97-013-01p

CALGENE

10 January 1997

Dr. John Payne
USDA APHIS
Acting Director
Unit 147
4700 River Road
Riverdale, MD 20737-12137

Subject: Petition for the Determination of Nonregulated Status,
BXN with Bt Cotton

Dear Dr. Payne:

It is important to the commercialization of BXN with Bt cotton that we are able to plant in the 1997 season, which begins in late-April, without any regulatory restrictions. Recognizing the time required to complete the USDA review and deregulation process, we have proposed and presented arguments in support of the use of two existing Environmental Assessments (EA) which may be combined to support the safety of BXN with Bt cotton. We are hereby requesting deregulation of BXN with Bt Cotton based upon the BXN cotton (Petition No. P93-196-01) and Bollgard cotton (Petition No. 94-308-01p) determinations. We believe that the Environmental Assessments for both these products are directly applicable to BXN with Bt cotton and that no new issues are raised with this product that are not addressed in these existing Environmental Assessments. We have submitted data in those areas that are specific to BXN with Bt cotton but for other areas related to the potential impacts on the environment from nitrilase or nptII (contained in BXN cotton) and the CryIA(c) protein (contained in Bollgard cotton), we propose that the positions and statements contained within the two existing Environmental Assessments can be applied directly to this product. The rationale for this approach is that it a) is logical and warranted based on the biology of the product, b) is consistent with USDA regulations and overall mission, and c) reduces unnecessary duplication of effort and saves time.

In conjunction with Monsanto, we have reached agreement with the Environmental Protection Agency (EPA) to add Calgene's CryIA(c) protein to

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the existing registration for Monsanto's CryIA(c) protein based upon a demonstration of structural and functional equivalency of the pesticidal portions of the two proteins. This process was completed with the submission of the notification letter, a copy of which is included here. The data generated in support of this position are included with this submission to support the application of the Bollgard deregulation to BXN with Bt cotton. The FDA consultation procedure for BXN with Bt cotton will be completed in March 1997.

The applicability of the BXN cotton EA to this product is supported by several points. The genetic construction used in the production of BXN with Bt cotton, pCGN4084, is identical to the genetic construction used in the production of BXN cotton with only the addition of the *cryIA(c)* gene and associated regulatory sequences. This results in the situation where all the genes in BXN with Bt cotton, except for the mac promoter, *cryIA(c)* gene and *mas* 3' terminator, were previously evaluated during the deregulation of BXN Cotton. The *mas* 3' terminator sequences were previously evaluated in conjunction with the deregulation of the FLAVR SAVR tomato. The mac promoter is a constitutive promoter that is a hybrid of the CaMV35S and *mas* promoters. Data specific to this promoter are included here. Therefore, the only element of this product that has not been directly considered in the context of a previous deregulation of a Calgene product is the *cryIA(c)* gene.

The applicability of the Bollgard EA to BXN with Bt cotton is based upon a demonstration of functional equivalency of the CryIA(c) protein in our product to that in the Bollgard product. Functional equivalency of the two genes has been accepted by the EPA. The Monsanto registration of Bt cotton has been amended to include Calgene's CryIA(c) protein. This position and the acceptance by the EPA is based on the equivalency of the trypsinized functional portions of the two CryIA(c) proteins. This position is supported in this submission by the inclusion of the amino acid sequences and a Western blot demonstrating the equivalency of the trypsinized fragments of the two CryIA(c) proteins.

We believe the applicability of the BXN cotton Deregulation and Environmental Assessment to BXN with Bt cotton is evident and does not require any further discussion. We have focused our discussion on the applicability of the Bollgard Deregulation and Environmental Assessment to this product.

The Environmental Assessment for Bollgard Cotton specifically addresses four areas significant to the Finding of No Significant Impact. The basis of these findings are discussed in greater detail in the "Determination" of June 1995 where six specific areas are addressed. The conclusions reached in these

two documents may be applied to this product. The manner by which these are applicable is discussed in the following.

Environmental Assessment

A. Potential for Lepidopteran-Resistant Cotton Lines to Exhibit Increased Weediness Relative to Traditionally Bred Cotton Varieties

The issue of potential increased weediness is the identical for Bollgard Cotton and BXN with Bt Cotton. This is essentially a cotton issue and not one related to the presence of the *cryIA(c)* gene. The introduced genes function the same in both products and those characteristics of cotton that may or may not contribute to weediness, are unchanged by the insertion of the genes.

B. Potential Impacts Associated with Potential Gene Introgression from Lepidopteran-resistant Cotton Lines to Sexually Compatible Plants (With Wild and Cultivated relatives)

The issue of potential gene introgression is identical for Bollgard Cotton and BXN with Bt Cotton. This is essentially a cotton issue and not one related to the presence of the Bt gene. The introduced genes function the same in both products and those characteristics of cotton that may or may not affect sexual compatibility, are unchanged by the insertion of the genes. Numerous factors affecting gene introgression, exclusive of the inserted gene, are discussed in the EA and it was then stated that "APHIS believes that it is these factors, rather than gene movement from cultivated cottons, that are of real significance to this species".

C. Potential Impact on Nontarget Organisms Including Beneficial Organisms such as Bees and Earthworms

The conclusion that "CryIA(c), expressed in Lepidopteran-resistant cotton lines, shows a strict host-range specificity for lepidopteran insects and has no deleterious effects on nontarget organisms" is stated in the EA. The major factor in reaching this conclusion is that nontarget organisms are "... not expected to contain the receptor protein found in the midgut of target insects".

The insecticidal property of CryIA(c) is contained within the trypsinized fragment that, in nature, occurs only within the midguts of the target insects. This is the condition that results in target specificity. The basis of the EPAs acceptance of adding Calgene's CryIA(c) to the Monsanto registration is the equivalency of the two trypsinized fragments.

Equivalency is supported here by the Western blot and the amino acid comparison. These data clearly support the functional equivalency of the two CryIA(c) proteins. This equivalency is further supported by the comparison of Calgene's CryIA(c) to Mycogen's MVP, a commercial genetically engineered Bt that is topically applied, and a comparison of Monsanto's CryIA(c) to Dipel, also a topically applied commercial Bt. Based on the demonstration of equivalency, these four products are essentially identical as to their target specificity and environmental safety. Additionally, the amino acid sequence of trypsinized CryIA(c) in BXN with Bollgard cotton is identical to the native CryIA(c) from *B.t.k.*, and both differ from the CryIA(c) in Monsanto's Bollgard cotton by 6 amino acids.

The other element of this assessment may be the consideration of any differences in potential risks represented by the difference in the size (number of amino acids) of the non-trypsinized CryIA(c) in the Monsanto and Calgene products. The full length CryIA(c) in Bollgard Cotton is 1178 amino acids in length while the CryIA(c) in BXN with Bt Cotton is 590 amino acids long. Native CryIA(c) is also 1178 amino acids in length. The trypsinized fragment of all three is 574 amino acids long. The trypsinized forms of CryIA(c) from Bollgard cotton, BXN with Bt cotton and MVP all appear at 64 kD on Western blots. Additionally, in the MVP Western blot, bands are also present at 120kD, 96kD and 70kD demonstrating that fragments of CryIA(c) greater than the 574 amino acids of the trypsinized form but less than full length are present in an existing commercial product and do not appear to alter the specificity or safety of the product. This supports the position that the size of the full length CryIA(c) is not a factor in this assessment.

D. Potential Impacts on Agricultural Practices Associated with the Cultivation of Lepidopteran-resistant Cotton Plants and the Development of Insect Resistance to the *Btk* Insect Control Protein

The areas addressed in this section are directly applicable to BXN with Bt Cotton in that both products will be used in exactly the same manner for insect control. Additionally, as a result of our relationship with Monsanto, Calgene's product, BXN with Bt cotton, will be sold as BXN with Bollgard and will fall under the Monsanto Resistance Management plans.

Determination

A. The introduced genes, their regulatory sequences and their products do not present a plant pest risk in the lepidopteran-resistant cotton line.

This section of the determination for BXN cotton may be applied directly to this section for BXN with Bt cotton with the inclusion of those elements related to CryIA(c) and associated regulatory regions. Those elements associated with the *cryIA(c)* gene are the same as contained in the Bollgard determination and may also be applied. The specific elements associated with the mac promoter and *mas3'* terminator are provided with this submission. The rational for the applicability of these two determination's has been previously stated.

B. Lepidopteran-resistant cotton has no significant potential to become a successful weed.

Again, as with the discussion under Environmental Assessment A, the elements in the Bollgard determination are directly applicable to BXN with Bt cotton.

C. Lepidopteran-resistant cotton will not increase the weediness potential of any other plant with which it can interbreed.

For those reasons previously stated for Environmental Assessment A and B, this is directly applicable to BXN with Bt cotton.

D. Lepidopteran-resistant cotton will not cause damage to processed agricultural commodities.

The general basis of this conclusion is applicable to BXN with Bt cotton. Product characteristic and compositional data on BXN with Bt cotton have been provided.

E. Lepidopteran-resistant cotton will not be harmful to beneficial organisms, including bees.

This is discussed in Environmental Assessment C and is directly applicable to BXN with Bt cotton as stated in E. 1) and 2).

F. Impacts on the current agricultural practices.

In that BXN with Bt cotton will be used exactly like Bollgard cotton for insect control and the pesticidal elements in both products have been

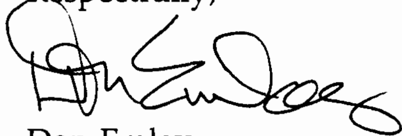
determined to be "substantially equivalent", all elements of this section are directly applicable.

Our objective in taking this approach to the safety assessment of BXN with Bt cotton is to provide the basis for an expedited review of the product without compromising any elements of safety. We believe this approach allows you to accomplish both.

We therefore request that a Determination of Nonregulated Status be made for BXN with Bt cotton and the petition process be expedited so the determination can be made by the end of April 1997.

We appreciate your consideration of our request.

Respectfully,



Don Emlay
Vice President, Regulatory Affairs

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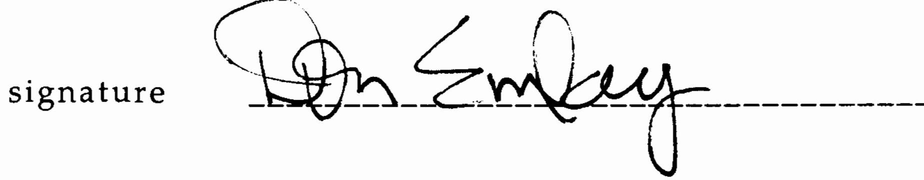
PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR BXN® with Bt Cotton (*Gossypium hirsutum* L.)

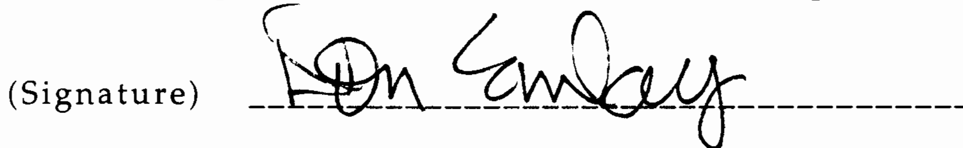
Calgene, Inc.
January 10, 1997

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

signature 

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.

(Signature) 

(Name of Petitioner) Calgene, Inc.
Don Emlay, Vice President Regulatory Affairs

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Petition for Determination of Nonregulated Status for BXN® with Bt Cotton

I. Introduction....1
A. Statement of Request for Determination....1
B. Standard of Identity of BXN with Bt Cotton....2
II. Cotton: The recipient plant....2
III. Description of the genetic modification....3
A. The transformation and vector system....3
B. Genetic constructs....3
C. Inserted genetic sequences and their sources....3
1. DNA sequences from nonregulated articles....3
2. DNA sequences from regulated articles....5
D. Genetic characterization....6
1. Number of inserts in BXN with Bt cotton....6
2. Lack of transfer beyond the T-DNA borders....6
3. Genetic stability....6
E. Equivalence of CryIA(c) protein in Bollgard cotton and BXN with Bt cotton....7
IV. Characteristics of BXN with Bt cotton plants....11
A. Field performance....11
B. Seed germination and dormancy....13
C. Lack of overwintering potential....13
D. Lack of plant pest characteristics....14
V. Potential for Environmental Impact by BXN with Bt Cotton....15
A. Herbicide tolerance trait....15
B. Insect resistance trait....15
Nontarget organism effects....15
Regulatory status at EPA....16
C. Lack of effect upon raw or processed agricultural commodities: Compositional Analysis of BXN with Bt Cotton....17
VI. Statement of Grounds Unfavorable....23
VII. Literature Cited....24
VIII. Appendices....26
1. Determinations of nonregulated status for BXN® cotton and Bollgard® cotton
2. Genetic characterization: construct maps, DNA sequence, Southern blots.
3. Equivalence of cryIA(c) in Bollgard cotton and BXN with Bt cotton: amino acid sequence comparison and Western blots
4. Field trial reports
5. Seed germination and dormancy
6. EPA Notification letter
7. Report on lack of CryIA(c) in pollen and nectar of BXN with Bt cotton
8. Report on lack of toxicity of BXN with Bt cotton to earthworms and Colembola.
9. Literature References

LIST OF TABLES

		Page
Table 1.	DNA sequences from nonregulated articles.	3
Table 2a.	DNA sequences from regulated articles.	5
Table 2b.	Characteristics of CryIA(c) gene sequences and proteins from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 (i.e. the native sequence) and in transgenic cotton plants.	10
Table 3.	Field trials with BXN with Bt cotton events 31807 and 31808 in the United States.	11
Table 4.	Examples of field performance characteristics of BXN with Bt cotton events 31807 and 31808 compared to a commercial variety used as control.	12
Table 5.	Mean Comparisons of BXN with Bt strains for germination percentages.	13
Table 6a.	Nutritional fiber analysis of BXN with Bt and control cottonseed, expressed as a percentage of fuzzy seed by weight.	18
Table 6b.	Fatty acid composition of refined cottonseed oil from BXN with Bt cottonseed and control varieties.	19
Table 6c.	Proximate analysis of BXN with Bt cottonseed meal.	20
Table 6d.	Amino acid profile of BXN with Bt and control cottonseed meal.	21
Table 6e.	Levels of CPFA in BXN with Bt cotton seeds and seeds of control cotton varieties.	22
Table 6f.	Levels of total gossypol in BXN with Bt cotton seeds and seeds of control cotton varieties.	22

LIST OF FIGURES

	Page
Figure 1. Plasmid Map of Calgene construct pCGN4084.	4

Petition for Determination of Nonregulated Status for BXN® with Bt Cotton

This petition contains no confidential business information.

