.3
MS114	Coker 312	8.2	25.6	51.0	50.8	1.3
	TAM66274	7.5	24.9	49.8	51.2	1.3
Site	**Treatment**	**2015 Studies** (Means)				
NC115	Coker 312	8.7	27.4	34.5	33.0	0.8
	TAM66274	7.4 †	24.4 †	33.9	33.1	1.0
NC315	Coker 312	7.4	27.6	34.1	33.1	1.0
	TAM66274	6.7 †	26.7 †	32.8	32.2	1.0
MS115	Coker 312	18.9	34.9	41.0	40.2	1.6
	TAM66274	17.4	31.7 †	37.3 †	37.6	1.6
MS315	Coker 312	12.2	30.9	39.8	38.6	1.6
	TAM66274	10.5	30.6	36.8	36.6	1.4
TX515	Coker 312	10.1	28.4	29.2	27.9	1.4
	TAM66274	9.7	28.3	30.5	29.7	1.4

* Plant height was measured as the distance in inches from the cotyledon leaf scar to the tip of the terminal meristem. Plant lodging was rated on a 1-9 scale: 1 = plants fully upright, 5 = plants leaning 45 degrees from ground, 9 = plants laying on soil surface.

† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-13. Reproductive development.
Comparison of reproductive development of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Reproductive development values are presented as the means of four replicated plots of each treatment at each field site.

		Reproductive Development*					
		Days to Bloom	**Seeds per Boll**	**Seed Index** (g/100 seed)	**Lint Percent**	**Lint Yield** (lb/A)	**Seed Yield** (lb/A)
Site	**Treatments**	**2014 Studies** (Means)					
NC114	Coker 312	57.3	25.48	12.38	41.85	1612	2239
	TAM66274	57.8	19.88 †	11.95	42.55	1592	2144
NC214	Coker 312	57.0	29.08	8.45	40.94	1577	2274
	TAM66274	57.3	29.07	8.75	42.04	1572	2164
MS114	Coker 312	55.5	27.48	9.45	36.94	949	1620
	TAM66274	55.5	29.82	9.70	37.13	853	1442
Site	**Treatments**	**2015 Studies** (Means)					
NC115	Coker 312	54.3	27.03	10.63	40.58	1154	1689
	TAM66274	54.5	25.53	9.73	38.88 †	1175	1842 †
NC315	Coker 312	54.8	27.50	10.13	40.98	1233	1776
	TAM66274	54.8	24.96	9.85	39.50 †	1250	1914
MS115	Coker 312	46.0	25.48	12.38	39.29	1484	2293
	TAM66274	46.0	19.88 †	11.95	37.58 †	1235 †	2052
MS315	Coker 312	50.3	22.62	12.48	37.10	1462	2476
	TAM66274	50.3	25.86 †	11.65	35.04 †	1424	2639
TX515	Coker 312	48.3	23.79	11.93	35.68	535	965
	TAM66274	50.0	20.19	11.78	32.33 †	469	983

* Days to bloom were the number of days after planting to the appearance of five white flowers in two rows. Seeds per boll were the average number of mature seeds per boll in a 25-boll sample. Seed index was the mass of 100 ginned, fuzzy seed from a 25-boll sample. Lint percent was determined by dividing lint weight by weight of seed cotton hand harvested from two rows. Yields (lb/A) were calculated based on the weight of seed cotton hand harvested from two rows, standardized to one acre.
† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-14. Fiber quality.
Comparison of fiber quality of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Values are presented as the means of four replicated plots of each treatment at each field site. Fiber quality was measured by HVI instrumentation calibrated using USDA-AMS fiber samples.

		Fiber Quality					
		Micronaire (mic units)	**Elongation** (%)	**Strength** (g/tex)	**UHM Length** (inch)	**Short Fiber Content** (%)	**Uniformity** (%)
Site	**Treatments**	**2014 Studies** (Means)					
NC114	Coker 312	4.08	5.20	27.60	1.195	6.90	85.75
	TAM66274	4.40	5.33	28.05	1.143 †	6.80	85.18
NC214	Coker 312	3.71	5.13	28.80	1.188	7.25	85.48
	TAM66274	4.11	5.20	28.30	1.125 †	6.68 †	85.83
MS114	Coker 312	3.99	4.58	28.25	1.215	6.98	85.53
	TAM66274	4.53 †	4.85	29.73	1.223	6.48 †	86.80
Site	**Treatments**	**2015 Studies** (Means)					
NC115	Coker 312	3.98	5.28	28.83	1.260	6.75	86.50
	TAM66274	4.15	5.73 †	28.28	1.190 †	6.40	86.45
NC315	Coker 312	3.51	5.28	28.93	1.278	6.55	86.08
	TAM66274	4.18	5.70 †	28.95	1.185 †	6.35	86.53
MS115	Coker 312	4.38	4.88	31.93	1.288	6.43	86.45
	TAM66274	4.26	5.13	32.03	1.218 †	6.55	86.30
MS315	Coker 312	4.06	5.28	30.15	1.283	6.48	86.20
	TAM66274	4.11	5.53	31.05	1.200 †	6.65	86.35
TX515	Coker 312	4.75	5.15	31.73	1.200	6.45	85.30
	TAM66274	4.68	5.65	30.23	1.128 †	6.63	85.60

† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-15. Plant mapping characteristics.
Comparison of plant mapping characteristics of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Values are presented as the means of four replicated plots of each treatment at each field site.

		Plant Mapping Characteristics*					
		Total Nodes	**Height to Node Ratio**	**Total Bolls**	**No. of First Position Bolls**	**No. of Second Position Bolls**	**Boll Type** (1-9 scale)
Site	**Treatments**	**2014 Studies** (Means)					
NC114	Coker 312	16.0	2.24	9.6	5.0	2.0	8.5
	TAM66274	17.3	2.04	9.4	6.4	2.0	7.5
NC214	Coker 312	16.6	2.06	9.6	5.0	2.7	4.5
	TAM66274	17.4	1.97	10.2	5.6	2.5	3.8
MS114	Coker 312	22.0	2.31	5.3	0.9	2.6	7.7
	TAM66274	21.9	2.35	4.2	0.6 †	2.2	8.0
Site	**Treatments**	**2015 Studies** (Means)					
NC115	Coker 312	14.7	2.26	7.8	4.7	1.6	5.8
	TAM66274	15.9 †	2.10	7.9	5.0	1.7	5.3
NC315	Coker 312	15.8	2.10	9.0	4.5	1.8	5.0
	TAM66274	16.2	2.00	9.1	4.1	1.8	4.5
MS115	Coker 312	18.3	2.20	9.5	3.2	2.8	4.8
	TAM66274	20.0 †	1.90 †	9.9	2.9	2.4	4.5
MS315	Coker 312	17.5	2.22	11.7	5.4	3.2	5.6
	TAM66274	18.8	1.95 †	12.0	6.3	3.2	5.2
TX515	Coker 312	16.1	1.75	9.1	4.5	1.6	7.5
	TAM66274	19.1	1.57 †	9.1	4.4	1.6	7.7

*Total nodes represent the total number of nodes on the main stem of the plant at maturity. Height to node ratio was calculated by dividing plant height by the total number of nodes. Total bolls represent the total number of fruiting and vegetative bolls. First and second position bolls represent the total number of bolls set on the first and second position, respectively, of fruiting branches. Boll type was rated on a 1-9 scale: 1 = loose bolls, 5 = intermediate tightness, 9 = stormproof bolls.
†Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-16. Disease incidence.
Comparison of plant disease susceptibility of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Values are presented as the means of four replicated plots of each treatment at each field site.

		Disease Severity*				
		14 DAP (1-9 scale)	**28 DAP** (1-9 scale)	**56 DAP** (1-9 scale)	**84 DAP** (1-9 scale)	**112 DAP** (1-9 scale)
Site	**Treatments**	**2014 Studies** (Means)				
NC114	Coker 312	1.0	1.0	1.0	1.5	2.0
	TAM66274	1.0	1.0	1.0	1.5	2.0
NC214	Coker 312	1.0	1.0	1.0	1.8	2.0
	TAM66274	1.0	1.0	1.0	1.3	2.0
MS114	Coker 312	1.0	1.0	1.0	2.0	2.0
	TAM66274	1.0	1.0	1.0	2.0	2.0
Site	**Treatments**	**2015 Studies** (Means)				
NC115	Coker 312	1.0	1.0	1.0	2.0	2.0
	TAM66274	1.0	1.0	1.0	2.0	2.0
NC315	Coker 312	1.0	1.0	1.0	3.0	2.0
	TAM66274	1.0	1.0	1.0	3.0	2.0
MS115	Coker 312	1.0	1.0	1.0	1.0	1.0
	TAM66274	1.0	1.0	1.0	1.0	1.0
MS315	Coker 312	1.0	1.0	1.0	1.0	1.0
	TAM66274	1.0	1.0	1.0	1.0	1.0
TX515	Coker 312	1.0	1.0	1.0	1.0	1.0
	TAM66274	1.0	1.0	1.0	1.0	1.0

* Disease severity was rated on a 1-9 scale: 1 = no symptoms, 5 = intermediate symptoms, 9 = severe disease.
† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-17. Insect damage.
Comparison of insect damage of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Values are presented as the means of four replicated plots of each treatment at each field site.

