United States Department
of Agriculture

Animal and
Plant Health Inspection
Service

November 5, 1996

Guide for Preparing and Submitting a Petition for Genetically Engineered Plants

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How to use this guide—

When an applicant has field tested a transgenic crop and accumulated enough data to show that it is free from any risk under 7 CFR 340, the applicant can petition the Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture that a transgenic crop should no longer be considered a regulated article. The pages that follow represent a suggested format for submission of field test data to APHIS for deregulation of a transgenic crop.

Comments and issues that may need to be addressed by the applicant are in italics in the left margin adjacent to the portions of the sample permit to which they pertain.

APHIS took certain liberties in preparing the sample information and data presented in this publication. With the agronomic performance data and certain of the molecular biology data, material was condensed to ensure that the guide was a reasonable length and that the reader would be able to ascertain the kind of information APHIS expects to be provided in a petition.

Sample Petition

U.S. Department of Agriculture
Animal and Plant Health Inspection Service
4700 River Road, Unit 147
Riverdale, MD 20782

PETITION FOR DETERMINATION OF REGULATORY STATUS

Enclosed is a copy of a petition for determination on the regulatory status of *Gossypium hirsutum* L. cultivar "Banjaran," which has been modified to be resistant to the herbicide glyphosate, which is currently deemed a "regulated article". Based on the data and information contained in the enclosed petition, we believe that there is no longer "reason to believe" that the modified cotton plant should be deemed to be a regulated article. The modified cotton plant does not present a plant pest risk and is not otherwise deleterious to the environment. The enclosed petition does not contain confidential business information.

The undersigned certifies that, to the best of his/her knowledge and belief, this petition includes all data, information, and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is the subject of the petition.

/s/Terry Smith
1234 Main Street
American Star, Inc.
Biotechnologies, Ltd.
Detroit, Michigan 46666
313-555-1212 VOICE
313-555-2121 FAX

Sample Application

Application for Determination of Nonregulatory Status for Banjaran: The Glyphosate-Tolerant Cotton

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Abbreviations and Scientific Terms

Cyanazine (herbicide)	Bladex®
Dinoseb	an insecticide
Diuron (herbicide)	Karmex®
DMSA (herbicide)	monosodium methylarsonic acid
DSMA (herbicide)	disodium methylarsonic acid
EPSP synthase	5-enolpyruvyl-3-phosphoshikimate synthase
Fluazifop (herbicide)	Fusalide®
Fluometuron (herbicide)	Cotoran®
Glyphosate (herbicide)	Roundup®
IPM	Integrated Pest Management
Linuron (herbicide)	Lorox®
NOS	nopaline synthase
NPT II	neomycin phosphotransferase
Prometryn (herbicide)	Caparol®
Sethoxydim (herbicide)	Poast®
Ti plasmid	tumor-inducing plasmid
Tn5	transposon 5
Triazine	a class of herbicides
Trifluralin (herbicide)	Treflan®

I. Rationale for Development of Banjaran

American Star Biotechnologies, Inc. has developed genetically transformed cotton plants that are tolerant to the herbicide glyphosate. The major weed pests of cotton in the Southern United States include morning glories, cocklebur, pigweeds, johnsongrass, nutsedges, prickly sida, and bermuda grass. The development of glyphosate-tolerant cotton will allow producers the option of applying glyphosate postemergence in an over-the-top application or replacing preemergence herbicides under appropriate conditions. Introduction of these plants will offer producers several advantages: several toxic herbicides, including arsenic compounds can be replaced with a herbicide that is more benign to the environment; glyphosate-tolerant cotton would be compatible with IPM schemes; and glyphosate does not have carryover problems and restrictions on application that some currently approved cotton herbicides have. No increase of the proportion of cotton acreage treated with herbicide is possible because herbicides are currently used on 99 percent of the acreage.

II. The Cotton Family

A. Cotton as a Crop

Four species of the genus Gossypium are known as cotton, which is grown primarily for the seed hairs that are made into textiles. Cotton is predominant as a textile fiber because the mature dry hairs twist in such a way that they can be spun into fine, strong threads. Other products, such as cottonseed oil, meal, and cotton linters are byproducts of fiber production.

Cotton, a perennial plant cultivated as an annual, is grown in the United States mostly in areas from Virginia southward and westward to California in an area often referred to as the Cotton Belt (McGregor, 1976).

B. Taxonomy of Cotton

The genus Gossypium, a member of the Malvaceae, consists of 39 species, 4 of which are generally cultivated (Fryxell, 1984).

The most commonly cultivated species is G. hirsutum L. Other cultivated species are G. arboreum L., G. barbadense L., and G. herbaceum L.

Four species of Gossypium occur in the United States (Fryxell, 1979; Kartesz and Kartesz, 1980). G. hirsutum is the primary cultivated cotton. G. barbadense is also cultivated. The other two species, G. thurberi Todaro and G. tomentosum Nuttall ex Seemann, are wild plants of Arizona and Hawaii, respectively. G. tomentosum is known from a few isolated locations very close to the ocean.

C. Genetics of Cotton

At least seven complete sets of genes, designated A, B, C, D, E, F, and G, are found in the genus (Endrizzi, 1984). Diploid species (2n=26) are found on all continents, and a few are of some agricultural importance. The A genome is restricted in diploids to two species (G. arboreum and G. herbaceum) of the Old World. The D genome is restricted in diploids to some species of the New World, such as G. thurberi.

By far the most important agricultural cottons are G. hirsutum and G. barbadense. These are both allotetraploids of New World origin and presumably resulted from an ancient cross between Old World A genomes and New World D genomes. How and when the original crosses occurred are speculative. Euploids of these plants have 52 somatic chromosomes and are frequently designated as AADD. Four additional New World allotetraploids occur in the genus, including G. tomentosum, the native of Hawaii. G. tomentosum has been crossed with G. hirsutum in breeding programs.

