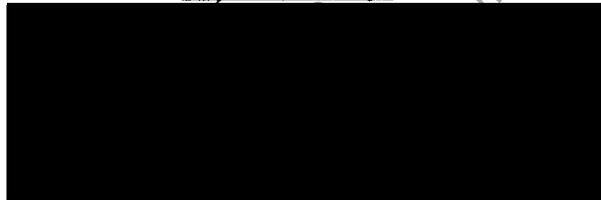


**Request for Extension of Determination of Nonregulated Status to the
Additional Regulated Article:**

Roundup Ready Corn Line NK603

**The undersigned submits this request under 7 CFR Part 340.6(e) to
request that the Director, BBEP, make an extension of determination
of nonregulated status that the article should not be regulated
under 7 CFR part 340.**

Submitted by:



**Maize Traits Lead - Regulatory Affairs
Monsanto Company
700 Chesterfield Parkway North, BB1K
St. Louis, MO 63198**



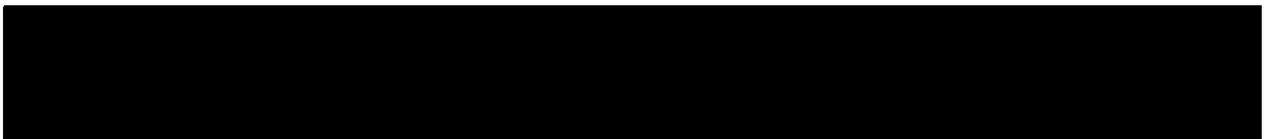
submitted January 7, 2000

#99-884-U

Prepared by:



Contributors:



Request for Extension of Determination of Nonregulated Status for Roundup Ready Corn Line NK603

Summary

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Federal Plant Pest Act (7 U.S.C. 150aa-150jj) and the Plant Quarantine Act (7 U.S.C. 151-167) to prevent the introduction and dissemination into the U.S. or interstate of plant pests. The regulations provide that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article. Section 340.6(e) of the regulations further provides that APHIS may extend a determination to additional regulated articles upon finding that the articles do not pose a potential for plant pest risk.

Monsanto Company is submitting this request to APHIS for extension of a determination of nonregulated status for Roundup Ready^{®1} corn line NK603 based upon the previous approval of Roundup Ready corn line GA21 (97-099-01p). The glyphosate tolerance of Roundup Ready corn line GA21 is imparted by the insertion of a modified corn 5-enolpyruvylshikimate-3-phosphate synthase (mEPSPS) into the corn genome. In comparison, Roundup Ready corn line NK603 (may also be referred to as Roundup Ready corn line 603) utilizes an EPSPS from *Agrobacterium* sp. strain CP4 to provide glyphosate tolerance. In both cases, when corn plants containing the inserted EPSPS gene are treated with glyphosate, the plants are unaffected since the continued action of the expressed tolerant EPSPS enzyme provides the plant's need for aromatic amino acids (OECD, 1999; Padgett *et al.*, 1996). It is on this basis that Monsanto requests this extension for the determination of nonregulated status of Roundup Ready corn line NK603.

Corn, *Zea mays* L., has been extensively characterized, genetically and as a crop, and has a long history of safe agricultural production. Seeds are the only known survival structures, and corn is not capable of surviving as a weed due to past selection as a result of its domestication. Corn is not found growing in fence rows, ditches, road sides, or unmanaged habitats in the U.S.

¹ Roundup Ready[®] is a registered trademark of Monsanto Company.

The donor organism, *Agrobacterium* sp. strain CP4, was used to supply the CP4 EPSPS gene for glyphosate tolerance. The CP4 EPSPS gene is well characterized and is homologous to plant and microbial EPSPSs which are widely prevalent and have a long history of safe use. The transformation vector, PV-ZMGT32L, containing the CP4 EPSPS gene and regulatory sequences, was introduced into the corn genome by a particle acceleration method to produce Roundup Ready corn line NK603. Genetic analysis of line NK603 was performed to characterize the single stable site of insertion into the corn plant genome. The insertion resulted in the expression of only the single additional protein of interest, CP4 EPSPS, which imparts tolerance to glyphosate, the active ingredient in Roundup^{®1} herbicide.

Agronomic, morphological and pest susceptibility observations have been recorded for Roundup Ready corn line NK603. As such, neither the CP4 EPSPS gene, including the regulatory sequences, nor the CP4 EPSPS protein, confer plant pest characteristics. Although DNA from organisms known to be pathogenic to plants were used in its development (i.e., *Agrobacterium* sp. and cauliflower mosaic virus (CaMV)), line NK603 and plants derived from it are not infected by these organisms nor can corn line NK603 induce disease in other plants.

