93-136-01p

CALGENE

July 14, 1993

Director, Biotechnology, Biologics, and
Environmental Protection
c/o The Deputy Director Biotechnology
Coordination and Technical Asistance
Animal and Plant Health Inspection Service
U.S. Department of Agriculture
6505 Belcrest Road
Hyattsville, MD 20782

PETITION FOR DETERMINATION OF NONREGULATED STATUS: BXN™ Cotton

Dear Sir or Madam:

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, BBEP, make a determination that BXN cotton should not be a regulated article under 7 CFR part 340.

Sincerely,

Keith Redenbaugh, Ph.D.
Regulatory Affairs

000001

1920 Fifth Street
Davis, California 95616
Telephone (916) 753-6313
Telex: 350370 CGENE
Fax: (916) 753-1510

Calgene, Inc.

Petition for Determination of Nonregulated Status under 7 CFR 340

BXN™ Cotton

Statement of Grounds for Decision

July 14, 1993

No CBI

Table of Contents

A. Statement of Grounds for Decision

This petition contains no confidential business information.

I. Introduction

BXN[1] cotton provides a valuable tool for cotton growers and a significant benefit to the environment as part of a sustainable agriculture system. After conducting and evaluating 67 field trials in the United States and three other countries, Calgene has established a workable and proven system for cotton production which maintains (or potentially increases) yield while significantly reducing the amount of herbicides used. BXN cotton varieties have been shown to be like other cotton varieties but with enhanced agronomic performance. Results from the field trials and additional evaluations demonstrate that BXN cotton is not a weed or plant pest and therefore should not be a regulated article under 7 CFR part 340.

A. BXN™ Cotton Definition

BXN™ cotton is defined as any cotton cultivar or progeny of a cotton line containing the BXN gene (Stalker et al. 1988a) with its associated 35S promoter (Gardner et al. 1981) and *tml* 3' terminator (Barker et al. 1983). It may also contain the *kan*^{r} gene (Beck et al. 1982) with its associated 35S promoter and *tml* 3' terminator, the ori pRi (Jouanin et al. 1985, McBride and Summerfelt 1990), the left T-DNA border (Barker et al. 1983), a Tn5 transposon segment (Auerswald 1981, Beck et al. 1982), a Lac Z' polylinker sequence (Yanisch-Perron 1985), the right T-DNA border (Barker 1983), and the ori 322 (Sutcliffe 1979, McBride and Summerfelt 1990). BXN cotton was produced using one of the *Agrobacterium*-derived binary vectors described in McBride and Summerfelt (1990). BXN cotton has been field tested under one or more of the following USDA APHIS permits: 92-106-01, 92-105-01, 91-357-01, 91-333-02, 91-329-04, 91-329-03, 91-329-02, 91-329-01, 91-107-06, 91-035-07, 90-303-02, 90-297-01, 90-016-04, 89-192-01, 89-047-07. The BXN gene was isolated from *Klebsiella pneumoniae* subsp. *ozaenae* (*Klebsiella ozaenae*) and encodes an enzyme (nitrilase) that degrades the herbicide bromoxynil (McBride and Summerfelt 1990; Stalker and McBride 1987; Stalker et al. 1988a and 1988b).

B. Rationale for Development

BXN cotton was developed by Calgene to improve weed management practices for cotton production. The BXN gene encodes the protein nitrilase which detoxifies the broadleaf herbicide Buctril®[2], containing the active ingredient bromoxynil. As a result, Buctril can then be sprayed "over the top"

---

[1]BXN is a trademark of Calgene, Inc.
[2]BUCTRIL is a registered trademark of RHÔNE-POULENC.

for broadleaf weed control in BXN cotton fields. The use of Buctril for weed control in BXN cotton has a dramatic effect on reduction in overall herbicide use. In one experiment, the use of a BXN cotton/Buctril herbicide system did not affect yield, while total herbicide could be decreased 31-64% (see Section VI, Environmental Consequences of Introduction of the Transformed Cultivars: Impact of Use of BXN Cotton on the Environment).

C. Removal from Regulated Status

Calgene requests that USDA APHIS, based on data and information presented in this document, determine that BXN cotton does not present a plant pest risk, is not otherwise deleterious to the environment, and is therefore not a regulated article under 7 CFR 340.

Currently, the BXN cotton is considered a regulated article because it may contain the following subspecies (DNA sequences) from the list of organisms in 7 CFR 340.2: the *tml* 3' terminator and the right and left border regions from *Agrobacterium tumefaciens*; the ori pRi from *A. rhizogenes*, and the 35S promoter region (CaMV35S) from cauliflower mosaic virus (CaMV)(Barker et al. 1983; Gardner et al. 1981).

Other components are not from organisms considered to be plant pests. *Gossypium hirsutum* L. is not a regulated article under 7 CFR 340 and the BXN gene (Stalker and McBride 1987) was isolated from *K. ozaenae* which is not a plant pest. The *kan*^{r} gene encoding APH(3')II (aminoglycoside 3'-phosphotransferase II) was isolated as a component of transposon Tn5 from a ColE1::Tn5 containing strain of *Escherichia coli* K12 (Garfinkel et al. 1981). The Tn5 (Auerswald et al. 1981), Lac Z' (Yanisch-Perron et al. 1985), and ori 322 sequences (Sutcliffe 1978) are from *E. coli* which is not a plant pest.

Although *A. tumefaciens* and CaMV are regulated articles under 7 CFR 340, the BXN cotton containing sequences from these regulated articles should not be classified as a plant pest risk, as deleterious to the environment (see Section VI, Environmental Consequences of Introduction of the Transformed Cultivars: Impact of Use of BXN Cotton on the Environment), nor as a regulated article under 7 CFR 340 for the following reasons:

1. Cotton is not a regulated article under 7 CFR 340.
2. Genetic sequences from regulated articles used to produce BXN cotton have been disarmed and do not pose a plant pest risk.
3. Genes from regulated articles, introduced into cotton, do not confer characteristics that would present BXN cotton as a plant pest risk (e.g. cause cotton to become a weed pest risk).
4. No new compounds have been detected in BXN cotton that pose a hazard or are deleterious to the environment.
5. BXN cotton varieties do not differ from other cotton varieties, except for resistance to the herbicide bromoxynil.

II. The Recipient Plant: Cotton

Cotton in general and BXN cotton specifically have very low or no potential for becoming weed pests for several reasons:

1) Cotton is not a weed pest (Weed Science Society of America 1989; Appendix 1).
2) Other *Gossypium* species are not considered weed pests (Weed Science Society of America 1989; Holm 1979; Appendix 1).
3) Cotton is a tropical/sub-tropical species with very low tolerance to frost. Although cotton is a perennial, it is grown as an annual in temperate latitudes, primarily because of its frost intolerance (Fryxell 1979).
4) Cultivated cotton has generally lost its seed dormancy (Fryxell 1979).
5) The BXN gene is specific for bromoxynil resistance and does not confer any selective advantage to cotton outside agricultural fields where the herbicide is applied.
6) Results from field trials with cotton containing the BXN gene have shown no increased weediness potential, no *Agrobacterium* disease symptoms and no change in any traits that could affect weediness potential.

These conclusions are discussed in depth in the following sections.

A. Cotton as a Crop

Cotton is a perennial subtropical plant grown commercially under a variety of environmental conditions which permit an economic yield to be obtained. The principle growing region in the United States extends across the South, from Virginia to California, an area often referred to as the Cotton Belt (McGregor 1976).

Throughout the world, cotton may be grown under irrigated or nonirrigated (dryland) production regimes. In the United States, irrigated production is prevalent in the west (California and Arizona) and has been increasing in the southern Mississippi and Delta regions. Dryland production is prevalent in the rest of the Cotton Belt. Cotton is grown in the U.S. generally in areas south of the 36° N. latitude where 200 frost-free days prevail. In California, cotton is planted north to nearly the 37° N. latitude, in the San Joaquin Valley (Waddle 1984).

In the United States, as in other cotton growing regions, the production of cotton is an intensively managed form of agriculture with high levels of pesticide inputs. In large part due to the tropical and semi-tropical climatic conditions to which cotton is especially adapted, much of the area in which cotton is produced is afflicted with a relatively high degree of competition from pests, including weeds, insects, fungi, bacteria, and nematodes.

Cotton is grown primarily for the fiber (lint) that is produced on its seedcoat. The mature dry fibers twist in such a way that they can be spun into

fine, strong thread. Harvested seed cotton is typically between 32% and 42% lint by weight (USDA 1990) with the remainder composed of cottonseed and waste. The lint is removed from the seed by ginning and has numerous uses, chiefly in textile manufacture. Raw (unprocessed) cottonseed may be fed to ruminant livestock and may be processed into numerous products, including food for humans and animals. There are four principle processed components derived from cottonseed: oil, meal, hulls (seedcoats), and linters. Refined cottonseed oil is the principle component consumed by humans worldwide, whereas meal and hulls are mainly animal feeds. Linters are the short fibers which remain on the hulls after removal of the lint and which have both nonedible and edible uses (National Cottonseed Products Association 1990).

Upland cotton (*Gossypium hirsutum* L.) is the predominant species grown in the U.S., averaging about 96.6% of the total harvested cotton acreage over the years 1988, 1989, and 1990. American-Pima (*G. barbadense*) accounted for the remaining 3.4% of harvested acreage. Texas was by far the leading cotton producing state, followed by California, Mississippi, Arkansas, Louisiana, Arizona, and eleven other states (USDA 1991). In the U.S. in 1990, cotton was harvested from an estimated 11.7 million acres, which yielded over 15.6 million bales (480 lb net weight bales) with a value estimated at over $5.1 billion. In addition, approximately 6 million tons of cotton seeds were produced, with a estimated value of $739 million (USDA 1991).

B. Taxonomy and Genetics of Cotton

The genus *Gossypium*, a member of the Malvaceae family, consists of 39 species, 4 to 5 of which are generally cultivated (Fryxell 1984). Cultivated forms of cotton are *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L., *G. herbaceum* L., and *G. lanceolatum* Todaro. The most important agricultural cottons in the United States are *G. hirsutum* and *G. barbadense*, which are allotetraploid, having 52 chromosomes (Endruzzi et al. 1984) and a haploid genome containing approximately 0.795 picograms (pg) or 7.3×10^8 base pairs (bp) of DNA (Walbot and Dure 1976). Two other species occurring in the U.S., *G. thurberi* Todaro (a diploid) and *G. tomentosum* Nuttall ex Seemann (a tetraploid), are wild plants of Arizona and Hawaii, respectively (Fryxell 1979).

C. Pollination of Cotton

G. 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 in the U.S. (McGregor 1976). Concentration of pollinators varies from location to location and by season and is considerably suppressed by insecticide use. If suitable bee pollinators are present, distribution of pollen decreases considerably with increasing distance. The isolation distances for

Foundation, Registered, and Certified seeds in 7 CFR Part 201 are 1320, 1320, and 660 feet, respectively. Results from outcrossing studies in transgenic and nontransgenic cotton show that pollen movement in BXN cotton is equivalent to that for other cotton varieties (Appendix 2).

The only wild tetraploid species in the U.S., *G. tomentosum*, seems to be pollinated by lepidopteran insects, presumably moths (Fryxell 1979). This is supported by its flowering characteristics. The stigma in *G. tomentosum* is elongated, so the plant seems incapable of self-pollination until acted upon by an insect pollinator. *G. tomentosum* flowers are unusual, too, because they stay open at night, unlike flowers of most *Gossypium* species which open in the morning and wither at the end of the day.

D. Basis for Risk Assessment

In 1988, the Biotechnology Science Coordinating Committee (BSCC), representing the U.S. Department of Agriculture, Environmental Protection Agency, National Institute of Health, National Science Foundation, and Food and Drug Administration, asked the National Research Council (NRC) of the National Academy of Sciences to evaluate scientific information pertinent to making decisions about the introduction of genetically modified microorganisms and plants into the environment. The NRC report (National Research Council 1989) discussed the relevant biological properties of genetically modified plants, including past experience with genetic modification and with introductions of plants modified by classical and molecular genetic methods. The environmental issue of potential weediness received special attention in the NRC report.

The following scientific conclusions are quoted from the "Executive Summary: Plants" section of the publication (National Research Council 1989):

1. Plants modified by classical genetic methods are judged safe for field testing on the basis of experience with hundreds of millions of genotypes field-tested over decades. They are, in the terms used by the plant subcommittee, "manageable by accepted standards." The committee emphasizes that the current means for making decisions are entirely appropriate and no additional oversight is needed or suggested in this report.
2. Crops modified by molecular and cellular methods should pose risks no different from those modified by classical genetic methods for similar traits. As the molecular methods are more specific, users of these methods will be more certain about the traits they introduce into the plants. Traits that are unfamiliar in a specific plant will require careful evaluation in small-scale field tests where plants exhibiting undesirable phenotypes can be destroyed.

3. At this time, the potential for enhanced weediness is the major environmental risk perceived for introductions of genetically modified plants. The likelihood of enhanced weediness is low for genetically modified, highly domesticated crop plants, on the basis of our knowledge of their morphology, reproductive systems, growth requirements, and unsuitability for self-perpetuation without human intervention.
4. Established confinement options are as applicable to field introduction of plants modified by molecular and cellular methods as to introductions of plants modified by classical genetic methods.

These conclusions are germane in consideration of whether a particular genetic modification of a particular crop species affects any weediness characteristic of the species. In this analysis, cotton (*G. hirsutum*) transformed to contain the BXN gene is considered.

E. Weed Characteristics and Environments

Evaluation of weediness potential requires a careful definition of terms. The term "weed" has been variously defined, depending on the different perspectives of ecologists, agronomists, and the public (Buchholtz 1967; Salisbury 1961). In this document, a weed is defined as "an unwanted or undesirable plant that persists in natural or human environments." A weed pest is a weed that "is considered a pest." These definitions reflect the concern that transgenic plants might become weed pests or cross-pollinate with weedy relatives enhancing their pest characteristics.

Assessment of weediness potential can be done at two levels. The first level is whether the target crop species is itself a weed pest under specific conditions and/or environments or is sexually compatible with weedy relatives. If either is true, then a second level of assessment is needed to examine specific properties of the crop, particularly those properties generally attributed to weeds such as seed dormancy, long soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersion, and long-distance dispersal of seeds (Baker 1974; Hill 1977).

Assessment of weediness potential can also be made based on the risk of a target crop contributing to weediness problems, with the risk being a function of the hazard times the exposure (risk = hazard x exposure)(Keeler 1989). If there is no exposure (i.e. the crop is not released into the environment) or if there is no hazard (i.e. the gene has a negative or neutral effect on fitness), then the risk of a target crop contributing to weediness problems is zero. As will be seen with cotton, only the first level of assessment is needed in determining its weediness potential because in the United States cotton is not a weed pest and has no sexually compatible weedy relatives, with the possible exception of *G. tomentosum* found in Hawaii outside the cotton production area. This latter case is discussed separately.

Environmental conditions are of concern, especially with respect to control mechanisms that hold a plant species in balance with a particular environment, whether it is natural or disturbed. A plant can become a weed if it escapes control by migrating to a new environment lacking the factors that controlled the plant in its original habitat. In most parts of the world, including the United States, the bulk of the weed pests are exotic plants (Holm et al. 1977; Mack 1985; Mack 1986). A second method for a plant to become a weed is when it remains in its original habitat but effectively escapes a particular control factor by gaining a trait that imparts to it the ability to overcome the control factor, such as acquiring resistance to disease (Pimentel et al. 1988). This is not the case for the BXN cotton, because the introduced trait has no selective advantage, except for resistance to the herbicide bromoxynil (Appendices 2, 3 and 4).

Weeds can be considered according to whether they invade natural or human environments:

1) Natural areas
2) Rangelands
3) Parks and roadsides
4) Crop production fields
5) Urban and suburban environments (backyard weeds).

Through natural selection, many weed pests have apparently adapted to conditions in disturbed environments. Weeds are able to escape from biotic control agents (predators, pathogens, and competitors) and persist, either vegetatively or by high seed production, in repeatedly disturbed areas such as cultivated fields (Harper 1965). Perhaps most successful (most widespread, persistent and abundant) are weeds that have not only immigrated but also have a long history of close association with human settlement (Baker 1974). Although weediness is primarily an economic concern for disturbed areas, it is also an ecological concern, particularly for natural areas. In determining the weediness potential for cotton, both environments are considered.

Weediness is determined by characteristics of the species and its relationship with biotic control agents (e.g. predators) and environmental conditions. The likelihood that a given species or line becomes a weed is very low (or zero) if at least one of the following is true:

1) The species (and sexually compatible relatives) does not have weediness traits,
2) There are clear and adequate natural control agents, or
3) The environment is not conducive for natural persistence of the particular species.

F. Weediness Potential in Cotton

Although cotton is not a weed pest (Weed Science Society of America 1989; Appendix 1), it is useful to address the concerns about introduction of genetically modified plants in the environment and the potential to inadvertently produce a new weed or increase the aggressiveness of existing weeds (Colwell et al. 1985; Tiedje et al. 1989). In this way, it can clearly be demonstrated that BXN cotton poses no additional weed pest risk than any other cotton varieties and that cotton itself is not a weed pest. Three aspects of weediness are of general concern (Keeler 1989):

1) Comparison of transformed crops with exotic species. Is the experience with the introduction of exotic plants into new environments (sometimes with the result that a weed problem is created) a valid analogy for the introduction of genetically modified plants?
2) Potential for transformed, domesticated crops to convert to a weedy state. For a given crop such as cotton, are there examples in which the crop has become weedy, such as due to plant breeding or movement of the species outside its center of origin?
3) Potential for hybridization between domesticated crops and wild relatives creating or enhancing weediness. Will crops such as cotton outcross with wild relatives and, if so, what is the potential for increased weediness?

Manasse and Kareiva (1991) state, "It is the prospect of uncontained spread that underlies many of the worries environmentalists express regarding biotechnology." The authors affirm "that an organism whose rate of spread is minimal poses negligible risk compared to an organism that can multiply its population and rapidly expand its range." In assessing the possibility of a transgenic crop or a sexually compatible weed pest becoming a greater weed problem, it is important to consider that weedy properties usually represent complicated, multigenic traits and generally do not result from single gene traits (Keeler and Turner 1991).

1. Comparison of Transformed Cotton with Exotic Species.

The analogy between the introduction of an exotic species into a new environment and the introduction of a genetically modified crop plant is tenuous (Fincham and Ravetz 1991) and is not applicable to BXN cotton. Introduced exotic plants that have become pests bring with them many traits that enhance weediness and, very importantly, leave behind control organisms (predators) and competitors. Genetically modified plants are altered in only a few, specific characteristics that relate to crop production characteristics (National Research Council 1989). Unlike exotic plant introductions, genetically modified crops will generally not be released into exotic environments but will be planted in typical growing regions for the specific crop. BXN cotton will be grown in areas currently under cotton

cultivation. Cotton has been grown in Africa, North and South America, Asia, Australia, and Europe (Munro 1987) without becoming a weed pest.

For the most part, introductions of exotic species have been environmentally harmless and economically beneficial; most North American crop plants are in fact exotic species. In rare situations, such as kudzu, introductions have resulted in environmentally undesirable consequences. In most cases, careful review of the organism's biology would have predicted the unfavorable consequences (Williams 1980) and the problem of weediness could have been avoided. In like manner, careful consideration of the biology of a transgenic crop (as addressed in this document) can alleviate any concern that the crop might respond like an exotic species that becomes established as a weed pest.

Recently, one report focused on the specific organisms that have become pests. Of an estimated 5,800 plant species intentionally introduced into the United States, 125 have become weed pests in some areas of the country (Pimentel et al. 1988). However, despite an exhaustive survey of plants, animals and insects, and the speculation that single gene changes in organisms could cause increased pest problems, the authors were unable to identify any examples of this occurring.

The likelihood of enhanced weediness is low for genetically modified, highly domesticated crop plants, on the basis of our knowledge of their morphology, reproductive systems, growth requirements, and unsuitability for self-perpetuation without human intervention (National Research Council 1989). Cotton has been highly characterized and is a well-defined major source of fiber and vegetable oil. Since cotton is an exotic species in the United States and has not become a weed pest, the concern of exotics becoming pests upon introduction into a new environment is not warranted for BXN cotton.

2. Potential for BXN Cotton to Become a Weed Pest.

As will be discussed in this section, there is very low or no potential for BXN cotton becoming a weed pest since the BXN gene does not confer any selective advantage outside agricultural fields where the herbicide Buctril is used.

Plant varieties are continually selected for improved resistance or tolerance to external factors that inhibit their inherent productivity using a wide range of plant breeding techniques. Plant varieties have been selected for 1) insect, disease and herbicide resistance, 2) better tolerance of environmental constraints to growth, such as heat, cold and drought tolerance, and ability to withstand high moisture, excessive alkalinity, excessive salts, iron deficiency and high aluminum content in soils; and 3) ability to prevail in competition with weeds through quick germination and extremely rapid growth in the seedling stage. In theory, such improved cultivars presumably are better adapted to persist in the presence of disease, insects, herbicides and a number of environmental constraints to growth.

However, plant breeders have a long history of incorporating these types of traits into crops without evidence of enhanced weediness (Appendix 3).

Similarly, it can be expected that crops modified by molecular and cellular methods should present no different risks in regard to weediness potential. Since molecular methods are highly specific in terms of what genes are being added, users of these methods will be more certain about the traits they introduce into plants (Appendix 3; National Research Council 1989) and the weediness potential may actually be less than using traditional breeding methods. Nevertheless, it is important to consider the effect of new, introduced genes on the potential of a crop to become a weed pest.

For new genes to be retained in a natural population, the genes must have at least one of three characteristics (Hauptli et al. 1985):

1) They must confer improved fitness to the first and resultant generations of the species.
2) They must have no negative effect on fitness.
3) The genes must be tightly linked to other genes conferring improved fitness.

If the genes do not confer improved fitness to the species, then individuals containing the genes will not have a selective advantage over those that do not.

Cotton. In agricultural areas, the cotton plant does not persist due to common crop practices and/or climatic conditions. According to Dr. Fred Bourland (Cotton Agronomist, University of Arkansas, personal communication) and Fryxell (1979), cotton would be a very poor competitor in most of the Southern and Southeastern United States cotton growing areas. In areas where freezing conditions occur, the cotton plant cannot overwinter and there is essentially no volunteerism from seed, because seed becomes nonviable after imbibition in cool soils (<50°F). In areas such as central Texas which lack freezing conditions, normal tillage prevents regrowth from the root stock. In milder climate areas, such as the Arizona low deserts, there is a use of "stubble cotton" return crop practices (in which the plant is regrown from its base the following season) is outlawed due to the possibility of overwintering plant parts harboring pests and diseases. A mandatory plow down date is set for specific areas as part of the USDA pink bollworm eradication program (Dr. Jeff Silvertooth, Cotton Agronomist, University of Arizona, personal communication). Volunteerism can be a sporadic problem in Arizona in areas under above-ground drip irrigation systems; however, this represents a very small percentage of the cotton acreage. In most cases, cultivation prevents volunteerism and the land is well worked due to the bed shaping required for irrigation. Irrigation is necessary for survival of the cotton plant in arid Western climates, thus cotton seed does not develop outside irrigated field areas (Silvertooth).

Upland cotton, *G. hirsutum*, does not outcross with weedy relatives in the continental United States, nor does it display significant potential to develop

into a weed itself (National Research Council 1989). *Gossypium* was not listed as a weed in the Weed Science Society of America, Composite List of Weeds (1989) nor in the following major references: Crockett (1977), Holm et al. (1977), and Muenscher (1980). It is not present on the lists of noxious weed species distributed by the State of California and the Federal Government (Appendix 1). In addition, cotton does not contain attributes associated with weed pests (Keeler 1989). Similarly, transformed cotton should have no increased tendency to convert to a weedy state since BXN cotton possesses the same agronomic characteristics as other cotton varieties, which have never been considered weeds, even in agricultural fields.

In general, cotton lacks many of the traits characteristic of weed pests and has not been considered a weed pest in the United States. The USDA has concluded in environmental assessments of transgenic field trial applications that cotton does not display significant potential to develop into a weed itself and that "the genus shows no particular weedy aggressive tendencies" (Appendix 3). This lack of weediness potential is for all environments, natural, disturbed, as well as in agricultural fields.

BXN cotton field trials. Calgene conducted extensive field trials with cotton containing the BXN gene in the principle cotton producing states in the U.S. (Table 1). In addition, cotton trials were conducted in Argentina, Bolivia, and South Africa. In none of the trials were any weediness problems nor *Agrobacterium* disease symptoms observed.

In plant breeding programs in general, broad variation is observed in early generations. For BXN cotton, variation was observed in the first two generations (T_1 and T_2), most likely a result of the tissue culture process and single plant descent. This variation is not different from variation derived through wide hybridization or other breeding methods, but is actually highly desirable. The next step was selection of lines possessing desirable traits, which was done through field trials. Lines demonstrating the desirable traits were selected and those with undesirable characteristics were discarded.

The BXN cotton trials were conducted to determine the effect of the BXN gene on agronomic and horticultural traits and to select desirable lines. In general, the trials showed that there were no changes in the selected lines that might affect weediness potential and that the cotton plants grew normally. Greenhouse and field observations conducted during all trials have shown that BXN cotton had the same agronomic traits as traditionally bred cotton. No unpredicted changes occurred (Appendix 4; Kiser and Mitchell 1991).

Table 1. Calgene's Field Trials with Cotton Containing the BXN Gene.

Permit #	States/Countries	Number of Sites	Date Issued	Trial Completed?	Report Enclosed[a]
92-106-01	AZ, SC	3	6/25/92	yes	yes
92-105-01	MS	1	6/9/92	yes	yes
91-357-01	AZ, SC, MS	4	4/17/92	yes	yes
91-333-02	MS	1	1/14/92	yes	yes
91-329-04	NC	1	3/6/92	yes	yes
91-329-03	AL, GA, MS, SC, TN	5	3/20/92	yes	yes
91-329-02	AR, LA, MO, TX	4	3/6/92	yes	yes
91-329-01	TX	1	3/6/92	yes	yes
91-107-06	SC	1	6/18/91	yes	yes
91-035-07	GA, SC	4	4/19/91	yes	yes
90-303-02	AL, AR, AZ, CA, GA, LA, MS, NC, SC, TN, TX	28	3/6/91	yes	yes
90-297-01	MS	1	3/6/91	yes	yes
90-016-04	MS	1	4/11/90	yes	yes
89-192-01	HI	1	10/10/89	yes	yes
89-047-07	MS	1	5/24/89	yes	yes
n/a[b]	South Africa	6	10/2/92	yes	yes
n/a[b]	Argentina	1	11/14/91	yes	yes
n/a[b]	South Africa	1	10/22/91	yes	yes
n/a[b]	Bolivia	1	8/29/91	yes	yes
n/a[b]	South Africa	1	11/5/90	yes	yes
Total number of sites		67			

[a] See Appendix 4.