		Insect Damage*				
		14 DAP (1-9 scale)	**28 DAP** (1-9 scale)	**56 DAP** (1-9 scale)	**84 DAP** (1-9 scale)	**112 DAP** (1-9 scale)
Site	**Treatments**	**2014 Studies** (Means)				
NC114	Coker 312	4.0	2.0	2.1	1.0	1.0
	TAM66274	4.0	2.0	2.1	1.0	1.0
NC214	Coker 312	4.0	3.0	2.0	1.0	1.0
	TAM66274	4.0	3.0	2.0	1.3	1.0
MS114	Coker 312	3.0	2.0	4.0	3.2	3.0
	TAM66274	3.0	2.0	4.0	3.1	3.0
Site	**Treatments**	**2015 Studies** (Means)				
NC115	Coker 312	3.0	3.3	3.0	2.0	2.0
	TAM66274	3.0	3.5	3.0	2.0	2.0
NC315	Coker 312	2.0	2.0	2.0	2.0	2.0
	TAM66274	2.0	2.0	2.0	2.0	2.0
MS115	Coker 312	3.0	2.0	3.1	2.1	2.0
	TAM66274	2.9	2.0	3.1	2.2 †	2.0
MS315	Coker 312	3.0	2.0	3.0	3.3	2.5
	TAM66274	3.0	2.0	3.0	3.2	2.5
TX515	Coker 312	1.0	1.1	1.3	1.0	1.0
	TAM66274	1.0	1.1	1.0	1.0	1.0

* Insect damage was rated on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage.
† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

Table F-18. Rodent damage.
Comparison of rodent damage of TAM66274 and non-transgenic cv. Coker 312. Plants were grown in three U.S. field locations in 2014, two independent sites in Perquimans County, North Carolina (NC114 and NC214), and one site in Washington County, Mississippi (MS114). Plants were grown in five U.S. field locations in 2015, two independent sites in Perquimans County, North Carolina (NC115 and NC315), two independent sites in Washington County, Mississippi (MS115 and MS315), and one site in Tom Green County, Texas (TX515). Values are presented as the means of four replicated plots of each treatment at each field site.

		Rodent Damage*
		Harvest (1-9 scale)
Site	**Treatments**	**2014 Studies** (Means)
NC114	Coker 312	1.0
	TAM66274	1.0
NC214	Coker 312	1.0
	TAM66274	1.0
MS114	Coker 312	1.0
	TAM66274	1.0
Site	**Treatments**	**2015 Studies** (Means)
NC115	Coker 312	1.0
	TAM66274	1.0
NC315	Coker 312	1.0
	TAM66274	1.0
MS115	Coker 312	1.0
	TAM66274	1.0
MS315	Coker 312	1.0
	TAM66274	1.0
TX515	Coker 312	1.0
	TAM66274	1.0

* Rodent feeding on mature seed was rated on a 1-9 scale: 1 = no damage, 5 = intermediate damage, 9 = severe damage.
† Mean values of TAM66274 compared to non-transgenic cv. Coker 312 at individual sites (orthogonal contrast) are statistically significantly different at $P<0.05$ (n=4).

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Appendix G.

USDA Notifications for TAM66274 Cotton

USDA Notification	Effective Date	Expiration Date	County, State	Number of Trials	Trial Report Submitted
16-081-101n	04/08/2016	04/08/2017	Burleson, TX	1	01/02/2017
15-054-101n	04/01/2015	04/01/2016	Washington, MS Perquimans, NC Tom Green, TX	2 2 1	04/14/2016
15-048-109n	04/01/2015	04/01/2016	Burleson, TX	1	01/22/2016
14-057-103n	04/01/2014	04/01/2015	Washington, MS Perquimans, NC	1 2	01/27/2015
13-071-104n	04/01/2013	04/01/2014	Washington, MS Perquimans, NC Hale, TX	1 1 1	01/27/2014
13-071-105n	04/15/2013	04/15/2014	Burleson, TX	1	04/03/2014
12-081-109n	04/17/2012	04/17/2013	Burleson, TX	1	02/28/2013