I. Introduction

BXN® with Bt cotton[1] provides a valuable tool for cotton growers and a significant benefit to the environment as part of a sustainable agricultural system. BXN with Bt cotton can be thought of as a second generation product, combining the benefits of its two predecessors, BXN cotton and Bollgard® cotton[2]. All of the potential environmental safety and plant pest issues relevant to the commercial production of BXN with Bt cotton have been addressed by USDA APHIS during the review of BXN cotton and Bollgard cotton. Results from field trials and additional evaluations demonstrate that BXN with Bt cotton poses no additional weed, plant pest or nontarget organism risks and therefore should not be a regulated article under 7 CFR part 340.

A. Statement of Request for Determination

Calgene requests that USDA APHIS, based on data and information presented in this document and based on previous determinations of nonregulated status for BXN cotton and Bollgard cotton, determine that BXN with Bt cotton lines derived from transformation events 31807 and 31808 do not present a plant pest risk, are not otherwise deleterious to the environment, and are therefore not regulated articles under 7 CFR part 340.

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[1] BXN is a registered trademark of Calgene, Inc.

[2] Throughout this document, the term "Bollgard cotton" will be used to designate Monsanto's cotton lines 531, 757, and 1076 which were removed from regulated status by USDA during 1995 (60 FR: 36096, July 13, 1995) and also the insect control protein as registered by EPA (EPA Reg. No. 524-478). Bollgard is a registered trademark of Monsanto Co.

B. Standard of Identity of BXN with Bt Cotton

BXN with Bt cotton is defined as any cotton cultivar or progeny of a cotton line containing

1) the BXN gene (Stalker et al., 1988a) with its associated 35S promoter (Gardner et al., 1981) and *tml* 3' terminator (Barker et al., 1983), and
2) the *cry*IA(c) gene (Hofte and Whitely, 1989; MacIntosh et al., 1990; Perlack et al., 1991) with its associated Mac promoter (*mas* and 35S promoter hybrid, Comai et al., 1990) and *mas* 3' terminator (Barker et al., 1983; Houck et al., 1990).

BXN with Bt cotton may also contain the *kan*^{r} gene (Beck et al,. 1982) with its associated 35S promoter and *tml* 3' terminator, 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) and the right T-DNA border (Barker, 1983). BXN with Bt cotton was produced using one of the *Agrobacterium*-derived binary vectors described in McBride and Summerfelt (1990).

The BXN gene was isolated from *Klebsiella pneumoniae* subsp. *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). The BXN gene contained in BXN with Bt cotton is identical to the BXN gene contained in BXN cotton. BXN cotton is not a regulated article under APHIS' regulation in 7 CFR part 340 (FR 59 [35]:8452, February 22, 1994).

The *cry*IA(c) gene was originally isolated from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 (Hofte and Whitely, 1989; MacIntosh et al., 1990; Perlack et al., 1991). The *cry*IA(c) gene in BXN with Bt cotton is a synthetic, truncated version of the full-length *cry*IA(c) gene contained in Bollgard cotton (Monsanto's cotton lines 531, 757 and 1076). After treatment with trypsin, the active CryIA(c) protein in BXN with Bt cotton is immunologically indistinguishable from the active CryIA(c) protein in Bollgard cotton, and they are the same size (574 amino acids). Bollgard cotton lines 531, 757 and 1076 are not regulated articles under APHIS' regulation in 7 CFR part 340 (FR 60 [1340]:36096, July 13, 1995).

II. Cotton: The recipient plant

BXN with Bt cotton was produced by transformation of the cotton species *Gossypium hirsutum* L. Cotton is not a weed pest in the United States, and the agency has previously determined that neither the bromoxynil tolerance nor insect resistance traits would cause the cotton crop to become weedy. All aspects of the biology, genetics and agronomy of the cotton crop relevant to this petition were previously submitted to the agency by Calgene in 1993 as part of the Petition for Determination of Nonregulated Status for BXN cotton (copy not attached). Conclusions of the agency regarding the cotton crop are found in the determination documents for BXN cotton and Bollgard cotton (Appendix 1).

III. Description of the genetic modification

A. The transformation and vector system

The transformation and vector system used to produce BXN with Bt cotton is identical to that used to produce BXN cotton, and thus has previously been reviewed by the agency. The following points briefly summarize the most critical aspects of the system.

1. Plant cells were transformed *in vitro* using disarmed (nonpathogenic) strains of *Agrobacterium tumefaciens.*
2. The vector system is binary, with the T-DNA insert on a separate plasmid from the *vir* genes required for gene transfer. The binary vectors are completely described in McBride and Summerfelt (1990).

B. Genetic constructs

Plasmid pCGN4084 was used to produce BXN with Bt cotton (Figure 1). The sequence of the inserted region of the construct is in Appendix 2.

C. Inserted genetic sequences and their sources

1. DNA sequences from nonregulated articles

BXN with Bt cotton, in addition to the cotton genome, contains added DNA sequences from several organisms which are not regulated articles (Table 1).

Table 1. DNA sequences from nonregulated articles.

DNA sequence	Source Organism	Literature reference
BXN gene	*Klebsiella pneumoniae*	Stalker and McBride, 1987, Stalker et al., 1988a
*cry*IA(c) gene	truncated gene based on active core sequence from *Bacillus thuringiensis var. kurstaki* HD-73	Hofte and Whitely, 1989; MacIntosh et al., 1990; Perlack et al., 1991
kan^r gene	*Escherichia coli* K12 containing Tn5	Beck et al., 1982
Lac Z' polylinker	*E. coli*	Yanisch-Perron, 1985
Tn5 transposon segment	Tn5	Auerswald, 1981; Beck et al., 1982

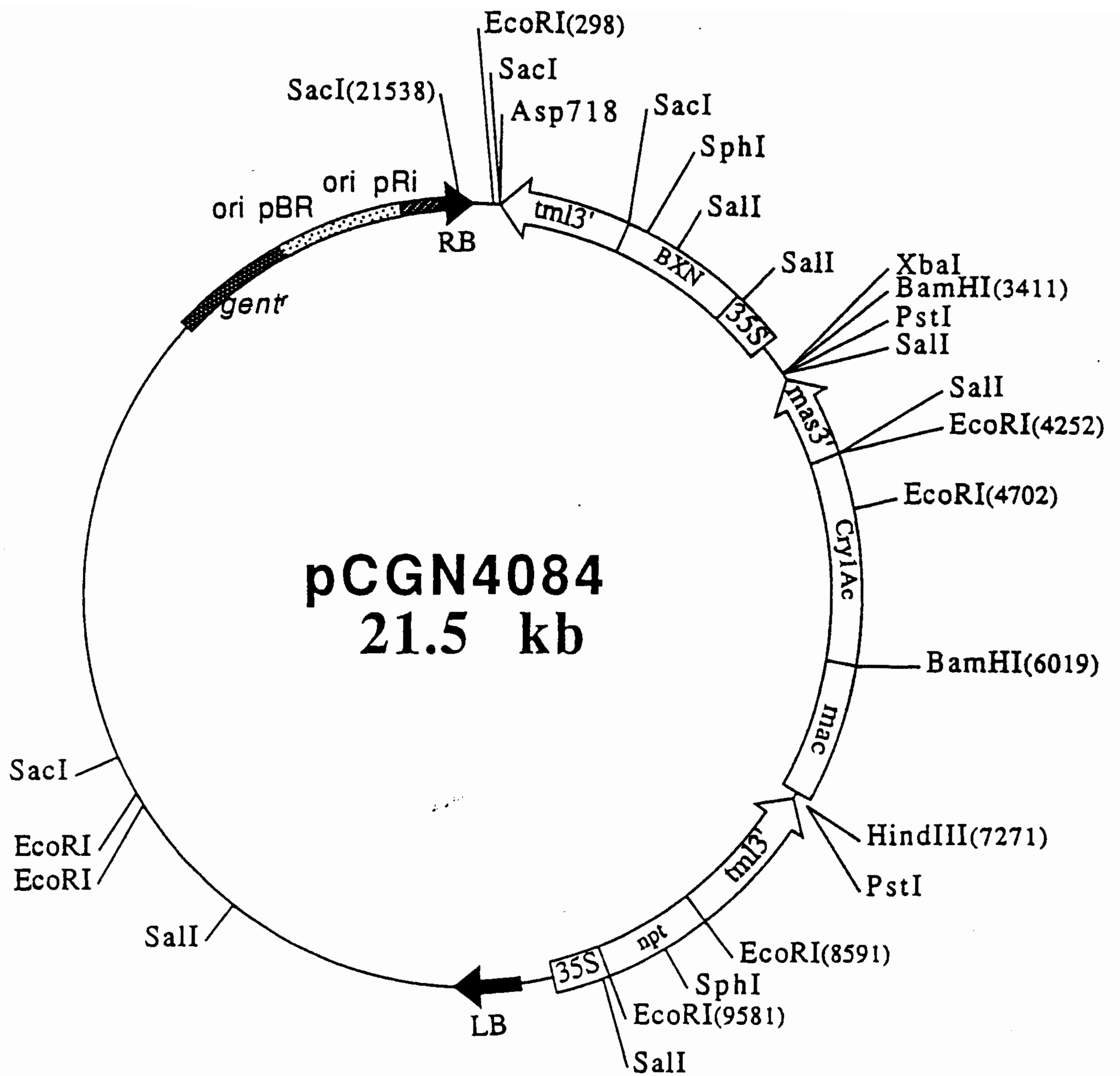

Figure 1. Plasmid Map of Calgene Construct pCGN4084

2. DNA sequences from regulated articles

BXN with Bt cotton contains added noncoding DNA sequences from two organisms which are considered regulated articles (Table 2a). Noncoding DNA sequences cannot confer plant pest characteristics upon the cotton plant by any known mechanism. These sequences function to regulate the extent of gene transfer from the *Agrobacterium* vector (T-DNA border regions) or to regulate gene expression in the cotton plant (all other sequences).

Table 2a. DNA sequences from regulated articles.

DNA sequence	Source Organism	Literature reference
Left and right T-DNA border	*Agrobacterium tumefaciens* pTiA6	Barker et al., 1983
tml 3'	*A. tumefaciens* pTiA6	Barker et al., 1983
35S promoter	Cauliflower mosaic virus (CaMV)	Gardner et al., 1981
mas 3'	*A. tumefaciens* pTiA6	Barker et al., 1983; Houck et al., 1990
Mac promoter (hybrid of *mas* 5' and 35S 5' regions)	*A. tumefaciens* and CaMV	Comai et al., 1990

With the exception of *mas* and Mac sequences, all of these DNA sequences are contained in BXN cotton, and thus have been reviewed previously by the agency and determined to not pose a plant pest risk specifically in cotton (USDA, 1994, App. 1).

The *mas* gene of *A. tumefaciens* pTiA6 codes for production of the enzyme mannopine synthase (Houck et al., 1990). The physical map of pTiA6 clearly demonstrates that the *mas* gene is separate from oncogenes (Houck et al., 1990). In addition, the production of mannopine cannot in and of itself cause crown gall tumors (DiRita and Gelvin, 1987). Finally, the coding region of the mannopine synthase gene is not part of the genetic construct in BXN with Bt cotton, which contains only noncoding, regulatory regions. Thus, there is no plant pest risk associated with the use of these noncoding *mas* sequences.

The Mac promoter is a hybrid of the *mas* and 35S promoter regions (Comai et al., 1990). In BXN with Bt cotton, Mac is used to drive the *cry*IA(c) gene. As described above, the 35S component of this promoter was previously evaluated by the agency and determined to not pose a plant pest risk. The *mas* component confers no plant pest risk, as discussed above. Thus, this noncoding DNA (Mac promoter) cannot confer plant pest characteristics on the cotton plant by any known or plausibly hypothesized mechanism.