[b] Field trials approved by appropriate government agencies in respective country.
n/a = not applicable.

Herbicide resistance. Of particular concern in the discussion of whether transgenic crops might have increased weediness is the effect of introduction of herbicide resistance. However, herbicide resistance is not an environmental concern for BXN cotton because BXN cotton is not a weed, is grown only in agricultural fields and does not persist in the environment. Neither does BXN cotton have weedy, sexually compatible relatives.

Several crops such as cotton, corn, sugarbeets, tobacco, and tomato that have been transformed to resist active ingredients of herbicides, such as glyphosate, bromoxynil, sulfonylurea and phosphinothricin, have been tested in the field. The potential benefit of such crop modifications is increased flexibility in weed control, such as greater efficacy, reduced cost to farmers, the opportunity to replace currently used chemicals with more environmentally compatible chemicals, and the reduction of overall herbicide usage (Boyce Thompson Institute for Plant Research 1987).

The key factors in evaluating the weediness risk of transgenic, herbicide-resistant plants are the possibility of volunteer plants becoming weeds in a subsequent year and the potential for introgression of herbicide resistance genes into weedy relatives. Corn, wheat, and sugarbeets are examples of crops which can become volunteer weeds but are controlled in subsequent crops by

cultivation and by different herbicide products. A glyphosate-resistant volunteer corn plant in a soybean field would be controlled by normally used pre-emergent or post-emergent herbicides. Similarly, a sulfonylurea herbicide-resistant wheat plant could be controlled in either rotational crops or on fallow land using today's normal cultural practices.

Of significant importance is past and current experience with breeding herbicide resistance into crops, such as metribuzin resistance in soybean, atrazine resistance in canola, and acetanilide resistance in corn. These nontransformed, herbicide tolerant crops have stable phenotypes, and the modifications have not increased the weedy characteristics of the crop. The primary U.S. crop targets into which herbicide resistance is being genetically engineered are corn, cotton, soybean, and tomato. None of these species outcrosses with weedy relatives in the United States or displays significant potential to develop into weeds (National Research Council 1989).

Herbicide resistance genes already exist in some weedy species. For a herbicide resistance gene to persist in a weed population, the gene must offer a selective advantage to those plants possessing it. This can occur when the herbicide in question is applied continuously for a number of generations, creating enough selective pressure to maintain the gene in a weedy population (Gessel 1984). This problem can be controlled using appropriate agricultural production practices, whether herbicide resistance is derived from classical breeding or from genetic engineering.

Bromoxynil resistance. Bromoxynil (trade name Buctril®) is a halogenated aromatic nitrile (3,5,-dibromo-4-hydroxybenzonitrile) and is registered for control of certain broadleaf weeds in small grains, field corn, cereals, popcorn, grain sorghum, seedling alfalfa, flax, garlic, onions, annual canarygrass, mint, and turf grown for sod (Appendix 5). The herbicide is applied at specified growth stages of the crops on which it is used. Many plants, especially grasses, corn, and small grains such as barley, possess natural resistance to the herbicide.

Bromoxynil was first registered for use as a herbicide in 1963 for small grains and some specialty crops such as garlic and mint. In 1983, this was expanded to include post-emergent corn; in 1985, to include sorghum and milo.

The use of bromoxynil for weed control in BXN cotton could dramatically reduce overall herbicide use in cotton production. In one experiment, for example, the use of a BXN cotton/Buctril herbicide system effectively controlled problem weeds using less herbicide and fewer herbicide applications without reducing crop yields (see Section VI, Environmental Consequences of Introduction of the Transformed Cultivars: Impact of Use of BXN Cotton on the Environment).

Introduced genes. None of the introduced genes pose any environmental risk. The BXN gene encodes the enzyme nitrilase which catalyzes detoxification of bromoxynil by hydrolyzing the active cyano moiety. Hydrolysis via nitrilases has been well characterized and results in 3,5-dibromo-4-hydroxybenzoic acid and free ammonia (Harper 1977a; Harper

1977b; Harper 1985; Mahadevan and Thimann 1964; McBride et al. 1986; Smith and Fletcher 1964; Thimann and Mahadevan 1964).

The nitrilase encoded by the BXN gene has a high substrate specificity for bromoxynil. As compared with bromoxynil metabolism, metabolism of 3-bromo-4-hydroxybenzonitrile and 4-hydroxybenzonitrile by this nitrilase was 6- and 75-fold less, respectively. Additionally, neither 3,5-dibromo-4-hydroxybenzamide nor benzonitrile were efficient substrates. In contrast, a *Nocardia* nitrilase has low substrate specificity for bromoxynil but high specificity for benzonitrile (McBride et al. 1986). The high substrate specificity indicates it is very unlikely that BXN coded nitrilase metabolizes other substrates.

The BXN gene does not confer any selective advantage on cotton which would enhance its survival outside an agricultural environment. The BXN gene only affects resistance to the herbicide bromoxynil and does not confer resistance to any other herbicide. In its environmental assessments, the USDA concluded, "neither the introduced genes, nor their gene products, confers on cotton any plant pest characteristics" (Appendix 3).

Other introduced gene sequences (*kan*^{r} gene and specific noncoding regulatory sequences) in the BXN cotton also do not confer any selective advantage which would enhance survival of cotton in the field. No characteristics of these sequences give any indication these genes would increase fitness of BXN cotton, and none of the sequences have any relationship to the traits that characterize weed pests (Keeler 1989). Therefore, even if transfer of genes among *G. hirsutum* plants did occur, there would be no change in weediness potential.

Seed germination. Transformed cotton lines were shown to be unchanged from control lines in regard to seed germination rate and frequency, as measured with seed harvested from the greenhouse and the field (Appendix 6). Empirical observations of seedling emergence and stand establishment made throughout the eight seasons of cotton field trials indicate Calgene's transgenic cotton strains do not show increased dormancy. In a greenhouse study, the germination rate was not affected by the transformation process, and the nongerminated seed showed no sign of morphological characteristics which would indicate dormancy.

Strict quality control (QC) standards are in place for seed production of commercial lines, since uniform, high germination frequency is essential for commercial cultivars. Cotton lines under product development are not finished varieties and therefore are not subject to strict QC measures such as high quality field production, screening/gravity table or size sorting, and germination tests. The plant breeding and product development process will eliminate or improve lines with slow germination rate or low germination frequency. Under commercial settings, all seed must have fast germination rate and a high germination frequency.

Seed survival. No enhanced survival of transgenic cotton seeds was found after extensive monitoring of 50 field trials conducted over a two-year period.

Thirty-two post-trial monitoring reports were received for the 1991 cotton trials using the BXN gene in construct pBrx45 (USDA APHIS Permit Numbers 90-297-01, 90-303-02 and 91-035-07)(Appendix 7). Twenty-nine trials reported no volunteer cotton plants within the trial sites as monitored during 1992. One site was replanted with transgenic cotton in 1992 (Washington County, MS). One site reported regrowth from stubble surviving a disking operation (Wharton, Texas) and one site reported germination of a few seeds (Barnwell, SC). For the latter two, the plants were rogued and no further volunteers were observed. Volunteer plants from seed were also observed and destroyed 1-2 months after trial destruction in Pemiscot County, MO and Darlington County, SC (Appendix 8). This low incidence of regrowth and volunteers is typical for these growing regions based on observations from cotton growers, cooperators and Calgene Product Development staff. Volunteers are easily controlled by cultivation or crop rotation.

Sixteen field trial notebooks (Appendix 9) were received for the 1992 cotton trials using the BXN gene in construct pBrx45 (USDA APHIS Permit Numbers 91-329-01, 91-329-02, 91-329-03, 91-329-04, 91-333-02, 91-357-01). No volunteers were reported at any of these sites except for a solitary plant in "extremely poor condition" which was pulled one month after the trial was destroyed in Arizona. This plant was not a volunteer from seed, but rather a plant which survived the disking during crop destruction.

Seed dormancy. No changes in seedling emergence, stand establishment or seed dormancy were observed over four years of trialing transgenic cotton.

Change in seed dormancy is a factor which may influence persistence in the environment. An impermeable seed coat, which confers temporary dormancy, is a common character in most members of the *Gossypieae* tribe (Fryxell 1979 pp. 128). Selection for rapid germination and thus, the loss of seed dormancy was part of the domestication of cotton (Fryxell 1979, pp 168-169). In cultivated cotton, the seed coat dormancy is broken by weathering in the field over the maturation period (Cherry and Leffler, 1984 pp 525-526) or abrasion of the seed coat in the ginning and delinting process.

Empirical observation of seedling emergence and stand establishment in the field over four years of advancing BXN cotton strains in a two season per year breeding program have not indicated any seed dormancy. Seed which is being planted rapidly after harvest is the most likely to show dormancy. We routinely plant BXN cotton strains grown in a winter nursery within four to six weeks of harvest and have observed no inhibition in emergence. Winter nursery seed has germinated in standard tests and compared favorably to commercial varieties. No evidence of increased seed dormancy or decline in ability to germinate in BXN cotton has been observed (Appendix 6).

Relative fitness. Agronomic fitness of BXN cotton lines were unchanged from nontransgenic controls (Table 2). A comparison of advanced BXN cotton strains in Stoneville Pedigreed Seed Company's breeding program with five current commercial varieties was conducted which provides an indication of potential environmental effects. Using data generated in the 1992 replicated yield trials, an analysis of variance was completed (Appendix

2). In field trials covering the three divergent growing regions of the US cottonbelt, the BXN cotton strains produced lint yields within the same range as the current commercial varieties. Comparative yield and quality trials give us the best measure of a strain or line's fitness as a variety: These trials also provide a good comparison of the variation existing in a set of transgenic strains to the variation present in a set of standard commercial varieties. The characteristics measured in these tests are those which make a variety valuable as a crop, and therefore some of these characteristics define the crop. These data support the position that BXN cotton lines exhibit variation within the range expected for cotton and are morphologically and agronomically similar to typical cotton varieties.

Table 2. Summary of Characteristics of Commercially Suitable BXN Cotton Lines Compared to Commercial Varieties Grown Under Field Conditions (Appendix 2).

Characteristic	Significantly different[a]	Not significantly different[a]
Agronomic Traits[b]		
Buctril herbicide resistance	+	
Seed germination %		+
Seedling vigor		+
Stand establishment		+
Maturity		+
Yield		+
Fiber Characteristics[a]		
Lint Percent		+
Length		+
Uniformity		+
Strength		+
Elongation		+
Micronaire		+

[a] Analysis of variance with statistical differences at 5% confidence level.
[b] Values for yield across three locations (4 replications); fiber quality data from two locations (2 replications); and maturity rating, one location (2 replications).

Since cotton is not considered weedy, relative rather than absolute measures are made. Traits that might indicate a greater potential for weediness include faster growth rate, consistently higher seed yield, increased seedling vigor or seed germination over a long period of time. However, our field tests provide measurements of crop performance in agricultural settings. The potential for movement of a crop or gene from a managed agricultural system into a natural community is largely attributable to its fitness advantage. This potential can be measured by quantification of the net replacement value and by persistence and invasiveness studies comparing

the parent and the transgenic crop. These studies must simulate non-managed, settings, that is, without common agronomic practices. Following the methodology used in the PROSAMO studies (Crawley (1992) net replacement values may be calculated. A value of less than 1 indicates the plant would become extinct without human intervention.

Estimates of this parameter were made for BXN cotton in collaboration with Dr. Peter Kareiva (Appendix 2). Following the fall harvest in 1992, fuzzy cotton seed (i.e. not delinted) were buried in paper cups (to aid retrieval) along the margin of the field site at the Stoneville breeding station, Washington County, Mississippi. The planting conditions were uncultivated to simulate field margins, with the density of weeds experimentally manipulated. The site was monitored for seedling emergence and as of July 7, 1993, no cotton seed had emerged and an indigenous weed population had established. Thus, the net replacement value for cotton in the Mississippi delta is zero, since no seed germinated and no new seed was produced. Under these conditions, BXN cotton is neither persistent nor invasive. Seed that may leave the field following harvest will be unable to reproduce and establish a feral population. The BXN gene provided no competitive advantage for cotton in field margins.

Although similar studies were not conducted in other states, it is expected that the BXN gene would also not provide a competitive advantage and BXN cotton would not be persistent or invasive. For example in Arizona and other arid Western climates, irrigation is required for cotton to survive and so it has not become established outside of managed agricultural areas (Silvertooth, personal communication). To become establish in unmanaged areas, cotton seed must survive overwintering, seedlings must survive and the plants must reproduce. However, in the U.S., wild populations of *G. hirsutum* occur only in Florida (USDA 1993).

Outcrossing. Outcrossing in BXN cotton was not altered over that observed in other cotton varieties. This was expected, since cotton is normally considered to be a self-pollinating crop, although natural outcrossing may range from zero to more than 50%, depending on location (Simpson 1954). Three studies provide information to compare outcrossing between transgenic cotton (Kareiva and Morris report in Appendix 2; and Umbeck et al. 1991) as compared with traditionally bred varieties (Vaissiere report in Appendix 2). The results show agreement among the three studies which were conducted at different locations using different genetic markers. All three studies had the same conclusion that outcrossing decreased very rapidly as the distance increased and that there was very little pollen transfer beyond 12 m (Kareiva and Morris report in Appendix 2; and Umbeck et al. 1991). These data provide evidence that the distance of pollen movement is unchanged in transgenic lines.

When considering outcrossing results at the same location (Stoneville, MS), two reports showed the same outcrossing frequency. Meredith and Bridge (1973) detected no outcrossing between adjacent plants in a study conducted in Stoneville, MS (the approximate limit of detection for the

sample size and methods was approximately 0.046%). Their observations were consistent with results obtained for BXN cotton (see Table 1 in Kareiva and Morris 1993 in Appendix 2), which showed no outcrossing in 4144 seeds at any of the 5 distances sampled.

These results support the conclusion that integration and expression of the BXN gene construct did not alter outcrossing behavior of transgenic cotton as compared to nontransgenic cotton. In the opinion of Kareiva, Morris and Jacobi (1993) the quantification of outcrossing should not be a priority of risk assessment. The authors conclude by recognizing that once a transgenic crop is determined not to be a regulated article under 7 CFR part 340 and is planted commercially, movement of transgenic pollen is inevitable. Thus, to assess the potential for environmental impact, it is important to understand the invasiveness potential of the new crop.

Summary of potential for BXN cotton to become a weed pest. Based on these relative fitness studies, germination results, outcrossing data, and observations made during 67 field trials, BXN cotton seed is not different from cotton seed produced through traditional breeding and is unaffected by the BXN gene and other inserted genes. There is no indication that the transgenic seed will be dispersed differently, last longer or be more competitive in new environments. Pollination characteristics also were unchanged. Therefore, there is no greater potential for BXN cotton, or any other cotton cultivar crossed with it, to become a weed pest risk than for traditionally bred cotton. A list of studies with BXN cotton to support these conclusions is provided in Table 3.

Table 3. Field Studies on BXN Cotton.

Study Report	Location
Relative Fitness in Agronomic Ecosystems	Appendix 2
Relative Fitness in Non-managed Ecosystems	Appendix 2
Outcrossing within *Gossypium*	Appendix 2
Seed Germination	Appendix 6

3. Potential for Hybridization Between BXN Cotton and Wild Relatives Creating or Enhancing Weediness.

Cotton has never been considered a weed pest itself, so any possible weediness problems would have to be a result of outcrossing with weed pest relatives. As discussed, cotton lacks sexually compatible weed pest relatives.

The various species of the genus *Gossypium* typically occur in relatively arid parts of the tropics and subtropics. The genus contains only one member, *G. tomentosum*, which has been considered by some in the past to be a weed. It is found only in the United States (Holm 1979), occurring in the Hawaiian archipelago (Fryxell 1984). Also called "native cotton or mao," it is endemic to Hawaii, where it occurs in dry coastal regions (Haselwood et al. 1983). According to Robert Hirano (Lyon Arboretum, Manoa, HI), who revised the

1983 edition of the *Handbook of Hawaiian Weeds*, *G. tomentosum* is no longer considered a weed, as populations are now limited and it is fairly rare on most of the Hawaiian islands. Populations on Molokai, for example, are known to occur in open kiawe forest and rocky coastal strand vegetation. Inland agricultural areas do not provide suitable habitat.

Native cotton has been reported on the leeward coast of Oahu, Lanai, Maui, and Kahoolawe (Wagner et al. 1990), and was never considered a weed in agricultural areas (Hirano, personal communication). D. R. Herbst (United States Dept. of Fish and Wildlife, Hawaii) maintains that *G. tomentosum* never was a weed; it is a native plant. This species is now considered vulnerable in Hawaii due to loss of its coastal habitat, which is under heavy development pressure (Joel Lau, Botanist, Nature Conservancy of Hawaii, personal communication).

According to Dr. Paul Fryxell (Texas A&M University), *G. hirsutum* is readily cross-compatible only with other tetraploid members of the genus *Gossypium*, which includes *G. tomentosum* in Hawaii, *G. darwinii* in the Galapagos, *G. mustelinum* in northeastern Brazil, *G. hirsutum* and *G. lanceolatum* in tropical/subtropical America, and *G. barbadense* in South America, as well as cultivated forms of *G. hirsutum* and *G. barbadense* (Fryxell 1979). Under natural conditions, chromosomal incompatibility of *G. hirsutum* with *G. tomentosum*, for example, leads to genetic breakdown of the F_2 generation, so the cross does not survive beyond the initial F_1/F_2 generations (Fryxell, personal communication). Even if such a hybrid survived and produced subsequent generations, the herbicide resistance trait would not provide any selective advantages in areas where *G. tomentosum* occurs naturally. Herbicide resistance does not provide selective advantage in natural habitats where herbicides are not used (Dale 1992). Therefore, genetic diversity of any wild cotton species will not be affected, since there would be no selective advantage from the herbicide gene in the wild species (Office of Biotechnology Iowa State University 1991). Furthermore, traditionally bred cultivars of *G. hirsutum* have been grown in Hawaii for counterseasonal seed increase and breeding purposes without restrictions and apparently without significant reports of outcrossing with *G. tomentosum*.

Upland cotton is not cross-compatible with wild diploid *Gossypium* such as *G. thurberi*, which occurs in Arizona, due to difference in ploidy level. Although *Gossypium* tetraploids and diploids have coexisted in the Americas for millennia, no natural hexaploids exist (Wendel 1989). Diploid cotton species have been crossed with tetraploids in breeding programs, but the vigor of the hybrid seed is low and the hybrid plants are usually infertile (Munro 1987). Even between cotton species which are closely related, they "maintain their identity when grown together; while vigorous hybrids may occur in the first generation, the later generation hybrids disappear from the mixture" (Munro 1987). Consequently, crossing of commercial cotton with *G. thurberi* is not a concern.

The habitat of the wild tetraploids in the tropics-subtropics is distinctive: coastal strand or seashore populations (Fryxell 1979). *G. hirsutum*, for

example, occurs in strand vegetation in tropical Americas rimming the Gulf of Mexico and extending into Florida, where it is an "outpost shrub" and is exposed to occasional salt spray. This ecological distribution places these wild tetraploids in virtual isolation from areas of agriculture.

Because cotton has no weed pest relatives, the possibility that a cross between transformed cotton and wild cotton would enhance weediness is zero. Also, the special case of *G. hirsutum* and *G. tomentosum* will not increase weediness because of low vigor of the hybrid and lack of significant weediness of *G. tomentosum*.

G. Mode of Gene Movement in Cotton

1. Pollination.

Movement of cotton genetic material by pollen is possible only to those plants with the proper chromosomal type, in this instance only to allotetraploids. In the United States this group would include only *G. hirsutum*, *G. barbadense*, and *G. tomentosum*. *G. thurberi*, the native diploid from Arizona, 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, lack of attractiveness to bees, intermediate pollinator-attractive plants, and other temporal or biological impediments would reduce the potential for pollen movement. Movement of genetic material to *G. tomentosum* is less well documented. The plants are chromosomally compatible with *G. hirsutum*, but some doubt exists 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 (McGregor 1976, Fryxell 1979, Fryxell 1984, Appendix 3).

Genes in cotton can move via pollination within the species *G. hirsutum* (Meredith and Bridge 1973). As with any other cotton line, seed purity will be maintained using standard breeding practices, such as for maintenance of pure seed stock. The BXN gene affects only resistance to bromoxynil, and this is not expected to have any effect on managed ecosystems (i.e. cotton production areas) as compared with current practices. Bromoxynil resistance will not increase the survival of cotton in a managed system, nor will it enhance the possibility that BXN cotton could become a weed.

Transfer of introduced genes to wild and weedy relatives of cotton will have no impact, because no sexually compatible weedy relatives are present in the United States. Gene transfer via pollination of endangered species is not a concern, since no sexually compatible relatives are endangered. Although it is possible that crosses between BXN cotton and *G. tomentosum* could occur in Hawaii, this possibility also exists for any other cotton variety and would have no different outcome. A cross between BXN cotton and *G. tomentosum* would provide no selective advantage to the hybrid unless

Buctril were used in unmanaged ecosystems. Consequently, it is extremely unlikely that BXN cotton will have any impact on unmanaged ecosystems.

Pollen movement and range of outcrossing are not affected by the presence of the BXN gene or the other gene sequences inserted using the binary vectors (McBride and Summerfelt 1990) and the BXN gene with its associated 35S promoter and *tml* 3' terminator (Appendix 2). Because the BXN gene confers no selective advantage to cotton except when sprayed with bromoxynil, there will be no danger that there would be reduced genetic variability in wild species should the unlikely event of outcrossing occur.

2. Potential Transfer of Genes from Plants to Microorganisms.

There is a theoretical possibility that a gene which was stably integrated in the cotton genome could be transferred to another organism, such as a soil microorganism. Such movement is termed "horizontal transfer." The possibility of horizontal transfer is of particular concern for antibiotic resistance genes because of the potential to expand the population of antibiotic resistant pathogens.

In their review of selectable marker genes, Flavell et al. (1992) concluded that transfer of transgenes from plant to pathogenic bacteria to humans or other species is highly unlikely and that potential gene movement from genetically modified plants will not cause unacceptable environmental damage.

Horizontal transfer is not a concern for the BXN gene because it is already naturally found in soil microorganisms, from which it was isolated (Stalker and McBride 1987).

The issue of horizontal transfer of the *kan*^{r} gene is discussed in Section VI of this document. In summary, horizontal gene flow from plants to microorganisms does not represent a risk because even if it could occur (as put forward in our hypothetical model), the probability of such a transfer posing a risk is extremely low. This conclusion is supported by the USDA in its environmental assessment of BXN cotton (Appendix 3). Furthermore, microorganisms containing *kan*^{r} and nitrilase-encoding genes are already present in soil, thereby mitigating any addition of these genes to the rhizosphere, if such transfer did occur.

H. Conclusions for Section II

1. The cotton genus *Gossypium* is not a weed pest risk. BXN cotton will not have enhanced weediness traits compared to nontransformed cotton.

2. There is little risk of genetic transfer to other *Gossypium* species because of natural outcrossing barriers and ploidy differences.

3. Commercial cotton is not sexually compatible with any weed species, nor are wild *Gossypium* species considered weed pests in the United States. Therefore, there is no potential for exchange of weedy traits by cross-pollination with weeds or weedy relatives.

4. The BXN gene will not confer weediness to cotton. There is no selective advantage to bromoxynil resistance except in situations where bromoxynil is used, which is generally crop production fields. Normal crop practices will control any persistence that might occur in cotton fields: cotton is grown as an annual, it is intolerant to frost, and in frost-free growing regions there are general requirements to prevent overwintering. In non-managed field margins, cotton seed were not persistent.

5. BXN cotton differs phenotypically from nontransformed cotton only for bromoxynil resistance and resistance to kanamycin.

III. The Transformation and Vector System

The vector system used to transfer the BXN gene to cotton is based on the Ti plasmid from *Agrobacterium tumefaciens* and is described in McBride and Summerfelt (1990). The vector system is "disarmed" or nonpathogenic, i.e. all the genes responsible for crown gall disease normally found in the T-DNA have been deleted. This system is also "binary" with the genes to be transferred on one plasmid and the genes encoding necessary functions for transfer, the *vir* genes, on a second plasmid. Genes on the second plasmid are not transferred to the transgenic plant.

The BXN cotton is considered a regulated article because it may contain the following subspecies (DNA sequences) from the list of organisms in 7 CFR 340.2: the *tml* 3' terminator and the right and left border regions from *Agrobacterium tumefaciens* (Barker et al. 1983), the ori pRi from *A. rhizogenes* (Jouanin et al. 1985, McBride and Summerfelt 1990), and the 35S promoter region (CaMV35S) from cauliflower mosaic virus (CaMV)(Gardner et al. 1981).

The binary vectors described in McBride and Summerfelt (1990) and the BXN gene with its associated 35S promoter and *tml* 3' terminator were used to produce the pBrx74 and pBrx75 constructs (Appendix 10). pBrx74 and pBrx75 were used to produce a number of BXN cotton varieties. The method used to co-cultivate cotton using disarmed *A. tumefaciens* (Appendix 11) is similar to the methods described for tomato cotyledonary explants by Fillatti et al. (1987) and for *Brassica napus* hypocotyl explants described by Radke et al. (1988). *A. rhizogenes* was not used as a vector for cotton transformation. Varieties will also be developed by traditional breeding using progeny of BXN cotton crossed with other cotton lines.

The USDA has written in environmental assessments that, "the vector used to transfer the resistance [BXN] genes to cotton plants has been evaluated for its use in this specific experiment [field trial] and does not pose a plant pest risk herein. The vector, although derived from a DNA sequence with known plant pathogenic potential, has been disarmed; that is, the genes that are necessary for producing plant disease have been removed from the vector" (Appendix 3).