The environmental consequences of the introduction of Roundup Ready corn line NK603 have been considered and there is no reason to believe that line NK603 would have a significant adverse impact on organisms beneficial to plants or to non-target organisms including threatened or endangered species. The safety of the EPSPS family of proteins, and specifically CP4 EPSPS as produced in a number of Roundup Ready crops including corn, soybean, canola, cotton and sugar beet, has been demonstrated. The environmental consequences of pollen transfer by wind pollination from Roundup Ready corn line NK603 to other corn is considered to be negligible due to the safety of the CP4 EPSPS protein and the lack of any selective advantage conferred on the recipient corn plant. The agronomic consequences of volunteer corn plants would be minimal as the plants are easily controlled by mechanical means, or by one of a number of other herbicides currently registered for corn.

There are no significant populations of sexually compatible related species of corn (e.g. teosinte; *Zea mays* ssp. *mexicana*) in the U.S. and its territories.

As herbicides are currently applied on 95% of the U.S. corn acreage (USDA, 1999), the use of Roundup Ready corn line NK603 is unlikely to have any significant adverse impact on agricultural practices in the U.S. Rather, the use of Roundup as applied to Roundup Ready corn is

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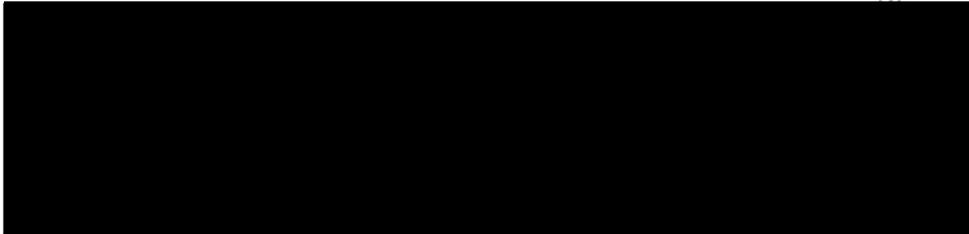
expected to have significant grower and public benefit due to increased adoption of conservation tillage systems and the favorable environmental characteristics of Roundup herbicide.

Data and information in this request demonstrate that Roundup Ready corn line NK603 does not represent a unique plant pest risk. Therefore, Monsanto requests an extension of determination of non-regulated status from APHIS that corn line NK603, any progenies derived from crosses between this line and other corn varieties, and any progeny derived from crosses of this line with transgenic corn varieties that have also received a determination of non-regulated status, no longer be considered regulated articles under regulations in 7 CFR part 340.

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Certification

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



Maize Traits Lead - Regulatory Affairs
Monsanto Company
700 Chesterfield Parkway North, BB1K
Chesterfield, MO 63198



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**Abbreviations Used in this Request for Extension of a
Determination of NonRegulated Status for
Roundup Ready Corn Line NK603**

APHIS	Animal Plant and Health Inspection Service
~	Approximately
CaMV	Cauliflower mosaic virus
CFR	Code of Federal Regulations
CP4 EPSPS	EPSPS derived from <i>Agrobacterium</i> sp. strain CP4
CTAB	Cetyltrimethylammonium bromide
CTP	Chloroplast transit peptide
DNA	Deoxyribonucleic Acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EcoRV	Restriction enzyme
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
e35S	Cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
FDA	Food and Drug Administration
fw	Fresh weight
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
LOQ	Limit of quantitation
mEPSPS	Modified corn EPSPS
<i>Mlu</i> I	Restriction enzyme
MW	Molecular weight
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
Na ₂ HPO ₄	Sodium phosphate dibasic
NFDM	Non-fat dried milk
NOS 3'	Nopaline synthase 3' polyadenylation sequence
<i>nptII</i>	The gene for the enzyme neomycin phosphotransferase type II
OD	Optical density (sample absorbance)
OECD	Organization for Economic Co-operation and Development
<i>ori</i>	Origin of replication

P-ract1/ract1 intron	Rice actin promoter and intron
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PMSF	Polymethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
PV-ZMGT32	Plasmid vector
PV-ZMGT32L	Linear fragment of PV-ZMGT32 used for transformation
S3P	Shikimate-3-phosphate
<i>SacI</i>	Restriction enzyme
<i>ScaI</i>	Restriction enzyme
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOP	Standard operating procedure
sp.	Species
SSC	Saline-sodium citrate buffer. 20X SSC is 3 M sodium chloride, 0.3 M sodium citrate
<i>StuI</i>	Restriction enzyme
TBA	Tris-borate with L-ascorbic acid
TBST	Tris buffered saline with Tween
TE buffer	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
TMB	(3,3',5,5'-Tetramethylbenzidine) peroxidase substrate
Tris	Tris (hydroxymethyl)aminomethane
tRNA	<i>Escherichia coli</i> transfer RNA
Tween 20	Polyoxyethylenesorbitan mono laurate
U.S.C.	United States Code
USDA	United States Department of Agriculture
UV	Ultraviolet
<i>XbaI</i>	Restriction enzyme
<i>Zmhsp70</i>	corn (<i>Zea mays</i>) <i>hsp70</i> gene (heat-shock protein)

Standard abbreviations, e.g., units of measure, will be used according to the format described in 'Instructions to Authors' in the Journal of Biological Chemistry.