D. Genetic characterization

1. Number of inserts in BXN with Bt cotton

BXN with Bt cotton lines derived from events 31807 and 31808 contain a single insert of the genetic construct, determined using Southern analysis. Full description of methods and results are in Appendix 2. Results from Southern analysis are consistent with segregation characteristics of these events indicating a single genetic locus (Appendix 2).

2. Lack of transfer beyond the T-DNA borders

DNA sequences from beyond the T-DNA borders were not detected in BXN with Bt cotton lines derived from events 31807 and 31808 using Southern analysis. A description of methods and results is in Appendix 2.

3. Genetic stability

Banding patterns in Southern blots were identical for generations T2 and T6 of transformation events 31807 and 31808. A description of methods and results is in Appendix 2. These DNA insertion events were inherited in Mendelian fashion demonstrated by resistance to Buctril® herbicide and were stable for a minimum of up to four generations of growth in the field. All evidence gathered to date indicate that BXN with Bt cotton lines derived from transformation events 31807 and 31808 are genetically stable.

E. Equivalence of CryIA(c) protein in Bollgard cotton and BXN with Bt cotton

BXN with Bt cotton contains a truncated, codon optimized version of the *cry*IA(c) gene. The *cry*IA (c) gene was originally isolated from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 (*B.t.k.*) (Hofte and Whitely, 1989; MacIntosh et al., 1990; Perlack et al., 1991). The gene product is a protoxin with very low toxicity to mammals and other nontarget organisms[3]. In the gut of the target pests (lepidopteran larvae) the protoxin is cleaved to its active form by trypsin. This trypsin resistant core fragment is the active, toxic form of the protein. The CryIA(c) protein in BXN with Bt cotton is composed of the trypsin resistant core, and little else (App. 3) All potential plant pest and nontarget organism concerns associated with this gene and its gene product have been adequately evaluated during the review of Bollgard cotton, since the active forms of the two proteins are equivalent in structure as well as identical in function and specificity. Monsanto's Bollgard cotton lines 531, 757 and 1076 are not regulated articles under APHIS' regulation in 7 CFR part 340 (FR 60 [1340]:36096, July 13, 1995). Data to support the equivalence of the CryIA(c) protein in BXN with Bt cotton and the CryIA(c) protein contained in Monsanto's Bollgard cotton lines 531, 757 and 1076 are summarized below. Thus, no additional issues pertinent to 7 CFR part 340 are posed by the presence of the *cry*IA (c) gene or the CryIA(c) protein in BXN with Bt cotton.

1. *cry*IA(c) gene sequences

The complete DNA sequence of the gene contained in BXN with Bt cotton is contained in Appendix 2. This sequence is virtually identical to that in Bollgard cotton in the internally conserved area (trypsin resistant core) of the gene which codes for the bioactive insecticidal protein. The gene in BXN with Bt cotton is 1770 nucleotides, which is approximately half the length of the native gene from *B.t.k.* (Table 2b). The version of the gene in BXN with Bt cotton has been truncated at both the N-terminus and C-terminus since the additional DNA in the native gene does not contribute to the insecticidal activity or host range specificity of the gene. The codon-optimized version of the gene in BXN with Bt cotton is synthetic (as it is also in Bollgard cotton), produced as described in Appendix 2.

2. Amino acid sequence of the bioactive proteins

The protein in BXN with Bt cotton contains amino acids 25 - 613 of the published (GenBank Accession No. M73248) amino acid sequence for CryIA(c) from *B.t.k.*, plus a methionine at the front of the protein (Appendix 3). After treatment with trypsin, the active CryIA(c) protein in BXN with Bt cotton and the active CryIA(c) protein in Monsanto's Bollgard cotton lines 531, 757 and 1076 differ by 6 amino acids out of a total of 590, and thus are 98.98% identical (Appendix 3). These 6 amino acids do not alter the pesticidal or other

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[3] Data and a literature review confirming the lack of toxicity of CryIA(c) protein to nontarget organisms was previously reviewed by the agency (USDA, 1995 in App. 1).

properties of the protein in any significant way (see below, Regulatory status at EPA). Further, the reported natural variation[4] of amino acid sequences for *Bacillus thuringiensis* within CryI crystal protein types is 0 to 7 amino acids in the toxin, and 0 to 24 amino acids in the protoxin (Hofte and Whiteley, 1989; Von Tersch et al., 1991). Thus, the active proteins in Bollgard cotton and BXN with Bt cotton are equivalent.

The amino acid sequence of trypsinized CryIA(c) in BXN with Bt cotton is identical to the native CryIA(c) from *B.t.k.*, and both differ from the CryIA(c) in Monsanto's Bollgard cotton by 6 amino acids (Table 2b and Appendix 3).

3. Protein size

The CryIA(c) protein produced in BXN with Bt cotton is approximately half the size of the full length CryIA(c) protein in Bollgard cotton (Table 2b). Due to the truncation of the DNA sequence as described above, the CryIA(c) protein in BXN with Bt cotton is shorter by 23 amino acids at the N-terminus, and shorter by 565 amino acids at the C-terminus. The deletions are located outside of the 2 trypsin sites in the protein (shown in Appendix 3).

After treatment of the plant extracts with trypsin, the active CryIA(c) protein in BXN with Bt cotton and the active CryIA(c) protein in Bollgard cotton are the identical size (64 kD, 590 amino acids).

4. Immunological characteristics of the proteins

Immunological assays, e.g. Western blots, are often used to detect, identify and quantify specific proteins. For example, in Monsanto's petition the CryIA(c) proteins in Bollgard cotton lines were shown to be equivalent to the CryIA(c) protein in *B.t.k.*, (e.g. Dipel) in a Western blot. Similarly, as a demonstration of equivalence of the active CryIA(c) protein in BXN with Bt cotton and in Bollgard cotton, we show that they are immunologically indistinguishable (Appendix 3). The trypsinized CryIA(c) proteins from both sources have an apparent molecular weight of 64 kD. As additional support for the assertion of equivalency, it is demonstrated in Appendix 3 that the active CryIA(c) protein in BXN with Bt cotton is also immunologically indistinguishable from the active CryIA(c) protein in Mycogen's MVP® formulation (a commercial, EPA registered preparation of bacterially produced *B.t.k.* CryIA(c) protein).

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[4] Variation in amino acid sequence of cryIVB in *B. t.* subspecies *israelensis* is much greater, up to 97 amino acids, but in the other crystal proteins examined, the amino acid sequences were much more uniform (Hofte and Whiteley, 1989; Von Tersch et al., 1991).

5. Activity against a target insect species

Equivalency of CryIA(c) activity in leaves of BXN with Bt cotton (example events) and in MVP® was determined using a *Heliothis virescens* bioassay (App. 8). Effects upon the target insect were identical with the two types of preparations, demonstrating dose-dependent equivalency of the CryIA(c) proteins from these two sources.

6. Regulatory status at EPA

EPA has determined that the CryIA(c) protein in BXN with Bt cotton and the CryIA(c) protein in Monsanto's Bollgard cotton lines 531, 757 and 1076 are the same active agent. The CryIA(c) protein in Calgene's BXN with Bt cotton lines are to be included in the same Bollgard registration as an alternate formulation for the same active agent (Appendix 6).

Table 2b. Characteristics of CryIA(c) gene sequences and proteins from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 (i.e. the native sequence) and in transgenic cotton plants.

	Source of CryIA(c)		
Parameter	**Native (*B.t.k.*)**	**Bollgard cotton**	**BXN with Bt cotton**
gene size	3534 bp	3534 bp	1770 bp
no. amino acids in full length protein	1178	1178	590
no. amino acids in trypsinized protein	574	574	574
deviations from native amino acid sequence in full length protein	not applicable	6 internal substitutions	0 internal subs. 23 N-term. del. 565 C-term. del.
deviations from native amino acid sequence in trypsinized protein	not applicable	6	0

bp = DNA base pairs
N-term., C-term. = the N-terminus and C-terminus of the protein, respectively
subs. = amino acid substitutions
del. = deletion

IV. Characteristics of BXN with Bt cotton plants

A. Field performance

BXN with Bt cotton plants have been field tested during the years 1994, 1995 and 1996 in a total of 11 states across the cotton belt (Table 3). Suitability for commercial introduction is dependent upon the plants demonstrating normal growth, development and yield characteristics, and producing fiber of good quality. BXN with Bt cotton lines in the breeding program all meet the minimum standards for commercial quality and demonstrate no novel characteristics other than those intentionally conferred by the genetic insertions (i.e. tolerance to bromoxynil and resistance to lepidopteran pests).

A sample of representative field data extracted from the field trial reports is provided in Table 4. Complete data are in the field trial reports (Appendix 4). The BXN with Bt plants (indicated as events 31807 and 31808) demonstrated normal growth, development and yield. Susceptibility to fungal and nematode pathogens was not altered in the transgenic cotton lines. There was no evidence of crown gall, hairy root or Cauliflower Mosaic Virus infection in any of the control or transgenic lines. Events 31807 and 31808 differed from controls in that 31807 and 31808 were tolerant to the herbicide bromoxynil, and they were resistant to damage by larvae of the target, lepidopteran insect pests *Heliothis virescens, Pectinophora gossypiella* and *Helicoverpa zea,* while the controls were susceptible. Susceptibility to and population levels of non-target insect pests such as cotton aphid and boll weevil were not altered in cotton events 31807 and 31808 (Appendix 4).

Thus, cotton events 31807 and 31808 displayed the intended, technical effect of the genetic transformation, and did not display unintended, or potentially deleterious effects. Significantly, cotton events 31807 and 31808 did not display any plant pest characteristics.

Table 3. Field trials with BXN with Bt cotton events 31807 and 31808 in the United States.

Year	Notification Number	Trial Type	States
1994	94-046-01N 94-074-01N	variety development/ efficacy	AZ, SC, MS
1995	95-039-06N 95-060-07N	nursery/variety/ efficacy	AR, AZ, GA, LA, MS, NC, SC, TN, TX
1996	96-051-11N	research/yield/ nursery	AL, AR, AZ, GA, LA, MS, NC, SC, TN, TX
1996	96-053-03N	research/yield	NC, TX
1996	96-089-01N	research	MS
1996	96-239-04N	yield	MO

Table 4. Examples of field performance characteristics of BXN with Bt cotton events 31807 and 31808 compared to a commercial variety used as control. Complete results are in the field trial reports (Appendix 4).

Evaluation Parameter	Control (eg. Coker 130)	Event 31807	Event 31808
% fruit damaged by *Helicoverpa zea* (percentages were transformed to square root of their arcsine)	12.87 a	1.88 b	0.61 b
Squares damaged by *Heliothis zea*	15.4 a	2.92 b	2.92 b
Crown gall incidence	0	0	0
Cauliflower Mosaic Virus infection	0	0	0
Susceptibility to *Phomopsis*, *Verticillium* and other normal fungal pathogens of cotton	within expected range	within expected range	within expected range
Levels of non-target insect pests, such as cotton aphid, tarnished plant bug, spider mite and boll weevil	within expected range	within expected range	within expected range
Bromoxynil tolerance	No	Yes	Yes
Seed germination	normal[1]	normal	normal
Plant morphology[2]	normal	normal	normal
Flowering period	normal	normal	normal
Yield	normal	normal	normal
Fiber quality	normal	normal	normal
Incidence of post-season volunteer cotton plants	0	0	0

[1] Normal growth parameters and pollination characteristics, including flowering period, for *Gossypium hirsutum* were discussed in detail in Calgene's petition for BXN cotton previously submitted to the agency (Calgene, 1993. Reference not attached).

[2] Numerical data on plant height, number of nodes, height/node ratio, node with first white flower etc are given in Table 8 of Field trial report dated 3/15/94 for Notification # 94-046-01N and in Table 1 of Field trial report dated 3/15/95 for Notification # 94-046-01N (Appendix 4).

B. Seed germination and dormancy

Germination studies from replicate samples of BXN with Bt cotton transformation events, including 31807, were performed to demonstrate that no significant changes in germination ability occurred as a result of transformation with the BXN with Bt constructs.

Seed harvested from all four replications of the 1995 Stoneville, MS yield trial was analyzed using the standard Stoneville Pedigreed Seed Co. germination protocol. Full description of methods and results are included in Appendix 5.

The germination percentages in this test demonstrate that no unusual rates of dormancy exist in the BXN with Bt strains in this study (Table 5). The differences seen between strains are considered normal variation for commercial germination testing. No increase in weediness potential due to seed dormancy characteristics would be expected in the BXN with Bt example strains.

Table 5. Mean Comparisons of BXN with Bt strains for germination percentages.

Event or Strain	N	Warm Germination Percentage		Cool Germination Percentage	
31707	4	99	a	92	a
31803	4	98	ab	86	b
31807A	3	97	ab	87	ab
31807C	4	97	ab	89	ab
Coker130	4	97	ab	92	a
ST474	4	95	b	88	ab
LSD(0.05)		2		5	
CV(%)		1.6		3.5	

C. Lack of overwintering potential

Results from field trials indicate that BXN with Bt seed have essentially no overwintering potential in the cotton belt. The seed demonstrate no significant level of dormancy (see above). No cotton volunteers have been detected at field trial sites following any trials with BXN with Bt cotton (App. 4).