The transferred genetic material in BXN cotton was shown to be genetically stable and segregate in a Mendelian fashion (Appendices 4 and 11). None of the transgenic cotton have shown any pest characteristics or evidence of infection by a pathogen, such as gall formation, even after several generations (Appendix 4, 8 and 9). All data generated on BXN cotton suggest the transgenic sequences and resultant selected plants are not a pest risk, nor have any negative results been produced on the safety of these cotton varieties (Appendix 4).

Conclusions for Section III

1. Vectors used in production of BXN cotton are derived from binary vectors described in McBride and Summerfelt (1990).
2. Vectors used in production of BXN cotton have been disarmed and do not pose a plant pest risk.
3. BXN cotton lines are genetically stable and do not exhibit any pest risk characteristics or evidence of infection by a pathogen, such as infection by *Agrobacterium*.

IV. Donor Genes from Organisms Considered Regulated Articles under 7 CFR 340

The BXN cotton is considered a regulated article under 7 CFR 340 because it may contain the following subspecies (DNA sequences) from the list of organisms in 7 CFR 340.2: the *tml* 3' terminator and the right and left border regions from *Agrobacterium tumefaciens* (Barker et al. 1983), the ori pRi from *A. rhizogenes* (Jouanin et al. 1985, McBride and Summerfelt 1990), and the 35S promoter region (CaMV35S) from cauliflower mosaic virus (CaMV)(Gardner et al. 1981). *A. tumefaciens*, *A. rhizogenes* and CaMV are considered plant pests.

A. Nonregulated Articles

Gossypium hirsutum L. is not a regulated article under 7 CFR 340. The BXN gene (a nitrilase gene isolated from *K. ozaenae*) is not a regulated article under 7 CFR 340 (Appendix 3). The *kan*^{r} gene encoding APH(3')II was isolated as a component of transposon Tn5 from a ColE1::Tn5 containing strain of *Escherichia coli* K12. The Tn5, Lac Z', and ori 322 sequences are from *E. coli* which is not a plant pest. These genes and gene sequences are contained in the binary vectors described by McBride and Summerfelt (1990) as well as the constructs identified as pBrx74 and pBrx75.

A description of the function of the genetic modification in the BXN cotton is provided in Appendices 4 and 12, and Kiser and Mitchell (1991). The nitrilase gene (the BXN gene) was identified, cloned, and inserted into the cotton genome. The expression product of the BXN gene, nitrilase, detoxifies bromoxynil by hydrolysis into 3,5-dibromo-4-hydroxybenzoic acid and free ammonia (Harper 1977a; Harper 1977b; Harper 1985; Mahadevan and Thimann 1964; McBride et al. 1986; Smith and Fletcher 1964; Thimann and Mahadevan 1964). The result is the cotton plants have greater resistance to bromoxynil than nontransgenic cotton, which is the direct intended technical effect of the BXN gene (Kiser and Mitchell 1991). Nitrilases are commonly found in many organisms (Appendix 13), but there are no reports of nitrilases in cotton, except for BXN cotton. The BXN nitrilase is highly substrate specific for bromoxynil (McBride et al. 1986), so it is very unlikely that BXN coded nitrilase metabolizes other substrates.

Descriptions of the *kan*^{r}, Lac Z' and ori 322 sequences are described in Beck et al. (1982), Yanisch-Perron et al. (1985), Sutcliffe (1978) and "*kan*^{r} Gene: Safety and Use in the Production of Genetically Engineered Plants" (Food Additive Petition No. 3A4364). These genes are important for the genetic engineering and transformation processes, allowing for selection of desired material.

The USDA has concluded that the BXN gene, its expression product (nitrilase), the *kan*^{r} gene, and its expression product (APH(3')II) do not confer "on cotton any plant pest characteristic" (Appendix 3).

B. Sequences from Organisms Considered Regulated Articles under 7 CFR 340

A description of the *tml* 3' terminator, the right and left border regions from *Agrobacterium tumefaciens*, the ori pRi sequence from *A. rhizogenes*, and the CaMV35S promoter from CaMV are provided in Barker et al. (1983), Jouanin et al. (1985) and Gardner et al. (1981). These sequences, isolated from their source organisms, do not present a plant pest risk.

Agrobacterium has a broad host range, generally defined within dicotyledonous plant species, but not strictly limited as such (Houck et al. 1990; White 1989).

Wildtype Ti plasmids contain T-DNA (transferred DNA) which is stably integrated into the plant nuclear genome. The Ti plasmid used in the production of BXN cotton was "disarmed" so that the plasmid no longer could re-direct plant cells into biosynthesis of phytohormones leading to tumor (or gall) formation. This was done by identifying an *A. tumefaciens* strain containing a deletion plasmid that did not retain any T-DNA (Ooms et al. 1982). This disarmed strain was transformed with the binary plasmid (pBrx74 or pBrx75) which has a T-DNA region containing the BXN gene. Because none of the T-DNA genes are involved in transfer and integration (Zambryski 1988), this integrated material (T-DNA containing the BXN gene) does not contain the necessary *A. tumefaciens* genes, such as the *vir* genes needed for transfer and infection (Fincham and Ravetz 1991).

The disarmed Ti plasmid contains DNA sequences that are not in the binary vector plasmid and so are not transferred to the transgenic plants. These Ti sequences include the *vir* genes, octopine or nopaline catabolism genes, *tra* functions (the ability for conjugal transfer of the Ti plasmid between bacteria), and origin of replication. These genes are not transferred to the recipient plant during transformation (Hohn and Schell 1987; Koukolíková-Nicola et al. 1987).

Following the use of the disarmed *Agrobacterium* strain (which contains the disarmed Ti plasmid) for plant transformation, the *Agrobacterium* are killed with carbenicillin so no subsequent infection or transformation can occur (Fillatti et al. 1987). The transformed plants are regenerated from sterile cultures and are then grown to produce seed. Because of these procedures, the original plant transformation vector (Ti plasmid) does not remain associated with the plants, and any further transfer of genes from such plasmids to humans, animals or the environment could not occur.

Segregation of the inserted gene according to predicted Mendelian ratios and continued expression of the phenotype over multiple generations confirm and support stability of the inserted genes. Southern analyses to identify gene copy number and demonstrate lack of gene movement and inserted nucleotide sequences are described in detail in Appendices 4, 10, 12, and 14.

1. Terminator.

The 3' end of the *tml* gene from *A. tumefaciens* (Barker et al. 1983) is used as a terminator for inserted genes in pBrx74 and pBrx75. The *tml* 3' sequences function only in expression of the *kan*^{r} and BXN genes. No *A. tumefaciens* disease symptoms were observed in any plants in any of the field trials (Appendix 4). These sequences, as used in producing BXN cotton, no longer function as regulated articles since they do not make BXN cotton a plant pest.

2. Border Regions.

The right and left border regions (Barker et al. 1983) are the only necessary *cis*-acting elements in T-DNA (Klee and Rogers 1989) for T-DNA transfer. The use of a binary vector system allows for other necessary elements to act in *trans* so only the border regions are required for integration of the T-DNA into the plant host genome (Zambryski 1988).

These border regions facilitated integration of the specific gene sequences into the cotton genome. No *A. tumefaciens* disease symptoms were observed in any plants in any of the field trials (Appendix 4). These sequences, as used in producing BXN cotton, no longer function as regulated articles since they do not make BXN cotton a plant pest.

3. Origin of Replication from pRiHRI.

The origin of replication (ori pRi or *ori*) from plasmid pRiHRI of *Agrobacterium rhizogenes* strain HRI allows the binary vector to be stably maintained in the disarmed *A. tumefaciens* strain without antibiotic selection (Jouanin et al., 1985). The binary vector was constructed using an 8 kb fragment of pRiHRI named Bam HI-11 since this fragment confers additional stability over just the *ori* sequence alone. This 8 kb fragment includes the *stb* locus (stability and copy number) as well as *ori* functions (Jouanin et al. 1985). Published data show that the *ori* sequences, and indeed the entire Bam HI-11 fragment, are both physically separated and functionally distinct from DNA sequences responsible for inducing plant disease.

HRI is an agropine strain. Agropine-type pRi plasmids harbor two T-regions (TL and TR) which can be transferred independently to the host plant genome, and can each independently induce proliferation of transformed roots (Vilaine and Casse-Delbart, 1987).

pRiHRI was mapped by Jouanin (1984). The *ori* and *vir* regions are nearly adjacent, but both are far removed from the T-DNA regions. The *vir* region ends within BamHI fragment 3 based on homology with *vir* regions from pTiAch5 and pRi8196 (Birot and Casse-Delbart, 1988). Four additional fragments, 19a, 9, 23 and 33, separate fragments 11 and 3 by at least 12 kb. Thus, it is virtually certain that BamHI fragment 11 does not contain any of the *vir* region. BamHI fragment 11 is even more distantly located from the two T-DNA regions.

Huffman et al., (1984) completely mapped the restriction enzyme sites in pRiA4b, another Ri plasmid. All agropine-type Ri plasmids examined are very similar and quite separate as a group from the mannopine-type plasmids

(Costantino et al., 1981; White and Nester, 1980). The origin of replication region in this Ri plasmid is also physically separated from the virulence region and both T-DNA regions, for example.

White et al. (1985) published a functional and molecular analysis of both T-DNA regions of pRiA4b. Neither of these regions contained the origin of replication sequences. Disarmed plasmids in nonpathogenic *Agrobacterium* strains contain *ori* sequences. Cosmids containing full length T-DNA's restored full virulence of such *Agrobacterium* strains and caused normal hairy root tumor morphology on Kalanchoe stems (White et al., 1985). Furthermore, *A. rhizogenes* was not used as a vector for transformation of cotton. These results demonstrate that *ori* sequences do not confer pathogenicity or virulence in *Agrobacterium.*

4. CaMV35S.

The 35S promoter region (CaMV35S) is derived from cauliflower mosaic virus (Gardner et al. 1981). Cauliflower mosaic virus is a double-stranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. Genome size is about 8 kb. CaMV35S has a very high constitutive strength as compared to other plant promoters, allowing it to be widely used as a promoter for high expression of genes (Gronenborn and Matzeit 1989).

The CaMV35S promoter sequence has not been shown to be a plant pest risk. Palukaitis (1991) concludes that, "while some of these plants [containing CaMV35S promoter] may have shown either unusual or abnormal responses, it has in every case been possible to delimit these host abnormalities to the expression of the gene and not to the presence of a promoter of viral origin. There is no evidence that the sequences of the CaMV promoters are in themselves inducers of pathogenicity. Thus, the major gene product rather than the well-characterized regulatory signals on the CaMV DNA are involved in the induction of pathogenicity in plants."

CaMV35S is the promoter region that drives the BXN gene (and the *kan*^{r} gene for some of the binary vectors). Expression of the BXN gene is described in detail in Appendix 14. No cauliflower mosaic virus symptoms were observed in any plants transformed using this promoter (Appendix 4). This sequence, as used in producing BXN cotton, does not cause these cotton lines to become plant pest risks.

C. Conclusions for Section IV

1. Data were generated to show that the regulated articles (7 CFR 340.2) –*tml* 3' terminator, the right and left border regions from *A. tumefaciens*, the ori pRi from *A. rhizogenes* and the double 35S promoter region from cauliflower mosaic virus – do not make the BXN cotton a plant pest risk. These sequences, isolated from their source organisms, do not present a plant pest risk in and of themselves.
2. Components of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes* that cause the organisms to be considered plant pests are not present in the BXN cotton.
3. In its Environmental Assessments the USDA concludes, "regulatory sequences, derived from plant pest organisms and necessary for the function of the inserted genes, have also been transferred to recipient cotton, but these sequences confer no plant pest property on the plants" (Appendix 23).

V. The Transgenic Plant

BXN cotton varieties differ from other cotton cultivars currently grown only in that they have the BXN gene and the protein product of the BXN gene, nitrilase. BXN cotton lines may also contain the *kan*^{r} gene, the protein product of the *kan*^{r} gene (aminoglycoside 3'-phosphotransferase II), and other inserted sequences as described by McBride and Summerfelt (1990). The BXN gene is responsible for making BXN cotton resistant to the herbicide Buctril, the active ingredient of which is bromoxynil (3,5-dibromo-4-hydroxybenzonitrile). Except for these changes, BXN cotton varieties do not differ from other cotton varieties. The use of bromoxynil for weed control in BXN cotton has a dramatic effect on reduction in overall herbicide use. In one experiment, the use of a BXN cotton/Buctril herbicide system did not affect yield, less herbicide could be used, and either the preemergence herbicide application could be eliminated or only one Buctril application was needed with a preemergent herbicide (see Section VI, Environmental Consequences of Introduction of the Transformed Cultivars: Impact of Use of BXN Cotton on the Environment).

The specific BXN gene, isolated from a selected strain of *Klebsiella pneumoniae* subsp. *ozaenae* (*K. ozaenae*), encodes nitrilase which degrades bromoxynil, the active ingredient in the herbicide Buctril. Buctril can then be sprayed "over the top" for broadleaf weed control in BXN cotton fields. Currently, no broad spectrum herbicides of this type exist on the market for use in weed control in cotton. Current weed control practices call for prophylactic pre-plant and pre-emergence herbicide application or post-directed herbicide sprays to avoid crop injury from the herbicides. The rationale for transforming cotton with the BXN gene is to allow farmers the flexibility to use the herbicide Buctril, after weed problems have been identified and only when needed. Appendix 5 contains a current Buctril label, describing its use on wheat, oats, barley, triticale, rye, seedling alfalfa, field corn, popcorn, sorghum, flax, garlic, onion, mint and grass grown for nonresidential turf seed or sod production and noncropland. Rhône-Poulenc Ag Company, the manufacturer of the herbicide Buctril, has submitted to the EPA an amended label and tolerance for Buctril use on transgenic cotton. Cotton varieties not containing the BXN gene are killed by low concentration sprays of Buctril.

The phenotype (resistance to the herbicide Buctril) was used as a screening tool throughout the selection program. The initial BXN cotton transformants, T_1 plants, were first screened with Buctril by dabbing a leaf with Buctril (on a cotton swab) at a rate which approximates 1.5 lb bromoxynil per acre (a.i./ac). Subsequent generations were sprayed with higher rates of Buctril, up to 4.5 lb a.i./ac, to select events with an adequate safety margin.

A. Field Trial Results

BXN cotton lines (T_2) were selected which showed segregation ratios of 3:1 and 15:1, which are consistent with one or two independent Mendelian loci (Table 4; Appendices 4 and 12). These segregation ratios support the conclusion that the BXN and *kan*^{r} genes were stably integrated. Once selected lines were determined to be resistant to Buctril, the lines entered a breeding and selection program identical to that used for any other cotton variety development, with the addition of a spray screen for Buctril resistance.

Extensive field trial data (Appendices 2 and 4) demonstrated the intended technical effect of cotton containing the BXN gene using both plants transformed with a prototype construct, pBrx45, and plants containing the BXN gene in commercial constructs (defined as BXN cotton, transformed using constructs pBrx74 or pBrx75). Transgenic plants produced using the prototype construct had the same phenotype (i.e. production of the nitrilase protein resulting in resistance to the herbicide Buctril) as BXN cotton. These data from pBrx45 lines are provided as examples of the testing and selection process used for BXN cotton. Trials were also conducted with pBrx45 lines to evaluate the efficacy of the herbicide under various field conditions with different weed spectra and to obtain residue data to support registration of the herbicide Buctril used on BXN cotton. Trials conducted with lines for commercialization were compared with standard varieties. Yield and quality traits were comparable. No evidence of increased seed dormancy in BXN cotton occurred (Appendices 2 and 6).

The BXN gene is identical in both 1) prototype lines transformed using pBrx45 and 2) BXN cotton. Each transformation event is unique, but the intended technical effect was the same. Only plants transformed with the binary vectors (McBride and Summerfelt 1990) and the BXN gene (with associated 35S promoter and *tml* 3' terminator) will be commercialized.

Outcrossing rates were measured in different environments using cotton containing the BXN gene (Kareiva and Morris report in Appendix 2). Outcrossing frequencies in transgenic cotton agree with results from another study conducted using nontransgenic cotton (Vaissiere report in Appendix 2). No characteristics that could promote the survivability of transgenic cotton over control cotton were observed. Seed germination percentage and flowering period were within the expected range (Appendix 2). These data support the conclusion that cotton containing the BXN gene does not have enhanced weediness characteristics.

No unexpected or abnormal cotton phenotypes were observed in advanced transgenic cotton lines and no *Agrobacterium* disease symptoms were observed in cotton in the field (Appendix 4). As in any breeding program, however, morphological variation among lines does occur. For example, T_2 lines showed variability in agronomically important characteristics such as height, uniformity and maturity date, as well as leaf size (Appendix 4). Since only lines with desirable characteristics were selected

for advancement (as in any breeding program), all off-types were removed from further consideration.

All cotton trials were screened for the presence of crown gall disease symptoms on transgenic and nontransgenic plants. No symptoms were found in any of the 67 trial sites tested to date (Appendix 4). Specific observations were made and recorded in Field Trial Notebooks in 1991 (Appendix 8) and in 1992 (Appendix 9). No symptoms or abnormal appearance in the cotton plants were reported at any of the sites. In 1991 Buctril damage was observed in the transgenic cotton plots, but this was due to a mixed seed lot containing nontransgenic and transgenic seeds.

B. Example Lines Produced Using Constructs pBrx74 and pBrx75

To provide data to support this exemption, progeny of several independent events of BXN cotton were selected for detailed molecular and compositional analysis. These events are representative of and support exemption for other BXN cotton events. The events were also selected based on agronomic potential which includes traits like earliness, location of fruit set, boll size, plant size, growth habit, vigor, yield, and fiber quality. Representative events are 10103, 10109, 10206, 10208, 10209, 10211, 10215, 10217, 10222, 10224 (Table 4). Only plants with superior agronomic characteristics will be carried forward for commercialization.

Table 4. Example BXN Cotton Events, T_2 Segregation Ratios and Levels of Nitrilase and APH(3')II in Leaves.

Event #	Total:Susceptible T_2 Plants[a]	Chi-Square Fit for 3:1 Ratio[b]	Chi-Square Fit for 15:1 Ratio[b]	% Nitrilase Protein in Leaves of T_3 Plants	% APH(3')II Protein in Leaves of T_3 Plants
10103	262:65	0.005	154.016	< 0.002	< 0.008
10109	173:42	0.048	95.954	< 0.002	< 0.008
10206	211:50	0.191	109.612	< 0.002	< 0.008
10208	241:51	1.893	91.459	< 0.002	< 0.008
10209	271:78	2.068	234.816	< 0.002	< 0.008
10211	241:53	1.163	101.922	< 0.002	< 0.008
10215	244:51	2.186	89.395	< 0.002	< 0.008
10217	223:7[c]	56.839	3.683	not measured	not measured
10222	240:52	1.422	97.351	< 0.002	< 0.008
10224	245:55	0.850	109.721	< 0.002	< 0.008

[a] Note that data are presented as "Total:Susceptible T_2 Plants."
[b] A chi-square value of <3.84 indicates a 3:1 (or 15:1) segregation ratio at a 95% confidence level.
[c] Data fit either a 15:1 two loci or a 63:1 three loci model. Two loci were subsequently confirmed by Southern analysis and additional segregation analysis.

C. Molecular Characterization

The number of BXN and *kan*^{r} genes integrated into the genomes of the independently derived examples of BXN cotton were determined by Southern analysis (Southern 1975). Specific information is provided on the structure of the T-DNA inserts and the number of insertion sites in the genome for each of the example events (Table 5; Appendix 14).

Homozygous T_3 progeny of each of the example events were analyzed by western blot analysis for levels of nitrilase and APH(3')II enzymes in leaf tissue, acid delinted cottonseed, decorticated seed kernels, seed hulls, and processed cottonseed meal (Table 5; Appendix 14).

Southern blot analysis of examples of BXN cotton indicated that the structures of the inserted BXN and *kan*^{r} genes were as shown in Figure 1. For each of these events, the BXN and *kan*^{r} genes were stably inserted into the cotton chromosomes at a single locus. For other lines, there were two loci. The primary structures of the characterized T-DNA regions in each of the example events fall into three categories: 1) those that have the same structure as the T-DNA region in the original plasmids, pBrx74 and pBrx75; 2) those with a DNA rearrangement giving a larger size hybridizing DNA fragment than expected; and 3) those with a DNA rearrangement giving a smaller size hybridizing DNA fragment than expected. There was also one event which has one complete copy of the T-DNA and one incomplete copy of the T-DNA inserted in tandem resulting in one copy of the BXN gene and two copies of the *kan*^{r} gene.

Additional Southern analyses were done to determine whether any other sequences from the binary vector had been transferred and to select BXN cotton lines (Appendix 15).

Table 5. Molecular Characterization of Example Lines of BXN Cotton.

Component	Quantitation
Number of *kan*^{r} genes (haploid)	<3
Levels of APH(3')II	<0.008 %[a]
Number of BXN genes (haploid)	1 or 2
Level of nitrilase	<0.002%[a]
Gene linkage between *kan*^{r} and BXN genes	yes
Number of insertion sites	1 or 2

[a] Percent of total protein in tissue.

D. Additional Data on BXN Cotton

To support the conclusion that BXN cotton is unchanged from other cotton varieties except for the intended technical effects, additional data were generated. These data are included in order to provide additional characterization and information on BXN cotton.

1. Seed Processing Characteristics.

Samples of BXN cotton seed were processed and found to be unchanged compared to other commercial cotton varieties (Table 6). For BXN cotton varieties to be commercially acceptable, processing characteristics must be as good as or better than other cotton varieties. Seed from the example events described above were selected to serve as examples for processing data. Processing fractions were made by the pilot processing plant at the Texas A&M University Engineering Biosciences Research Center (College Station, TX). Delinted seeds were fractionated into linters, linter motes, decorticated seed, kernels, hulls, crude oil, and meal. The processed meal was further analyzed for percent total protein, percent nitrogen and percent oil. A summary of the weight of each of these components for the samples is presented in Table 6.

Table 6. Small Scale Processing – Summary of Cottonseed Fraction Data Comparing Example Lines with Coker C315 Controls.

Sample	Line	Fuzzy Seed	Linters[c]	Hulls	Crude Oil	Meal	Losses
1	C315 rep 1	44.2[a]	5.9(13.3)[b]	11.0(24.9)	6.3(14.3)	14.1(31.9)	6.9(15.6)
2	C315 rep 2	45.1	6.4(14.2)	10.7(23.7)	6.2(13.7)	15.1(33.5)	6.7(14.9)
3	10211	45.5	8.1(17.8)	10.2(22.4)	6.2(13.6)	12.5(27.5)	8.5(18.7)
4	10217	49.1	9.8(20.0)	3.1(6.3)	7.7(15.7)	18.2(37.1)	10.3(21.0)
5	10222	50.3	14.2(28.2)	2.3(4.6)	6.7(13.3)	14.7(29.2)	12.4(24.7)

[a] Sample weights are in pounds.
[b] Numbers in parenthesis are percent yield of each component based on the initial weight of the fuzzy seed sample.
[c] Linters includes linter motes.

2. Fiber (Lint) Characteristics.

Fiber characteristics were unchanged for BXN cotton as compared to other cotton lines (Appendix 4). Fiber characteristics were determined in replicated trials from prototype lines of pBrx45 transformed cotton as compared to Coker 315 controls (Baldwin et al. 1992; Kiser and Mitchell 1991). These data compared fiber characteristics across a number of lines (see specifically Table 1 in Kiser and Mitchell 1991). The data for lint percentage and elongation were significantly greater for all the transformed lines as compared to Coker 315 controls. Since all of these plants were progeny of a single regenerated plant, it is likely that the fiber improvement was due to variation within the seed lot used for transformation. Fiber characteristics commonly measured are micronaire, length, uniformity ratio, strength, elongation, leaf index, color factors (RD, +B, and color index). These measured fiber characteristics varied between the lines but were within the range of the controls. As in any breeding program, cotton lines containing desirable fiber characteristics will be selected to develop BXN cotton varieties.

3. Biochemical Composition of Oil and Meal.

Oil and meal composition were unchanged for BXN cotton as compared to other cotton varieties (Tables 7, 8, and 9). Three lots of Coker 315 nontransformed seed and a refined food grade cotton seed oil ("wok" oil) served as controls.

Table 7. Summary of Cottonseed Oil Composition Data from Example BXN Cotton Events, Three Coker 315 Controls, and an Oil Control.

FATTY ACID COMPOSITION (% wt/wt)

			CONTROLS			BXN COTTON LINES								
	Stand.[a]	Wok Oil[b]	1 C315	2 C315	3 C315	4 10103	5 10109	6 10206	7 10208	8 10209	9 10211	10 10215	11 10222	12 10224
C<14	<0.1	0.07	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.05	0.04	0.03	0.03
C 14:0	0.4-2.0	0.90	0.70	0.88	0.89	0.70	0.76	0.72	0.67	0.64	0.69	0.68	0.72	0.70
C 16:0	17.0-31.0	22.53	25.68	26.26	26.36	24.05	26.10	24.67	24.49	24.28	24.50	24.93	24.65	24.54
C 16:1	0.5-2.0	0.63	0.52	0.56	0.58	0.47	0.53	0.48	0.46	0.45	0.46	0.51	0.47	0.48
C 18:0	1.0-4.0	2.62	2.82	2.64	2.69	2.76	2.63	2.64	2.80	2.97	2.78	2.67	2.83	2.60
C 18:1	13.0-44.0	19.65	15.51	15.58	15.79	15.24	14.21	14.40	15.03	14.63	14.28	14.84	13.72	14.31
C 18:2	33.0-59.0	52.37	53.87	53.05	52.65	55.83	54.68	56.12	55.73	56.11	56.30	55.49	56.72	56.48
C 18:3	0.1-2.1	0.43	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.19	0.17	0.18	0.18
C 20:0	<0.7	0.33	0.31	0.34	0.35	0.31	0.36	0.33	0.31	0.31	0.33	0.30	0.30	0.31
C 20:1	<0.5	0.11	0.07	0.08	0.08	0.08	0.09	0.08	0.08	0.09	0.09	0.08	0.08	0.09
C 22:0	<0.5	0.21	0.16	0.20	0.20	0.18	0.20	0.18	0.17	0.21	0.21	0.17	0.17	0.19
C 22:1	<0.5	0.03	0.04	0.03	0.03	0.03	0.05	0.03	0.01	0.03	0.03	0.03	0.04	0.02
C 24:0	<0.5	0.07	0.11	0.13	0.14	0.12	0.13	0.13	0.00	0.00	0.00	0.00	0.00	0.00

[a] These are expected ranges for standard, edible cottonseed oil, according to Codex Stan 22-1981.
[b] Example of commercial refined cottonseed oil product (House of Tsang Wok Oil also contains natural flavors of garlic, onion, ginger, coriander and black pepper).