The New World allotetraploids are peculiar in the genus because the species, at least in their wild forms, grow near the ocean as invaders in the constantly disturbed habitats of strand and associated environs. It is from these “weedy” or invader species that the cultivated cottons developed (Fryxell, 1979).

D. Pollination of Cotton

Gossypium hirsutum is generally self-pollinating, but in the presence of suitable insect pollinators it can cross-pollinate. Bumble bees (Bombus spp.), Melissodes bees, and honey bees (Apis mellifera) are the primary pollinators (McGregor, 1976). Concentration of suitable pollinators varies from location to location and by season, and is considerably suppressed by herbicide use. If suitable bee pollinators are present, distribution of pollen decreases considerably with increasing distance. McGregor (1976) reported results from an experiment in which a cotton field was surrounded by a large number of honeybee colonies, and movement of pollen was traced by means of fluorescent particles. At 150 to 200 feet from the source plants, 1.6 percent of the flowers showed the presence of the particles. The isolation distance for Foundation, Registered, and Certified seeds in 7 CFR Part 201 are 1,320, 1,320, and 660 feet, respectively.

Unlike G. hirsutum, G. tomentosum seems to be pollinated by lepidopterans, presumably moths (Fryxell, 1979). The stigma in G. tomentosum is elongated, so that the plant seems incapable of self-pollination until acted upon by an insect pollinator. The flowers are unusual, too, because they stay open at night; most Gossypium flowers are ephemeral—they open in the morning and wither at the end of the same day.

E. Weediness of Cotton

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

F. Modes of Gene Escape in Cotton

Genetic material of G. hirsutum may escape from a planting site by vegetative material, by seed, or by pollen.

Description of the biology of the nonmodified recipient organism should include taxonomy, genetics, pollination, evidence of reported weediness (e.g., noting whether the crop or sexually compatible species is listed in the relevant publications of the Weed Society of America), and discussion of sexual compatibility with wild and weedy free-living relatives in natural crosses or crosses with human intervention. The applicant should provide the source of recipient (cultivar name or accession number) and the weed status of its sexually compatible relatives.

Vegetative propagation is not a common mechanism by which cotton reproduces. Movement of genetic material by pollen is possible only to those plants with the proper chromosomal type, in this instance only to those allotetraploids with AADD genomes. In the United States this group would include only the cultivated species G. hirsutum, G. barbadense, and the wild species G. tomentosum. G. thurberi, the native diploid from Arizona with a DD genome, is not a suitable recipient. Movement to G. hirsutum and G. barbadense is possible if suitable insect pollinators are present and if there is a short distance from transgenic plants to recipient plants. Physical barriers, intermediate pollinator-attractive plants, and other temporal or biological impediments would reduce the potential for pollen movement.

The applicant should explicitly identify the lines to be considered in the petition and the cultivars from which they are derived. If there are multiple lines, each line must be given a unique identifier and listed in the application. For virus-resistant plants, applicants should provide in an additional section the following information on the nature of the virus that provided the sequences encoding the resistance phenotype:

i) the taxonomic name of the virus including family, genus, and strain designation including any synonyms; ii) the type of nucleic acid contained in the virus; iii) whether the infection is systemic or tissue specific; iv) whether the virus is associated with any satellite or helper viruses; v) the natural host range of the virus; vi) how the virus is transmitted; vii) if transmitted by a vector, the identity of the vector including mode of transmission (e.g., persistent or nonpersistent); viii) whether any synergistic or transcapsidation interactions with other viruses under field situations have been reported in the literature, and situations have been reported in the literature; and ix) the location and the name of the host from which the plant the virus was originally isolated.

The above information can be provided in a table format (see Table 1). This information can be supplemented by listing references that report the host range, insect vectors, etc., for the virus.

Movement of genetic material to G. tomentosum is less well documented. The plants are chromosomally compatible with G. hirsutum, but there is some doubt as to the possibility for pollination. The flowers of G. tomentosum seem to be pollinated by moths, not bees, and the flowers are receptive at night, not in the day. Both these factors would seem to minimize the possibility of cross-pollination. However, Fryxell (1979) reports that G. tomentosum may be losing its genetic identity from introgression hybridization of cultivated cottons by unknown means.

G. Characteristics of Nontransformed Cultivar

G. hirsutum L. cv. "Stoneville 825" is the cultivar that we genetically transformed. This cultivar is widely grown in the United States and was specially developed for introduction in the Mississippi Delta region. American Star Inc. has received a U.S. patent on the specific herbicide tolerant gene that has been transformed into this cultivar, and the transformed cultivar has received additional protection under the Plant Variety Protection Act of 1970. American Star Inc. intends to introduce the new traits into other cotton cultivars by traditional breeding techniques. The cultivar that has been transformed to be herbicide tolerant is called "Banjaran."

III. Description of Transformation System

For Agrobacterium-based transformation protocol, the applicant must indicate how Ti plasmid based vector was disarmed (i.e., all tumorigenic DNA was removed). Applicants can provide citations that describes the transformation procedure. However, any significant modifications of transformation, strain designation, etc. should be described.

For other methods of transformation, the applicant can describe the sources of various components of the plasmid (or other DNA including possible carrier DNA) and method of transformation by citation. However, any significant modifications in transformation, tissue regeneration, etc. should be described.

The vector system (pVST1) used to transfer the genes to cotton plants is based on the Ti plasmid from Agrobacterium tumefaciens (Fig. 1). The DNA becomes incorporated into the plant cell chromosome (Klein et at., 1989). Although some of the DNA sequences used in the transformation process were derived from the plant pathogen A. tumefaciens, the genes which cause crown gall disease were first removed, and therefore the recipient plant does not have crown gall disease (Zambryski, 1988; Klee and Rogers, 1989). Once the introduced genes are inserted into the chromosome of the recipient, they are maintained in the same manner as any other genes.