**Request for Extension of Nonregulated Status of
Roundup Ready Corn Line NK603**

TABLE OF CONTENTS

	<u>Page</u>
Title Page	1
Summary	2
Certification	5
Abbreviations	6
Table of Contents	8
I. Rationale for Submission of Request for Extension	11
A. Basis for request for extension of determination of nonregulated status under 7 CFR Part 340.6(e)	11
B. Roundup Ready corn line NK603	12
II. The Corn Family	13
A. Characteristics of the recipient corn materials	14
III. Description of the Transformation System	14
IV. Donor Genes and Regulatory Sequences	14
A. The vector PV-ZMGT32L	14
B. The CP4 EPSPS gene	19
C. The chloroplast transit peptide (CTP2)	20
D. Regulatory sequences	21
V. Genetic Analysis and Agronomic Performance	23
A. Molecular characterization of Roundup Ready corn line NK603	23
1. Materials and methods	23
a. Test substance	23
b. Control substance	23
c. Reference substances	24
d. DNA extraction	24
e. DNA quantitation and restriction enzyme digestion	25
f. Preparation of DNA probes	25
g. Southern blot analysis	26
h. Verification of the 5' and 3' flanking sequences.	26
2. Results and discussion	27
a. Determination of insert number.	27
b. Determination of copy number	28
c. Integrity of inserted gene cassettes	28
i. P-ract1/ract1 intron	29
ii. CTP2-CP4 EPSPS sequence	29

iii. e35S promoter.....	30	
iv. NOS 3' polyadenylation sequence.....	30	
d. Analysis for backbone fragments	31	
e. Verification of sequences at the 5' and 3' ends of line NK603 insert.....	31	
3. Conclusions.....	32	
B. Segregation Data and stability of gene transfer of Roundup Ready corn line NK603.....	42	
1. Segregation data and stability of line NK603	42	
2. Corn line NK603 generation stability: Southern blot analysis.....	43	
C. Expression of the inserted CP4 EPSPS gene.....	45	
1. Introduction.....	45	
2. Expression levels of the CP4EPSPS protein in Roundup Ready corn line NK603.....	45	
D. CP4 EPSPS protein specificity and homology to EPSPSs derived from a variety of plant and microbial sources	47	
E. Disease and pest susceptibilities	48	
F. Lack of toxicants in corn	50	
G. Agronomic characteristics of Roundup Ready corn line NK603	50	
H. Conclusion.....	51	
VI. Environmental Consequences of Introduction of Roundup Ready Corn Line NK603	52	
VII. Adverse Consequences of Introduction	53	
VIII. References.....	54	
List of Figures		
Figure 1	Linear map of PV-ZMGT32L..... 16	
Figure 2	Plasmid map of PV-ZMGT32..... 17	
Figure 3	Deduced amino acid sequence of the CP4 EPSPS protein	20
Figure 4	Development of Roundup Ready corn line NK603	22
Figure 5	Southern blot analysis of line NK603: determination of insert number.....	33
Figure 6	Southern blot analysis of line NK603: determination of copy number	34
Figure 7	Southern blot analysis of line NK603: P-ract1/ract1 intron.....	35
Figure 8	Southern blot analysis of line NK603: CTP2-CP4 EPSPS sequence	36
Figure 9	Southern blot analysis of line NK603: e35S promoter.....	37

Figure 10	Southern blot analysis of line NK603: NOS 3' polyadenylation sequence	38
Figure 11	Southern blot analysis of line NK603: backbone analysis	39
Figure 12	PCR verification of sequences at the 5' and 3' ends of line NK603 insert	40
Figure 13	Schematic representation of the line NK603 insert.....	41
Figure 14	Southern blot analysis of NK603: stability of the inserted DNA	44

List of Tables

Table 1	Summary of DNA components of the plasmid PV-ZMGT32	18
Table 2	Segregation data and analysis of progeny of Roundup Ready corn line NK603.....	43
Table 3	Summary of CP4 EPSPS protein levels measured by the ELISA in tissues of NK603 corn plants	46
Table 4	USDA Notifications relevant to the field testing of Roundup Ready corn line NK603.....	49
Table 5	Agronomic comparison of Roundup Ready corn line NK603 and control.....	51
Appendix	Expression of the CP4 EPSPS Protein	58

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I. Rationale for Submission of Request for Extension

A. Basis for request for extension of determination of nonregulated status under 7 CFR Part 340.6(e)

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has been given the responsibility, under the Federal Plant Pest Act (7 U.S.C. 150aa-150jj) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction and dissemination into the United States or interstate of plant pests. Under this authority, APHIS has published regulations found in 7 CFR part 340 entitled "Petition for Determination of Nonregulated Status", which provide that a person may petition APHIS to evaluate submitted data to determine that a particular regulated article does not represent an increased plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a risk of introduction or dissemination of a plant pest, the petition will be granted, thereby allowing unrestricted introduction of the article.