Table 8. Total Protein, Total Nitrogen and Residual Oil Content of Cottonseed Meal from Example BXN Cotton Events and Three Coker 315 Controls.

Sample	Line	Residual Oil	Total Nitrogen	Total Protein
1	C315 rep 1	2.65%	7.52%	47.01%
2	C315 rep 2	2.70%	7.72%	48.23%
3	10211	3.16%	8.60%	53.73%
4	10217	1.87%	6.91%	43.17%
5	10222	1.26%	6.41%	40.08%

Table 9. Summary of Amino Acid Analysis of Cottonseed Meal.[a]

Sample #		Cysteine	Proline	Aspartic Acid	Serine	Threonine	Glutamic Acid	Glycine	Alanine	Valine
1	C315	1.74	3.57	10.02	5.15	3.91	20.78	4.26	.29	5.92
2	C315	1.70	3.69	10.14	4.86	3.55	21.79	3.89	.39	5.33
3	C315	1.72	3.69	10.11	5.12	3.44	21.83	3.92	.34	5.48
4	10103	1.80	3.70	9.98	4.94	3.64	21.72	3.90	.89	5.21
5	10109	1.78	3.76	10.15	4.69	3.50	21.79	3.93	3.86	5.11
6	10206	1.73	3.73	10.19	5.00	3.51	21.67	3.81	3.74	5.23
7	10208	1.73	3.68	10.05	5.11	3.65	21.50	3.86	3.84	5.41
8	10209	1.76	3.72	10.03	5.16	3.67	21.71	3.86	3.81	5.31
9	10211	1.73	3.66	10.52	4.98	3.47	21.47	3.89	3.81	5.22
10	10215	1.78	3.67	10.20	4.85	3.58	21.69	3.95	3.90	5.48
11	10222	1.76	3.67	10.26	4.95	3.52	21.83	3.90	3.87	5.23
12	10224	1.77	3.61	10.54	5.18	3.62	21.75	3.91	3.87	5.31
Standards										
Solvent[b]		-	-	-	-	3.4	-	5.1	-	6.2
Prepressed solvent[b]		-	-	-	-	3.2	-	4.1	-	4.5
Mechanical[b]		-	-	-	-	3.2	-	5.0	-	4.7

Sample #		Methionine	Isoleucine	Leucine	Tyrosine	Phenyl-alanine	Histidine	Lysine	Arginine
1	C315	1.62	3.59	6.58	3.21	5.64	3.02	5.53	11.15
2	C315	1.47	3.49	6.32	3.09	5.98	3.01	4.85	12.95
3	C315	1.46	3.56	6.25	2.97	5.92	3.01	4.88	12.78
4	10103	1.67	3.56	6.32	3.33	5.79	3.01	5.05	12.47
5	10109	1.59	3.52	6.32	3.02	5.85	3.03	4.98	13.13
6	10206	1.52	3.50	6.16	3.11	5.78	3.08	4.87	13.39
7	10208	1.55	3.68	6.24	3.14	5.77	2.98	5.03	12.80
8	10209	1.48	3.59	6.23	3.17	5.86	2.97	4.85	12.82
9	10211	1.25	3.61	6.21	3.31	5.78	2.96	4.84	13.28
10	10215	1.49	3.54	6.27	3.32	5.70	3.00	4.84	12.72
11	10222	1.46	3.39	6.15	3.58	5.67	3.01	4.92	12.84
12	10224	1.37	3.53	6.15	3.03	5.63	2.97	4.93	12.84
Standards									
Solvent[b]		1.5	3.8	6	-	5.2	2.7	4.1	10.3
Prepressed solvent[b]		1.3	3.2	-	-	5.4	2.6	4.1	11.1
Mechanical[b]		1.4	3.7	5.6	-	5	2.5	3.8	9.9

[a] % wt amino acid in cottonseed meal protein.
[b] Standards from Ensminger et al. (1990).

4. Nitrilase Levels.

The level nitrilase is expressed in cotton impacts the intended technical effects. Nitrilase functions in the cotton plant, detoxifying the herbicide Buctril at the point of contact. Since Buctril is not generally transported within the plant, any nitrilase present in plant tissues which are not directly in contact with Buctril will probably not contribute significantly to detoxification.

Tests for adequate levels of bromoxynil detoxification were done at 4.5 lb a.i./ac of Buctril. This level was chosen because 0.5 lb a.i./ac of Buctril (over the entire surface area of the field) is the highest spray rate intended for commercial use. Commercial field applications will be done as a concentrated band of Buctril over the top of the cotton. The band could be applied to only 25% of the total surface area in the field. Thus, the effective rate applied to the cotton plants would be up to 2.0 lb a.i./ac. To accommodate a safety margin for this maximum spray rate, tests for adequate protection are done at 4.5 lb a.i./ac.

The amount of nitrilase was quantitated using an immunological assay which detects both active and inactive nitrilase protein. The technique is known as Western blot analysis and involves electrophoretic separation of proteins through a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Laemmli 1970), transfer to a solid support medium (nitrocellulose), and incubation with a rabbit antibody specific for the Klebsiella-derived, bromoxynil-specific nitrilase (Stalker et al. 1988 a&b). The nitrilase protein/antibody complex is then incubated with secondary anti-rabbit antibodies coupled to alkaline phosphatase and detected using the appropriate color reagents. The Western blot procedure is described by Towbin et al. (1979) and its application to transgenic plants in Stalker et al. (1988 a&b).

Nitrilase protein levels were determined in leaf tissue, acid delinted cottonseed, decorticated seed kernels, seed hulls, and processed cottonseed meal.

Samples of leaf tissue from T_3 homozygous plants of each of the example BXN cotton events were assayed for the level of nitrilase protein by Western blot analysis (Figures 2 to 4). The cotton nitrilase signal consists of a single band at 37 kilodaltons (kd). There was no signal, as expected, in the Coker 315 nontransformed control lanes, while 3.5 ng of nitrilase was detected when added to these controls. Nitrilase was detected in all example events. The intensity of the signal varied among samples and between replicates of the same samples on different filters, so each assay was repeated at least three times to establish an average level. The intensity of the nitrilase signal for each of the example BXN samples ranged up to 3.5 ng nitrilase for 200 µg total leaf protein. Therefore the maximum level of nitrilase measured in these example BXN cotton lines was approximately 0.002% of total leaf protein (Table 4).

Samples of acid delinted seed, decorticated seed and seed hulls from homozygous T_2 individuals (T_3 seed) of the example BXN cotton lines were assayed for nitrilase by Western blot analysis (Figures 5 to 9). The nitrilase

signal in these tissues was near the limit of detection for this antibody preparation under these conditions. Nitrilase signals when 600 µg of BXN cottonseed protein were loaded in one lane were much less than seen in the lane containing 3.5 ng pure nitrilase. Therefore the concentration of nitrilase was less than 0.0006% of the total seed protein.

Assuming seed protein is approximately 22% of seed weight (Ensminger 1990), the maximum amount of nitrilase in 1 gram of unprocessed cottonseed is

= (0.22 g protein/g seed)(0.0006% nitrilase)
= 1.3×10^{-6} g nitrilase/g cottonseed
= 1.3 µg nitrilase/g cottonseed

Cottonseed meal samples were subjected to Western blot analysis (Figures 10 and 11). The nitrilase signals from 600 µg of meal protein in the example BXN cotton events were approximately equal to the 0.35 ng pure nitrilase signal in these western blots. Therefore the concentration of nitrilase was approximately 0.00006% of the total meal protein.

Assuming meal is approximately 41% protein (Ensminger 1990), the maximum amount of nitrilase in 1 gram of processed cottonseed meal is

= (0.41 g protein/g meal)(0.00006% nitrilase)
= 2.5×10^{-7} g nitrilase/g cottonseed meal
= 0.25 µg nitrilase/g cottonseed meal

At most, the levels of nitrilase present in the example lines were approximately 0.002% of the total protein in leaf tissue (Table 4):

% of Leaf Protein	% of Unprocessed Seed Protein	% of Processed Meal Protein
<0.002%	< 0.0006%	<0.00006%

All of these events have been tested by Buctril application at 4.5 lb a.i./ac with no symptom development or detectable changes in growth. This level of nitrilase was sufficient to achieve the intended effect of Buctril resistance.

5. APH(3')II Levels.

The amount of APH(3')II in tissues was also quantitated using Western analysis with a rabbit antibody specific for APH(3')II. This assay detects both active and inactive APH(3')II protein. APH(3')II protein levels were determined in leaf tissue, acid delinted cottonseed, decorticated seed kernels, seed hulls, and processed cottonseed meal.

Samples of leaf tissue from T_3 homozygous plants of each of the example BXN cotton lines were assayed for the level of APH(3')II protein by Western blot analysis (Figures 12 and 13). The APH(3')II signal consists of a single band at 29 kd. There was no signal, as expected, in protein extracts from the

nontransformed Coker 315 control, while 16 ng APH(3')II was detected when added to this control. APH(3')II was detected in all example BXN cotton events. The intensity of the signal varied between samples and between replicates of the same samples on different filters, so each assay was repeated at least three times to establish an average level. The intensity of the APH(3')II signal for each of the example BXN cotton samples ranged up to 16 ng APH(3')II for 200 µg total leaf protein. Therefore the maximum level of APH(3')II measured in these example BXN cotton lines was approximately 0.008% of total leaf protein.

Samples of acid delinted seed, decorticated seed and seed hulls from homozygous T_2 individuals (T_3 seed) of the example BXN cotton lines were assayed for APH(3')II by Western blot analysis (Figures 14 to 18). The APH(3')II signal when 600 µg of BXN cottonseed protein was loaded in one lane was less than seen in the lane containing 16 ng pure APH(3')II. Therefore, the concentration of APH(3')II was less than 0.003% of the total seed protein.

Assuming seed protein is approximately 22% of seed weight (Ensminger et al. 1990), the maximum amount of APH(3')II in 1 gram of unprocessed cottonseed is

= (0.22 g protein/g seed)(0.003% APH(3')II)
= 6.6×10^{-6} g APH(3')II/g cottonseed
= 6.6 µg APH(3')II/g cottonseed

Cottonseed meal samples were subjected to Western blot analysis (Figures 19 and 20). The APH(3')II signals from 600 µg of meal protein in the example BXN cotton events were less than the 8 ng purified APH(3')II signal in these western blots. Therefore, the concentration of APH(3')II was less than 0.0014% of the total meal protein.

Assuming meal is approximately 41% protein (Ensminger et al. 1990), the maximum amount of APH(3')II in 1 gram of processed cottonseed meal is

= (0.41 g protein/g meal)(0.0014% APH(3')II)
= 5.8×10^{-6} g APH(3')II/g cottonseed meal
= 5.8 µg APH(3')II/g cottonseed meal

Based on these measurements, the highest level of APH(3')II was less than 0.008 % of the protein in leaves (Table 4):

% of Leaf Protein	% of Unprocessed Seed Protein	% of Processed Meal Protein
<0.008%	< 0.003%	< 0.0014%

6. Analysis for Gossypol and Cyclopropenoid Fatty Acids in Transgenic Cottonseed.

Gossypol and cyclopropenoid fatty acids are antinutritional compounds found in standard cotton varieties. Processed meal and oil fractions from the examples of BXN cotton and three examples of standard cotton were analyzed for levels of gossypol in the meal and presence of cyclopropenoid fatty acids in the oil using standard methods. Total and free gossypol were measured at the Engineering Biosciences Research Center of Texas A&M University by spectrophotometry (AOCS Official Methods Ba 7-58 and Ba 8-78) in processed meal (Appendix 16).

Comparison of cyclopropenoid fatty acids levels in the crude oil from each of the representative BXN cotton events and the three Coker 315 controls was also made using the AOAC Method 974.19 (1990) and JAOAC Methods 55, 1288 (1972)(Appendix 17).

Gossypol levels were unchanged in BXN cotton as compared with controls. Gossypol levels in meal samples from example events ranged from 0.92 to 1.72%) and from the Coker 315 controls, 0.98 to 1.25%. All measured levels of gossypol were within the range (or lower) of 1.2-2.0% reported for meal prepared from hexane-extracted kernels in traditional cotton varieties (Lawhon et al. 1977, Jones and King 1990).

Cyclopropenoid fatty acids levels were unchanged in BXN cotton as compared with controls (Table 10). Natural ranges are from 0.005% sterculic equivalent in refined and deodorized cottonseed oil to 0.62% malvalic equivalent in cottonseed salad oil; and from 0.08% sterculic equivalent to 0.98% malvalic equivalent in crude oil (Jones and King 1990). For the analysis in Table 12, comparisons of cyclopropenoid fatty acids in crude oil of BXN cotton lines and Coker control lines were made. Refined food grade cottonseed and corn oils were analyzed to show the reduction in cyclopropenoid fatty acid levels in refined oil as compared to crude cottonseed oil.

Table 10. Cyclopropenoid Fatty Acids[a] in Crude Cottonseed Oil from the Example Events, Three Coker 315 Controls, a Flavored Cottonseed Oil Control[b] and Corn Oil[c].

Sample	Type of Oil	Event	Oil (g)	abs 547 nm	abs (dil) 547 nm	abs (cor)[d] 547 nm
Blank A				0.00	0.01	-0.03
Blank B				0.00	0.00	-0.04
1A	Crude	Coker 1	0.1009	0.89	0.63	0.60
1B	Crude		0.0995	0.86	0.68	0.65
2A	Crude	Coker 2	0.1000	0.88	0.70	0.67
2B	Crude		0.0995	0.88	0.70	0.66
3A	Crude	Coker 3	0.0998	0.91	0.74	0.71
3B	Crude		0.0997	0.90	0.73	0.69
4A	Crude	10103	0.0993	0.94	0.79	0.76
4B	Crude		0.0992	0.92	0.77	0.73
5A	Crude	10109	0.1003	0.91	0.75	0.72
5B	Crude		0.1005	0.91	0.74	0.70
6A	Crude	10206	0.1009	0.93	0.79	0.75
6B	Crude		0.1009	0.93	0.79	0.75
7A	Crude	10208	0.1006	0.90	0.72	0.68
7B	Crude		0.0998	0.90	0.73	0.70
8A	Crude	10209	0.1009	0.90	0.73	0.70
8B	Crude		0.0997	0.89	0.70	0.67
9A	Crude	10211	0.0999	0.93	0.79	0.76
9B	Crude		0.1003	0.92	0.76	0.73
10A	Crude	10215	0.0999	0.92	0.77	0.73
10B	Crude		0.1000	0.90	0.72	0.68
11A	Crude	10222	0.1010	0.90	0.73	0.70
11B	Crude		0.0997	0.89	0.71	0.68
12A	Crude	10224	0.0996	0.93	0.79	0.76
12B	Crude		0.0994	0.93	0.79	0.75
Corn A	Refined		0.0993	0.00	0.05	0.01
Corn B	Refined		0.1009	0.00	0.02	-0.02
Cotton A	Refined		0.1004	0.27	0.13	0.10
Cotton B	Refined		0.0992	0.24	0.14	0.11

[a] The trading rules for cottonseed oil do not set a standard for CPFA levels, only that there is a positive reading in a Halpen test. These tests compare CPFA in crude oil of controls and transgenic lines, showing that there were no differences.
[b] Cottonseed oil was "House of Tsang" wok oil, which is a refined food grade cottonseed oil product which contains oils from other plant sources. The low level of the cyclopropenoid fatty acids found in this oil is due to the refining process.
[c] Corn oil was Mazola.
[d] Corrected abs = (sample abs - corn abs).

7. Impact of Use of BXN Cotton.

The principle effect of use of BXN cotton will be the availability of better weed management practices. In general, the data generated demonstra[te] that BXN cotton lines do not differ from other cotton varieties except fo[r] very specific traits that are predictable from the function of the nitrilase gene. This is not surprising given the small genetic changes and the high de[gree] of substrate specificity of the BXN nitrilase (McBride et al. 1986). [In] all characteristics measured, unintended effects were not observed. Th[e] data and observations suggest that breeding and development of BXN c[otton] are directly analogous to traditional cotton breeding methods, and [that] BXN cotton poses no greater risk than other cotton cultivars.

The use of BXN cotton will not affect current agricultural practices for cotton production, except for increased flexibility in weed management systems. BXN cotton will allow the use of the post-emergence, broad-leaf weed control herbicide Buctril which will be a major benefit to cotton growers, by providing them with more options for weed control and by reducing overall herbicide use. As discussed in Section VI (Environmental Consequences of Introduction of the Transformed Cultivars: Impact of Use of BXN Cotton on the Environment), the use of a BXN cotton/Buctril herbicide system does not affect yield, allows less herbicides to be used, and either eliminates the preemergence herbicide application or reduces the number of postemergent herbicide applications. This change should have no impact on floral communities, faunal communities, endangered or threatened species, health of plants or animals, or genetic resources of cotton.

All greenhouse and field observations to date show that there were no deleterious effects on humans involved in cultivation or post-harvest production of BXN cotton.

E. Conclusions for Section V

1. The BXN cotton differs from other cotton cultivars only in the presence of the novel BXN and *kan*^{r} genes and their expression products: nitrilase and APH(3')II, respectively. Agronomic and developmental traits, and antinutritional compounds (gossypol and cyclopropenoid fatty acids) are unchanged.
2. BXN cotton lines were shown to be genetically stable. The inserted genes were shown to be immobile and segregated according to Mendelian predictions.
3. No *Agrobacterium* or cauliflower mosaic virus disease symptoms were observed in any plants in any of the field trials (Appendix 4). The regulated articles (sequences), as used in producing BXN cotton, no longer functioned as regulated sequences since they were isolated from their source organisms which are considered plant pests and since they did not change BXN cotton, and did not make them a plant pest risk.
4. Seed germination rate and frequency of BXN cotton are equivalent to traditionally bred varieties. No changes in flowering time, outcrossing characteristics, seed production, or controlled pollination were measured. Yield of fiber from BXN cotton is equivalent to traditionally bred controls. Except for weed management, current agricultural practices will not be affected by cultivation of the BXN cotton.
5. Composition of BXN cotton seed is essentially unchanged in terms of seed processing characteristics (fuzzy seed, linters, hulls, crude oil and meal), fatty acid profile, total protein, total nitrogen, residual oil, amino acid profile, and fiber characteristics.
6. Although variation may occur in experimental cotton lines derived via transformation or plant breeding, variety development will result in lines selected for desirable agronomic characteristics. The probability of incurring pleiotropic effects as a result of the transformation process is low and likely not significantly different than the probability of pleiotropic effects resulting from other plant breeding techniques.
7. That traditional plant breeding and selection processes used to develop commercial BXN cotton varieties will be adequate to assure no unintended changes in these varieties will affect their safety.

VI. Environmental Consequences of Introduction of the Transformed Cultivars

In his review on the ecology of transgenic plants, Crawley (1992) concluded:

- "There is a view that the ecology of genetically engineered organisms is somehow different from the ecology of conventional organisms, and that the intentional release of genetically engineered organisms poses a greater threat to the Balance of Nature than do other kinds of organisms bred by man. This view is mistaken. ... The ecological rules are the same for transgenics as for non-transgenics."
- "Release of genetically engineered crop plants does not pose substantial new threats to the environment."

A. Stability of Gene Products in the Environment

Use of the BXN cotton will not affect the weediness status of cotton or any related species (see section II).

Calculations were made for potential release of APH(3')II into the soil from cotton debris (Appendix 18). Although these calculations are for potential levels of APH(3')II, they are also applicable to nitrilase, which is present at one fifth the amount of APH(3')II in BXN cotton (1.3 µg nitrilase/g cottonseed vs. 6.6 µg APH(3')II/g cottonseed). It is not expected that such release of APH(3')II or nitrilase will pose an environmental risk, since soil bacteria naturally produce APH(3')II and nitrilase, and much of these proteins are likely sequestered or degraded in the soil. The only effect of nitrilase in soil would be to enhance degradation of the herbicide bromoxynil.

B. Horizontal Gene Flow

Concerns have been expressed about potential transfer of DNA from transgenic plants to microorganisms. To date, examples of such horizontal gene flow are very few and difficult, if not impossible, to prove. The probability of horizontal gene flow is vanishingly small for the following reasons (IFBC 1990):

1. The introduced genes are permanently incorporated into the plant chromosome.
2. Transgenic plants do not contain genes required for transport of DNA into bacteria.
3. If bacteria acquired the DNA through passive uptake from decomposed plant tissue, the bacterial cell could not "read" the genes since they do not have bacterial promoters.

In addition, no mechanism for transfer of genes from plants to microorganisms is known and no cases of such transfer have been adequately documented. Carlson and Chelm (1986) argued for an eukaryotic (plant) origin of glutamine synthetase II in bacteria, albeit over an evolutionary time period. They suggested that this was evidence that horizontal gene flow from plants to microorganisms had occurred at one point in evolution. However, their paper was directly refuted by Shatters and Kahn (1989) who concluded that "the GS [glutamine synthase] proteins are highly conserved and the divergence of these proteins is proportional to the phylogenetic divergence of the organisms from which the sequences were determined. No transfer of genes across large taxonomic gaps is needed to explain the presence of GSII in these bacteria." Other "evidence" that horizontal gene flow occurs from plants to microorganisms involves transient changes (nonheritable) such as transencapsidation of chloroplast DNA (Rochon and Siegel 1984) or possibly endocytosis (Bryngelsson et al. 1988), neither of which have been shown to result in actual transfer of genes from plants to microorganisms. No mechanism by which plant DNA could be incorporated from plants into the genomes of the microorganisms has been proposed. For *Agrobacterium*-mediated transformation, Zambryski et al. (1982) provide evidence that once inserted DNA is integrated into the plant host genome, it cannot be remobilized even if acted on again by *vir* genes.

Recently, Smith et al. (1992) presented arguments for the occurrence of horizontal gene transfer. Two cases of transfer from eukaryotes to prokaryotes, and two cases of transfer from prokaryotes to eukaryotes were considered by the authors as likely to have occurred over an evolutionary time period. If their assertions are true (and these authors admit that "horizontal gene transfers are difficult to prove" and that additional data "may also lead us to reconsider some of the cases that at present appear to be sound"), these occurrences can still be considered to be extremely rare.

1. *kan*^{r} Gene.

One particular concern is the use of antibiotic resistant marker genes which could pose a special situation if horizontal gene transfer were possible. Calgene has addressed this issue previously (Redenbaugh et al. 1993) and provides a summary here.

Even if we were to assume that transfer of the *kan*^{r} gene to bacteria could occur easily, the consequences of such transfer would be insignificant. There would be no significant increase in the numbers of resistant bacteria present relative to background numbers: the worst case scenario predicts that background levels of kanamycin resistant organisms would be 7 orders of magnitude greater than the numbers of organisms resistant through incorporation of the *kan*^{r} gene from plant tissues.

Using worst case probability estimates for hypothetical gene transfer, the additive effect of a *kan*^{r} gene entering the microbial flora from transgenic plants is insignificant when compared to the population of kanamycin

resistant microorganisms naturally present. For worst case calculations, Calgene's assumptions were that free plant DNA containing an intact *kan*^{r} gene could become disassociated from a plant cell, exist long enough in the soil to be taken up by a soil bacterium, incorporated into the bacterial genome (including bacterial plasmids), and be expressed by the bacteria (even though the kanamycin resistance gene in the plant does not contain bacterial promoter sequences). Even using these unrealistic assumptions, the impact of increased numbers of kanamycin resistant bacteria in humans, animals, or environment is insignificant compared to the population of naturally occurring resistant bacteria.

Calgene's worst case calculations assume that soil transformation rates would be as high as those achieved under ideal laboratory conditions. These calculations suggest that, at worse, only 9×10^5 bacteria per hectare per year would become resistant compared to 7.2×10^{12} kanamycin resistant bacteria per hectare already present naturally in the environment (Henschke and Schmidt 1990). Under the worst case scenario, the increase in background population would be an infinitesimal 1.25×10^{-5}%.

For risk assessment, we assigned probabilities to such a scientifically unjustifiable event and generated calculations for worst case scenarios based on existing scientific literature and data. When data were unavailable, the worst case probability (usually 1) was used. To generate a worst case risk assessment for horizontal transfer of genes from plants to microorganisms, we assumed that such transfer could occur and used ideal laboratory conditions as a basis for probability calculations. Consequently, the probability estimates are greatly exaggerated on the side of safety and the actual risk, if any, is much less. The following risks were considered:

Likelihood that the genes will move from the plants to soil bacteria. The worst case estimate, assuming DNA could be transferred from plant debris to soil microorganisms, would result in a contribution of 1/10,000,000 to the *kan*^{r} microorganisms already present in soil.

These are significantly low probabilities as compared to the natural levels of kanamycin resistant microorganisms in the soil, estimated at 7.2×10^{12} per hectare (Henschke and Schmidt 1990). A more realistic estimate is that *kan*^{r} bacteria resulting from transformation from plant debris (if such transformation were even possible) would represent no more than 1.4×10^{-11}% of the *kan*^{r} soil microorganisms.

Likelihood the soil bacteria might persist or be selected for in the soil. This possibility is no greater than that for any other naturally occurring *kan*^{r} bacteria. Because kanamycin is not used for controlling microorganisms in the soil, there would be no selective advantage to any specific *kan*^{r} bacteria which result from transformation from plant debris compared to those normally resistant.

Likelihood that the genes may be transferred to yet other bacteria. This possibility would be no greater than that for genes from any other *kan*^{r} bacteria. Because the frequency of natural *kan*^{r} bacteria is far greater than estimated levels of *kan*^{r} bacteria resulting from transformation from plant debris, there would be no substantial increase in the population of *kan*^{r} bacteria.

No mechanisms of natural transformation have been demonstrated for transfer of DNA sequences from eukaryotic cells eaten as food to microorganisms found in the human gut. However, to assess risk, Calgene calculated a worst case scenario for transformation of human gut bacteria with the *kan*^{r} gene from fresh transgenic tomatoes. This calculation resulted in a frequency of 2.6×10^{-3} transformed bacteria/person. This represents 10^{-13}% of susceptible gut bacteria in a human. The population of *kan*^{r} bacteria which normally inhabit the human gut is substantial (e.g. 75% of *Streptococcus faecalis* bacteria, Atkinson 1986), and the impact, if any, of transfer of the *kan*^{r} gene from transgenic tomatoes to human gut bacteria would be insignificant.