The T-DNA, which includes the glyphosate resistance and nptII genes, was transferred into the genomes of individual cotton hypocotyl sections as described by Umbeck et al. (1987). Plants were regenerated as described by Trolinder and Goodlin (1987).

The applicant must provide a detailed restriction map of the plasmid that is sufficient to be used in the analysis of Southern data. Description of added restriction sites is helpful in interpretation of Southern data and should be provided (see Fig. 1).

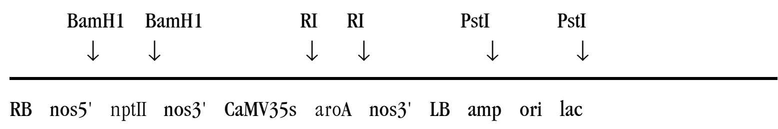

Fig 1—pVST1: A linear schematic illustration of the plasmid vector (7.8kb) that was conjugated into *Agrobacterium tumefaciens* (Malik and Wahab, 1993). Various components of pVST1 have been described in Table 1. Map reflects the gene order but not the sizes. The plasmid does not have restriction sites for *SpI* and *NdeI*, but has one site for *SmaI* and *NotI*.

Indicate the functions of the gene(s), promoters, leader sequences, enhancers, introns, and any other sequences that are used for gene expression in the plant and a reference describing from where the sequences were obtained. Discussion should include whether the inserted sequences are responsible for disease or injury to plants or other organisms. The nucleotide sequence(s) of the plant-expressed gene(s) should be provided by citation and not submitted in the application. If there has been a significant modification to sequences and the modified sequence has not been published, provide the complete sequence highlighting the modifications. If there have been minor modifications to the sequence of the plant-expressed gene, they should be provided. For example, if in a chemically synthesized Bt gene amino acid 23 was changed from methionine to alanine it should be stated without providing the complete sequence.

Table 1—Summary of DNA components in pVSTI

Genetic element	Source	Function
RB	A. tumefaciens	Right border of the Ti plasmid of A. tumefaciens LB 444 (White, 1992)
nos 5'	Ti plasmid of A. tumefaciens	Promoter for transcription of the nptII gene (White, 1992)
nptII	E. coli	Neomycin phosphotransferase type II enzyme that confers resistance to kanamycin and allows for selection in plant cells (Beck et al., 1982)
nos 3'	A. tumefaciens	A 3' nontranslated region of the nopaline synthase gene involved in transcription termination and polyadenylation (Depicker et al., 1982)
CaMV35s	Cauliflower mosaic virus	Promoter that directs transcription of cryIA(B) gene (Odell et al., 1982)
aroA	Salmonella typhimurium	5-Enolpyruvyl-3-Phosphoshikimate Synthase Gene (Pittard, 1987)
nos 3'	see above	see above
LB	A. tumefaciens	Left border of the Ti plasmid of A. tumefaciens (White, 1992)
amp	E. coli	Resistance to ampicillin expressed only in E. coli (White, 1992)
Ori	Col E1 plasmid of E. coli	The origin of replication for the PUC plasmids that allows for plasmid replication in E. coli (Vieira and Messing, 1987)
lac	E. coli	β-galactosidase of E. coli (White, 1992)

IV. Donor Genes and Regulatory Sequences

A. 5–Enolpyruvyl Shikimate Phosphate (EPSP) Synthase Gene

The donor organism used to supply the glyphosate tolerance gene, aroA, was the bacterium Salmonella typhimurium. S. typhimurium is a well-characterized enteric bacterium with homology to E. coli (Ochman and Wilson, 1987). Some strains of S. typhimurium are known to cause a disease in susceptible mice and humans, but there is no evidence that strains of the bacterium are plant pests (Le Minor, 198l). Salmonella species may be associated with vegetation as free-living organisms whenever these plants have been contaminated with fertilizers of fecal origin or when they have been irrigated with polluted water. Salmonella organisms do not seem to multiply significantly in the natural environment (outside of digestive tracts), but they can survive several weeks in water and several years in soil if the conditions of temperature, humidity, and pH are favorable (Delage, 1960).

The aroA gene, which encodes the sequence for the enzyme EPSP synthase, has no known inherent plant-pest characteristics nor direct involvement in human and animal disease. EPSP synthase is one of the enzymes in the biosynthetic pathway leading to chorismate, an intermediate in the formation of aromatic amino acids and their derivatives (Pittard, 1987). This pathway is found in most plants, many single-celled organisms, and some lower forms of animals. The aroA gene is constitutively expressed in both E. coli (Tribe et al., 1976) and S. typhimurium (Gollub et al., 1983). The gene was mutated in the bacterium to provide glyphosate resistance by the classical genetic techniques of mutation and selection (Comai et al., 1983). The description of the modified aroA gene, including the method of isolation and complete gene sequence, is contained in two published papers (Comai et al., 1983 and Stalker et al., 1985). The EPSP synthase gene was fused to the CaMV35s promoter and NOS termination/polyadenylation sequences as previously described (White, 1992). Confirmation that this gene was indeed inserted into the cotton chromosome was provided by the following: Southern gel analysis, Mendelian inheritance, measurement of expression levels of

gene product, and demonstration that the whole plant is resistant to applied glyphosate (see subsequent sections).

B. The Selectable Marker Gene: Neomycin Phosphotransferase

In addition to the aroA gene, the nptII gene from transposon Tn5 of the bacterium E. coli has been introduced in cotton to be used as a selectable marker. This gene codes for the enzyme neomycin phosphotransferase which confers resistance to the common aminoglycoside antibiotic kanamycin (Fraley et al., 1986). The DNA sequence of the gene has been determined (Beck et al., 1982). The lack of risk to humans of the nptII gene can be supported by its use in the first human gene therapy trials (Anonymous, 1990). The nptII coding region is under the control of the nos promoter and nos terminator.