D. Lack of plant pest characteristics

USDA APHIS has previously determined that neither BXN cotton nor Bollgard cotton present a plant pest risk (Appendix 1). There is no evidence or reason to suspect that combining the phenotypic traits in these two types of cotton varieties would pose a plant pest risk, since the parental types do not pose such a risk. Indeed, if Calgene had developed BXN with Bt cotton by cross breeding BXN cotton with Bollgard cotton, rather than by transformation, the resulting genetic lines would not be regulated articles.

We have demonstrated in the data shown above, as well as in data contained in the Appendices, that BXN with Bt cotton does not display any plant pest characteristics, nor is it weedy. Similarly, BXN with Bt cotton is not infected with either of the plant pest organisms which contributed noncoding DNA to the inserted genetic construct (i.e. *Agrobacterium tumefaciens* and Cauliflower Mosaic Virus). Following this line of reasoning, there is no reasonable mechanism whereby BXN with Bt cotton could transfer plant pest, weed or pathogenic characteristics to any plant with which it might interbreed. Finally, given that BXN with Bt cotton has normal yield and fiber characteristics, it has no reasonable potential to harm any agricultural commodities.

V. Potential for Environmental Impact by BXN with Bt Cotton

A. Herbicide tolerance trait

Tolerance of the cotton crop to the herbicide bromoxynil via the mechanism of production of the enzyme nitrilase has been determined previously to not pose an environmental, nontarget organism or plant pest risk (see FR 59 [35]:8452, February 22, 1994, and text of determination for BXN cotton in Appendix 1).

B. Insect resistance trait

Resistance of the cotton crop to lepidopteran insect larvae via the mechanism of production of the *Bacillus thuringiensis* protein toxin CryIA(c) has been determined previously to not pose an environmental, nontarget organism or plant pest risk (see FR 69 [134]:36096, July 13, 1995, and text of determination for Bollgard cotton in Appendix 1). Since the CryIA(c) protein in BXN with Bt cotton is equivalent to the active CryIA(c) protein in Bollgard cotton, no hazards attributable to the insect resistance trait are posed by BXN with Bt cotton.

Nontarget organism effects

Potential for a negative impact on nontarget organisms, particularly beneficial organisms such as honey bees and earthworms, by cotton plants producing the CryIA(c) protein was adequately addressed during review of Bollgard cotton (see FR 69 [134]:36096, July 13, 1995, and text of determination for Bollgard cotton in Appendix 1). The basis for lack of nontarget organism effects is two-fold:

1. CryIA(c) is host specific. Since the amino acid sequence of the active protein has not been altered, there will be no change in host range of the protein. The sequence of the active portion of CryIA(c) in BXN with Bt cotton is identical to the active portion of the native, bacterial protein from *Bacillus thuringiensis* subsp. *kurstaki* (which is present in formulations registered as pesticides by the Environmental Protection Agency) and differs by only 6 amino acids from the active protein portion of that in Bollgard cotton, and thus will have the identical host range and activity.

2. A receptor for the CryIA(c) protein has never been identified in beneficial invertebrate organisms, such as earthworms, or in any vertebrate organisms. Thus, there is no known mechanism by which the CryIA(c) protein could cause adverse effects in earthworms or nontarget insects, fish or mammals.

There is no apparent link between size of the protoxin (i.e. protein before treatment with trypsin) and toxicity to nontarget organisms, or to host specificity. Although the native full-length CryIA(c) contains 1178 amino acids, the CryIIB protein from the same species (*B.t.k.*) has only 633 amino acids and retains specificity to lepidopteran larvae (Hofte and Whiteley, 1989) and lack of nontarget effects. The commercial insecticide MVP® contains several lengths of proteins which are antigenic to the CryIA(c) antibody, one of which is only slightly larger than the protein in BXN with Bt cotton (Appendix 3). MVP contains the *cry*IA(c) gene and no other genetic material which codes for an insecticidal protein, yet produces several sizes of CryIA(c) proteins, demonstrating once again the natural variation inherent in these proteins, and the lack of significance *per se*, of the size of the protein outside of the trypsin-resistant core fragment. Thus, there is no reason to suspect that the truncated CryIA(c) protein in BXN with Bt cotton would be less safe to nontarget organisms than the full length protein in Bollgard cotton.

Safety of the CryIA(c) protein in BXN with Bt cotton for beneficial and other nontarget organisms has also been demonstrated in the following ways:

1. There is no detectable CryIA(c) protein in pollen or nectar of BXN with Bt cotton events 31807 and 31808 (Appendix 7). Thus there is no mechanism for exposure of honeybees to the protein. The level of detection for the Western blot assay (2 ppm) is below the level of CryIA(c) necessary for deleterious effect upon a sensitive insect (10 ppm). Thus, even if CryIA(c) protein were present in pollen and nectar at some level which could not be detected, that level would be insufficient to produce a deleterious effect.

2. Leaf tissue of BXN with Bt cotton (example event 31707) demonstrated no toxicity to earthworms during a 14-day feeding study conducted by Springborn Laboratories (Appendix 8). Similarly, BXN with Bt cotton (example event 31707) demonstrated no toxicity to *Colembola* (Appendix 8). Since the CryIA(c) protein in the example event is identical to that in events 31807 and 31808, we conclude that BXN with Bt cotton has no toxicity to either of the nontarget organism species tested.

Regulatory status at EPA

As discussed above, EPA has agreed that Calgene's CryIA(c) protein as expressed in BXN with Bt cotton represents an alternate formulation of Monsanto's EPA registered CryIA(c) protein as contained in Bollgard cotton. Thus, EPA has not required an independent environmental review of CryIA(c) protein in BXN with Bt cotton before adding it to the existing Monsanto registration. BXN with Bt cotton will be regulated by EPA under Monsanto's existing registration, including the Bt resistance management program (see Appendix 6).

C. Lack of effect upon raw or processed agricultural commodities: Compositional Analysis of BXN with Bt Cotton

BXN with Bt cotton will not have any adverse effects upon agricultural commodities since it is compositionally equivalent to other cotton varieties.

Materials & Methods

Cottonseed was harvested from yield trials planted in Washington County, MS and consisted of 5 lb. lots of fuzzy seed from five BXN with Bt example events and four commercial varieties (Coker 130, Stv. 474, Stv. LA887, and DPL 50). Seed was processed into fractions at Texas A&M University. Oil fractions were retained at Calgene, Inc. for fatty acid analysis.

Toasted cottonseed meal samples as well as whole unginned seed were sent to Woodsen-Tenent Laboratories (Little Rock, AR). Whole seed was analyzed for crude fiber, acid detergent fiber and neutral detergent fiber. Cottonseed meal was analyzed for moisture, protein, ash, fat and amino acid profile.

Analysis for cyclopropyl fatty acids (CPFA) were conducted at Corning-Hazelton Labs in Madison, WI using High Performance Liquid Chromatography. In previous studies, it has been observed that the levels of CPFA are highly variable differing significantly even in replications from the same field. The analysis was therefore conducted on replicated samples of T_5 and T_6 seed from the listed events of BXN with Bt cotton and controls. Results were analyzed by the JMP Statistical Program.

Total gossypol was analyzed at Woodsen-Tenant Laboratories by Spectrophotometric assay. Samples were taken from the same lots used for the CPFA analysis but the samples were not replicated. It has been observed that environmental conditions have much less effect on variability of gossypol levels than they do on CPFA levels. Variability in gossypol is more due to varietal differences and differences in processing of seed. This seed was not processed so the only variability present would presumably be varietal.

Results

Tables 6a - 6f show the results of the compositional analysis of Calgene BXN with Bt cotton seed, refined oil, and cottonseed meal. The data provided demonstrate that Calgene BXN with Bt cottonseed does not differ significantly from commercially available cottonseed varieties in these important components.

Table 6a. Nutritional fiber analysis of BXN with Bt and control cottonseed, expressed as a percentage of fuzzy seed by weight.

Event	Crude Fiber	ADF	NDF
31707	30.2	39.7	49.4
31803	31.8	36.8	45.7
31807	32.1	42.1	48.8
31808	31.4	40.4	47.5
42317	31.4	41.8	48.5
Coker 130	31.8	38.1	46.3
Stv. 474	30.4	40.4	47.6
St. LA887	32.5	42.4	49.0
DPL 51	33.8	41.4	48.6

Table 6b. Fatty acid composition of refined cottonseed oil from BXN with Bt cottonseed and control varieties. Each fatty acid is expressed as the percentage of total fatty acids by weight.

SAMPLE	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
31707	0	0	0	1.17	25.84	0.62	3.28	16.80	51.66	0.31
31803	0	0	0	0.98	26.97	0.56	3.72	15.22	52.21	0.30
31807	0	0	0	1.04	25.72	0.58	3.43	17.01	51.55	0.41
31808	0	0	0	1.04	26.39	0.58	3.39	16.09	51.83	0.35
42317	0	0	0	0.94	25.39	0.60	3.34	15.98	53.41	0.04
Coker 130	0	0	0	1.04	26.03	0.59	3.41	16.19	52.36	0.04
Stv. 474	0	0	0	1.11	26.76	0.70	3.40	17.27	50.41	0.05
Stv.LA887	0	0	0	0.77	23.40	0.80	3.29	18.91	52.22	0.35
DLP 51	0	0	0	1.18	26.05	0.67	3.17	16.71	51.90	0.05

Sample	20:0	20:1	20:2	22:0	22:1	22:2	24:0	24:1	Total saturates
31707	0.32	0	0	0	0	0	0	0	30.61
31803	0.04	0	0	0	0	0	0	0	31.71
31807	0.26	0	0	0	0	0	0	0	30.45
31808	0.34	0	0	0	0	0	0	0	31.15
42317	0.29	0	0	0	0	0	0	0	29.97
Coker 130	0.32	0	0	0	0	0	0	0	30.81
Stv.474	0.30	0	0	0	0	0	0	0	31.57
Stv.LA887	0.27	0	0	0	0	0	0	0	27.72
DLP 51	0.27	0	0	0	0	0	0	0	30.67

Table 6c. Proximate analysis of BXN with Bt cottonseed meal. Values are the percent (w/w) of meal.

Event	Moisture	Crude Fat/Oil	Protein-Kjeldahl	Ash
31707	2.98	2.53	49.52	6.82
31803	2.00	2.42	49.41	6.36
31807	1.69	2.14	53.31	7.16
31808	2.38	2.27	51.02	6.55
42317	1.69	1.73	49.17	6.53
Coker 130	3.14	2.39	53.10	7.14
Stv. 474	2.34	2.13	44.92	6.18
Stv. LA 887	3.05	2.45	46.12	6.44
DPL 51	2.74	3.01	45.54	6.92

Table 6d. Amino acid profile of BXN with Bt and control cottonseed meal. Values are the percent (w/w) of meal.

	31707	31803	31807	31808	42317	Coker 130	Stv. 474	Stv. LA 887	DPL 51
Tryptophan	0.61	0.59	0.60	0.58	0.55	0.61	0.49	0.49	0.49
Aspartate	4.42	4.24	4.63	3.95	4.46	4.43	3.90	3.91	3.69
Threonine	1.61	1.54	1.66	1.42	1.60	1.58	1.50	1.49	1.33
Serine	2.13	2.08	2.25	1.95	2.13	2.12	2.03	2.04	1.79
Glutamic Acid	9.90	9.60	10.48	8.80	9.88	9.80	8.78	8.51	8.29
Proline	1.91	2.13	1.82	1.68	1.81	1.74	1.76	1.63	1.66
Glycine	2.01	1.96	2.12	1.81	2.01	2.04	1.83	1.84	1.70
Alanine	1.88	1.88	2.01	1.73	1.82	1.93	1.71	1.74	1.59
Cystine	0.67	0.66	0.65	0.66	0.61	0.72	0.50	0.59	0.51
Valine	2.08	2.04	2.20	1.88	2.06	2.16	1.81	1.85	1.78
Methionine	0.79	0.76	0.83	0.67	0.68	0.79	0.62	0.68	0.60
Isoleucine	1.46	1.42	1.49	1.30	1.49	1.51	1.23	1.32	1.24
Leucine	2.89	2.80	3.07	2.58	2.92	2.93	2.51	2.58	2.51
Tyrosine	1.20	1.20	1.05	1.05	1.11	1.00	0.95	1.09	0.99
Phenyl-alanine	2.62	2.62	2.79	2.43	2.58	2.61	2.28	2.37	2.23
Histidine	1.53	1.55	1.74	1.47	1.56	1.55	1.45	1.45	1.36
Lysine	2.07	1.96	1.78	1.83	1.98	2.07	1.72	1.88	1.79
Arginine	5.57	5.63	5.76	5.31	5.52	5.66	4.52	4.48	4.64

Table 6e. Levels of CPFA in BXN with Bt cotton seeds and seeds of control cotton varieties as a percent of total fatty acids.

Event	N	Malvalic		Sterculic	Total CPFA
31707	4	0.36	a	0.28	0.64
42317	4	0.40	ab	0.31	0.71
31803	4	0.41	ab	0.30	0.71
Coker130	4	0.41	ab	0.33	0.74
STV474	4	0.41	ab	0.30	0.71
DPL51	4	0.42	ab	0.25	0.67
31808	4	0.43	bc	0.50	0.93
31807	4	0.49	cd	0.50	0.99
STVLA887	4	0.51	d	0.34	0.85
CV		7.60%		82.30%	
LSD		0.06		NS	

Table 6f. Levels of total gossypol in BXN with Bt cotton seeds and seeds of control cotton varieties as a percentage of seed weight.