Experiments were conducted to demonstrate that APH(3')II is degraded in the human gut and that APH(3')II would not compromise oral dosage of kanamycin (Petition for Determination: FLAVR SAVR™ Tomato as a Non-Regulated Article under 7 CFR 340, USDA APHIS #P92-157-01, May 31, 1992).

These calculations assume that transfer of DNA from a plant source is possible, although no mechanisms for such transformation are known. However, even with worst case calculations, the probability of a plant source of the *kan*^{r} gene impacting humans, animals, and the environment is insignificant compared to the naturally occurring reservoir of kanamycin resistance genes.

2. BXN Gene.

The discussion presented above is also directly applicable to the case of the BXN gene. Nitrilase was isolated from soil microbes. Because the nitrilase-encoding gene is naturally found on a bacterial plasmid (Stalker and McBride 1987), it is much more likely that the gene would move from bacteria to bacteria via conjugation. No mechanism is known for transfer of genes from plants to microorganisms. Even if such transfer could occur, the result would be of no concern since the BXN gene was originally isolated from soil microbes.

In its Environmental Assessments the USDA concludes, "horizontal movement of the introduced genes is not known to be possible. No mechanism that can transfer an inserted gene from a chromosome of a transformed plant to a chromosome of another organism has been shown to exist in nature" (Appendix 3).

C. Impact of Use of BXN Cotton on the Environment

1. Reduction in Herbicide Use.

The use of Buctril for weed control in BXN cotton can dramatically reduce overall herbicide use. Data presented in Appendix 19 and in Tables 11-13 provide examples of weed control of hemp sesbania and common cocklebur. Other weeds can also be controlled in a similar fashion using a BXN cotton/Buctril system.

Weed control has been a major problem in cotton production, evolving from hand hoeing prior to 1920, to mechanical tillage in the 1930's, to biological control with geese in the 1940's and fungi more recently, to herbicides in the 1950's (Buchanan 1992). Today, weed control depends heavily on herbicides, with supplemental use of hand hoeing, mechanical tillage and biological control. However, loss to weeds is still extensive: "Due to its unique growth and development, the cotton plant is greatly influenced by weeds, resulting in a $300 million crop loss per year" (Abernathy and McWhorter 1992). Chandler and Cooke (1992) place the losses at $200 million per year, despite the expenditure of $400 million annually to control weeds. Weed control costs vary depending on state, ranging from $19.31 to $67.18 per acre (Chandler and Cooke 1992). Without herbicides, hand labor to control weeds could cost up to $300 per acre (Abernathy and McWhorter 1992). Growing cotton without any herbicides is very difficult and currently is limited to certain geographical locations and requires extensive mechanical tillage (which compacts the soil and increases erosion) and/or expensive hand hoeing (Gordon 1993). Weed control scientists, university extension agents, farmers, chemical companies and others are continually seeking methods to lower costs while at the same time reducing the use of chemicals. The use of herbicide resistant crops (HRCs) has the potential to accomplish these objectives and, in all likelihood, will lead to promotion of integrated pest management (IPM) of weeds.

Field trials with BXN cotton have provided data to move the discussion of HRCs beyond the theoretical to the actual. Standard weed control practices in cotton were compared with BXN cotton/Buctril herbicide systems in 1991 and 1992 field trials conducted by university weed scientists. Different combinations of herbicides were tested on BXN cotton using Buctril at a 0.38 a.i./acre equivalent (either broadcast or banded).

In addition, effectiveness of the herbicide regime in weed control and lint yield was measured in one location in 1992 (Appendix 19). In this study, cotton lint yield did not vary significantly for treatments 2, 5, 6, 10, 13, 14 (all with at least one Buctril treatment), and 8 and 16 (non-Buctril standards). These treatments were also effective in weed control (Appendix 19). Because yield and weed control were equivalent for a number of the treatments, comparisons of the herbicide regimes were made (Table 11). For example, a standard weed control regime (#16) used 0.5 lb a.i./acre Treflan, 2.0 lb Cotoran, 0.6 lb Bladex, and 4.0 lb MSMA, while the best yielding Buctril treatment (#13) used 0.5 lb Treflan, 1.0 lb Cotoran, and 1.25 lb Buctril (Treflan is a broadcast

herbicide; the others were applied on a 12 inch band). Table 11 also lists a standard herbicide regime for Stoneville, MS (Chandler and Cooke 1992) for comparison, since Mississippi is the one of the principle cotton producing states.

Several conclusions can be drawn from the data in Appendix 19 and Table 11:

1) The use of a BXN cotton/Buctril herbicide system did not negatively affect yield.
2) Less herbicide could be used without decreasing yield in BXN cotton. In fact, there is the potential to actually increase yield in cases where other herbicides would cause damage to the cotton crop.
3) The preemergence herbicide application could be eliminated for control of hemp sesbania and common cocklebur if two applications of Buctril were made.
4) With a preemergent herbicide, only one Buctril application was needed for control of hemp sesbania and common cocklebur.

Based on these field trial results and Calgene's experience that similar weed control can be accomplished for other weed pests, the use of a BXN cotton/Buctril herbicide system could significantly reduce the total amount of herbicides applied during the growing season.

Table 11. Weed Control Treatments and Amount of Herbicides Used (from Appendix 19).

Treatment	Herbicide	lb a.i./acre/trial (broadcast equivalent)
2 (Buctril)	Treflan Buctril (broadcast)	0.50 0.38 = 0.88 total
4 (Buctril)	Treflan Buctril (banded, two applications)	0.50 0.79 = 1.29 total
5 (Buctril)	Treflan Buctril (banded)	0.50 0.38 = 0.88 total
6 (Buctril)	Treflan Buctril (banded, two applications)	0.50 0.79 = 1.29 total
10 (Buctril)	Treflan Cotoran (banded) Buctril (broadcast)	0.50 0.32 0.38 = 1.20 total
13 (Buctril)	Treflan Cotoran (banded) Buctril (banded)	0.50 0.32 0.38 = 1.20 total
14 (Buctril)	Treflan Cotoran (banded) Buctril (banded, two applications)	0.50 0.32 0.79 = 1.61 total
8 (standard herbicide regime)	Treflan Cotoran (banded) MSMA (banded) Bladex (banded)	0.50 0.32 1.26 0.19 = 2.27 total
16 (standard herbicide regime)	Treflan Cotoran (banded) MSMA (banded) Bladex (banded)	0.50 0.63 1.26 0.19 = 2.58 total
Chandler and Cooke (1992) Mississippi weed control (Table 10 of reference)	Treflan (broadcast) Norflurazon (broadcast and banded) Cotoran (banded) MSMA (banded) Prometryn (banded) Cyanazine (broadcast)	0.50 0.60 0.51 0.50 0.13 1.25 = 3.49 total

The actual reduction in total amount of herbicides realized with a BXN cotton/Buctril herbicide weed control system will depend on a broad spectrum of components and will require a number of years of research and actual usage before reliable data are available. However, it is possible to provide estimates of potential herbicide reduction, based on published reports and the data in Appendix 19 which is summarized in Table 11. Two estimates

can be made based on conclusions in Appendix 19 for successful weed control using a BXN cotton/Buctril system in the field trials: 1) elimination of preemergence herbicide application (Table 12), and 2) elimination of one of the post-direct applications (Table 13).

Treatments 6 and 8 had good weed control and equivalent yield, without significant differences and without use of a preemergent herbicide (Appendix 19). These treatments can be compared with a typical herbicide regime in Mississippi (Chandler and Cooke 1992) and calculations made on the total herbicide a.i. used per year in that state (Table 12). Reduction in herbicide use from 2.8-3.8 million pounds per year down to 1.6 million, in Mississippi for example, would be of significant environmental benefit.

Table 12. Estimated Herbicide Reduction Using a BXN Cotton/Buctril System: Elimination of the Preemergence Herbicide Application.

Treatment (see Appendix 19)	Herbicide	lb a.i./acre/trial (broadcast equivalent)		lb a.i./year in Mississippi[a]
6 (Buctril, no preemergent)	Treflan	0.50		
	Buctril (banded)	0.38		
	Buctril (banded)	0.38	= 1.29 total	1,606,000
8 (standard herbicide regime, no preemergent)	Treflan	0.50		
	Cotoran	0.32		
	+ MSMA (banded)	0.63		
	Bladex	0.19		
	+ MSMA (banded)	0.63	= 2.27 total	2,826,000
Chandler and Cooke (1992) (Table 10 of reference, pre-emergent herbicide deleted)	Treflan +	0.50		
	Norflurazon (broadcast)	0.48		
	Cotoran +	0.20		
	MSMA (banded)	0.50		
	Prometryn (banded)	0.13		
	Cyanazine (broadcast)	1.25	= 3.06 total	3,810,000

[a] 1,245,000 acres planted in 1991 in Mississippi (USDA 1992).

A second example in herbicide reduction is the elimination of one of the post-directed applications, which is one of the conclusions in Appendix 19. For this example, data on the efficacy of weed control and the lint yield is only available for treatment 13, the BXN cotton/Buctril system, since there were no examples of the standard herbicide system which had eliminated one of the post-directed applications (Appendix 19). However, treatment 13 had weed control and lint yield at least as good as the best standard treatment, #16, which had two post-directed applications. In order to make the comparison of total herbicide usage in the example state of Mississippi, one post-directed application was eliminated in the calculations for treatment 16 and for Chandler and Cooke's (1992) data (Table 13). Again, the reduction in herbicide

use is dramatic, dropping from a range of 4.2 to 2.2 million pounds per year in Mississippi to 1.5 million.

Table 13. Estimated Herbicide Reduction Using a BXN Cotton/Buctril System: Elimination of One of the Post-Directed Applications.

Treatment (see Appendix 19)	Herbicide	lb a.i./acre/trial (broadcast equivalent)		lb a.i./year in Mississippi[a]
13 (Buctril, one treatment)	Treflan	0.50		
	Cotoran (banded)	0.32		
	Buctril (banded)	0.38	= 1.20 total	1,494,000
16 (standard herbicide regime)	Treflan	0.50		
	Cotoran (banded)	0.32		
	Cotoran +	0.32		
	MSMA (banded)	0.63		
	~~Bladex +~~	~~0.19~~		
	~~MSMA (banded)~~[b]	~~0.63~~	= 1.77 total	2,204,000
Chandler and Cooke (1992) Mississippi weed control (Table 10 of reference)	Treflan +	0.50		
	Norflurazon (broadcast)	0.48		
	Cotoran +	0.31		
	Norflurazon (banded)	0.12		
	Cotoran +	0.20		
	MSMA (banded)	0.50		
	~~Prometryn (banded)~~[b]	~~0.13~~		
	Cyanazine (broadcast)	1.25	= 3.36 total	4,183,000

[a] 1,245,000 acres planted in 1991 in Mississippi (USDA 1992).
[b] One of the standard post-directed applications was eliminated.

Even if there was no reduction in the number of herbicide applications, there would still be reduction in total amount of herbicides used (Table 14). Treatment 14 represents a conservative weed management regime (post directed herbicides replaced with Buctril, no decrease in number of herbicide applications) that was the most commonly accepted protocol by the weed scientists in the study. Even without reduction in number of herbicide treatments, herbicide reduction using BXN cotton/Buctril herbicide weed control ranged from 31 to 64% (Table 14).

Table 14. Estimated Herbicide Reduction Using a BXN Cotton/Buctril System: No Change in Number of Herbicide Applications.

Treatment (see Appendix 19)	Herbicide	lb a.i./acre/trial (broadcast equivalent)		lb a.i./year in Mississippi[a]
14 (Buctril)	Treflan	0.50		
	Cotoran (broadcast)	0.32		
	Buctril (banded)	0.38		
	Buctril (banded)	0.38	= 1.61 total	2,004,000
16 (standard herbicide regime)	Treflan	0.50		
	Cotoran (banded)	0.32		
	Cotoran +	0.32		
	MSMA (banded)	0.63		
	Bladex +	0.19		
	MSMA (banded)	0.63	= 2.58 total	3,212,000
Chandler and Cooke (1992) Mississippi weed control (Table 10 of reference)	Treflan +	0.50		
	Norflurazon (broadcast)	0.48		
	Cotoran +	0.31		
	Norflurazon (banded)	0.12		
	Cotoran +	0.20		
	MSMA (banded)	0.50		
	Prometryn (banded)	0.13		
	Cyanazine (broadcast)	1.25	= 3.49 total	4,345,000

[a] 1,245,000 acres planted in 1991 in Mississippi (USDA 1992).

2. Integrated Weed Management.

Critics of herbicide resistant crops often argue that use of herbicide resistant crops will increase herbicide use and that there are already sufficient nonchemical methods available to control weeds. However, for cotton there are not sufficient nonchemical methods. Mechanical tillage is already used between the rows in most production fields. Hand hoeing within the rows is very expensive and generally not practical as the primary weed control method in most cotton growing regions in the U.S.

Growing cotton organically is extremely difficult and is limited to specific areas in the U.S. , such as the high plains of Texas and the upper west corner of Tennessee where natural phenomenon of higher altitude and early freezes allow farmers to grow cotton without defoliants or in certain parts of California where insect pressure is decreased (Gordon 1993).

Duke et al. (1991) in the CAST report state,

> "There is growing pressure to reduce or eliminate tillage associated with weed management to reduce soil erosion and to increase soil fertility ... For example, weed pressure in some crops such as cotton in the southeastern United States is so great that conservation tillage is very difficult. A good broad-spectrum, postemergence herbicide to

> which cotton would be resistant would help ... The creation of cotton resistant to postemergence herbicides with appropriate traits could greatly enhance soil conservation in cotton production."

Minimum tillage and conservation tillage systems are becoming more prevalent, especially in areas of highly erodible soils (Holmes 1993). Over-the-top Buctril application using BXN cotton will likely become an important component of minimum tillage and conservation tillage systems.

The use of BXN cotton should reduce the overall amount of herbicides used in cotton production. In addition, there is the potential to increase yield in cotton in cases where other herbicides would cause damage to the cotton crop. Reduction in herbicide amount and increased yields would enhance competitiveness in world markets. Recently, Krimsky and Wrubel (1993) published an extensive review on herbicide resistant crops and concluded,

> "Acceptance of HRCs currently being tested should actually lead to reductions in quantities of herbicide used, at least in the short term, for several reasons. First, the application rates of some of the herbicides being promoted with HRCs are dramatically less than those they would replace ... Second, it is argued that HRCs may allow the use of a more effective herbicide that could replace several herbicides now being applied concurrently for control of the spectrum of weeds associated with a particular crop ... Third, HRCs can promote the use of post-emergence weed control, which can result in reduced quantities of herbicides applied, fewer applications per year, and in some situations reduction of the number of acres treated. Pre-emergence herbicides are applied before the crop and often before the weeds have appeared in the field as insurance against having to treat a weed problem once the crop is up. This is the antithesis of an integrated approach to weed management, which is based on treating a weed problem only when it is known to exist above the economic threshold for the crop ... Having post-emergence weed control options available to treat weed problems once they are identified is necessary to convince farmers that they do not need to rely on pre-emergence herbicide treatment."

Based on these arguments and Calgene's field trial results, the use of BXN cotton should be able to accomplish the objective of reduction in herbicide use as recommended in *Harvest of Hope* published by the Natural Resources Defense Council (Curtis et al. 1991). The integrated approach to weed management supported in *Harvest of Hope* and described by Krimsky and Wrubel (1993) can likely be accomplished using BXN cotton.

3. Bromoxynil Herbicide.

Buctril has been used for 25 years on over 6 million acres without any documented health, safety or environmental incidents. Buctril does not persist in the environment. It has a half life of less than 5 days and generates

no residues in any of the food crops on which it is used. It breaks down into components with no adverse human health effects or environmental impacts. In 1993, the EPA approved an EUP for Buctril on BXN cotton (Appendix 20).

Recently, Bromoxynil came under review because of questions arising from studies which suggested a relationship between exposure to bromoxynil and birth defects in laboratory animals. In 1992, the U.S. Environmental Protection Agency (EPA) determined after an extensive review that restricted-use status on bromoxynil was unwarranted, and restricted-use classification was dropped (Appendix 5). In addition, Rhône-Poulenc conducted a study of "real-world" bromoxynil use to identify the potential for worker exposure. In the study – the largest and most comprehensive of its kind in the industry – applicators and mixers/loaders at 23 sites were fitted with skin patches and breathing apparatus to determine the degree, if any, of exposure to bromoxynil. Findings submitted to federal regulators show that protective measures *do* protect applicators and that a more-than-adequate margin of safety exists when label instructions are followed. There is no evidence that suggests the health of any mixers, loaders or applicators of bromoxynil has been affected by use of bromoxynil.

D. Conclusions for Section VI

1. There is no increased risk to the environment from the gene products, APH(3')II or nitrilase.
2. There is no scientific basis to suggest that horizontal gene movement could occur from plants to microorganisms.
3. If there were a mechanism for horizontal gene transfer from plants to microorganisms, the effect would be insignificant, a contribution of less than 1/10,000,000 to the *kan*^{r} microorganisms already present in soil. The effect would likewise be insignificant for the BXN gene since it was originally isolated from a soil microbe.
4. Based on data presented in this document, there are no negative environmental consequences of introduction of BXN cotton into agricultural production.
5. The use of Buctril for weed control in BXN cotton could dramatically reduce overall herbicide use in cotton production. Not only could the environmental impact of weed control in cotton be greatly reduced, but it is anticipated that the cost to farmers for weed control would also decrease.

VII. Statement of Grounds Unfavorable

Unfavorable information: NONE. No data have been produced to date which reflect negatively on this petition.

VIII. List of Appendices

IX. List of References

Abernathy, J. and C. McWhorter. 1992. Evolution of weed control in cotton. In: *Weeds of Cotton: Characterization and Control.* (C. McWhorter and J. Abernathy, ed.). Memphis, TN, The Cotton Foundation. p. 1-8.

AOSCA Certification Handbook. 1971. No. 23. Raleigh, NC, Association of Official Seed Certifying Agencies.

Atkinson, B. 1986. Species incidence and trends of susceptibility to antibiotic in the United States and other countries: MIC and MBC. In: *Antibiotics in Laboratory Medicine.* (V. Lorian, ed.). Baltimore, MD, Williams and Wilkins. p. 995-1162.

Auerswald, E.-A., G. Ludwig and H. Schaller. 1981. Structural analysis of Tn5. Cold Spring Harbor Symp. Quant. Biol. 45:107-113.

Baker, H. 1974. The evolution of weeds. Ann. Rev. Ecol. Syst. 5:1-24.

Baldwin, G., J. Kiser, J. Mitchell and A. Germany. 1992. Impacts of Buctril® herbicide spray on Stoneville cotton containing the BROMOTOL™ gene. 1992 Proc. Beltwide Cotton Conf. 2:555-556.

Bandyopadhyay, A., T. Nagasawa, Y. Asano, K. Fujishiro, Y. Tani and H. Yamada. 1986. Purification and characterization of benzonitrilases from *Arthrobacter* sp. strain J-1. Appl. Environ. Microbiol. 51:302-306.

Barker, R., K. Idler, D. Thompson and J. Kemp. 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Bartling, D., M. Seedorf and E. Weiler. 1991. Expression of a nitrilase from an Arabidopsis cDNA synthesizing the plant hormone indoleacetic acid. The International Society for Plant Molecular Biology, 3rd Int'l Congress, Molecular Biology of Plant Growth and Development. 855.

Beck, E., G. Ludwig, E. Auerswald, B. Reiss and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene. 19:327-336.

Birot, A.-M. and Casse-Delbart, F. 1988. Map location on *Agrobacterium* Root-inducing plasmids of homologies with the virulence region of tumor-inducing plasmids. Plasmid 19: 189-202.

Boyce Thompson Institute for Plant Research. 1987. *Regulatory considerations: Genetically engineered plants.* San Francisco, Center for Science Information. 106.

Brady, N. 1974. *The nature and properties of soils.* 8th, ed. New York, Macmillan Publishing Co., Inc.

Bridge, R. 1980. Variation of cotton flower morphology. Proc. Beltwide Cotton Prod. Res. Conf. 64-66.

Bryngelsson, T., M. Gustafsson, B. Green and C. Lind. 1988. Uptake of host DNA by the parasitic fungus *Plasmodiophora brassicae.* Physiol. Molec. Plant Path. 33:163-171.

Buchanan, G. 1992. Trends in weed control methods. In: *Weeds of Cotton: Characterization and Control.* (C. McWhorter and J. Abernathy, ed.). Memphis, TN, The Cotton Foundation. p. 47-72.

Buchholtz, K. 1967. Report from the nomenclature committee. Weed. 15:388-389.

Buckland, J., R. Collins and E. Pullin. 1973. Metabolism of bromoxynil octanoate in growing wheat. Pestic. Sci. 4:149-162.

Carlson, T. and B. Chelm. 1986. Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. Nature. 322:568-570.

Chandler, J. and F. Cooke Jr. 1992. Economics of cotton losses caused by weeds. In: *Weeds of Cotton: Characterization and Control.* (C. McWhorter and J. Abernathy, ed.). Memphis, TN, The Cotton Foundation. p. 85-116.

Cherry, J.P. and Leffler, H.R. 1984. Seed. In *Cotton* (Kohel, R.J. and Lewis C.F. eds.). American Society of Agronomy. Madison. p. 525-526.

Collins, P. and C. Knowles. 1983. The utilization of nitriles and amides by *Nocardia rhodochrous*. J. Gen. Microbiol. 129:711-718.

Colwell, R., P. Brayton, D. Grimes, D. Roszak, S. Huq and L. Palmer. 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for the release of genetically engineered microorganisms. Bio/Technology. 3:817-820.

Costantino, P., Mauro, M.L., Mitcheli, G., Risuleo, G., Hooykaas, P.J.J. and Schilperoort, R.A. 1981. Fingerprinting and sequence homology of plasmids from different virulent strains of *Agrobacterium rhizogenes*. Plasmid 5: 170-182.

Crawley, M.J. 1992. The comparative ecology of transgenic and conventional crops. *Proceedings of 2nd International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Micro-Organisms.* Goslar 1992.

Crockett, L. 1977. *Wildly Successful Plants: North American Weeds.* Honolulu, University of Hawaii Press. 609.

Cullimore, D. and M. Kohout. 1974. Isolation of a bacterial degrader of the herbicide bromoxynil from a Saskatchewan soil. Can. J. Microbiol. 20:1449-1452.

Curtis, J., L. Mott and T. Kuhnle. 1991. *Harvest of Hope.* New York, Natural Resources Defense Council. 124 pages.

Dale, P. 1992. Spread of engineered genes to wild relatives. Plant Physiol. 100:13-15.

Davies, J. 1986. Aminoglycoside-aminocyclitol antibiotics and their modifying enzymes. In: *Antibiotics in Laboratory Medicine.* (V. Lorian, ed.). Baltimore, MD, William and Wilkins. p. 790-809.

Duke, S., A. Christy, F. Hess and J. Holt. 1991. Herbicide-resistant crops. Council for Agricultural Science and Technology. May. 24 pages.

Endruzzi, J., E. Turcotte and R. Kohel. 1984. Qualitative Genetics, Cytology, and Cytogenetics. In: *Cotton.* (R. Kohel and C. Lewis, ed.). Madison, Wisconsin, American Society of Agronomy, Inc., Crop Science Society of America, Inc. Soil Science Society of America, Inc. p. 81-139.

Ensminger, M. E., J. E. Oldfield and W. W. Heinemann. 1990. Excerpts with reference to cottonseed and cottonseed components. In: *Feeds and Nutrition*. (M. E. Ensminger, ed.). Clovis, California, Ensminger Publishing Company. p. 252, 386-387, 404, 406-407, 440-441, 452, 474.

Fillatti, J., J. Kiser, R. Rose and L. Comai. 1987. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technology. 5:726-730.

Fincham, J. and J. Ravetz. 1991. Genetic engineering of agricultural plants. In: *Genetically Engineered Organisms Benefits and Risks*). Toronto, University of Toronto Press. p. 48-70.

Flavell, R., E. Dart, R. Fuchs and R. Fraley. 1992. Selectable marker genes: Safe for plants? Bio/Technology 10:141-142, 144.

Fryxell, P. 1979. *The Natural History of the Cotton Tribe.* College Station, Texas A&M Press. 245.

Fryxell, P. 1984. Taxonomy and germplasm resources. In: *Cotton*. (R. Kohel and C. Lewis, eds.). Madison, American Society of Agronomy. p. 605.

Gardner, R., A. Howarth, P. Hahn, M. Brown-Luedi, R. Shepherd and J. Messing. 1981. The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucl. Acids Res. 9:2871-2888.

Garfinkel, D., R. Simpson, L. Ream, F. White, M. Gordon and E. Nester. 1981. Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell. 27:143-153.

Gessel, J. 1984. Evolution of herbicide-resistant weeds. In: *Origins and Development of Adaptation (Ciba Foundation Symposium 102).* (Anonymous, ed.). London, Pitman Books. p. 73-93.

Gordon, M. 1993. Overview of the supply/demand factors for organic cotton. 1993 Proceed. Beltwide Cotton Conf. 1399-1400.

Gronenborn, B. and V. Matzeit. 1989. Plant gene vectors and genetic transformation: plant viruses as vectors. In: *Cell Culture and Somatic Cell Genetics of Plants. Molecular Biology of Plant Nuclear Genes.* (J. Schell and I. Vasil, eds.). Vol. 6. San Diego, Academic Press, Inc. p. 69-100.

Halevy, J. 1976. Growth rate and nutrient uptake of two cotton cultivars grown under irrigation. Agron. J. 68:701-705.

Handbook Seed Certification Regulations. 1989. Mississippi Seed Improvement Assoc., Mississippi State.

Harper, D. 1977a. Fungal degradation of aromatic nitriles: enzymology of C=N cleavage by *Fusarium solani.* Biochem. Journal. 167:685-692.

Harper, D. 1977b. Microbial metabolism of aromatic nitriles: enzymology of C=N cleavage by *Nocardia* sp. (*rhodochorus* group) N.C.I.B. 11216. Biochem. Journal. 165:309-319.

Harper, D. 1985. Characterization of nitrilase from *Nocardia* sp. *rhodochorus*) group) N.C.I.B. 11215 using p-hydroxybenzonitrile as sole carbon source. Int'l. J. Biochem. 17:677-683.