None of the introduced genes has any inherent plant pest characteristics or poses a risk to plant health when introduced into the modified plants.

V. Genetic Analysis and Agronomic Performance

In general, it is always prudent to analyze data statistically when such analysis is possible. When unpublished information or an opinion has been supplied by a scientific expert, a letter communicating the information should be included in the petition. If the unpublished information provided is data resulting from scientific research then these data can be provided as a personal communication either in a letter from the researcher or in the text of the petition. In either case the materials and methods, data analysis, and discussion of the data analysis should be provided in detail. Unsupported assertions about the results of the experiment are not acceptable.

Applicants must report any differences noted between transgenic and nontransgenic plants that are not directly attributed to the expected phenotype. Differences observed could include changes in leaf morphology, pollen viability, seed germination rates, changes in overwintering capabilities, insect susceptibilities, diseases resistance, yield, agronomic performance etc. Applicants must also note the types of characteristics that were compared between transgenic and nontransgenic plants and found to be unchanged.

The applicant should describe whether data submitted are from inbred or hybrid plants; if hybrid plants, state which generation.

State whether data with respect to plant performance were generated in a greenhouse or field environment. If from the field, indicate how many sites, states and number of years the data represents.

Seed germination, seed dormancy, seed production, growth rate, and other data relating to the plant's performance will be required when the nature of the gene and the biology of the plant (including sexually-compatible relatives) warrant such data. This type of data will usually not be required for plants that have some of the following attributes: are highly domesticated (e.g., corn), are exclusively self-pollinating (e.g., soybean), are male sterile, and have high seed germination rates (>90%), and whose phenotypes are unlikely to affect performance with respect to weediness or fitness (e.g., delayed ripening or oil seed modification). Phenotypes that might require performance data (depending on the plant) include but are not limited to the following: cold tolerance, salt tolerance and tolerance or resistance to other biotic or abiotic stresses.

A. Southern Gel Analysis

The identity of the genetic material that was integrated into the genome of the transgenic plant was probed by Southern hybridization. In order to determine the number of insertion events, the DNA from the transgenic regulated plant and the parental Stoneville 825 recipient lines were digested with the restriction enzyme Nde1, which does not cleave within the pVST1 DNA. The hybridizations done with three probes indicated that the foreign DNA integrated at one site to yield the transgenic plant. This is supported by the presence of one hybridizing fragment of 20 kb that is present in the transgenic DNA but is absent from the parent.

The following restriction fragments were labeled and hybridized to the Southern blots:

1. a 300 bp EcoR 1 fragment of the aroA gene
2. a 400 bp BamH 1 fragment of the nptII gene
3. a 950 bp Pst 1 fragment with the amp and lac genes that are not expressed in plants and should not be integrated into the plant

Hybridization analysis of genomic DNA was performed following the method of Firoozabady et al. (1987). The results are shown in figure 2 for the aroA, nptII and Amp marker genes. Both aroA and nptII probes hybridize to the 20Kb fragment in lane 1. The data support the Mendelian results (shown below) that only one expressed copy of the aroA gene is present in the engineered cultivar and that a single copy of the npt II marker gene is present. No hybridization with the ampicillin or lac probe was detected.

Southern blot analysis supports the conclusion that the amp+lac sequences which lie outside the Ti plasmid left and right borders, were not integrated in to the genome of Banjaran, while the sequences inside the Ti left and right borders were.

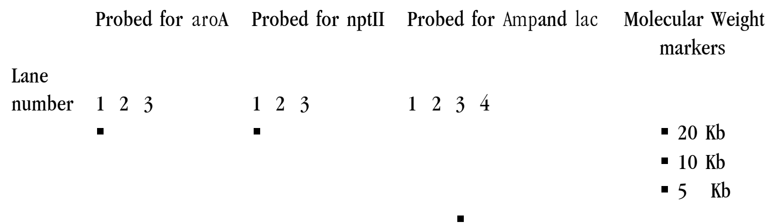

Fig. 2—Southern blot analysis of DNA: Detection of aroA and nptII genes in the Banjaran. Line lanes 1 and 2 contain 10 µg DNA of Banjaran and Stoneville 825 DNA respectively digested with NdeI. Lane 3 is Sal1 digested pUC 18 DNA as a positive control for amp and lac sequences. Lane 4 is a HindIII digest of phage lambda DNA, used as molecular weight markers. All four DNA samples were electrophoresed on three different 1% agarose gels and transferred to nitrocellulose filters. One nitrocellulose filter was hybridized with ^{32}P-labeled AroA probe, a second filter with the nptII probe and a third one with the amp+lac probe. A similar hybridization pattern was obtained when Southern blots were hybridized with either AroA or nptII probes. The PstI fragment (amp+lac) hybridized to pUC 18 DNA but not to the plant genomic DNA. This data is consistent with the presence of a single insertion of the genes between the LB and RB incorporated in the genome of the transgenic plant.

Southern analysis should include DNA isolated from nonmodified recipient, all or selected transformed lines, and the vector. Parental plasmid DNA (eg PUC 18) not containing intended donor genes may be labeled and hybridized to Southern blots to demonstrate that only the intended sequences have been incorporated in the genome of the transgenic plant. Restriction enzymes to be used might include enzymes that do not cut within the transforming plasmid but will cut the entire insert into one fragment from the DNA of the transgenic plant.

In the case of an Agrobacterium-based transformation system, the applicant should determine if genes that reside outside the LB/RB are inserted in the genome of the regulated cultivar. If a complete copy of any of these genes is present, the applicant should determine whether it is expressed in the plant. For direct transformation systems, applicants should determine which sequences are inserted in transgenic plants and whether they are expressed. PCR analysis may be used to prove that only the targeted DNA has been incorporated. Sequencing of the transgene in plant and adjacent sequences is not required. Determination of the number of copies of integrated transgenes is not required, but the number of insertions may be used to support analysis of inheritance data.