Event or variety	Total Gossypol
31707	0.84
31803	0.98
31807	0.78
31808	0.88
42317	0.92
Coker 130	0.97
St. LA887	1.20
St. 474	1.22
DPL 51	1.01

VI. Statement of Grounds Unfavorable

No data have been produced to date which reflect negatively on this petition.

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Addendum to Calgene Report Number BT009

Additional figures have been added as further characterization of events 31807 and 31808. No other events shone in these figures are in the current breeding program nor are there any plans to commercialize any of these other events.

A typographical error occurred in the figures depicting the insertions in events 31807 and 31808 (Figures 2 and 3 of BT009). Replacement pages are included here. The terminator for the NPTII gene should be listed as tml3' not *mas3'*. The circular map shown in Figure 4 is correct.

The following figures show that the genes contained on plasmid pCGN4084 have been inserted and maintained in the Bxn with Bt plants for up to five generations. Figure 1 shows the Bxn gene in events 31807 and 31808.

Figure 2 shows the same blot probed with the kan DNA fragment as a probe.

Figure 3 shows a blot of events 31807 and 31808 (along with others) hybridized with the 1532 probe confirming that no plasmid DNA sequences beyond the left transfer border occurred in either of these events.

These events were not hybridized specifically with a probe for *cryIAc*. As explained in the report BT009, when genomic DNA of a plant transformed with the pCGN4084 construct is digested with EcoRI and hybridized with the mac promoter fragment, bands of 3.9 and 3.6 kb are predicted if the 4084 construct in inserted into the host DNA intact. The front half of the *cryIAc* gene is present on the 3.6 kb EcoRI fragment and the Bxn gene is present within the 3.9 kb EcoRI fragment. Both the 31807 and 31808 events exhibited 3.9 and 3.6 kb EcoRI fragments thus confirming the presence and intactness of the Bxn and *cryIAc* genes. Western blots for both Bxn and *cry!Ac* gene products resulted in the expected size polypeptides in multiple tissues of events 31807 and 31808 confirming that both genes are intact and are expressed in the plant. The final argument for the presence and intactness of both these genes is data from the field showing resistance to Buctril herbicide and efficacy against Lepidopteran pests.

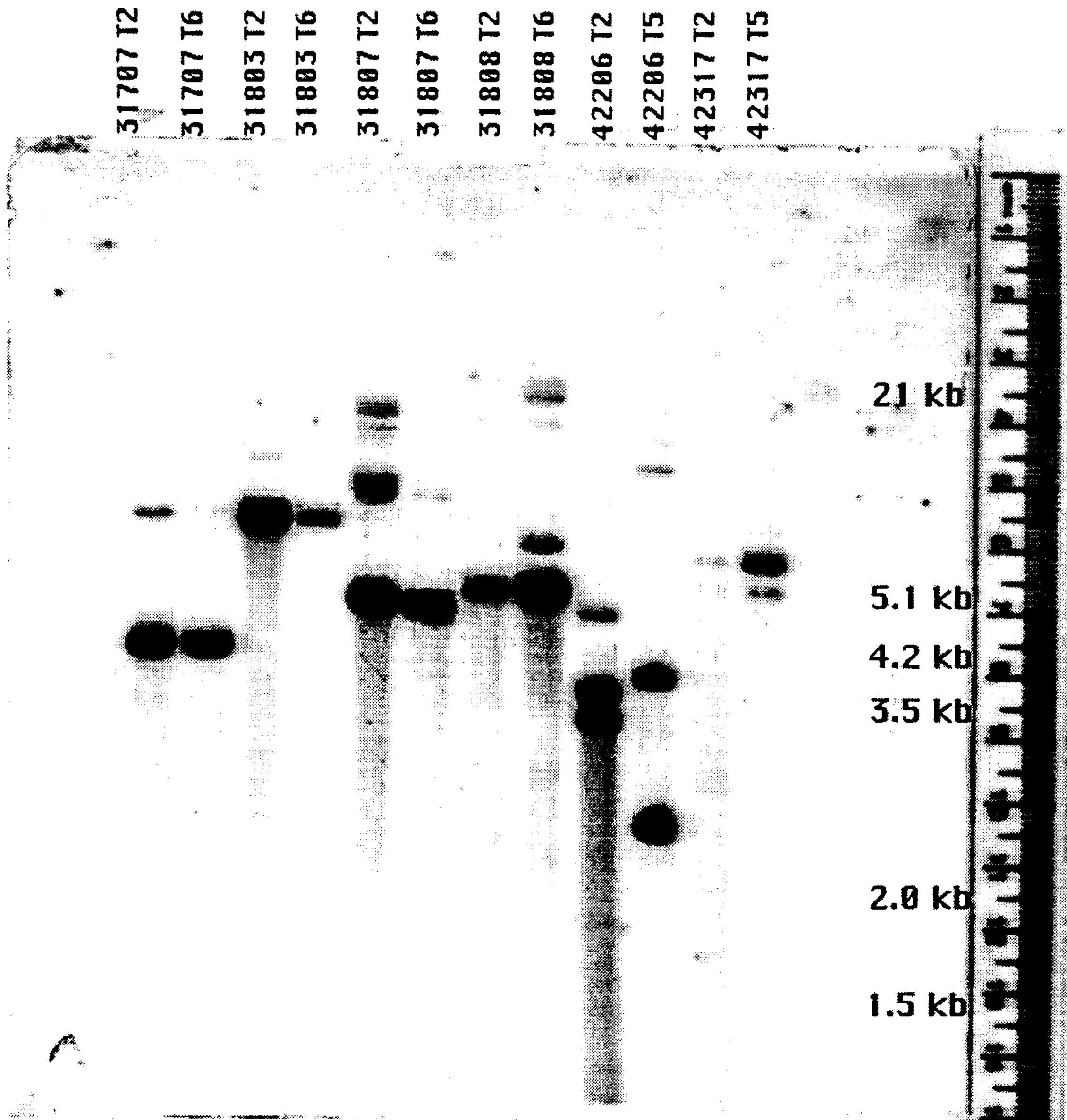

Figure 1. Genomic DNA from example events digested with BamHI/HindIII and probed with the Bxn probe. For events 31807 and 31808 a border fragment of greater than 3.1 kb containing the Bxn gene was predicted. Event 31807 has a band of about 5.1 kb and event 31808 has a band of about 5.3 kb. The extra band seen in the 31807 T2 lane is residual radiation from a previous hybridization.

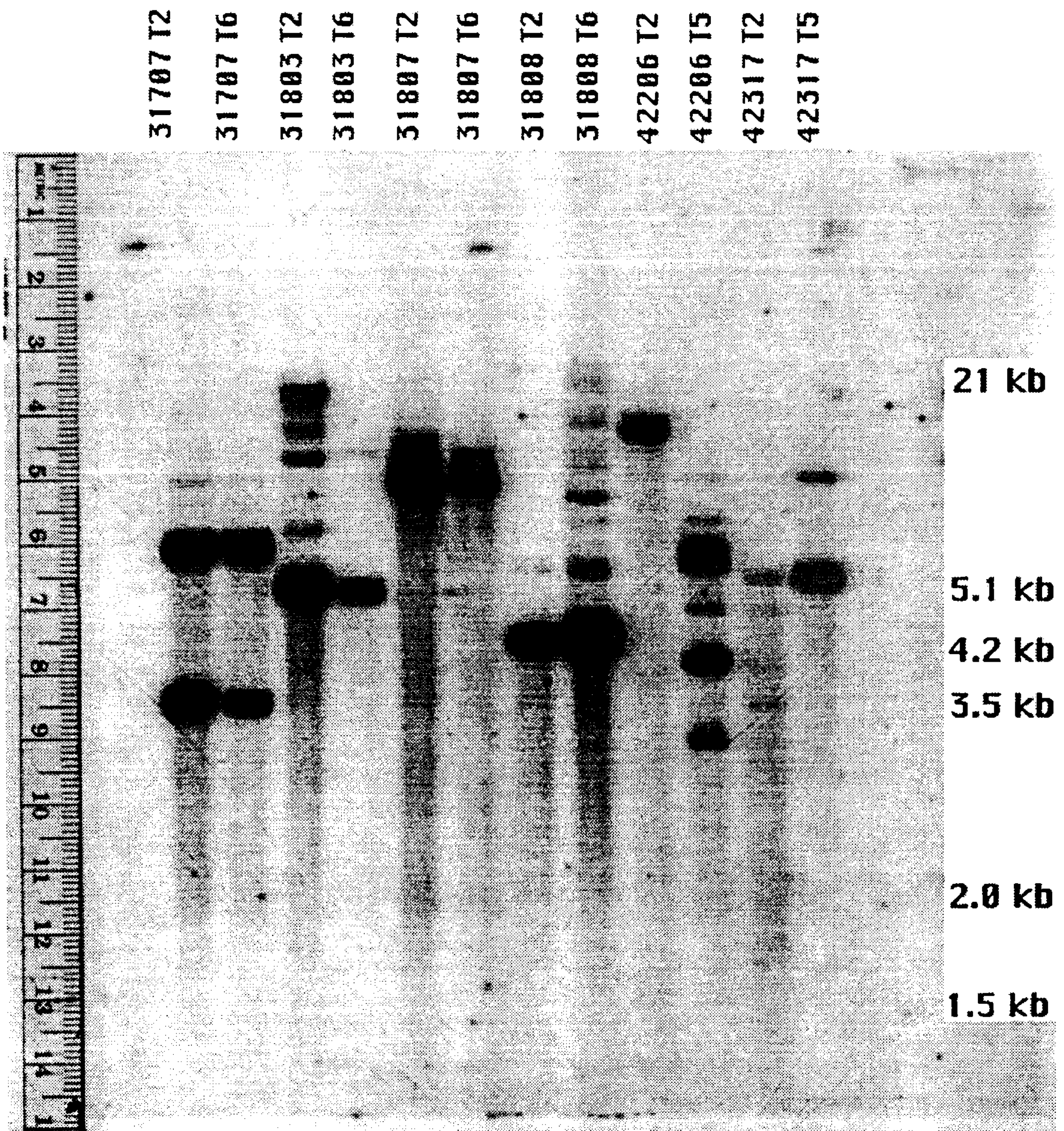

Figure 2 Genomic DNA from example events digested with BamHI/HindIII and probed with the Kan probe. For events 31807 and 31808 a border fragment of greater than 2.8 kb containing the kan^r gene was predicted. Event 31807 has a band of about 10 kb and event 31808 has a band of about 4.3 kb. The extra bands seen in the 31808 T6 lane are residual radiation from a previous hybridization.

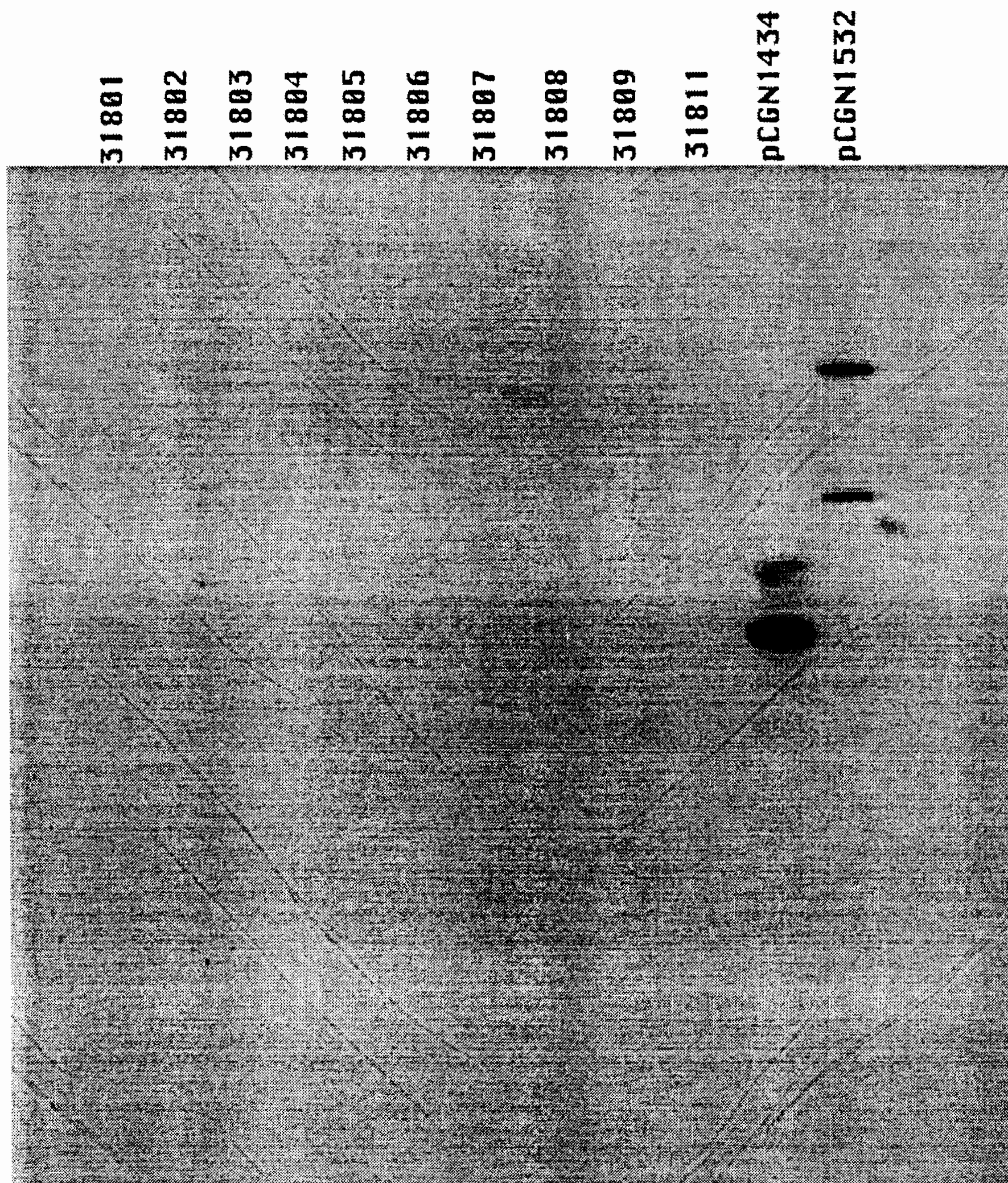

Figure 3 Genomic DNA from example events digested with EcoRI and probed with the 1532 probe. No hybridization was expected. pCGN1434 is the plasmid backbone minus the *gent*^{r} gene. pCGN1532 is the plasmid backbone including the *gent*^{r} gene.