In terms of a request for extension, Section 340.6(e) of the regulations provides that APHIS may extend a determination of nonregulated status to additional regulated articles, upon finding that the additional article does not increase the potential for plant pest risk, and should therefore not be regulated. Such a finding would be made based on an evaluation of the similarity of the additional regulated articles to an antecedent organism. The Agency has provided the following example in its guidance as a "molecular manipulation that would yield a regulated article that APHIS believes is unlikely to pose new risk issues beyond those that would have been considered in the initial determination of nonregulated status":

- *Modifications in which the antecedent organism and the regulated article in question contain different donor genes, but the donor gene used in producing the antecedent organism and the donor gene used in producing the regulated article in question encode enzymes catalyzing the same biochemical reaction (i.e., molecules that have the same substrates and products).*

When applying this guidance it is clear that a request for an extension of determination of nonregulated status for Roundup Ready^{®1} corn line NK603 as based upon the previous approval of Roundup Ready corn line GA21 (97-099-01p) is appropriate. The glyphosate tolerance of Roundup Ready corn line GA21 was imparted by the insertion of a modified corn (*Zea mays* L.) EPSPS (mEPSPS) into the corn genome.

¹ Roundup Ready[®] is a registered trademark of Monsanto Company.

In comparison, Roundup Ready corn line NK603 (may also be referred to as Roundup Ready corn line 603) utilizes an EPSPS from *Agrobacterium* sp. strain CP4 to provide glyphosate tolerance. In both cases, the EPSPS enzyme catalyzes the reversible reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate-3-phosphate (EPSP) and phosphate, a critical step in the biosynthesis of aromatic amino acids. The EPSPS enzyme is known to be present in plants, bacteria and fungi, but not in animals (OECD, 1999; Padgett *et al.*, 1996). It is on this basis that Monsanto requests this extension for the approval of Roundup Ready corn line NK603.

The specific differences between Roundup Ready corn line GA21 previously granted nonregulated status and corn line NK603, the basis for this submission, are discussed in the appropriate sections below. The name of the new line to be considered under extension is line NK603.

B. Roundup Ready corn line NK603

Monsanto Company has developed Roundup Ready corn line NK603 which is tolerant to glyphosate (the active ingredient in Roundup^{®1} herbicide) at the whole plant level. Similar to mEPSPS, the EPSPS from *Agrobacterium* sp. strain CP4 is functionally similar to plant EPSPS enzymes but has a greatly reduced affinity for glyphosate (Padgett *et al.*, 1996). In nontransgenic plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids thereby preventing plant production of these essential compounds (Steinrucken and Amrhein, 1980; Padgett *et al.*, 1996). In Roundup Ready corn, metabolic requirements for normal growth and development are met by the continued action of the glyphosate-tolerant CP4 EPSPS enzyme in the presence of glyphosate.

There are no changes in rationale from Section I.B of the previously approved petition number 97-099-01p, which briefly discusses the benefits of Roundup Ready corn. In brief, the use of Roundup herbicide for effective control of weeds in Roundup Ready corn allows the grower to take advantage of the herbicide's favorable environmental properties. The use of this technology benefits the farmer by providing (1) a broad-spectrum weed control option in corn, (2) a novel herbicidal mode of action for in-season corn weed control, (3) increased flexibility to treat weeds on an "as needed" basis, (4) cost-effective weed control and (5) an excellent fit with reduced-tillage systems. In turn, a number of environmental benefits arise from the

¹ Roundup[®] is a registered trademark of Monsanto Company.

use of conserved tillage, including improved soil quality, improved water infiltration, reductions in erosion and sedimentation of water resources, reduced runoff of nutrients and pesticides to surface water, improved wildlife habitat, increased carbon retention in the soil, reduced fuel use and encouragement of sustainable agricultural practices.

Before commercializing Roundup Ready corn line NK603, Monsanto will seek the following regulatory approvals in the United States:

1. Extension of the existing determination of nonregulated status granted for Roundup Ready corn line GA21 (97-099-01p) to Roundup Ready corn line NK603 and all progenies from crosses between this line and other corn varieties. As a result, this corn line and progenies would no longer be regulated articles according to 7CFR §340.6. A brief description of USDA APHIS regulatory authority is provided above in Section A.
2. As a result of consultations on Roundup Ready corn with the FDA since March, 1995, Monsanto will provide a summary of the food and feed safety and nutritional assessment of Roundup Ready corn line NK603 to the Agency prior to commercial distribution. Corn line NK603 is within the scope of the FDA policy statement concerning regulation of products derived from new plant varieties, including those genetically engineered, published in the Federal Register on May 29, 1992.