B. Mendelian Inheritance

The primary transformants that expressed the NPT II marker gene and the EPSP synthase gene were allowed to self-pollinate, and the seeds were collected. These seeds (T_1) were planted in a single 25-foot row. Seedlings were thinned to a density of two plants per foot. Seedlings were sprayed with one application of glyphosate at a rate of 8 oz/acre. Symptoms of bleaching or necrosis appeared 8 to 10 days after application and were compared to the symptoms of nontransformed plants that received an identical herbicide application. The number of resistant and sensitive plants in three separate rows was counted (Table 2). If primary transformants were expressing the EPSP gene from a single insertion site (genetic loci), the expected segregation would be 3:1. The segregation ratio totals for all 3 rows was 110:40, which fits the Mendelian prediction of 112½:37½ ($\chi^2 = 0.22$) well. The nontransformed plants were fully susceptible to glyphosate.

If the inserted DNA sequence order is complex, as is often the case for plants engineered via direct transformation systems (e.g. electroporation, polyethylene glycol transformation of protoplasts, or particle bombardment techniques), the applicants should summarize the data by providing the following information for the all genes (whether under the direction of plant or bacterial promoters). Is there a complete copy of the gene present in regulated article? Is the protein expressed in the plant? If multiple complete copies of a gene are present, applicants do not have to determine if each copy of the gene is expressed. It is very helpful to provide a table, like the one shown below, that summarizes the results and indicates where specific data is to be found.

Gene	Is a complete copy present?	Is protein expressed in plant?	Location of detailed data
ß-lactamase	yes	no	Fig. 3, p. 7
EPSP synthase	yes	yes	Fig. 4, p. 20
nptII	yes	yes	Fig. 8, p. 21
chloramphenicol acetyltransferase	no	—	Fig. 9, p. 31

Mendelian inheritance data and Chi square analysis for at least 2 generations are appropriate to demonstrate whether the transgene is stably inserted and inherited in Mendelian fashion. Such data are generally not necessary for infertile vegetatively propagated crops such as male-sterile potatoes.

Table 2—Segregation ratios of progeny of the seeds of the primary transformants

	Ratio of Herbicide Tolerant to Susceptible Plants		
Variety	Observed	Expected	χ^2
Banjaran	36:14	37½:12½	0.24
Banjaran	39:11	37½:12½	0.24
Banjaran	35:15	37½:12½	0.67
Stoneville 825	0:50	0:50	—

RNA—Northern analysis is generally not required except for virus-resistant plants. However, such analysis may be necessary for ribozyme, truncated sense, or antisense constructs, when protein levels can not be provided.

PROTEINS—Expression levels of gene(s) of interest and marker genes in various tissues, developmental stages of plant, and experimental conditions (induced or noninduced) are required. Assays can be of enzyme activity. Serology, ELISA, and Western blots may also be used. Describing the source of the immunogen is critical for serological analysis.

For virus resistant plants, the amount of viral transgene RNA produced should be determined and compared to the amount of the RNA produced by the viral gene in an infected nontransgenic plant. Applicants should address whether the transgene RNA (or protein) is present in the same tissues as are infected during natural infections. In addition, provide the amount of both coat proteins (i.e. from the transgene and the naturally infecting virus) produced in the transgenic plant singly infected with the widely prevalent viruses in the U.S. that normally infect the recipient plant (contact APHIS for the list of these viruses). For comparison, provide the amount of both coat proteins produced in the nonengineered plant in mixed infections of the virus from which the coat protein gene was derived and the same widely prevalent viruses used in the single infection study. Provide description of symptoms of infected plants in all cases

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C. Expression of Inserted Genes

The production of the desired proteins in Banjaran was confirmed by immunoassay. Banjaran has been modified by the insertion of the *aroA* gene imparting the glyphosate resistance trait. In addition to glyphosate resistance, Banjaran expresses the selectable marker protein, *nptII*. As measured by immunoassay, the *aroA* and *nptII* proteins were expressed at low and relatively consistent levels in Banjaran across all field sites (see data reports 94–000–02, 94–000–03). The mean from all tests reported in 10 states at 50 sites showed that Banjaran contained in leaf and seed tissue respectively approximately 12.6 µg and 17.3 µg 5-enolpyruvyl-3-shikimate phosphate synthase protein/gram fresh weight of tissue (fwt), and 6.9 and 3.3 µg *nptII* protein/gram fwt. At the one field site at which expression was evaluated over time, the EPSP protein levels varied less than five fold in young leaf tissue collected over the growing season with the highest levels observed early in the season (see data report 94-000-05).

The above data was based on leaf and seed. However, the levels of EPSP and *nptII* proteins in whole plant tissue were much lower, on a fresh weight basis, than in leaf or seed tissues. EPSP is present in whole Banjaran plants from 1.1 to 1.7 µg/g fwt of the whole plant respectively; NPTII protein levels are 14.6 µg/g/fwt for Banjaran plants. These measured concentrations were used to estimate the amount of EPSP and NPTII protein that could enter the environment due to post-harvest incorporation of 5000 mature Banjaran plants into the soil. Predicted values are 23.4 g EPSP protein/acre and 183 g NPTII protein/acre, Nectar and pollen collected from Banjaran contain very low levels of EPSP protein. The expression of EPSP protein in pollen collected from Banjaran greenhouse-grown plants were 37.8 ng/g fresh wt, respectively. The EPSP protein levels in nectar were 0.88 ng/g fresh wt collected from Banjaran plants. Thus, pollen and nectar produced by Banjaran present a low source of potential EPSP protein exposure to nontarget organisms.