CALGENE

Appendix 2

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Study Title

Genomic Southerns of Calgene BXN with Bt Transgenic Cotton Plants

Author

Eric Aasen

Study Completed

20 February 1996

Performing Laboratory

Calgene, Inc.
1920 Fifth Street
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Laboratory Project ID

BT009

Study Number: **BT009**

Title: Genomic Southerns of Calgene BXN with Bt Transgenic Cotton Plants

Facility: Calgene, Inc., Davis, California

Study Director: Dave Stalker

Principal Investigator: Eric Aasen

Study Initiation Date: 3 January 1994

Experimental Termination Date: 20 February 1996

Records Retention: All study specific raw data, protocols, final reports and facility records will be retained at Calgene, Davis, California.

Signatures of Approval

____ Date:
Dave Stalker, Study Director

____ Date:
Eric Aasen, Principal Investigator

____ Date:
Lori Malyj, Sponser

Table of Contents

Statement Of No Data Confidentiality Claims 2

Statement Of Compliance 3

Signatures Of Approval 4

Table of Contents 5

Definitions 6

Objectives 7

Materials & Methods 7
- Southern Analsysis of BXN with Bt Cotton DNA 7
- Probes and Restriction Digestions for Analysis of BXN with Bt Cotton 8

Results 9

Conclusions 11
- Figure 1 Southern blot analysis of 6 example events 12
- Figure 2 Schematic representation of event 31807. 13
- Figure 3 Schematic representation of event 31808 14
- Figure 4 Plasmid map of Calgene construct pCGN4084 15

Appendix A Isolation of Genomic DNA from Cotton Leaf Tissue 16

Definitions

bp	Base pairs
C	Centrigrade
cpm	Counts per minute
cry1Ac	Class I (Lepidoptera-specific) crystal protein gene
kb	Kilobase pairs
gent^r	Confers resistance to gentamicin
kan^r	Confers resistance to kanamycin
µg	Microgram
µm	Micrometer
ml	Milliliter
mg	Milligram
PCR	Polymerase Chain Reaction
SOP #CGN003	Isolation of Genomic DNA from Cotton Leaf Tissue
T_1 plants	Original transformant
T_2 progeny	Plants grown from seeds harvested off a T_1 plant
tDNA	Transfer DNA from *Agrobacterim tumefaciens*
uv	Ultraviolet
volt-hrs	Volt hours

Genomic Southerns of Calgene BXN with Bt Transgenic Cotton Plants

Objectives

Analysis of genomic DNA from BXN with Bt cotton plants transformed with Calgene constructs pCGN4083 and pCGN4084 was performed to meet the following objectives.

1. Determine the number and structure of inserts for examples lines with potential commercial value.
2. Analyze the lines for integration of DNA from outside the tDNA borders, ie. plasmid DNA.
3. Assess the stabilty of the inserted DNA by analysis of subsequent generations from the same transformation event.

Materials & Methods

A.Southern Analsysis of BXN with Bt Cotton DNA

Cotton genomic DNA was isolated according to Protocol #CGN003 from non-transformed control *Gossypium hirsutum* (var. Coker 130) plants and pooled leaf tissue from T_2 progeny of T_1 BXN with Bt cotton plants which had been transformed with Calgene constructs pCGN4083 and pCGN4084. The DNA samples (10 μg) were digested to completion with restriction endonucleases according to instructions supplied by the vendor (Boehringer Mannheim Biochemicals, Indianapolis, IN). One sixth volume of loading buffer (0.25% bromophenol blue, 40% sucrose in H_20) was added to each sample before loading onto 0.8% agarose gels. The gels were electrophoresed for approximately 375-400 volt-hrs, photographed, and prepared for transfer to 0.45 μm nylon membranes (Nytran+, Schleicher & Schuell). Preparations for transfer consisted of gentle shaking in a denaturing solution (Maniatis 1982) twice for 15 minutes each time and in neutralizing solution (Maniatis 1982) twice for 15 minutes each. DNA in the gels was then transferred to membranes overnight by capillary action using 10 X SSC (Maniatis 1982). Following transfer, the nylon membranes were crosslinked by UV (Stratagene, Inc.) for 30 seconds and pre-hybridized for 2-3 hours at 42°C in 25 ml of a solution containing 50% formamide, 6X SSPE, 5X Denhardt's solution (Maniatis 1982), 1% SDS, and 100 μg/ml denatured salmon sperm DNA.

The membranes were hybridized overnight in solutions identical to those described for pre-hybridizations, with the exception that the hybridization solutions also contained a denatured hybridization probe which had been radiolabeled with ^{32}P to a specific activity of 10^8 cpm/mg by the random primer method (Prime-It II, Stratagene, Inc.). After hybridization, the filters were washed in a solution of 2X SSC, 0.5% SDS at 55°C for 10 minutes followed by washing in 0.5X SSC, 0.5% SDS at 55°C for 20-30 minutes. The membranes were then wrapped in plastic wrap and exposed to Amersham HyperFilm using an intensifying screen at - 70°C in a light-proof cassette. Exposure time was generally 1-4 days. Membranes were stripped of radiolabeled probe by washing for 4 hours at 65°C in 250 ml of a solution containing 1.25 ml 1 M Tris.Cl pH 8.0, 0.5 ml 50X Denhardt's solution (Maniatis 1982), 0.2 ml 0.25 M EDTA and 0.125 g sodium pyrophosphate in 1 liter of water. The membranes were then ready for rehybridization with a new probe. The hybridization probes and restriction enzymes used for analysis of BXN with Bt cotton are described below.

Probes and Restriction Digestions for Analysis of BXN with Bt Cotton

The DNA probes used to analyze BXN with Bt cotton for number and structure of inserts were the *mac* probe, a 1.2 kb promoter fragment isolated by a BamHI/XhoI digest of pCGN1434 and the 35S probe, a 678 bp fragment isolated from CaMV 35S RNA digested with PstI/XbaI. The kan probe was a 1.0 kb fragment isolated by EcoRI digestion of Calgene plasmid pCGN552. The bxn probe was a 300 bp PCR fragment obtained by PCR from Calgene plasmid pBrx74 with primers 2991 and 2983. The probe used to analyze the genomic DNA for integration of DNA from outside the tDNA borders was the entire Calgene plasmid pCGN1532 digested with BamHI and EcoRI. This plasmid consists of all DNA located outside the tDNA borders of Calgene constructs pCGN4083 and 4084 including the gentamicin resistance gene ($gent^r$).

When the genomic DNA from events transformed with pCGN4083 was digested with EcoRI and probed with the *mac* probe, fragments of 3.9, 3.2, and a border fragment of greater than 0.7 kb were expected for a single insert event. When the same probe and digest were used to analyze events transformed with pCGN4084, fragments of 3.9, 3.6, and a border of greater than 0.7 kb were expected. In practice, the 3.9 and 3.6 kb bands were not resolved by electrophoresis. The BXN gene is contained on the 3.9 kb fragment.

The *cryIAc* gene is contained on the 3.2/3.6 kb fragments. The 35S probe was used to analyze the inserts for stability after growth in the field for up to five generations. When the genomic DNA from events transformed with pCGN4083 was digested with BamHI and HindIII together and hybridized with the 35S probe, a border fragment of greater than 3.1 kb containing the BXN gene and an internal fragment of 1.2 kb were predicted, with the bxn probe only the border fragment would be seen. When probed with the kan probe a border of greater than 2.8 kb was predicted. When the genomic DNA from events transformed with pCGN4084 was digested with BamHI and HindIII together and probed with the 35S probe, a border fragment of greater than 3.1 containing the Bxn gene, an internal fragment of 1.2 kb, and a border fragment of greater than 2.8 kb were predicted. When probed with the bxn probe a border of greater than 3.1 kb should be seen. When probed with the kan probe, 4084 events should have a border of greater than 2.8 kb. Comparison of border fragment sizes from T_2 and later generations confirms stability of the inserted sequences. To analyze the transgenic plants for integration of DNA from outside the tDNA borders, the genomic DNA was digested with EcoRI and probed with the 1532 probe. This probe comprises all the DNA outside the tDNA borders as well as the *gent^r^* gene and can confirm the presence or absence of this gene as well as any beyond the border transfer of DNA.

Results

Five example events were chosen for analysis based on their potential for commercialization. One of the events (31707) had been transformed with Calgene construct pCGN4083. Four of the events (31803, 31807, 31808, and 42317) were transformed with Calgene construct pCGN4084. When digested with EcoRI and probed with the *mac* probe event, 31707 had two bands of the correct size (3.9 and 3.2 kb) along with a border fragment of approximately 4.5 kb. In addition there were several much lighter bands seen in this event. When digested with BamHI/HindIII and probed with the 35S probe this event had the expected pattern with one band of approximately 5 kb and the internal fragment of 1.2 kb. When probed with the bxn probe the same 5.0 kb border was seen. When probed with the kan probe two borders of approximately 4.0 and 6.5 kb were seen indicating a partially inserted second copy of the construct containing the *kan^r^* gene.

A possible explanation for the light bands seen with the EcoRI digest is provided by the description of this restiction enzyme (NEB 1995 catalog pg. 211). Under non-standard conditions some enzymes including EcoRI recognize altered target sites for cleavage (star activity). These sites are much less specific than the original six base pair site and are therefore more frequent. More frequent sites would lead to more frequent cutting and more hybridizing bands on the gel. Conditions which contribute to star activity include large amounts of enzyme and long digestions, both of which were commonly used in digestion of BXN with Bt cotton genomic DNA ie. greater than 50 units of enzyme and digestion overnight. Probing DNA from the T_6 generation of this event with the 35S probe revealed the same pattern as the original DNA. No hybridization was seen with the 1532 probe. The events transformed with pCGN4084 (31803, 31807, 31808, 42317) all had predicted banding patterns when digested with EcoRI and probed with the *mac* probe and when digested with Bam/Hind and probed with the 35S, bxn, and kan probes. Event 42317 was shown to be a double insert at a single locus by all the probes. All progeny of these events had the same patterns as the original DNA. Figure 1 shows the Southern blot analysis of the T_2 DNA from six example BXN with Bt events digested with Bam/Hind compared to digested DNA from the same events of a later generation. It is quite clear that for events 31707, 31803, 31807, and 31808 there has been no change in the sizes of border fragments over several generations. In event 42206 it appears that a seed mix-up has occured as the border fragments from the later generation no longer match those of the T_2 generation. The T_2 lane of event 42317 is very light due to low DNA concentration, however the bands are the same. No hybridization with the 1532 probe was seen with any of these events. Events 31707, 31803, 42206, and 42317 have been dropped from the breeding program and will not be comercialized. Events 31807 and 31808 remain in the breeding program. Figure 2 is a schematic of the tDNA insertion of event 31807. Figure 3 is a schematic of the tDNA insertion of event 31808. See the figure captions for full explantations. Figure 4 is a plasmid map of Calgene construct pCGN4084 used to produce Calgene BXN with Bt cotton.

Conclusions

Southern analysis is an important and accurate tool for studying transgenic organisms. Information which can be obtained by Southern analysis includes, the structure and number of integrated DNA sequences, the presence or absence of genes of interest, and the stability of the DNA over many generations after the integration event. Using Southern analysis as described, the structure of the inserts for five example events transformed with pCGN4083 and pCGN4084 has been determined. One of the events

(31707) was classified as a single insertion with a partial insertion of a second copy resulting in two *kan*^{r} genes. Three of the events (31803, 31807, and 31808) were classified as single insertions of known structure. One (32317) was characterized as a double insertion event with both copies of the inserted DNA at the same locus. These DNA insertion events were inherited in Mendelian fashion demonstrated by resistance to Buctril® herbicide and were stable for a minimum of up to four generations of growth in the field. None of the events chosen as example events had hybridizing bands when probed with the 1532 probe confirming that no beyond the border transfer of DNA had occured. Events 31807 and 31808 remain in the breeding program.