The initial registration of Roundup Ultra^{®1} herbicide (EPA Reg. No. 524-475) for use over-the-top of Roundup Ready corn was granted by the EPA with label approval on March 28, 1997 (62 FR 17723-17730). Subsequent label amendments have been submitted and approved by the Agency as recently as July 13, 1999. The U.S. EPA has authority over the use of all pesticide substances, including herbicides, under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended (7 U.S.C. 136 *et seq.*).

II. The Corn Family

There are no changes from the previously approved petition submission with regard to the characteristics of corn (*Zea mays* L.). To date, a consensus document on the biology of corn has not been developed as a part of the Organization for Economic Co-operation and Development (OECD) Series on Harmonization of Regulatory Oversight in Biotechnology.

¹ Roundup Ultra[®] is a registered trademark of Monsanto Company.

A. Characteristics of the recipient corn materials

The corn plant tissue which was the recipient of the introduced DNA was embryogenic corn cells from a proprietary inbred line designated (AW x CW). Roundup Ready corn line NK603 was selected by its ability to survive on medium and grow in the presence of glyphosate, the active ingredient in Roundup herbicide.

III. Description of the Transformation System

The antecedent organism, Roundup Ready corn line GA21, was produced using a particle acceleration method. Roundup Ready corn line NK603 was produced using the same particle acceleration method (Klein *et al.*, 1987; Gordon-Kamm *et al.*, 1990), as identified in Section III of the previously approved submission for Roundup Ready corn line GA21 (97-099-01p).

IV. Donor Genes and Regulatory Sequences

The antecedent organism, Roundup Ready corn line GA21 (97-099-01p), was generated using a particle acceleration transformation system with a gel-isolated *NotI* DNA restriction fragment of plasmid vector pDPG434 containing the mEPSPS gene. In comparison, line NK603 used a gel-isolated *MluI* fragment, PV-ZMGT32L, containing the EPSPS gene from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). Both genes encode a tolerant form of EPSPS which confers glyphosate (Roundup) tolerance at the whole plant level (Figures 1 and 2).

The plasmid vector PV-ZMGT32 contains two plant gene expression cassettes, each containing a single copy of the CP4 EPSPS gene. A summary of DNA components of the plasmid PV-ZMGT32 is provided in Table 1.

A. The vector PV-ZMGT32L

The plant expression plasmid vector, PV-ZMGT32, was developed by Monsanto Company, St. Louis, Missouri. As with line GA21, transformation of corn cells using particle acceleration was performed at DEKALB Genetics in Mystic, Connecticut. The sources of the genetic materials used to construct the plasmid are listed in Table 1.

The plasmid vector PV-ZMGT32 contains two plant gene expression cassettes each containing a single copy of the CP4 EPSPS gene. The vector also contains an *nptII* bacterial selectable marker gene encoding kanamycin resistance allowing selection of bacteria containing the

plasmid, and an origin of replication (*ori*) necessary for replicating the plasmid in *E. coli* (Table 1). The agarose gel-isolated *Mlu*I restriction fragment of plasmid vector, PV-ZMGT32L, utilized for transformation of Roundup Ready corn line NK603 contains only the CP4EPSPS plant gene expression cassettes and does NOT contain the *nptII* selectable marker gene or origin of replication (Figure 1).

The rice actin 1 promoter and first intron (McElroy et al., 1990) from the rice actin gene and the NOS 3' sequence for termination of transcription and direction of polyadenylation were previously described in the transformation of Roundup Ready corn line GA21 (97-099-01p). The enhanced 35S promoter from CaMV with an enhanced duplicator region, corn *hsp70* intron, chloroplast transit peptide from *Arabidopsis thaliana* and the CP4 EPSPS gene sequence are described in Table 1 and were previously reviewed by the Agency in the granting of nonregulated status for Roundup Ready/European corn borer protected line MON 802 (96-317-01p). No new DNA elements are included in line NK603 which have not been reviewed by the Agency in previous decisions for nonregulated status.