For all diseases and pathogens surveyed, names of the diseases and the scientific names of the pathogens should be provided. Data from field tests in foreign countries are acceptable. If the data on diseases and pests were obtained in the foreign country, the applicant should submit information about the distribution of those pests, disease or pathogens in U.S.. Disease and pathogen susceptibility on wild type and transgenic plants should be determined preferably from natural infestations. However, if applicant must use direct inoculations; i.e., with virus resistant transgenic plants, the source and taxonomic classification of the virus should be provided.

D. Disease and Pest Resistance Characteristics

The transformed cotton plants were field-tested for 3 years at ten sites in five States (see data reports 93–1, 94–1, 95–1 in appendix). Based on field observation at these sites, pathogen-susceptibility or resistance characteristics of the transformed cultivar were unchanged when compared to those of the nontransformed cultivar. The transformed cultivar remains resistant or tolerant to bacterial blight (*Xanthomonas campestris* pv. *malvacearum*), Anthracnose boll rot, and *Fusarium* wilt–nematode complex rot. The transformed cultivar remains susceptible to *Alternaria* leaf spot, *Cercospora* leaf spot, and powdery mildew, as was the nontransformed cultivar.

E. Mycotoxins

Aflatoxins are most commonly found in food and feed commodities contaminated by *Aspergillus flavus*. Aflatoxins are the only contaminants of feeds and food routinely monitored. Banjaran was not any more susceptible to mold infection than its parent cultivar and was not going to be a source of mycotoxins.

F. Gossypol

Gossypol is a yellow pigment that occurs in various parts of the cotton plant. Cotton seed usually contains 0.4 percent to 1.7 percent gossypol (Abou-Donia, 1976). When present in untreated cottonseed meal, gossypol is toxic to animals. When cottonseed meal is processed under heat and moisture, most of the free gossypol is removed by solvent extraction or detoxified by the condensation of the aldehyde groups of gossypol with the free amino groups of proteins to form nonextractable (bound) gossypol. Flavanoids which are not major constituents in cotton were also measured because they can be toxic if eaten in large amounts. The amount of free or bound gossypol in the meal did not differ significantly between the transformed and nontransformed cultivars (Table 2).

Certain plants have minute quantities of known toxicants which may adversely impact nontarget organisms and beneficial insects; e.g., tomatine in tomatoes, cucurbitin in cucurbits, gossypol in cotton etc. If such plants are recipients of transgenes, the applicant should provide information as to whether the level of toxicants is altered. If the plant produces no known toxicant, the applicant should state so and provide the reference to support the claim. Plant toxins can be assessed by the tests and criteria that plant breeders traditionally use in the crop. In some instances, this may be done qualitatively, e.g. taste testing of cucurbits.

Table 2—Mean toxicant content in cottonseed of transformed and nontransformed cultivars grown at four sites

Quality factors[1]	Banjaran	Line 825
Free gossypol	0.75a	0.75a
Total gossypol	1.0a	1.0a
Flavonoids	1.84a	1.76a

Free and total gossypol and flavonoids are given as percent of kernel weight assayed according to the methods of Cherry (1983) and Hedin (1988) respectively. Within each column, means followed by the same letter are not significantly different according to the Newman-Kreuls multiple range test.

G. Characteristics of Glyphosate-Tolerant Cotton

We determined that the minimum level of glyphosate needed to control morning glory, cotton's major weed pest, was 8 oz/acre. At this level, the glyphosate-tolerant cotton was undamaged by the herbicide. This concentration is generally also adequate for the control of Morningglory, Common cocklebur, Pigweed, Johnsongrass, Nutsedges and Bermudagrass which are all important weeds in cotton cultivation.

Glyphosate-tolerant cotton is still susceptible to two other broad-spectrum herbicides, sulfonylurea and bromoxynil, as is its progenitor cultivar. Thus, the transformed cultivar can be eliminated using herbicides with a different mode of action from glyphosate if that is desired.

VI. Environmental Consequences of Introduction of the Transformed Cultivar

A. The Herbicide Glyphosate

N-(phosphonomethyl)glycerine (glyphosate) is an extremely effective broad-spectrum herbicide. The primary mode of action of the herbicide appears to be competitive inhibition of 5-enolpyruvylshikimic acid-3-phosphate (ESPS) synthase, an enzyme in the shikimic acid pathway of aromatic amino acid biosynthesis. Glyphosate provides effective control for the majority of the world's worst weeds. It is translocated in the plant via both phloem and xylem. The broad-spectrum herbicidal activity is evident only when glyphosate is applied to foliage because there is little penetration of bark or woody stems (Franz 1983). Glyphosate becomes nontoxic upon contacting soil. Its degradation appears to be mainly microbial. Glyphosate is essentially nontoxic to mammals and birds (Anonymous 1983). Environmental impact studies indicate that the herbicide has little direct effect on animal communities (Sullivan and Sullivan 1979, 1981, 1982). However, some bird communities may show decreased population densities due to destruction of habitat caused by use of the herbicide (Morrison and Meslow 1984). Fish and invertebrates are more sensitive to the herbicide, especially to the commercial formulations, as a result of the surfactants in the formulation (Anonymous 1983). Effects of the herbicide on soil invertebrates in field situations appear to be minor (Eijsackers 1985). Although there are numerous reports of the effects of glyphosate on microbial respiration, nitrogen cycling, and cellulolytic activity in soils, no significant effects on any of these microbial processes should be observed at recommended field application rate of the herbicide (Carlisle and Trevors 1988). There have been no reports of groundwater contamination problems (Goldburg et al. 1990).

B. Current Uses of Herbicides on Cotton

Glyphosate is generally used as a foliar-applied herbicide. It is most effective for the control of perennial weeds. It is usually applied before planting to kill winter weeds or used as a spot spray at any time throughout the growing season. Glyphosate is also used for destroying weeds growing adjacent to the field.