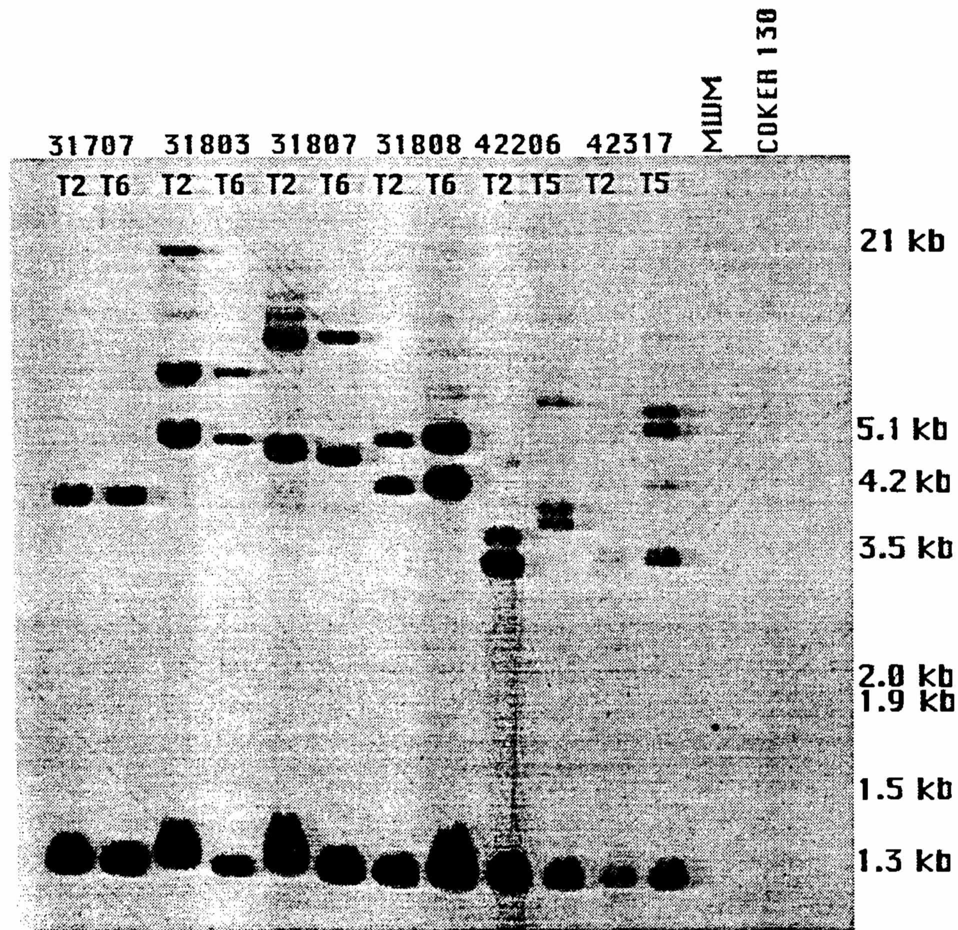

Figure 1: Southern blot analysis of 6 example events showing the BamHI/HindIII digested T_2 DNA next to digested DNA of a later generation of the same event. For events 31707, 31803, 31807, 31808, and 42317 the patterns are the same. The T_2 lane of event 42317 is very light due to low DNA concentration, however the bands are the same. Event 42206 does not have the same pattern of border fragments. This is likely a result of seed handling errors in the field. Only events 31807 and 31808 remain in the breeding program.

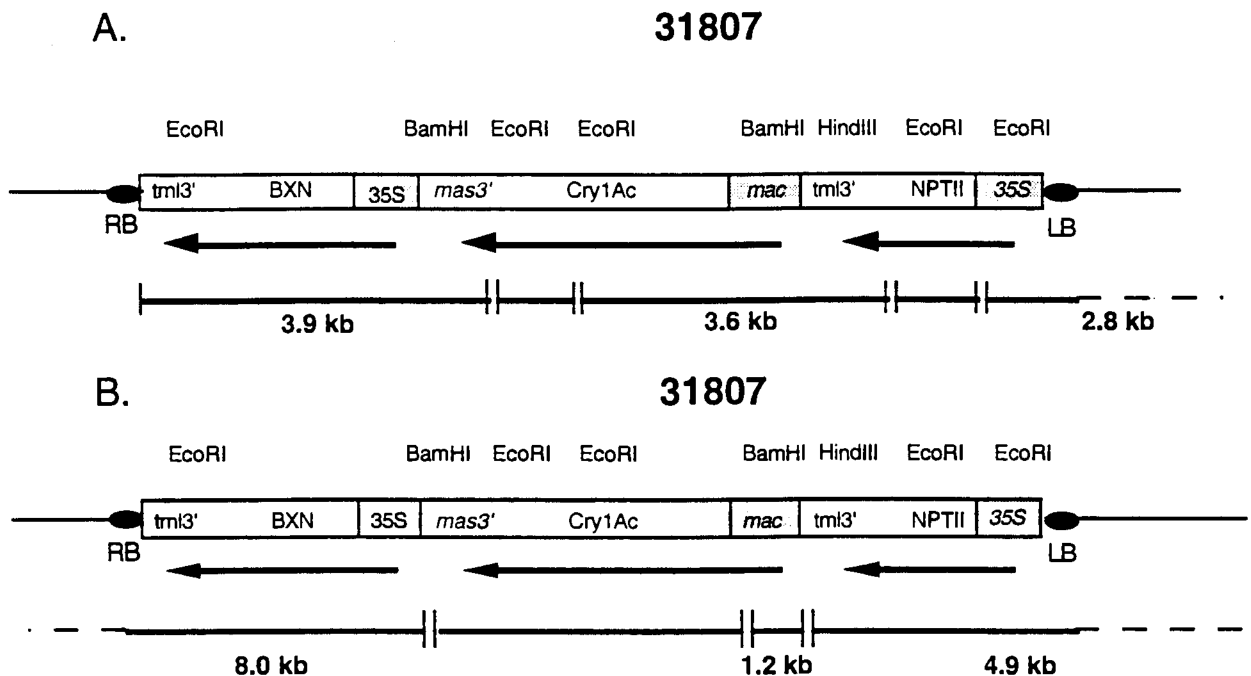

Figure 2: Schematic representation of event 31807. **A.** Event 31807 digested with EcoRI and probed with the *mac* probe. **B.** Event 31807 digested with BamHI and HindIII and probed with the 35S probe. Band sizes for each probe are given in kilobase pairs. Stipled areas depict the region of homology between the probe and the inserted sequence.

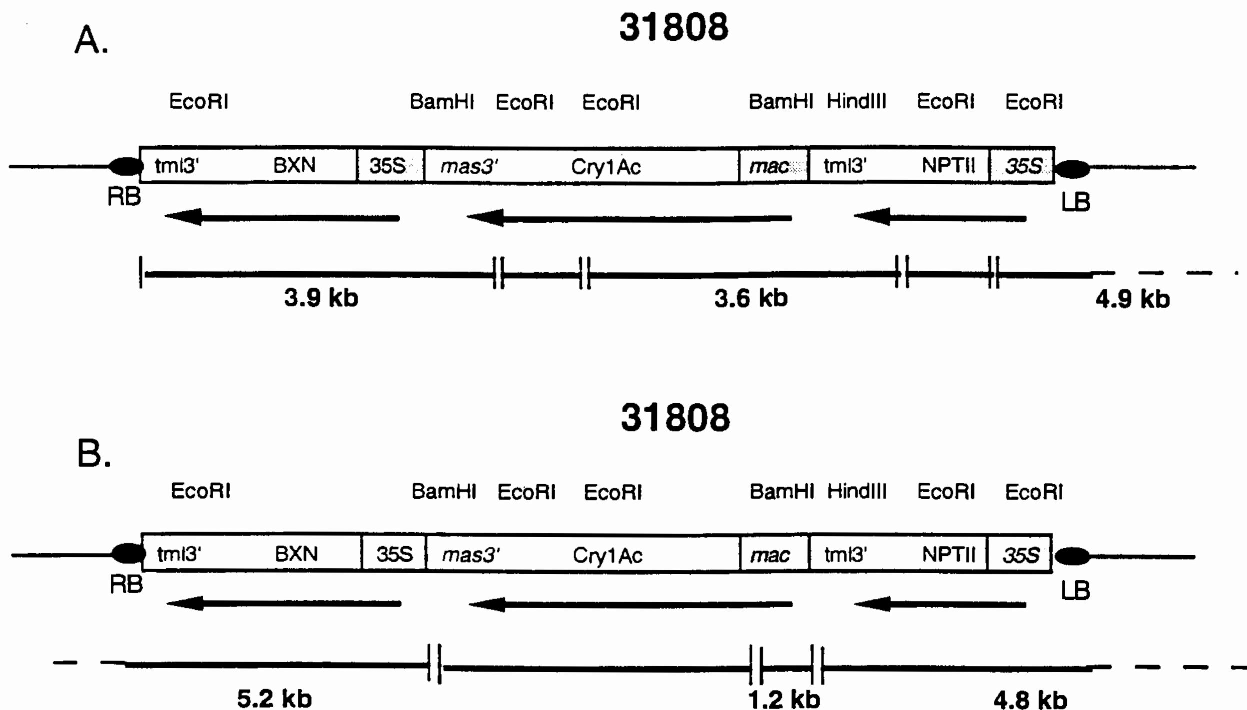

Figure 3: Schematic representation of event 31808. **A.** Event 31808 digested with EcoRI and probed with the *mac* probe. **B.** Event 31808 digested with BamHI and HindIII and probed with the 35S probe. Band sizes for each probe are given in kilobase pairs. Stipled areas depict the region of homology between the probe and the inserted sequence.

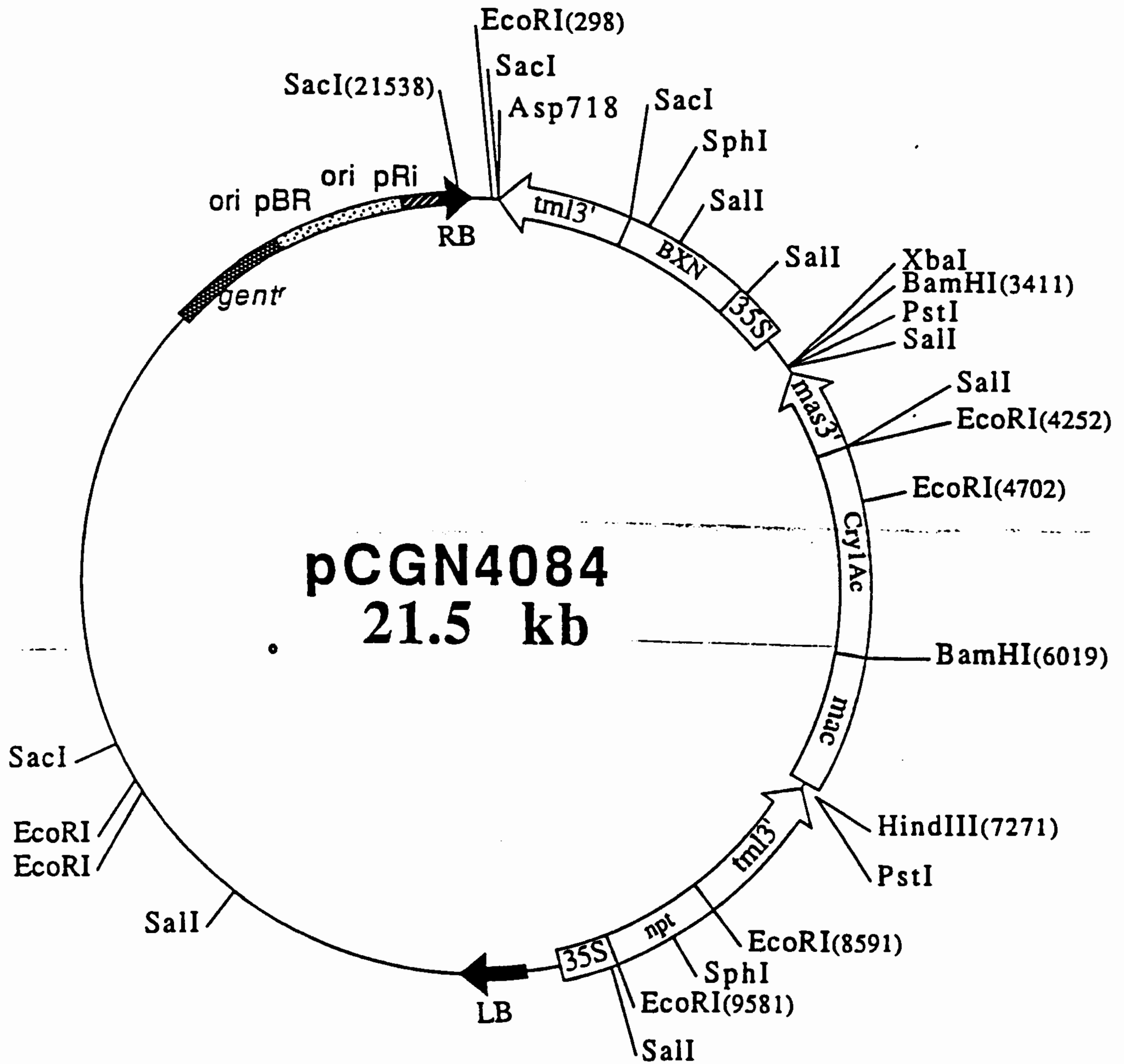

Figure 4 Plasmid map of Calgene construct pCGN4084. Transfered DNA lies between the left and right borders