Harper, J. 1965. Establishment, aggression, and cohabitation in weedy species. In: *The Genetics of Colonizing Species*. (H. Baker and G. Stebbins, eds.). New York, Academic Press. p. 245-265.

Haselwood, E., G. Motten and R. Hirano. 1983. *Handbook of Hawaiian Weeds*. Honolulu, University of Hawaii Press. 491.

Hauptli, H., N. Newell and R. Goodman. 1985. Genetically engineered plants: environmental issues. Bio/Technology. 3:437-442.

Henschke, R. and F. Schmidt. 1990. Plasmid mobilization from genetically engineered bacteria to members of the indigenous soil microflora in situ. Current Microbiol. 20:105-110.

Hill, T. 1977. *The Biology of Weeds*. Studies in Biology. London, Edward Arnold.

Hohn, T. and J. Schell. 1987. *Plant DNA Infectious Agents*. New York, Springer-Verlag.

Holland, B., A. Welch, I. Unwin, D. Buss, A. Paul and D. Southgate. 1991. *McCance and Widdowson's the Composition of Foods*. 5th, ed. Boca Raton, FL, CRC Press, Inc. 400.

Holm, L., D. Plucknett, J. Pancho and J. Herberger. 1977. *The World's Worst Weeds*. Honolulu, The East-West Center, University of Hawaii. 609.

Holm, L. 1979. *A Geographical Atlas of World Weeds*. New York, John Wiley and Sons. 391.

Holmes, B. 1993. Can sustainable farming win the battle of the bottom line? Science. 260:1893-1895.

Hook, R. and W. Robinson. 1964. Ricinine nitrilase. II. Purification and properties. J. Biol. Chem. 239:4263-4267.

Horsch, R., J. Fry, N. Hoffmann, D. Eichholtz, S. Rogers and R. Fraley. 1985. A simple and general method for transferring genes into plants. Science. 227:1229-1231.

Houck, C., D. Shintani and V. Knauf. 1990. *Agrobacterium* as a gene transfer agent for plants. In: *Frontiers Appl. Microbiol.* (K. Mukerji and V. Singh, ed.). Vol. 4. Meerut, India, Rastogi & Co. p. 1-17.

Huffman, G.A., White, F.F., Gordon, M.P. and Nester, E.W. 1984. Hairy-root inducing plasmid: Physical map and homology to tumor-inducing plasmids. J. Bacteriol. 157: 269-276.

Hsu, J. and N. Camper. 1976. Isolation of ioxynil degraders from soil-enrichment cultures. Can. J. Microbiol. 22:537-543.

Hsu, J. and N. Camper. 1975. Degradation of ioxynil and bromoxynil as measured by a modified spectrophotometric method. Canad. J. Microbiol. 21:2008-2012.

International Food Biotechnology Council (IFBC). 1990. Biotechnologies and food: assuring the safety of foods produced by genetic modification. Regul. Toxic. Pharm. 12 (3) Part 2 of 2 Parts:S1-S196.

Jouanin, L. 1984. Restriction map of an agropine-type Ri plasmid and its homologies with Ti plasmids. Plasmid 12: 91-102.

Jouanin, L., Vilaine, F. d'Enfert, C. and Casse-Delbart, F. 1985. Localization and restriction maps of the replication origin regions of the plasmids of *Agrobacterium rhizogenes* strain A4. Mol. Gen. Genet. 201: 370-374.

Joint FAO/WHO Consultation. 1991. *Strategies for assessing the safety of foods produced by biotechnology*. Geneva, World Health Organization. 59.

Jones, L. and C. King, eds. 1990. *Cottonseed Oil*. Memphis, TN, National Cottonseed Products Association, Inc. 60 pages.

Jorgensen, R., C. Snyder and J. Jones: 1987. T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol. Gen. Genet. 207:471-477.

Keeler, K. 1989. Can genetically engineered crops become weeds? Bio/Technology. 7:1134-1139.

Keeler, K. and C. Turner. 1991. Management of transgenic plants in the environment. In: *Risk Assessment in Genetic Engineering*. (M. Levin and H. Strauss, ed.). New York, McGraw-Hill, Inc. p. 189-218.

Kiser, J. and J. Mitchell. 1991. Bromoxynil safened cotton: Trialing history, yield and quality. Beltwide Cotton Production Research Conferences. 566-568.

Klee, H. and S. Rogers. 1989. Plant gene vectors and genetic transformation: plant transformation systems based on the use of *Agrobacterium tumefaciens*. In: *Cell Culture and Somatic Cell Genetics of Plants. Molecular Biology of Plant Nuclear Genes*. (J. Schell and I. Vasil, ed.). Vol. 6. San Diego, Academic Press, Inc. p. 1-23.

Kobayashi, M., T. Nagasawa and H. Yamada. 1991. Characterization of nitrile-degrading enzymes and their application. The Tenth International Symposium on the Life Sciences (1991). 72-75.

Koukolíková-Nicola, Z., L. Albright and B. Hohn. 1987. The mechanism of T-DNA transfer from *Agrobacterium tumefaciens* to the plant cell. In: *Plant DNA Infectious Agents*. (T. Hohn and J. Schell, eds.). New York, Springer-Verlag. p. 109-148.

Krimsky, S. and R. Wrubel. 1993. *Agricultural Biotechnology: An Environmental Outlook*. Medford, MA, Dept. Urban and Environ. Policy, Tufts Univ. 71 pages.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

Lawhon, J. T., C. M. Carter and K. F. Mattil. 1977. Evaluation of the food use potential of sixteen varieties of cottonseed. JOACS. 54(February):75-80.

Mack, R. 1985. Invading plants: Their potential contribution to population biology. In: *Studies on Plant Demography*. (J. White, ed.). London, Academic Press. p. 127-142.

Mack, R. 1986. Alien plant invasions into the intermountain west: A case history. In: *Ecology of Biological Invasions of North America and Hawaii*. (H. Mooney and J. Drake, ed.). Vol. 58. New York, Springer-Verlag. p. 191-213.

Mahadevan, S. and K. Thimann. 1964. Nitrilase. II. Substrate specificity and possible mode of action. Arch. Biochem. Biophys. 105:133-141.

Manasse, R. and P. Kareiva. 1991. Quantifying the spread of recombinant genes and organisms. In: *Assessing Ecological Risk of Biotechnology*. (L. Ginzburg, ed.). New York, Butterworth-Heinemann. p. 215-231.

Maniatis, T., E. Fritsch and J. Sambrook. 1982. *Molecular cloning a laboratory manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory. 545.

McBride, K., J. Kenny and D. Stalker. 1986. Metabolism of the herbicide bromoxynil by *Klebsiella pneumoniae* subsp. *ozaenae*. Appl. Environ. Microbiol. 52:325-330.

McBride, K. and K. Summerfelt. 1990. Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol. Biol. 14:269-276.

McGregor, S. 1976. Cotton. In: *Insect Pollination of Cultivated Crop Plants, Ag. Handbook #496*. ed.). U.S. Government Printing Office. p. 171-189.

Meredith, W. and R. Bridge. 1973. Natural crossing in cotton (*Gossypium hirsutum* L.) in the delta of Mississippi. Crop Sci. 13:551-552.

Miller, J. and C. Knowles. 1984. The cellular location of nitrilase and amidase enzymes of *Brevibacterium* R312. FEMS Microbiol. Lett. 21:147-151.

Muenscher, W. 1980. *Weeds*. Ithaca, Cornell University Press. 586.

Munro, J. 1987. *Cotton*. New York, John Wiley & Sons, Inc. 436.

National Cottonseed Products Association. 1990. *Cottonseed and Its Products. Ninth Edition*. Memphis, TN

National Research Council. 1989. *Field testing genetically modified organisms: framework for decisions*. Washington, D.C., National Academy Press. 170.

Niles, G. and C. Feaster. 1984. Breeding. Cotton, Agronomy Monograph (ASA, CSSA, SSA). 24:201-231.

Office of Biotechnology Iowa State University. 1991. A benefit/risk assessment for the introduction of herbicide tolerant crops in Iowa. Iowa State Univ. Biotech. Update. 5:1-14.

Ooms, G., P. Hooykaas, R. V. Veen, P. V. Beelen, T. Regensburg-Tuïnk and R. Schilperoort. 1982. Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid. 7:15-29.

Palukaitis, P. 1991. Virus-mediated genetic transfer in plants. In: *Risk Assessment in Genetic Engineering*. (M. Levin and H. Strauss, eds.). New York, McGraw-Hill, Inc. p. 140-162.

Pimentel, D., M. Hunter, J. LaGro, R. Efroymson, J. Landers, F. Mervis, C. McCarthy and A. Boyd. 1988. Benefits and risks of genetic engineering in agriculture. Cornell University, Dept. Entomology internal report. 57.

Radke, S., B. Andrews, M. Moloney, M. Crouch, J. Kridl and V. Knauf. 1988. Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Genet. 75:685-694.

Redenbaugh, K., T. Berner, D. Emlay, B. Frankos, W. Hiatt, C. Houck, M. Kramer, L. Malyj, B. Martineau, N. Rachman, L. Rudenko, R. Sanders, R. Sheehy and R. Wixtrom. 1993. Regulatory issues for commercialization of tomatoes with an antisense polygalacturonase gene. In Vitro Cell. Dev. Biol. 29P:17-26.

Rochon, D. and A. Siegel. 1984. Chloroplast DNA transcripts are encapsidated by tobacco mosaic virus coat protein. Proc. Natl. Acad. Sci. USA. 81:1719-1723.

Salisbury, G. 1961. *Weeds and Aliens.* London, Collins.

SAS Institute Inc. SAS/STAT® User's Guide, Release 6.04 Edition. Cary, NC:SAS Institute Inc., 1988. GLM pp. 549-640.

Shatters, R. and M. Kahn. 1989. Glutamine synthetase II in *Rhizobium*: Reexamination of the proposed horizontal transfer of DNA from eukaryotes to prokaryotes. J. Mol. Evol. 29:422-428.

Simpson, D. 1954. Natural cross-pollination of cotton. U.S. Tech. Bull. 1094:1-17.

Smith, A. and D. Cullimore. 1974. The in vitro degradation of the herbicide bromoxynil. Can. J. Microbiol. 20:773-776.

Smith, M., D.-F. Feng and R. Doolittle. 1992. Evolution by acquisition: The case for horizontal gene transfers. Trends in Biochem. Sci. 17:489-493.

Smith, J. and W. Fletcher. 1964. 3,5-dihalogeno-4-hydroxybenzonitriles and soil microorganisms. Hort. Res. 4:60-62.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Stalker, D. and K. McBride. 1987. Cloning and expression in *Escherichia coli* of a *Klebsiella ozaenae* plasmid-borne gene encoding a nitrilase specific for the herbicide bromoxynil. J. Bacter. 169:955-960.

Stalker, D., L. Malyj and K. McBride. 1988a. Purification and properties of a nitrilase specific for the herbicide bromoxynil and corresponding nucleotide sequence analysis of the *bxn* gene. J. Biol. Chem. 263:6310-6314.

Stalker, D., K. McBride and L. Malyj. 1988b. Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science. 242:419-423.

Stotzky, G. 1989. Gene transfer among bacteria in soil. In: *Gene Transfer in the Environment.* (S. Levy and R. Miller, ed.). McGraw Hill, Inc. p. 165-222.

Sutcliffe, J. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43:77-90.

Thimann, K. and S. Mahadevan. 1964. Nitrilase 1. Occurrence, preparation, and general properties of the enzyme. Archives Biochem. & Biophy. 105:133-141.

Tiedje, J., R. Colwell, Y. Grossman, R. Hodson, R. Lenski, R. Mack and P. Regal. 1989. The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology. 70:298-315.

Towbin, H., T. Staeheiin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

UK Dept. Agriculture. 1977. The biodegradation of herbicides (dichlobenil and bromoxynil) by soil micro-organisms. Ann. Rep. Res. Tech. Work Dept. Ag. North. Ireland 1975.

Umbeck, P., K. Barton, E. Nordheim, J. McCarty, W. Parrott and J. Jenkins. 1991. Degree of pollen dispersal by insects from a field test of genetically engineered cotton. J. Econ. Entomol. 84:1943-1950.

USDA. 1993. Genetically engineered organisms and products; notification procedures for the introduction of certain regulated articles; and petition for nonregulated status. Fed. Reg. 58:17044-17059.

USDA. 1992. *Agricultural Statistics 1992*. Washington, D.C., U.S. Government Printing Office. p. 62.

USDA. 1991. *Agricultural Statistics 1991*. Washington, D.C., U.S. Government Printing Office. p. 135, 479.

USDA. 1990. *Agricultural Statistics 1990*. Washington, D.C., U.S. Government Printing Office. p. 61-73.

USDA. 1989. *Agricultural Statistics 1989*. Washington, D.C., U.S. Government Printing Office.

Van Elsas, J. and M. Pereira. 1986. Occurrence of antibiotic resistance among bacilli in Brazillian soils and the possible involvement of resistance plasmids. Plant and Soil. 94:213-226.

Vilaine, F. and Casse-Delbart, F. 1987. Independent induction of transformed roots by the TL and TR regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes*. Mol. Gen. Genet. 206: 17-23.

Waddle, B. A. 1984. Chapter 8. Crop Growing Practices. In: *Cotton*. (R. J. Kohel and C. F. Lewis, ed.). Vol. 24. Madison, Wisconsin, American Society of Agronomy Inc., Crop Science Society of America, Inc. Soil Science Society of America, Inc. p. 233-263.

Wagner, L., D. Herbst and S. Somer. 1990. *Manual of the Flowering Plants of Hawaii*. Honolulu, Bishop Museum Press.

Walbot, V. and L. Dure. 1976. Developmental Biochemistry of Cotton Seed Embryogenesis and Germination, VII. Characterization of the Cotton Genome. J. Mol. Biol. 101:503-536.

Weed Science Society of America. 1989. Composite List of Weeds. WSSA, Champaign, IL. 1-3, 21-20.

Wendel, J. 1989. New World tetraploid cottons contain Old World cytoplasm. Proc. Natl. Acad. Sci. USA. 86:4132-4136.

White, F. 1989. Vectors for gene transfer in higher plants. In: *Plant Biotechnology*. (S.-d. Kung and C. Arntzen, eds.). Boston, Butterworths. p. 3-34.

White, F.F. and Nester, E.W. 1980. Relationship of plasmids responsible for hairy root and crown gall tumorigenicity. J. Bacteriol. 144: 710-720.

White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P. and Nester, E.W. 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J. Bacteriol. 164: 33-44.

Williams, M. 1980. Purposefully introduced plants that have become noxious or poisonous weeds. Weed Sci. 28:300-305.

Yanisch-Perron, C., J. Vieira and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene. 33:103-119.

Zambryski, P. 1988. Basic processes underlying *Agrobacterium-mediated* DNA transfer to plant cells. Ann. Rev. Genet. 22:1-30.

B. Certification

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

Date July 14, 1993

Signature Keith Redenbaugh

Name of Petitioner Dr. Keith Redenbaugh

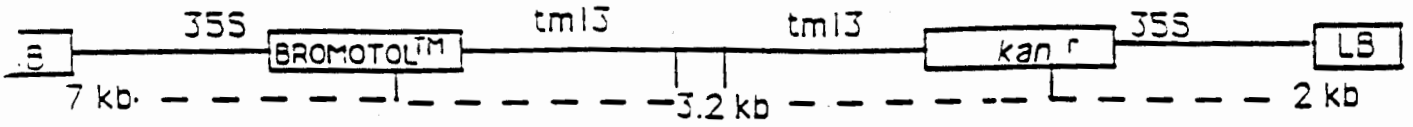
Mailing Address Calgene, Inc.
1920 Fifth Street
Davis, California 95616

Telephone (916) 753-6313

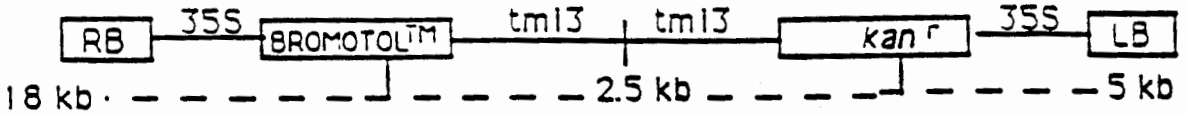
Figure 1. Maps of T-DNA based on data in Appendix 14 from cotton lines transformed with pBrx74 and pBrx75.

LB = Left border from the T-DNA of pTiA6 (Barker et al. 1983).
35S = 35S promoter region from CaMV35S transcript (Gardner et al. 1981).
kan^r = *kan*^{r} gene. Encodes aminoglycoside 3'-phosphotransferase II (APH(3')II) from transposon Tn5 (Beck et al. 1982).
tml 3' = Polyadenylation region of *tml* gene from pTiA6 (Barker et al. 1983).
BROMOTOL = BXN gene, which encodes nitrilase (Stalker et al. 1988a).
RB = Right border from the T-DNA of pTiA6 (Barker et al. 1983).

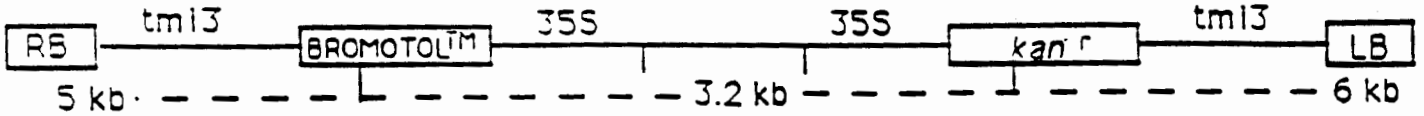
10103



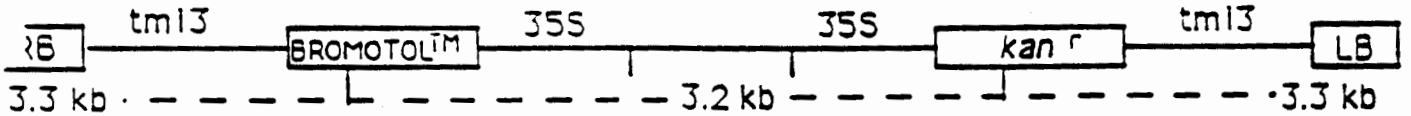
10109



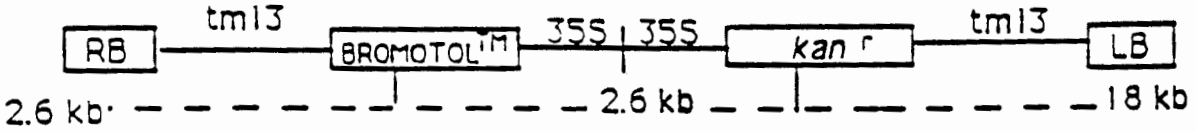
10206



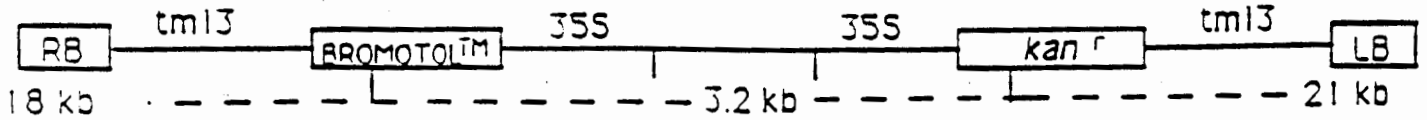
10208



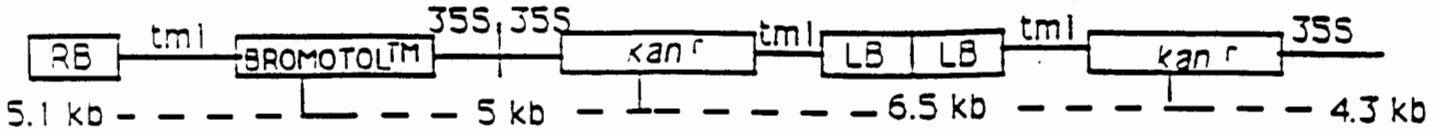
10209



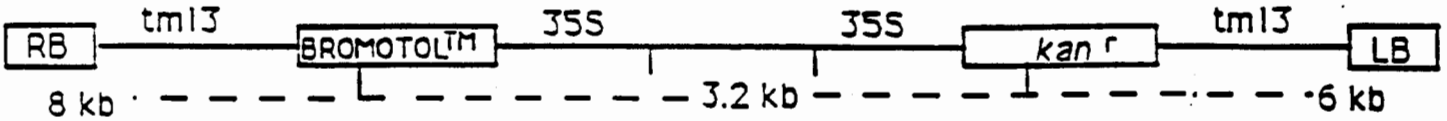
10211



10215



10222



10224

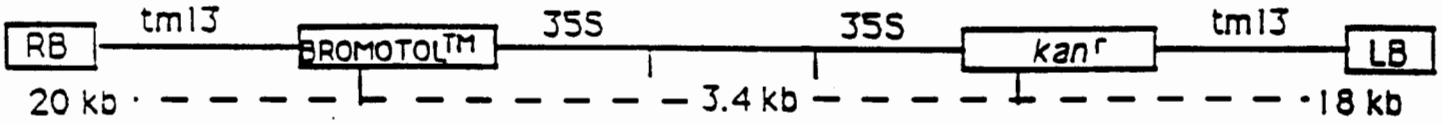


Figure 2. Nitrilase in leaves. Western blot analysis. Samples were prepared as described in Section D.3.2. The nitrilase signal consists of a single band at 37 kd. BROMOTOL cotton lines are 10103, 10107, 10109, and 10111. 1B-207 is a prototype line developed using pBrx45.

MWM = molecular weight marker.
Coker = Stoneville Pedigreed Seed Company Coker cotton varieties.
kd = kilodalton.

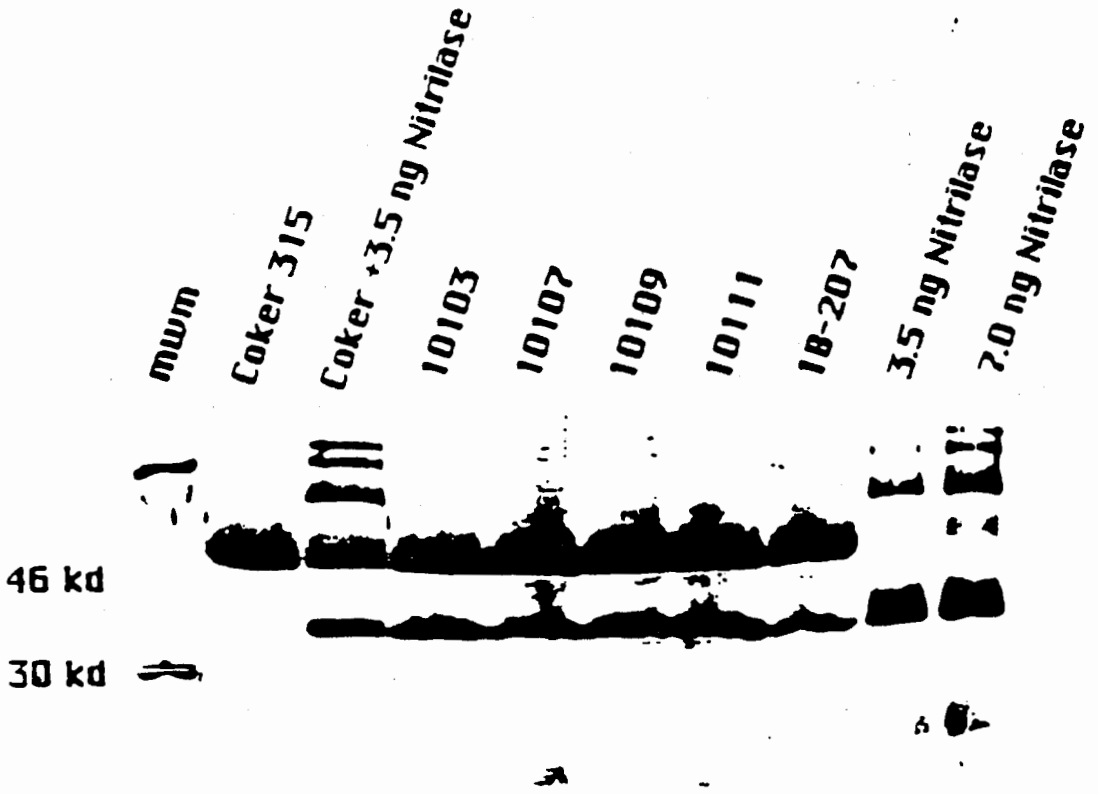


Figure 3: Nitrilase in leaves
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10206, 10208, 10209, 10211 and 1B-207

Figure 4: Nitrilase in leaves
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10215, 10222, 10224, 10107, and 1B-207

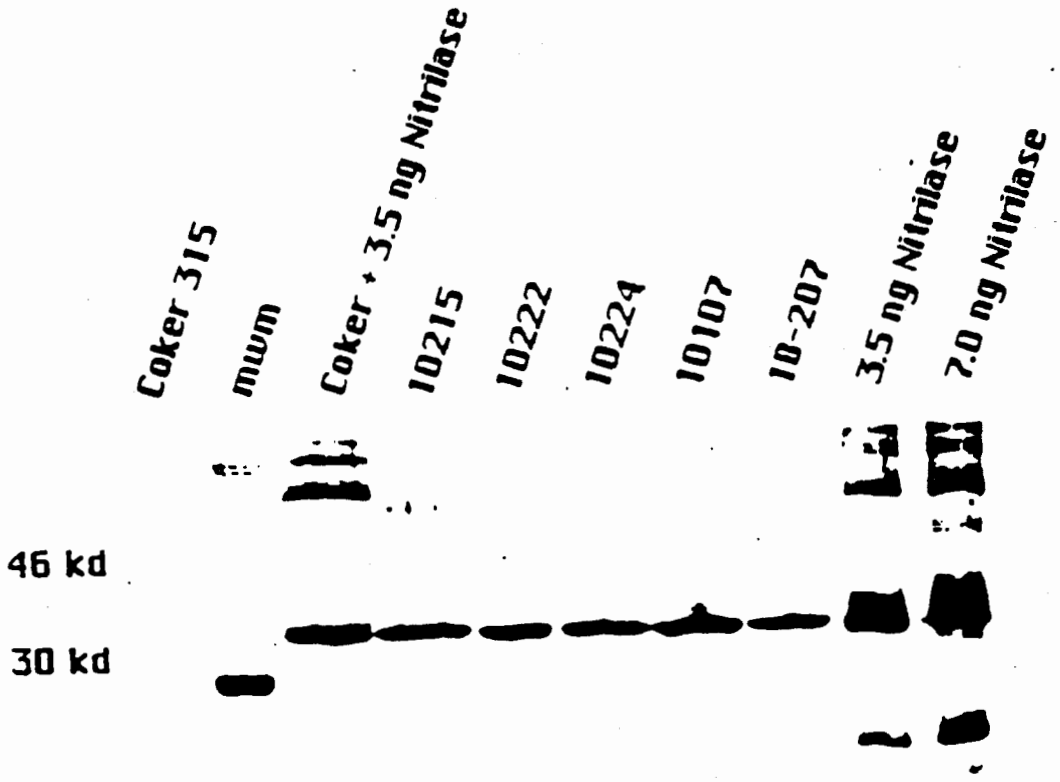


Figure 5: Nitrilase in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10103 and 10109
Very faint nitrilase signal in 10103 seeds lane