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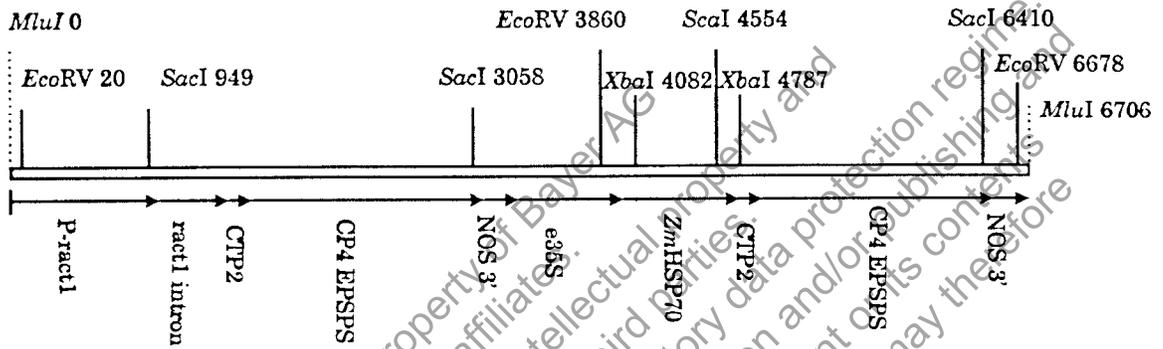


Figure 1. Linear map of PV-ZMGT32L. The DNA fragment PV-ZMGT32L was used to generate Roundup Ready corn line NK603 by particle acceleration technology. The dashed lines represent the remaining *Mlu*I sites following digestion of PV-ZMGT32.

The *Xba*I 4082 site is not active due to methylation.

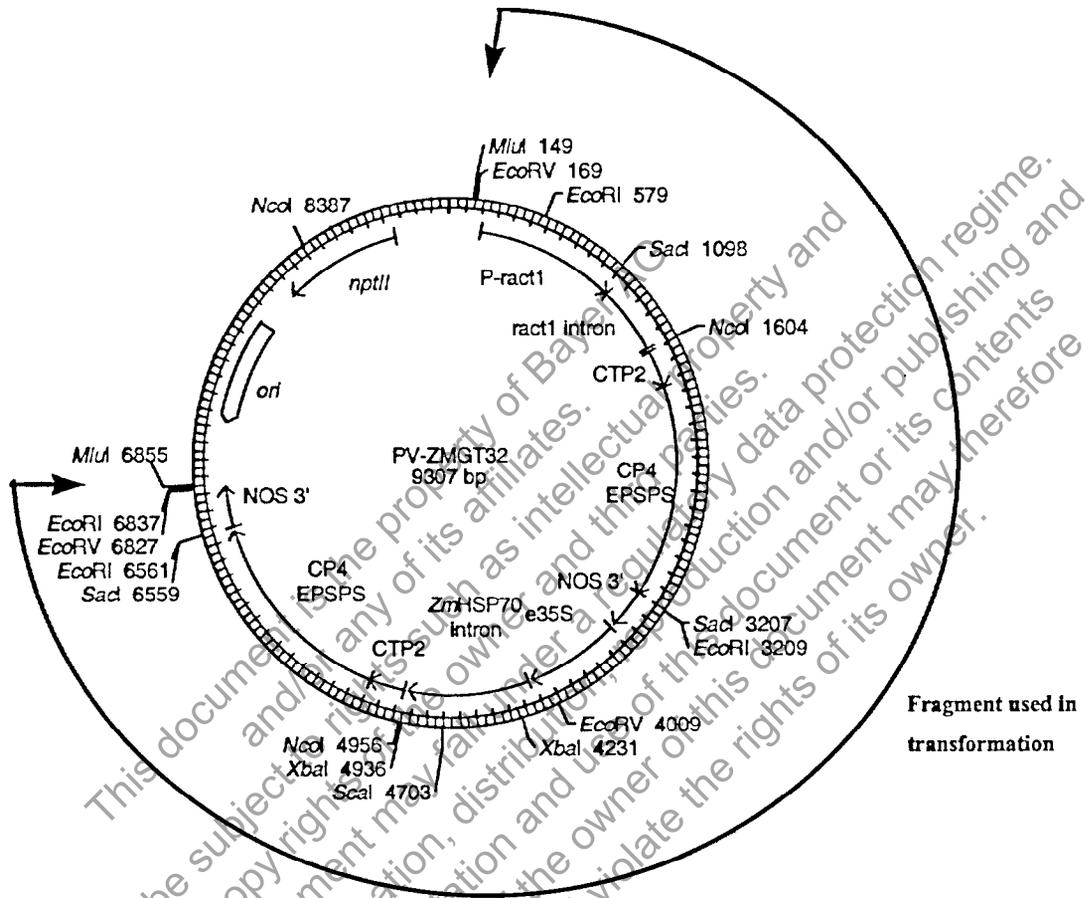


Figure 2. Plasmid map of PV-ZMGT32. The plasmid PV-ZMGT32 was used to prepare the *MluI* fragment used in the transformation of corn line NK603 by excision and discarding of the origin of replication (*ori*) and antibiotic resistance marker (*nptII*) sequences.