Appendix A

Title: **Isolation of Genomic DNA from Cotton Leaf Tissue**

Prepared by: ______________________________
Signature Title Date

Approved by: ______________________________
Signature Title Date

Isolation of Genomic DNA from Cotton Leaf Tissue

Fresh, frozen leaf tissue (3-5 g) is ground in liquid nitrogen with a mortar and pestle. Powdered tissue is added to clean 30 ml Oak Ridge (Nalgene; Rochester, NY) tubes containing 0.3-0.5 g insoluble polyvinylpyrrolidone. Twenty milliliters of extraction buffer is added and the tubes are incubated at 65°C for 10 min, agitating every few minutes. Potassium acetate (7 ml) is added and the tubes are incubated for 30 min on ice with frequent agitation. The tubes are then centrifuged at 20,000 x g for 20 min at 4°C. The supernatant fluid is filtered through two layers of Miracloth® (Calbiochem, La Jolla, CA) into a new tube. Tubes are then centrifuged as before. The supernatant is again filtered through two layers of Miracloth into a new tube. Isopropanol (15 ml) is added and the tubes are slowly inverted until the DNA precipitates. The tubes are centrifuged at 20,000 x g for 25 minutes to pellet the DNA. The DNA is suspended in exactly 4 ml TE. Optical grade CsCl (3.88 g) and 300 µl ethidium bromide (10 mg/ml) are added to the suspended DNA. The solution is placed in an ultracentrifuge tube (Beckman, Palo Alto, CA) and the top is heat-sealed. The samples are spun overnight at 65,000 rpm in a vTi80 rotor at 20°C. After centrifugation, bands of genomic DNA are collected through an 18 gauge needle attached to a 5 cc syringe and placed in 15 ml Falcon tubes (Becton Dickinson, Lincoln Park NJ). The solution is extracted with 2-4 volumes of water-saturated 1-butanol to remove the ethidium bromide. This is repeated until the aqueous layer is colorless. The volume of the aqueous layer is adjusted to 4 ml with water and the DNA is precipitated by adding two volumes of 100% ethanol at room temperature. Falcon tubes are then centrifuged at 8,000 x g for 25 minutes. The DNA pellets are washed with 70% ethanol and the tubes are again centrifuged for 10 minutes at 8,000 x g. The DNA pellets are air-dried and suspended in 100 µl water. The Falcon tubes are washed with 100 µl water. This wash is pooled with the pellet fraction giving 200 µl of genomic DNA. Alternatively, the DNA may be spooled out on a heat sealed Pasteur pippete, dipped in 70% ethanol and placed in 200 µl water.

Reagents and Solutions

Extraction Buffer
200 mM CHES/NaOH pH 9.1
200 mM NaCl
100 mM EDTA pH 9.0
2% SDS
0.5% sodium deoxycholate
2% Nonidet NP-40
20 mM β-mercaptoethanol

Potassium acetate (5 M, pH 6.5)

TE
10 mM Tris.HCl
1 mM EDTA

pCGN4084: Sequences which may be inserted into BXN with Bt Cotton

Location	Description
21347-71	Right border from the T-DNA of pTiA6 (Barker *et al,* 1983)
72-308	LacZ' gene segment with polylinker sequence (Yanish-Peron, 1985)
323-1459	*tml* 3'. Polyadenylation region of *tml* gene pTiA6 (Barker *et al,* 1983)
1478-2632	*bxn* gene. Encodes nitrilase (Stalker *et al,* 1988a)
2670-3069	35S promoter region from the CaMV35S transcript (Gardner *et al,* 1981)
3434-4244	*mas* 3'. Polyadenylation region of *mas* (Transcript 7) gene from T-DNA of pTiA6 (Barker *et al,* 1983)
4245-6017	*cry1Ac* gene. Encodes the N-terminal portion (truncated) of the active Bt toxin (Hofte & Whitely, 1988; MacIntosch *et al,* 1990; Perlak *et al,* 1991)
6024-7257	Mac promoter. Hybrid of the *mas* and 35S promoter regions (Comai *et al,* 1990)
7448-8588	*tml* 3'. Polyadenylation region of *tml* gene pTiA6 (Barker *et al,* 1983)
8597-9575	*kan*^{r} gene. Encodes aminoglycoside 3'-phosphotransferase II (APH(3')II) from transposon Tn5 (Beck *et al,* 1982)
9587-10009	35S promoter region from the CaMV35S transcript (Gardner *et al,* 1981)
10010-10326	Tn5. Transposon segment (Auerswald *et al,*1981; Beck *et al,* 1982)
10340-10927	Left border from T-DNA of pTiA6 (Barker *et al,* 1983)

Construction of a truncated synthetic *Bacillis thuringensis cry*IAc gene for plant expression.

To effect high level expression of the *Btk cry*IAc gene in transgenic cotton plants, we undertook the resynthesis of the portion of the *cry*IAc gene that encodes only the toxic fragment of the full length CryIAc protein. The codon optimization for the proposed synthetic gene was based on the codon usage of a gene encoding small subunit (SSU) of carboxylase (RUBISCO), a well characterized plant protein that is expressed at high levels in all plants. SSU is highly homologous at the protein level for all the plant species for which it has been characterized and was a rational choice to utilize as a model for plant nuclear codon optimization of the *cry*IAc gene segment. Resynthesis of the truncated portion of the gene was undertaken in the following manner:

The proposed 1.8 kilobase gene encoding toxic segment was resynthesized as three distinct segments: a 500 bp *Bam*HI to *Eco* RI restriction fragment, a 600 bp *Eco*RI to *Apa*I restriction fragment and a 700 bp *Apa*I to *Sal*I restriction fragment. For each of these three fragments, groups of 70-90 bp complementary overlapping oligonucleotides were generated, annealed and ligated together. The ligated fragments of the correct size, 500, 600 and 700 bp respectively, were gel purified and each fragment was PCR amplified and separately cloned. Clones containing the correct DNA sequence were then identified for each fragment. The three fragments were then assembled together in the correct order to form a 1.8 kb *Bam*HI to *Sal*I DNA segment. This truncated *Bt* gene is 1770 nucleotides in length and encodes the 590 amino acid CryIAc toxic fragment of the full length protein which was then cloned into a promoter cassette to effect CryIAc expression in plants.

Segregation Data and Fit to Mendelian Ratios For T2 Populations of BXN/Bt cotton.

		1994 Field Data				Greenhouse Data			
			Chi-square value					Chi-square value	
Event	Total	Sus*	3 to 1^	15 to 1†		Total	Sus	3 to 1	15 to 1
31701	173	47	0.43	129.19		93	18	1.58	27.26
31702	215	64	2.61	202.94		90	26	0.73	78.72
31703	140	35	0.00	84.00		48	10	0.44	17.42
31704	126	8	23.38	0.00					
31705	113	22	1.84	33.70		96	19	1.39	30.04
31706	187	52	0.79	148.32		156	26	5.78	28.89
31707	180	34	3.59	49.07		96	22	0.22	45.51
31708	67	9	4.78	5.90					
31709	143	8	28.72	0.10		89	0	29.67	5.93
31801	62	6	7.76	1.24		90	3	22.53	1.31
31802	104	21	1.28	34.50		90	16	2.50	20.41
31803	207	45	1.17	84.76		190	48	0.01	117.22
31804	196	41	1.74	71.97		95	29	1.55	95.55
31805	151	39	0.06	98.78		183	36	2.77	56.27
31806	205	53	0.08	134.45		172	39	0.50	79.19
31807	**176**	**44**	**0.00**	105.60		**93**	**19**	**1.04**	31.91
31808	**220**	**63**	**1.55**	188.16		**284**	**74**	**0.17**	190.14
31809	226	64	1.33	187.85		86	23	0.14	61.65
31810	169	69	22.58	344.86		94	51	42.91	-369.70
31811	226	56	0.01	132.42		94	18	1.72	26.69
31812	173	43	0.00	102.21		114	33	0.95	100.23
31813	249	65	0.16	167.52		93	16	3.01	19.05
31814	170	47	0.64	132.83		71	17	0.04	37.94
31815	133	42	3.07	145.62		75	22	0.75	68.20
31816	165	46	0.73	131.73		57	8	3.65	5.90
31817	210	14	37.64	0.06		95	0	31.67	6.33
31901	104	32	1.85	106.71		88	25	0.55	73.75
31902	143	25	4.31	30.79		86	20	0.14	42.45
32001	101	23	0.27	47.06		73	20	0.22	55.72
32101	131	10	21.07	0.43		87	5	17.20	0.04

* Sus- number of Buctril® sensitive plants

^ 3 to1:Chi-Square Value for best fit to an expected ratio of 3 to 1 (single insertion site)

† 15 to 1: Chi-Square Value for best fit to an expected ratio of 15 to 1.

Chi-Square (0.05) = 3.84. Values less than 3.84 are said to fit the expected value.

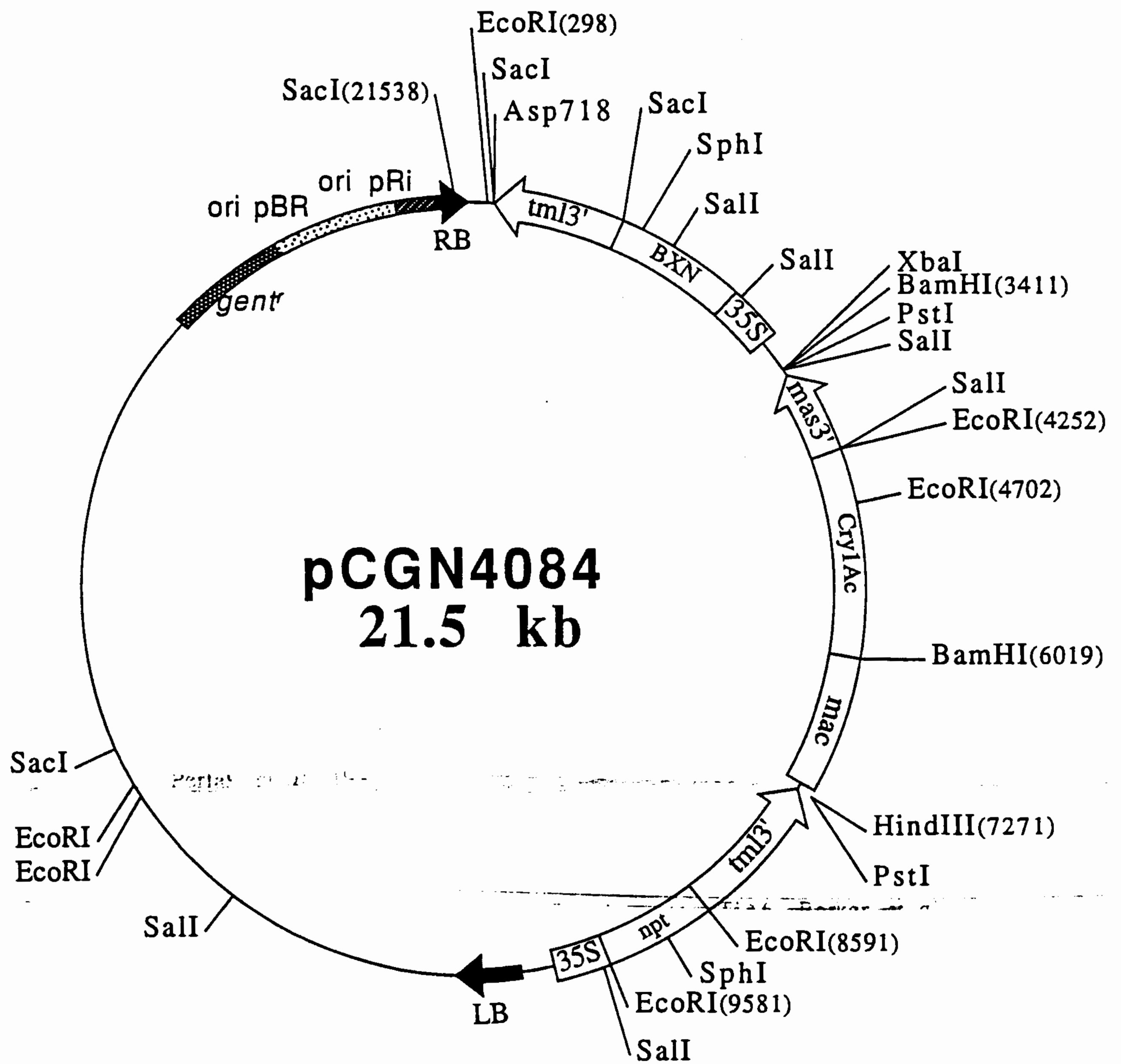

Figure 1. Plasmid Map of Calgene Construct pCGN4084

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Pages 61-79