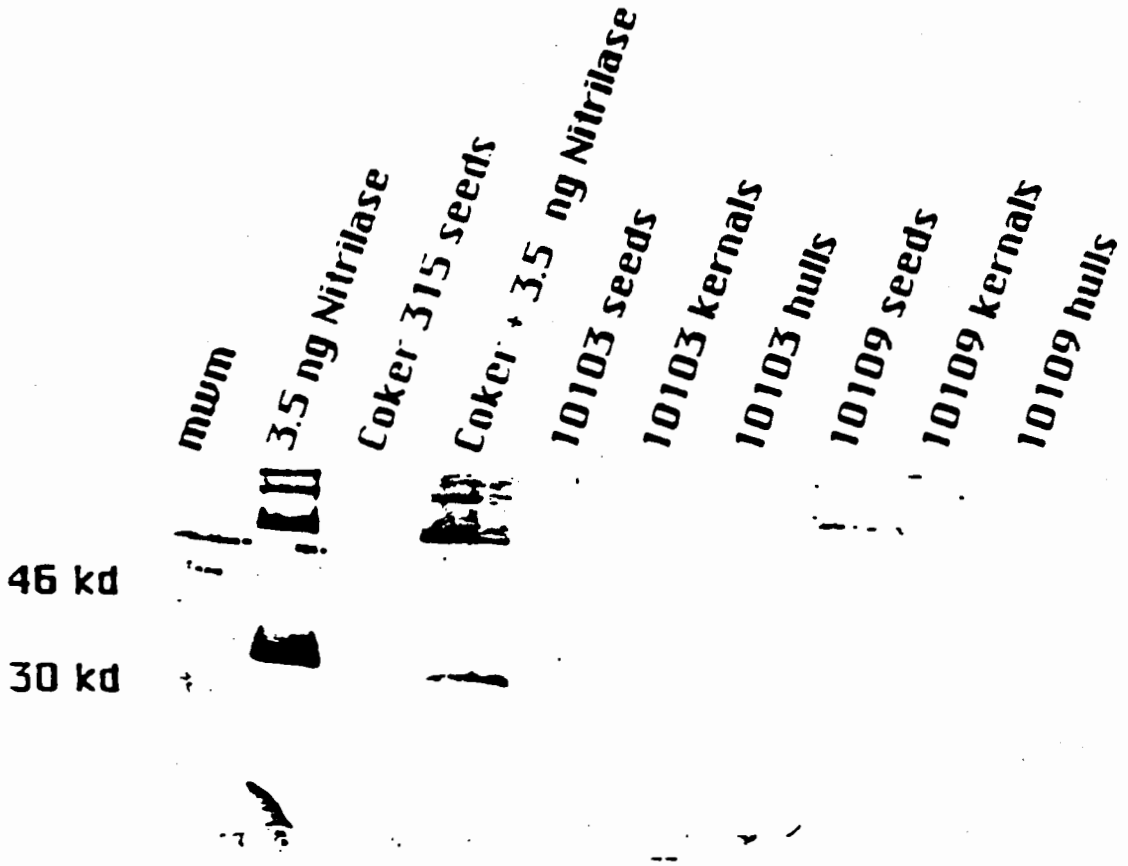


Figure 6: Nitrilase in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10206 and 10208
Nitrilase signals in 10206 seeds and kernals lanes and all 10208 lanes

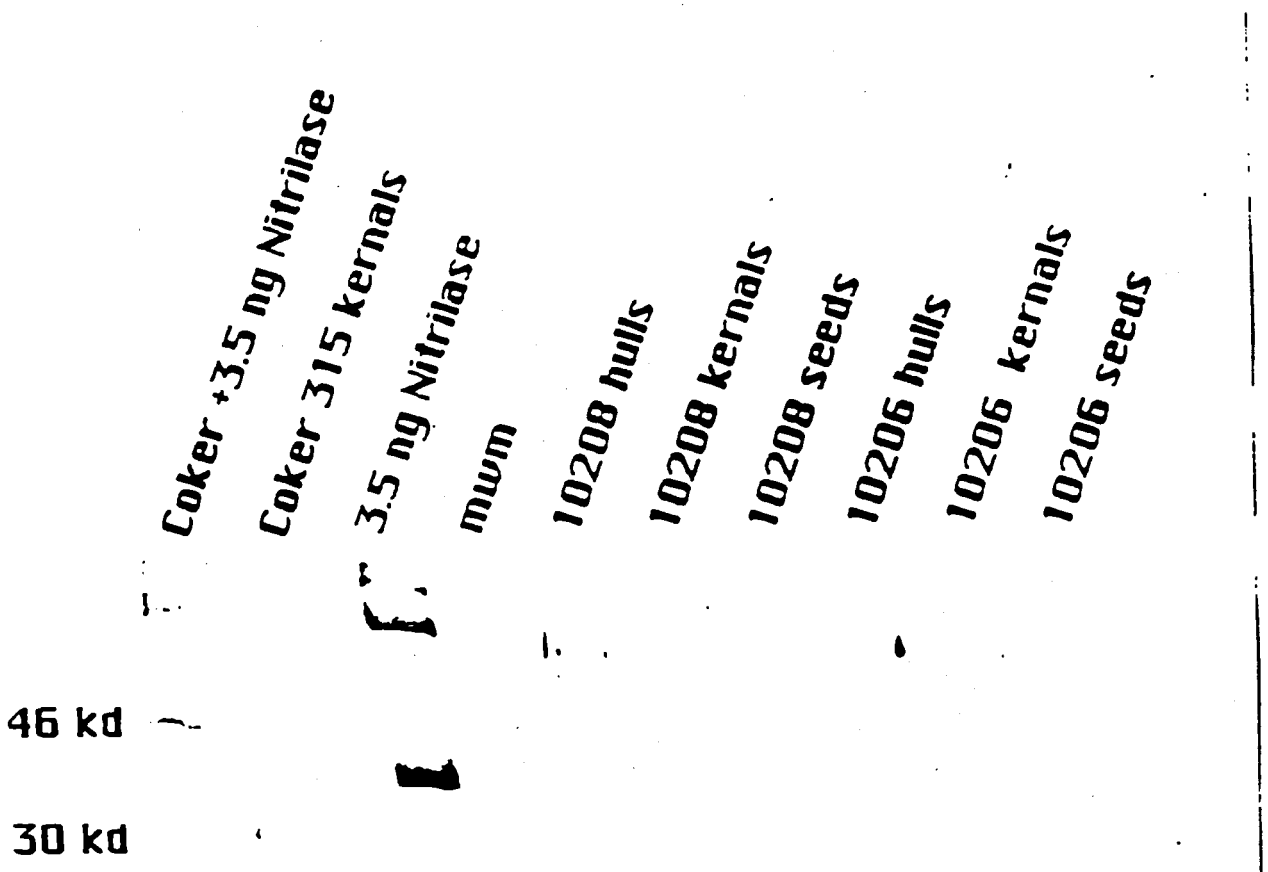


Figure 7: Nitrilase in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10211 and 10209
No Nitrilase signals.

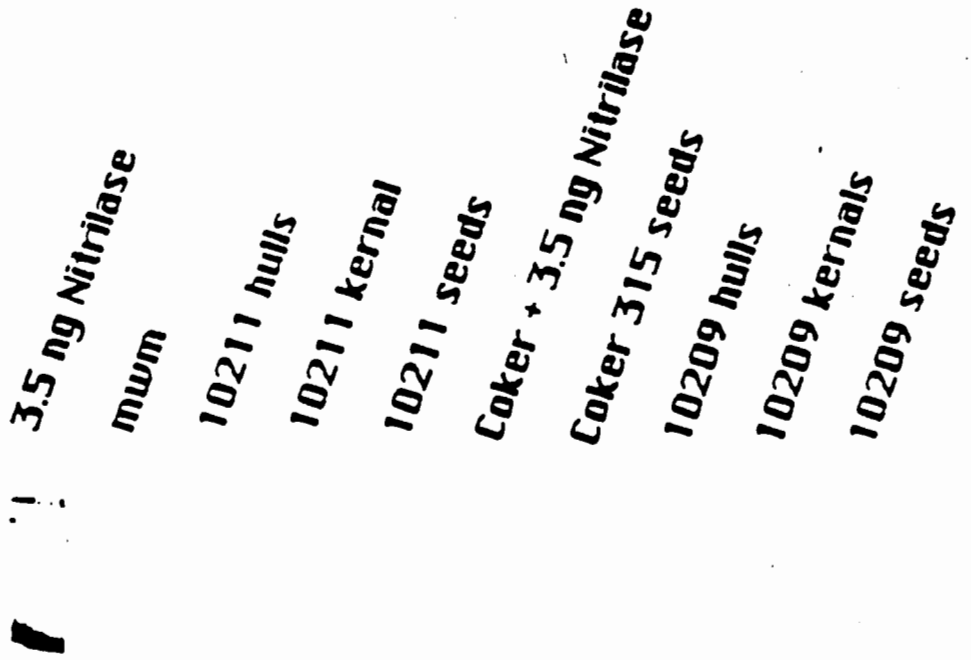


Figure 8: Nitrilase in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10215 and 10222
Nitrilase signals in all samples in both lines.
Slight background in Coker 315 hulls nontransformed control lane, most likely due to the overloading of lane 5, the 3.5 ng nitrilase spike in Coker 315 hulls extract. The overloading is shown by the double width of lane 5.

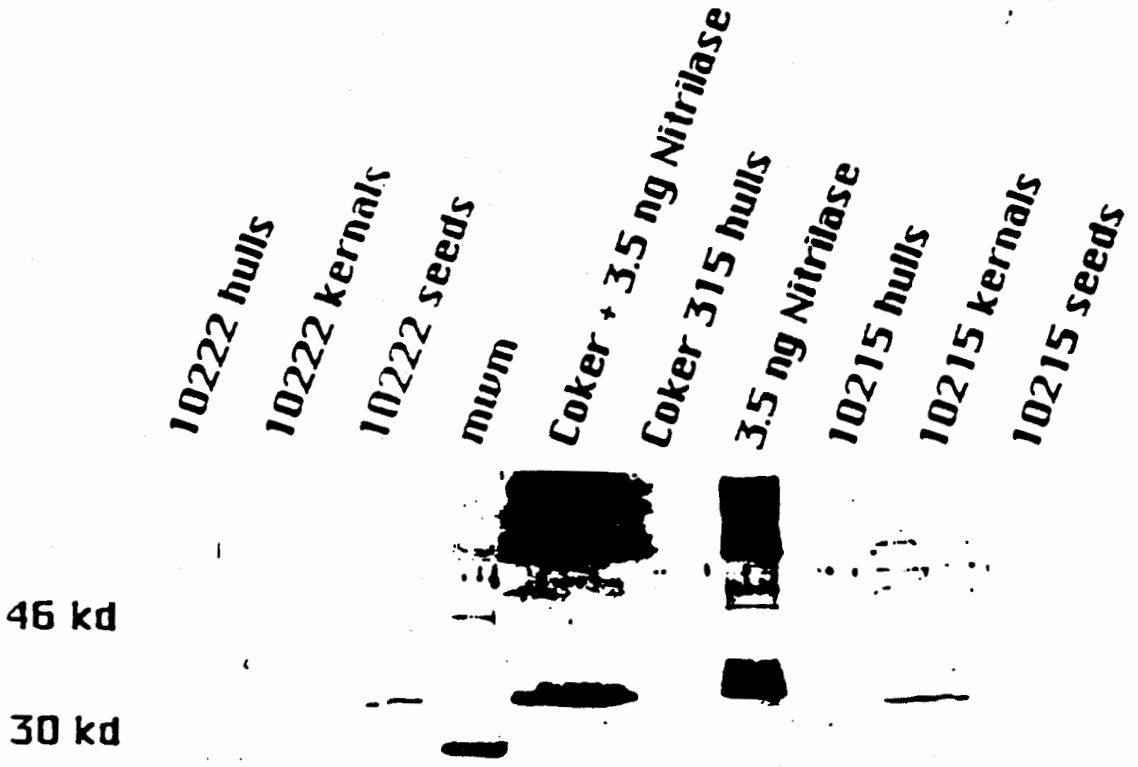


Figure 9: Nitrilase in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10211 (repeat) and 10224
Nitrilase signals in 10211 kernals and 10224 seeds and [illegible]nals lanes.
No background signal in Coker 315 hulls nontransformed control lane.

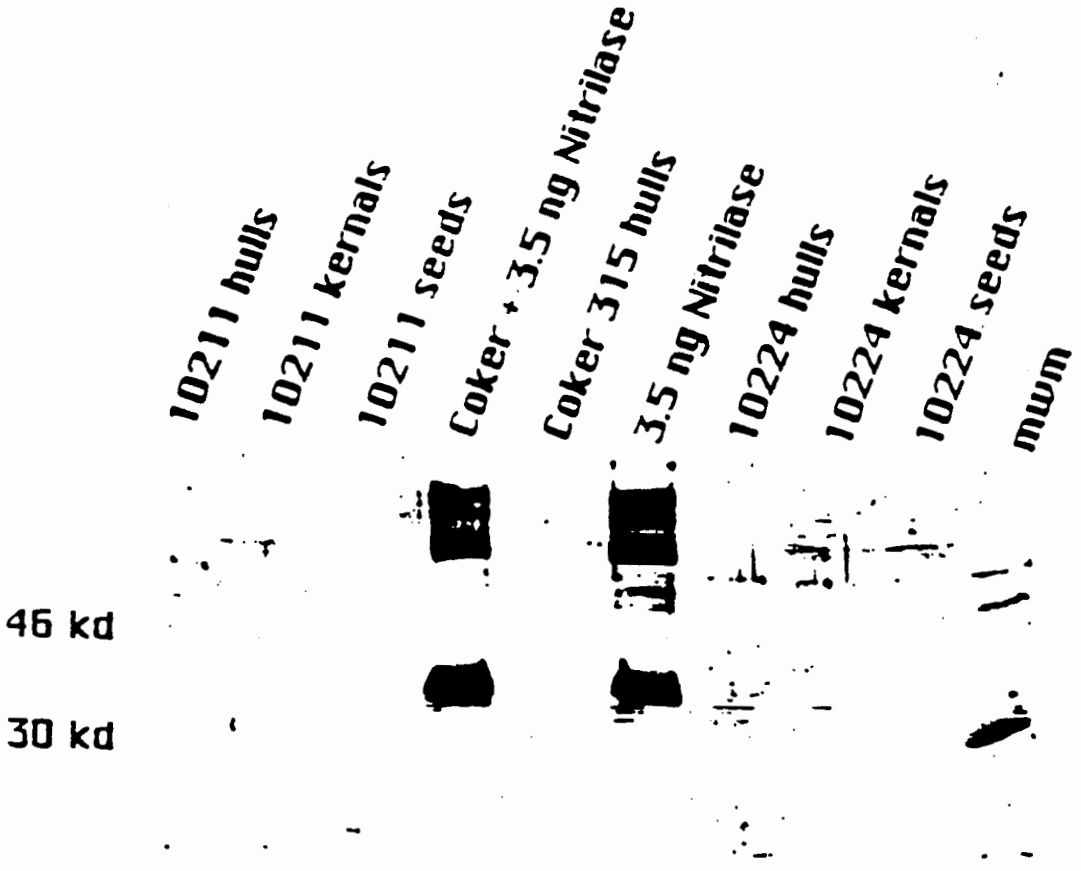


Figure 10: Nitrilase in processed cottonseed meal
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10103, 10109, 10206, 10208, and 10209
Nitrilase signals in all test lines

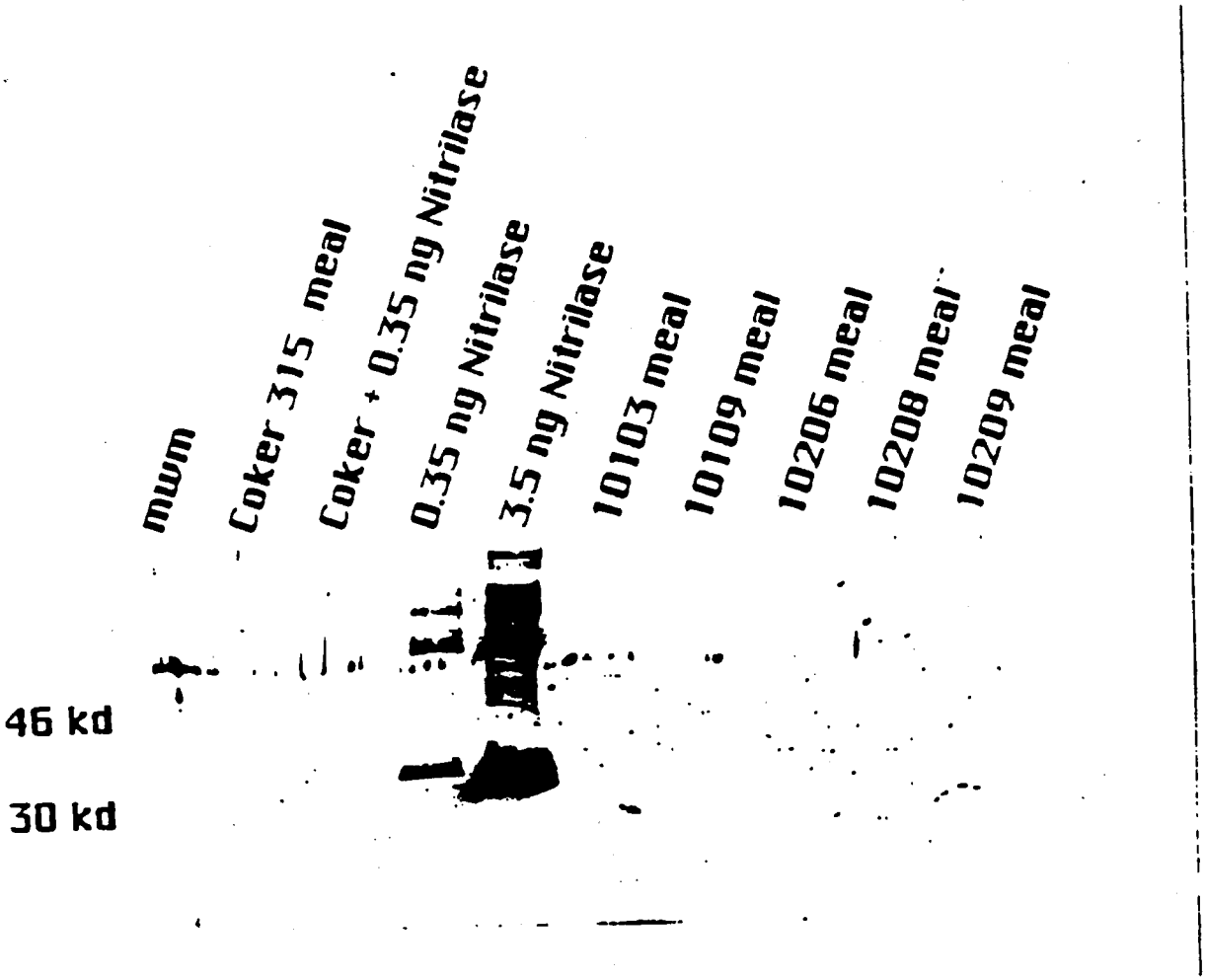


Figure 11: Nitrilase in processed cottonseed meal
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The nitrilase signal consists of a single band at 37 kd
Lines 10211, 10215, 10222, 10224
Very faint nitrilase signals in 10222 and 10224

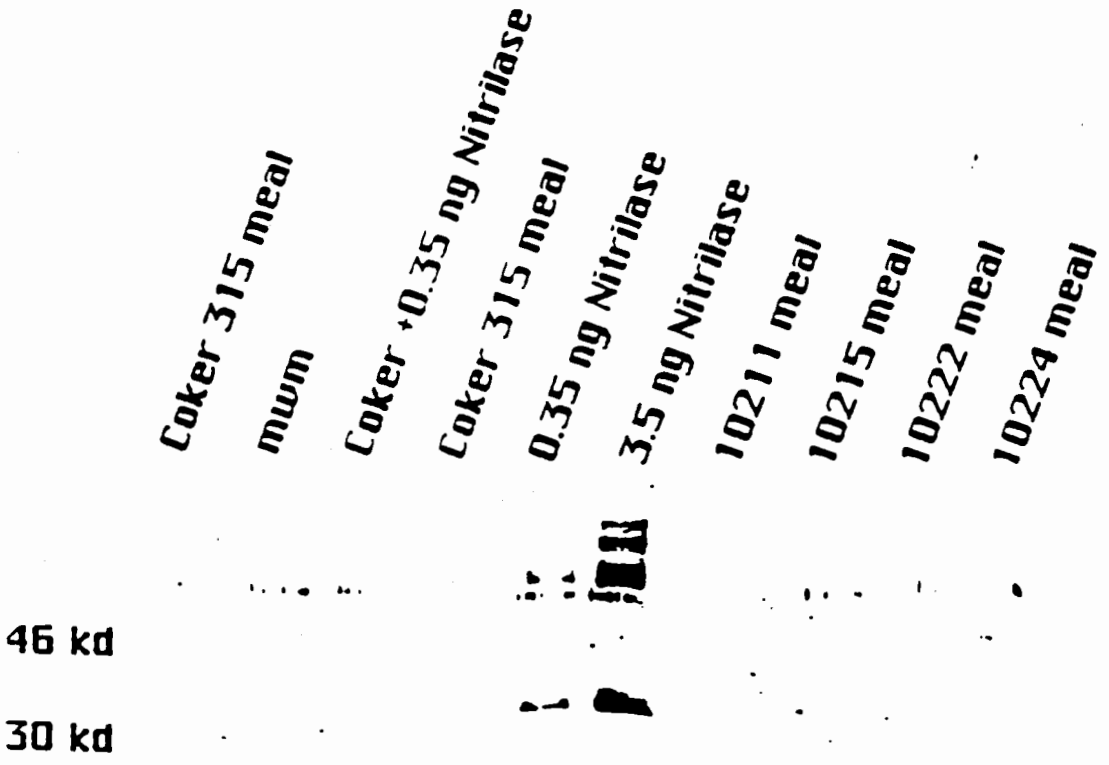


Figure 12: APH(3')II in leaves
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10103, 10109, 10206, 10208 and 10209

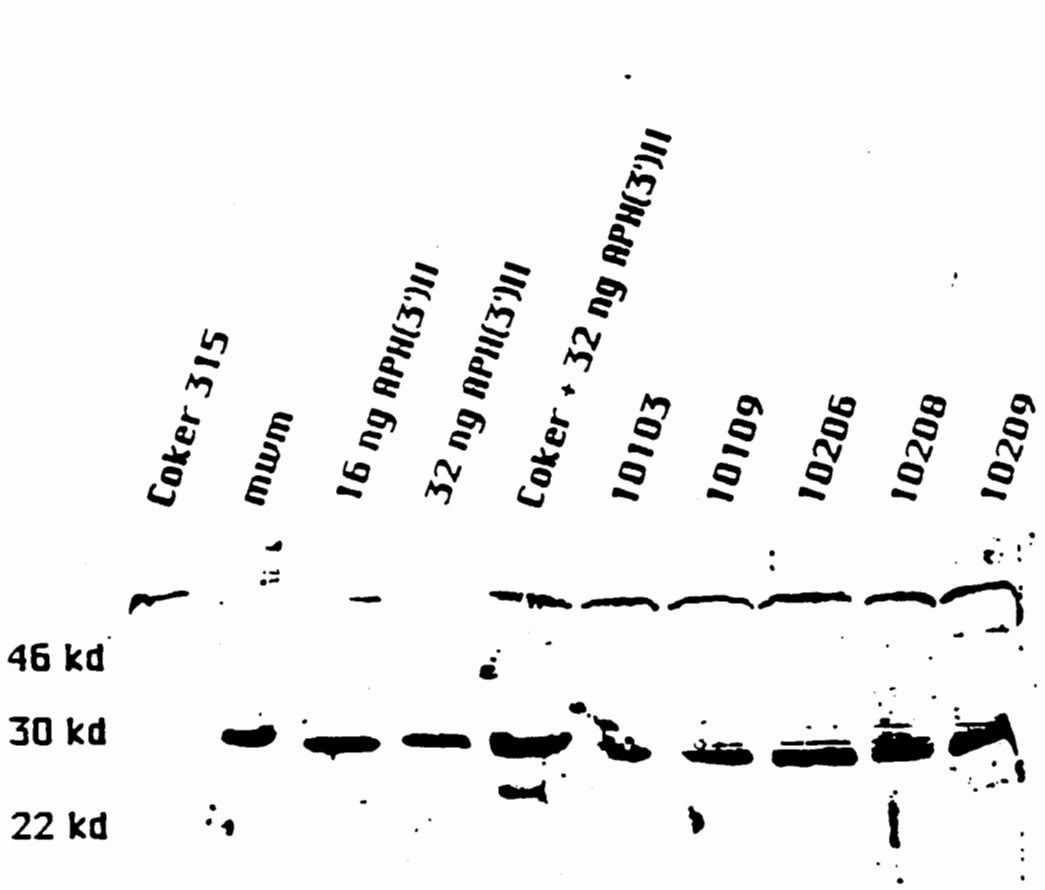


Figure 13: APH(3')II in leaves
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10211, 10215, 10222, 10224, and 1B-207