Herbicides are applied to cotton before planting (to preplant weed foliage or in soil-incorporated applications), at planting (preemergence applications), or after seedlings emerge (postemergence directed or over-the-top). Herbicides were used on 99 percent of the cotton acreage (2.7 million acres) in the Delta region of the United States in 1990, on average from 3.6 to 4.1 treatments per acre. The severe weed pressure in the Delta is demonstrated by the large proportion of the cotton acreage receiving three or more herbicide treatments per season. At least one-quarter of the acreage in the Delta is treated with arsenic-based herbicides (DMSA or MSMA), singly or in combination with other herbicides. The total amount of arsenic-based herbicides applied to cotton in 1990 was approximately 3.5 million pounds.

Several postemergence herbicides are registered for use in cotton. They are usually applied when the plants are 3 to 6 inches high. These herbicides include diuron, fluometuron, prometryn, and cyanazine, to which MSMA or DMSA is added to broaden the spectrum. One additional application of the mixture is often made during the season since there is a limit of two applications of the arsenical herbicides. Late postemergence herbicides are sometimes applied at or near the time of the last cultivation ("layby"). Direct applications are usually placed between the rows, in order to maintain cotton seed quality. A few over-the-top herbicides are available. The two used are sethoxydim and fluazifop. Both are specific for control of grass weeds and have little effect on broadleaf plants. Before the introduction of sethoxydim and fluazifop, glyphosate was used to control grasses. Because glyphosate is nonselective, special

application methods were devised.

In the various cotton-growing regions of the country, cotton producers manage weeds differently. The following summarizes typical practices for the mid-South region (Frans and Chandler 1989).

1. Disk twice and broadcast and incorporate trifluralin before planting.
2. At planting, apply fluometuron preemergence on bands.
3. Cultivate and postdirect fluometuron plus MSMA on bands.
4. Cultivate and postdirect prometryn plus MSMA on bands.
5. Spot spray with fluazifop.
6. Cultivate and postdirect cyanazine on bands.
7. Hand hoe, cultivate, and postdirect dinoseb on bands.

Recently yields of cotton lint have declined, and continued herbicide use is strongly implicated, especially where cotton is grown continuously and the same herbicides are applied yearly (Frans and Chandler, 1989). Rogers et al. (1985, 1986) summarized results from a long-term experiment in which herbicides were applied to cotton at different levels for 6 to 7 years. No reduction in cotton yield occurred following continuous use of a minimum set of herbicide practices. When intensive practices were used (trifluralin preplant incorporated, fluometuron preemergence, two postemergence directed applications of fluometuron plus MSMA, and linuron applied at last cultivation), yields dropped on average up to 8 percent. Of the rotation crops planted on these areas, corn and sorghum suffered the least damage while soybeans and rice were severely injured.

Herbicide residues in the cotton crop have also been a concern, especially those of organic arsenicals. Both DMSA and MSMA are used postemergence for control of

grasses. Although most producers apply arsenicals in a directed manner, some apply them over the top. In the latter case, there is the possibility of high residue levels occurring in cotton, especially if applications are made during the early reproductive stage of cotton growth and if there are multiple applications (Frans and Chandler 1989).

C. Banjaran: The Glyphosate-Tolerant Cotton

Environmentally desirable feature of the introduction of glyphosate-tolerant cotton include

- It offers producers the option of replacing with glyphosate several herbicide combinations that include arsenical compounds.
- Glyphosate is less likely to lead to the development of resistant weeds than many other herbicides (Benbrook 1991).
- The introduction of glyphosate-tolerant cotton is compatible with IPM. Producers could apply the herbicide only if needed, thus reducing the use of preemergence herbicides.
- The most damaging components of glyphosate are its inert components (Goldburg et al., 1990). Recently, the Environmental Protection Agency has given glyphosate an "E" carcinogenicity (noncarcinogen) rating.
- Glyphosate does not have carryover problems.
- The introduction of glyphosate-tolerant cotton could aid in the development of minimum-till practices that would result in reduced soil erosion.

Glyphosate-tolerant cotton could enable producers to apply herbicide on an as-needed basis, a key principle of all IPM systems. If a farmer planted a field with a herbicide-tolerant variety, the farmer could cut back the initial herbicide application or try to control weeds with mechanical cultivation. If chemical weed control became necessary, herbicide could be applied over the top to the entire field or by spot application in the areas of field where weeds were threatening.

D. Appearance of Glyphosate-Resistant Weeds

A decade ago, herbicide-resistant weeds were virtually unknown. Today there are some 109 herbicide-resistant weed biotypes and more than half of them are resistant to triazine (LeBaron, 1991). Certain herbicide characteristics and application regimes favor the development of resistant weeds: a single target site and a specific mode of action, broad spectrum of activity, long residual activity the capacity to control weeds through out the year, and frequent applications without rotation to other herbicides or cultural control practices. Current application data suggest that glyphosate is unlikely to engender the development of resistance in target vegetation (Benbrook 1991).

E. Weediness of Banjaran

Will the introduction of the herbicide-tolerance genes to a plant increase its weediness? Exactly which characters define a weed is debatable, but a general consensus of the traits shared by many weeds was developed by Baker (1974). They include (1) the ability to germinate in many different environments; (2) discontinuous germination and great longevity of seed; (3) rapid growth through vegetative phase to flowering; (4) continuous seed production for as long as growing conditions permit; (5) self-compatibility but partially autogamous and apometic; (6) ability to be cross-pollinated by unspecialized visitors or wind-pollinated; (7) high seed output in favorable environments and some seed production in a wide range of environments; (8) adaption for short and long-distance dispersal; (9) vegetative production or regeneration from fragments and brittleness (hard to remove from the ground); and (10) ability to compete interspecifically by special means (e.g., rosette formation and presence of allelochemicals). Weeds need not have all these characteristics to be successful. G. hirsutum cv. Stoneville 825 that was genetically transformed is not considered a weed and has few of the 10 weedy traits. Introduction of the herbicide tolerance gene into this cultivar did not significantly change its weedy characteristics. No change was noted with transformed cultivar in the number of seeds

produced, germination and overwintering characteristics of seeds, or the number of days from planting until first boll production or flowering. The herbicide-tolerant cultivar's sensitivity to all commonly used, registered cotton herbicides was not altered except for glyphosate tolerance.