Table 1. Summary of DNA components of the plasmid PV-ZMGT32

Genetic Element	Source	Size (Kb)	Function
Genetic elements present in the <i>Mlu</i>I restriction fragment, designated PV-ZMGT32L, used for transformation:			
CP4 EPSPS Gene Cassette (1)			
P-ract1/ ract1 intron	<i>Oryza sativa</i>	1.4	5' region of the rice actin 1 gene containing the promoter, transcription start site and first intron (McElroy <i>et al.</i> , 1990).
CTP2	<i>Arabidopsis thaliana</i>	0.2	DNA sequence for chloroplast transit peptide, isolated from <i>Arabidopsis thaliana</i> EPSPS (Klee and Rogers, 1987), present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
CP4 EPSPS	<i>Agrobacterium</i> sp. strain CP4	1.4	The DNA sequence for CP4 EPSPS, isolated from <i>Agrobacterium</i> sp. strain CP4 (██████████ 1993; Padgett <i>et al.</i> , 1996) which imparts tolerance to glyphosate.
NOS 3'	<i>Agrobacterium tumefaciens</i>	0.3	A 3' nontranslated region of the nopaline synthase gene from <i>Agrobacterium tumefaciens</i> T-DNA which ends transcription and directs polyadenylation (Fraleigh, <i>et al.</i> , 1983) of the mRNA.
CP4 EPSPS Gene Cassette (2)			
e35S	<i>Cauliflower mosaic virus</i>	0.6	The cauliflower mosaic virus (CaMV) promoter (Odell <i>et al.</i> , 1985) with the duplicated enhancer region (Kay <i>et al.</i> , 1985).
Zmhsp70	<i>Zea mays</i> L.	0.8	Intron from the corn <i>hsp70</i> gene (heat-shock protein) present to stabilize the level of gene transcription (Rochester <i>et al.</i> , 1986).
CTP2	<i>Arabidopsis thaliana</i>	0.2	DNA sequence for chloroplast transit peptide, isolated from <i>Arabidopsis thaliana</i> EPSPS (Klee and Rogers, 1987), present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
CP4 EPSPS	<i>Agrobacterium</i> sp. strain CP4	1.4	The DNA sequence for CP4 EPSPS, isolated from <i>Agrobacterium</i> sp. strain CP4 (██████████ 1993; Padgett <i>et al.</i> , 1996) which imparts tolerance to glyphosate.
NOS 3'	<i>Agrobacterium tumefaciens</i>	0.3	A 3' nontranslated region of the nopaline synthase gene from <i>Agrobacterium tumefaciens</i> T-DNA which ends transcription and directs polyadenylation (Fraleigh, <i>et al.</i> , 1983) of the mRNA.

Table 1. Summary of DNA components of the plasmid PV-ZMGT32 (continued)

Genetic Element	Source	Size (Kb)	Function
Genetic elements present in the PV-ZMGT32 plasmid backbone, but not present in the <i>Mlu</i>I restriction fragment (PV-ZGMT32L) used for transformation:			
<i>ori</i>	<i>Escherichia coli</i>	0.65	The origin of replication from the <i>E. coli</i> high copy plasmid pUC119 (Vieira and Messing, 1987).
<i>nptII</i>	<i>Transposon Tn5</i>	0.8	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid (Beck <i>et al.</i> , 1982).

B. The CP4 EPSPS gene

The CP4 EPSPS gene has been shown to have the potential to provide high levels of resistance to glyphosate inhibition when introduced into plants (Padgett *et al.*, 1993; OECD, 1999). Glyphosate binds to and blocks the activity of its target enzyme, EPSPS, an enzyme of the aromatic amino acid biosynthetic pathway. The CP4 EPSPS gene from *Agrobacterium* sp. strain CP4, has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgett *et al.*, 1996). The CP4 EPSPS gene with its CTP2 is approximately 1.6 kb in size. The deduced amino acid sequence of the CP4 EPSPS with the CTP2 transit peptide is shown in Figure 3.

The CP4 EPSPS protein is one of many different EPSPSs found in nature (Schulz *et al.*, 1985). CP4 EPSPS is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most EPSPSs (Barry *et al.*, 1992; Padgett *et al.*, 1993; Padgett *et al.*, 1996). Plant cells expressing the CP4 EPSPS protein are tolerant to glyphosate when present in growth medium because the continued EPSPS enzyme activity meets the needs for aromatic compounds. The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. There is no human or animal pathogenicity known from *Agrobacterium* species, nor is the EPSPS

gene a determinant of *Agrobacterium* plant pathogenesis. The CP4 and native corn EPSPS enzymes are functionally equivalent, except for their affinity to glyphosate.

The amino acid sequence of the CP4 EPSPS protein produced in Roundup Ready corn line NK603 is identical to that of the CP4 EPSPS protein as produced in a number of other Roundup Ready crops previously reviewed and granted nonregulated status by the Agency, including corn (96-317-01p) as well as soybean (93-258-01p), cotton (95-045-01p), and sugar beet (98-173-01p).