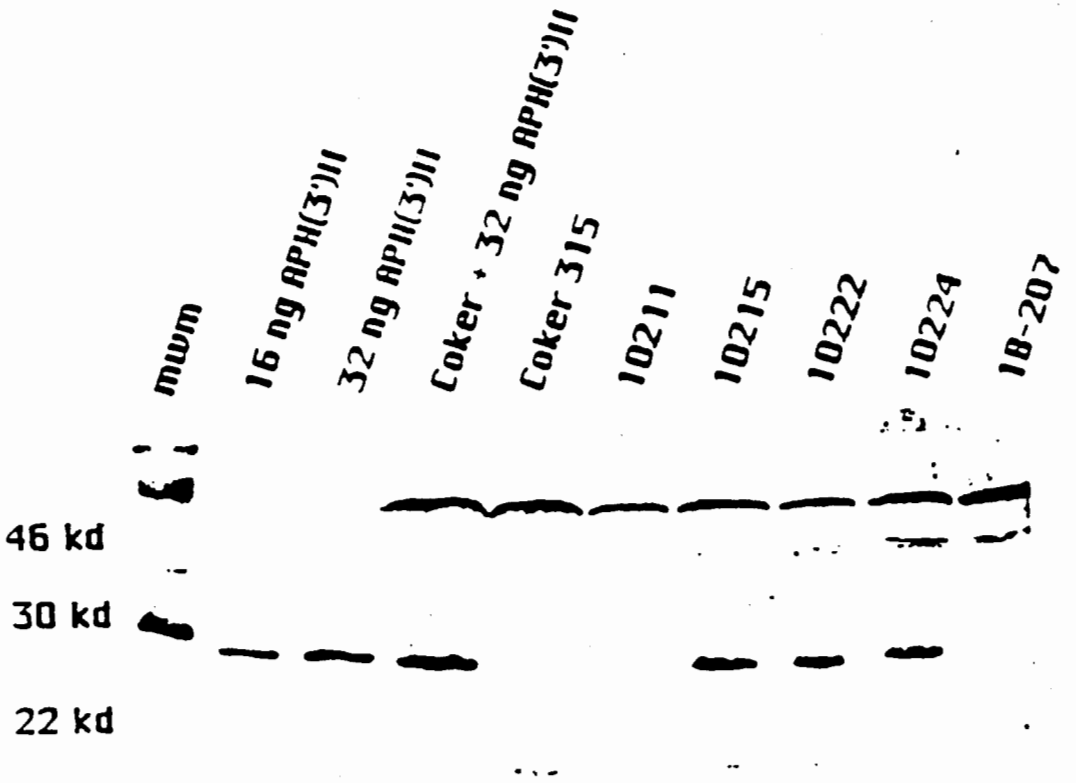


Figure 14: APH(3')II in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10103 and 10109
APH(3')II signals in all samples in both lines

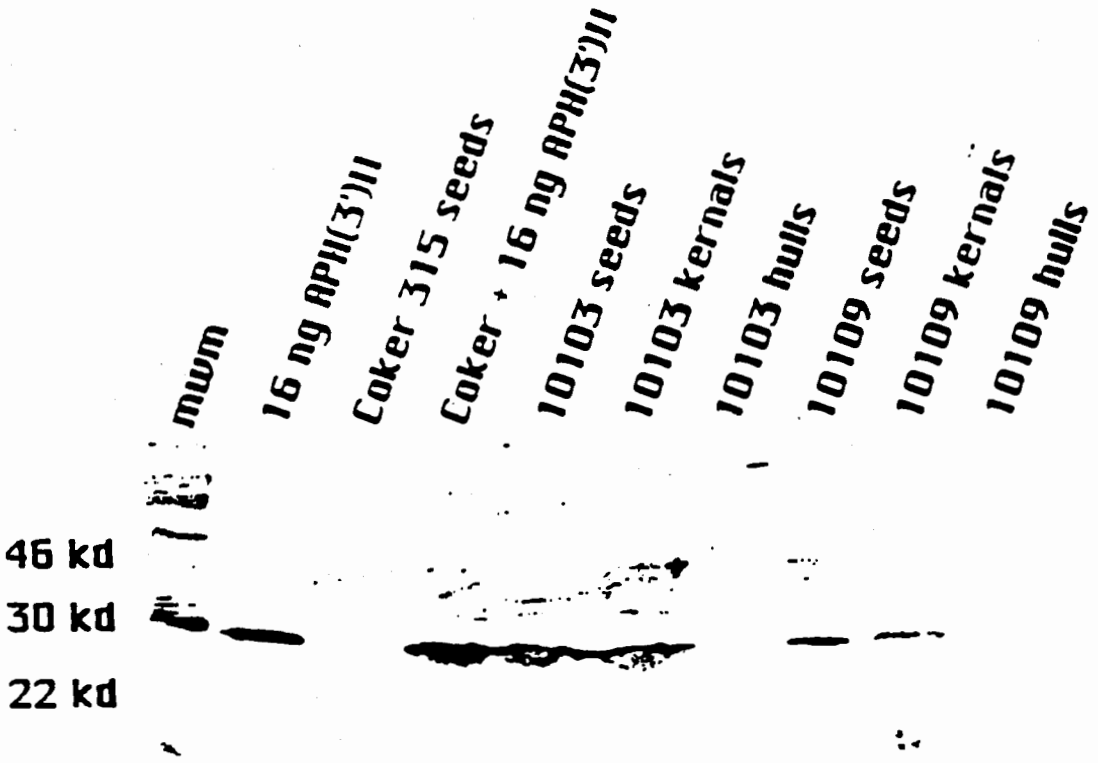


Figure 15: APH(3')II in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10206 and 10208
APH(3')II signals in all samples in both lines

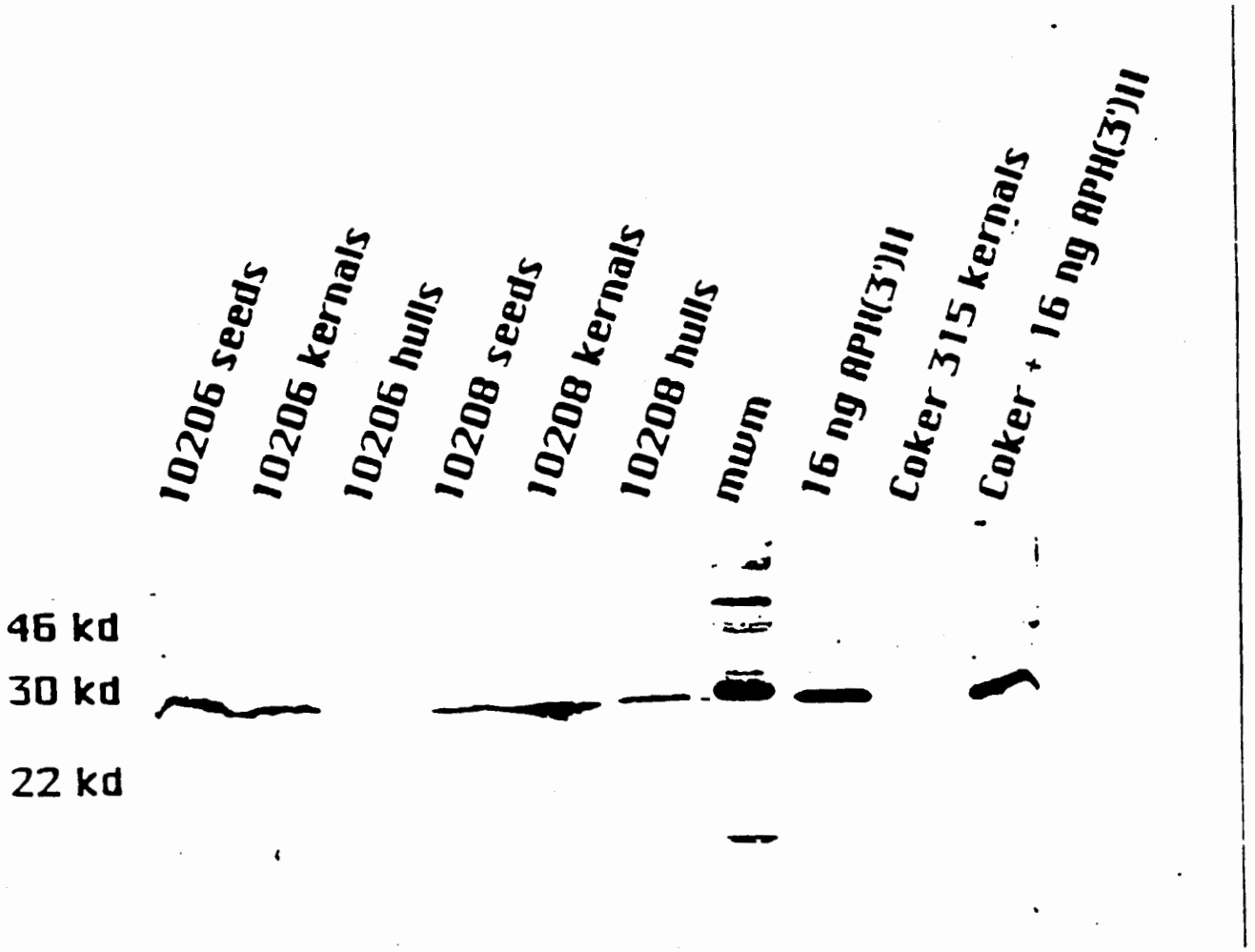


Figure 16: APH(3')II in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10209 and 10211
APH(3')II signals in all samples in both lines
There is a very slight signal in the Coker 315 hulls nontransformed control. This does not show in the Coker 315 seed or kernal extracts.

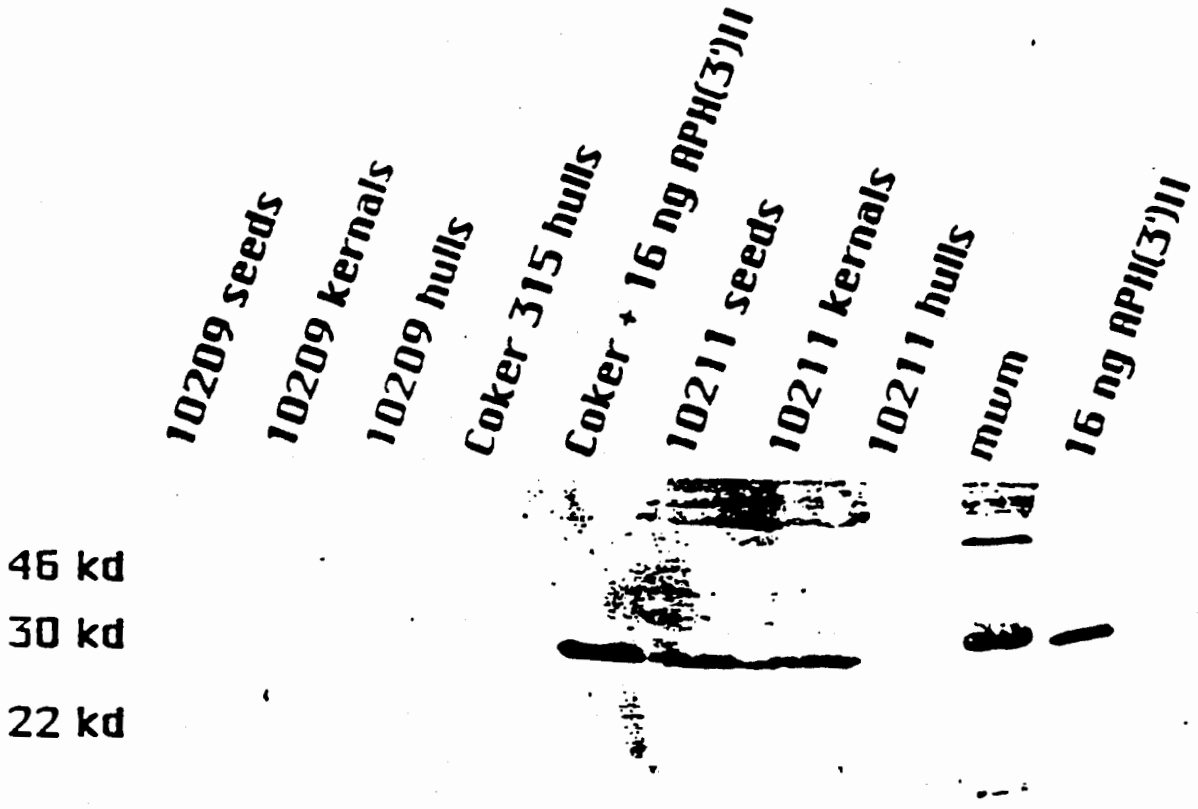


Figure 17: APH(3')II in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10215 and 10222
APH(3')II signals in all samples in both lines

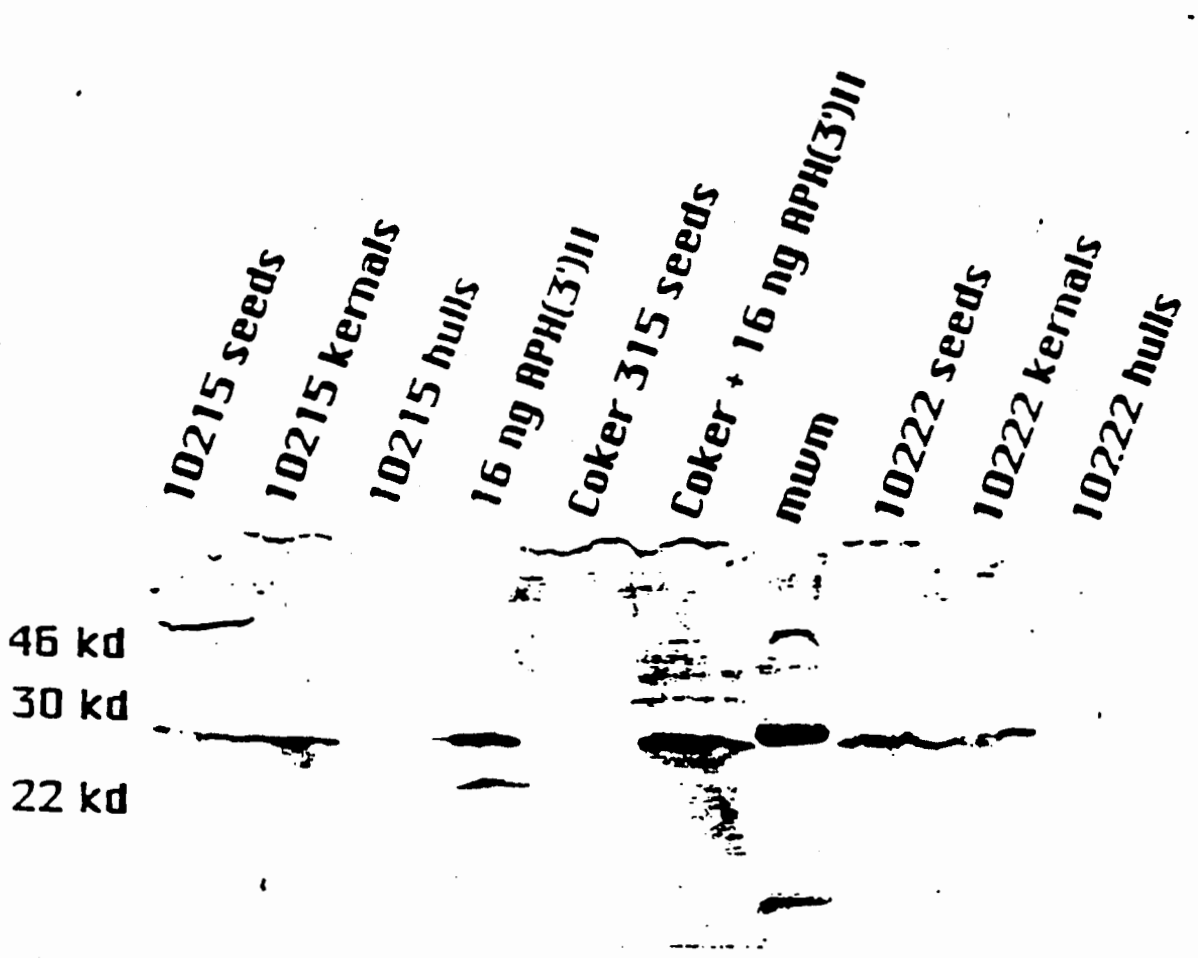


Figure 18: APH(3')II in cottonseed
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10211(repeat) and 10224
APH(3')II signals in all samples in both lines
There is a very slight signal in the Coker 315 hulls nontransformed control. This does not show in the Coker 315 seed or kernal extracts.

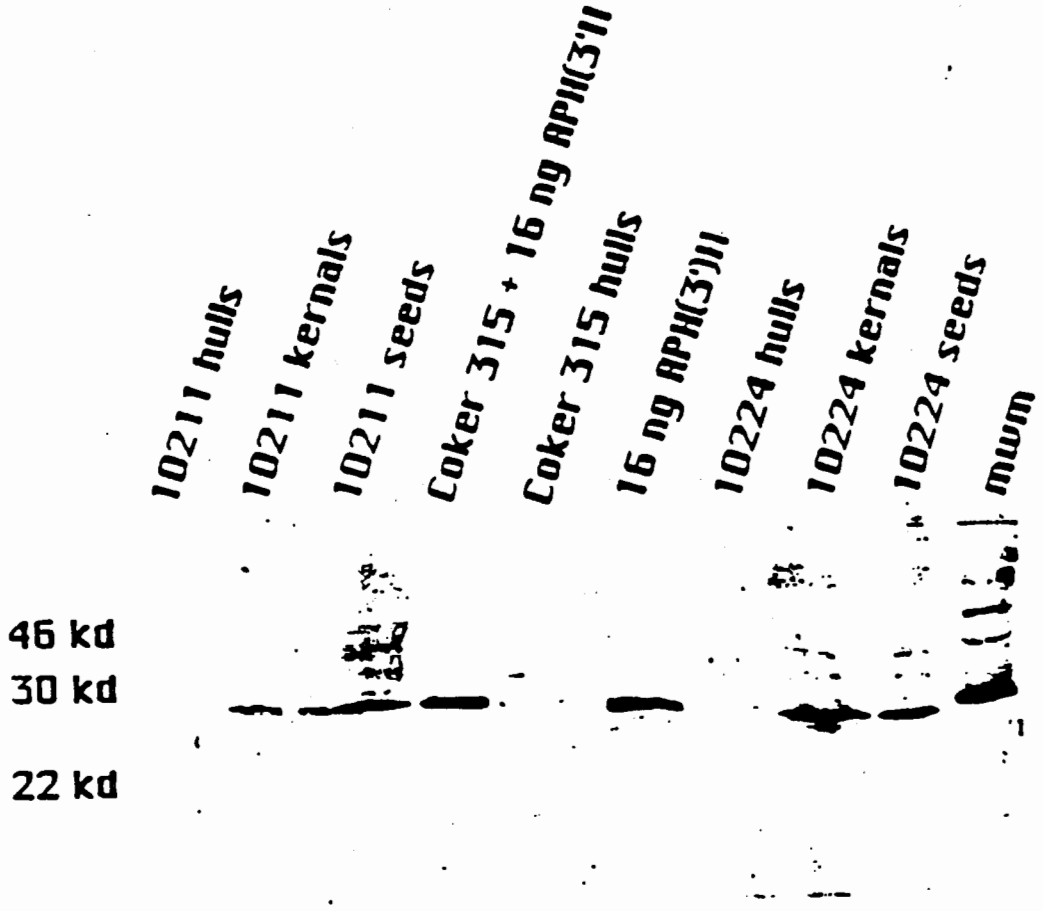


Figure 19: APH(3')II in processed cottonseed meal
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10103, 10109, 10206, 10208, and 10209
APH(3')II signals in all test lines

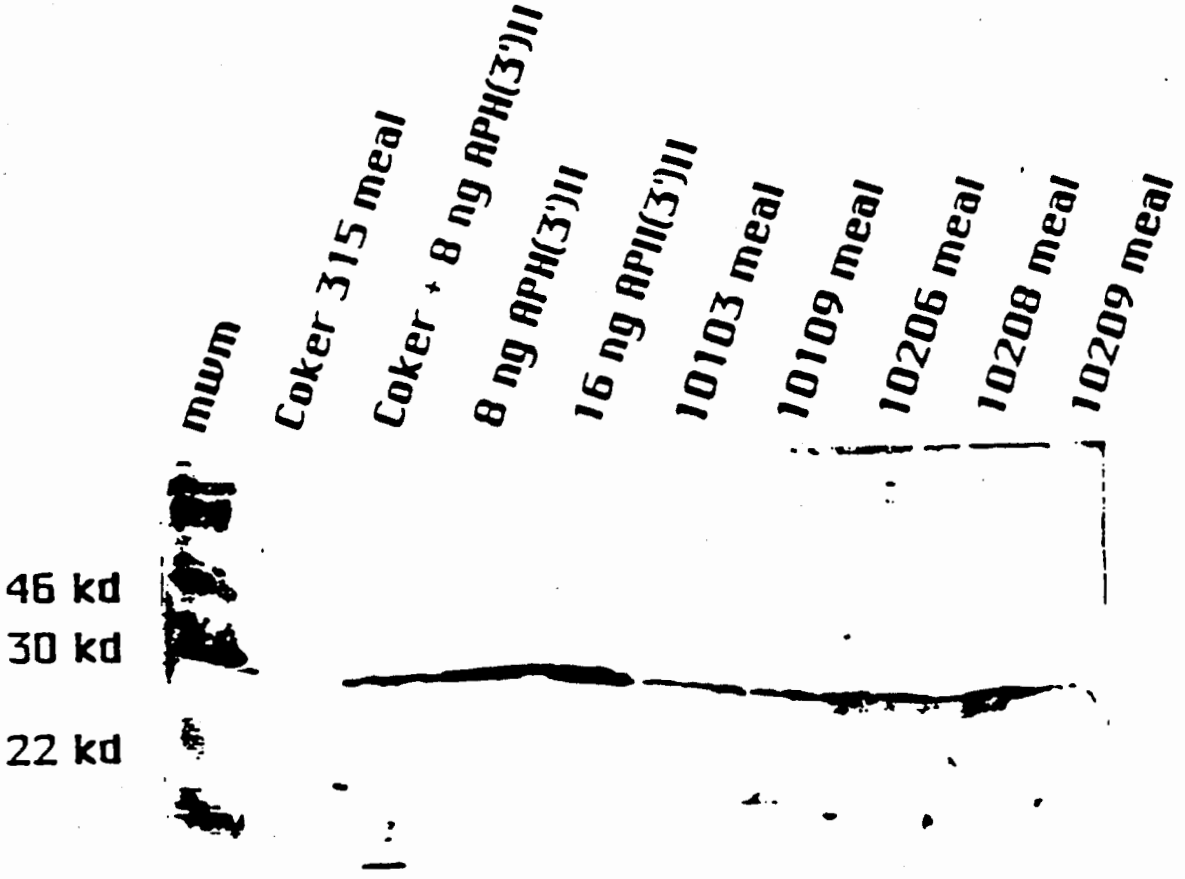
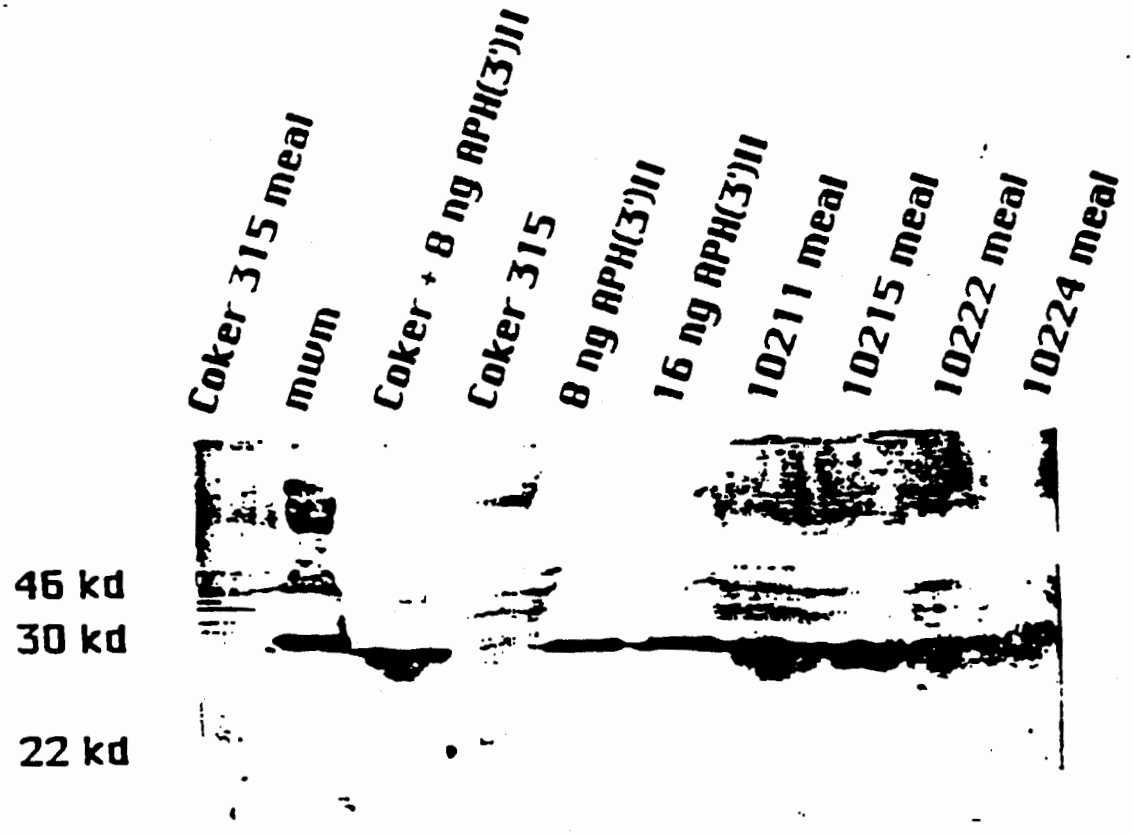


Figure 20: APH(3')II in processed cottonseed meal
Western blot anlaysis:
Samples were prepared as described in Section D.3.2
The APH(3')II signal consists of a single band at 29 kd
Lines 10211, 10215, 10222, and 10224
APH(3')II signals in all test lines



Appendix 1.

Weed Science Society of America. 1989. Composite List of Weeds. WSSA, Champaign, IL. 1-3, 21-20.

State of California, Department of Food and Agriculture, Division of Plant Industry, "Pest Ratings of Noxious Weed Species and Noxious Weed Seed," 1/8/90.

Federal Noxious Weed Regulation, CFR 360.