F. Vertical Transfer of the New Genes

It is apparent from the data that outcrossing from the transformed cultivar to other domestic cottons does and will occur. Of course, this kind of gene transfer happens in nature constantly. Because cotton producers purchase new seed every year, the cross-pollination phenomenon does not have a significant impact on the quality or nature of seed produced in a field where cross pollination has occurred. Seeds from all transgenic cotton cultivars will still have to meet existing certification requirements for cotton seed production.

The noncultivated Gossypium spp. found in the Southwestern United States and Hawaii are not considered weeds, and introgression of the new genes into these species would not significantly increase any of the 10 characteristics of weeds unless selection pressure favors these characteristics. With regard to glyphosate-tolerant cotton, we believe that introgression of this trait into noncultivated Gossypium spp. would not be highly favored in the absence of herbicide application. Herbicide application is likely only in agricultural settings, not in wild stands. In addition, in Hawaii, where G. tomentosum is found, cotton is not commercially produced. The great majority of cotton grown in that State is in experimental plots where cotton breeding programs operate. Cotton breeders generally bag or clip the flowers when performing crosses between plants. This practice significantly reduces the chance that flowers will be visited by pollinating insects and thus reduces the likelihood of gene movement.

G. Horizontal Transfer of the New Genes

Nonsexual, horizontal transfer of transgenes from genetically engineered plants into other organisms is not well documented and is difficult to measure (Harding, 1995). Horizontal gene transfer of transgenes from higher transgenic plants via the soil to a soil microorganism (the filamentous fungus Aspergillus niger), however, has been reported (Hoffmann et al., 1994). Genetic transfer across taxa of eukaryotes is suggested in only a few cases (Lewin, 1982; Houck et al., 1991), and of these the only one suggesting a transfer, even over evolutionary time scale (excepting Agrobacterium) from unrelated taxa to higher plants is with the case of vertebrate hemoglobin and legume hemoglobin (Wiborg et al., 1983). During the field testing of these plants, there was no evidence of horizontal transfer of the transformed genes to adjacent nonsexually compatible plants. This observation is based on sensitivity to glyphosate of weeds in the nearby fields.

VII. Adverse Consequences: New Cultivar Introduction

Assuming that the levels of known toxicants in the regulated article reported in Section V are in acceptable range; that there were no notable differences reported in Section V between transgenic and nontransgenic plant; and that the gene(s) engineered into the recipient plant have no known reported toxic properties; then, toxicological data on effects of the plant on nontarget organisms and threatened and endangered species will usually not be required

With respect to the herbicide-tolerant cotton, the use of glyphosate may increase if the transgenic cultivar is widely accepted by farmers. The increased use of glyphosate will be offset by the decreased use of organic arsenate-based herbicides used in conjunction with other herbicides.

The example given does not refer to a plant with a new pesticidal phenotype. For such plants, however applicants should indicate to APHIS whether they have applied for or been granted registration of the pesticide with the Environmental Protection Agency.

Applicants should also consult with APHIS on data the agency would deem to be appropriate and sufficient to demonstrate no significant impact on threatened and endangered species and beneficial nontarget organisms. This data should be submitted in the appendix of the petition application. However, brief summaries of data should appear in the petition application.

A separate petition should be submitted for each category/phenotype combination. For example, a petition for Coleopteran insect-resistant potatoes or PVY-resistant potatoes should be submitted separately. However, when a single plant contains more than one phenotype modification, submit only one petition. For example, one petition should be submitted for potatoes that are both PVY and PVX resistant.

Below is an example of an acceptable summary of a test of BT cotton pollen on a beneficial nontarget organism.

Springborn Laboratories, Inc. (Wareham, MA) conducted a 48-hour static-renewal test with Bt cotton pollen (homozygous for the cryIA(b) gene) and isogenic pollen on *Daphnia magna*, according to EPA Guideline No. 72-2. Details of these studies have been submitted in the registration package to the USEPA (see appendix). Daphnids were <24 hours old at the time of study initiation. For the definitive test, dose levels of 19, 32, 54, 90, and 150 mg pollen/L (containing 5.87 mg CryIA(b)/g pollen) were employed. These levels are a hundredfold higher than the LC_{50} for target insects. In addition, isogenic controls at the same pollen concentrations as the treatment group were tested along with a negative control group. Each test or control concentration consisted of two replicates of 10 daphnids each for a total of 20 daphnids/ concentration or control group. Daphnids were exposed for 48 hours with complete renewal of the test solutions after 24 hours.

Mean survival was 100 percent for each of the transgenic, isogenic, and negative control groups. All daphnids in the transgenic, isogenic, and negative control groups appeared normal during the study. No immobilization or sublethal signs of toxicity were observed. The only effect noted was a decrease in dissolved oxygen in the higher test concentrations of both pollen groups. Dissolved oxygen concentrations were inversely related to the concentration of pollen tested and were similar in equivalent concentrations of the transgenic and isogenic groups. The decrease in dissolved oxygen had no effect on the survival of the daphnids. Higher concentrations for both types of pollen were cloudy and some daphnids were observed to be coated with pollen. At 48 hours, the EC_{50} based on immobilization was >150 mg pollen/L for both the transgenic and isogenic groups. Based on these results, the NOEC was 150 mg transgenic or isogenic pollen/L (the highest concentration tested).

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