Figure 3. Deduced amino acid sequence of the CP4 EPSPS protein. Sequence includes the CTP2 transit peptide (amino acids 1-76 are the transit peptide).

1 MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
51 LKKSGMTLIG SELRPLKVMV SVSTACMLHG ASSRPATARK SSGLSGTVRI
101 PGDKSISHRS FMFGGLASGE TRITGLLEGE DVINTGKAMQ AMGARIRKEG
151 DTWIIDGVGN GLLAPEAPL DFGNAATGCR LTMGLVGVYD FDSTFIGDAS
201 LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP ITYRVPMASA
251 QVKSAVLLAG LNTPGITTVI EPIMTRDHTK KMLQGFGANL TVETDADGVR
301 TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT IILNVLMPTR
351 TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM
401 IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNQVDCDE
451 GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501 DATMIATSFP EFMDLMAGLG AKIELSDTKA A

C. The chloroplast transit peptide (CTP2)

In both plant gene expression cassettes, the CP4 EPSPS coding sequence is fused to chloroplast transit peptide (designated CTP2) whose sequence is based on the CTP isolated from *Arabidopsis thaliana* EPSPS (Klee *et al.*, 1987). This CTP directs the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis (Kishore and Shah, 1988). CTPs are typically cleaved from the "mature" protein following delivery to the plastid (della-Cioppa *et al.*, 1986).

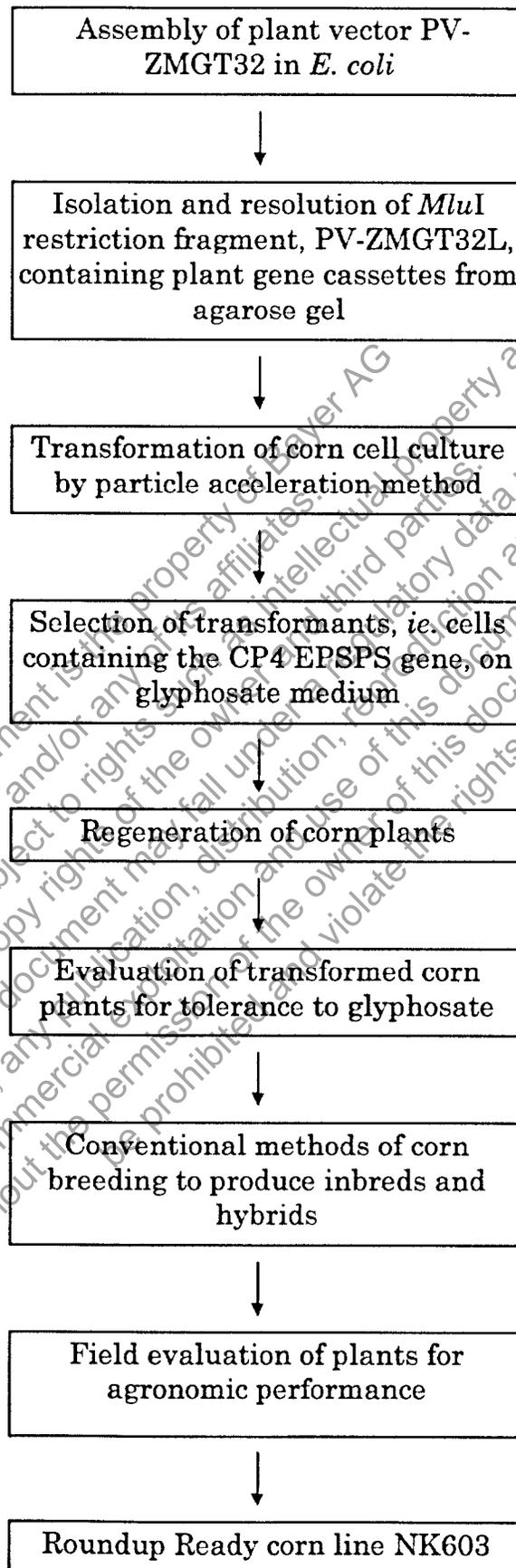
D. Regulatory sequences

In the first gene cassette, the CP4 EPSPS coding sequence is under the control of the 5' non-coding end of the rice actin 1 sequence (*ract1*) containing the promoter and first intron (McElroy *et al.*, 1990) introduced upstream of the CTP sequence. The second cassette contains the CP4 EPSPS coding sequence under the control of the enhanced CaMV 35S promoter (*e35S*) (Kay *et al.*, 1985; Odell *et al.*, 1985), which is approximately 0.6 kb in size. Located between the *e35S* promoter and the CP4 EPSPS sequence is the 0.8-kb intron from the corn *hsp70* (heat shock protein), present to increase the levels of gene transcription (Rochester *et al.*, 1986). In each cassette, the CP4 EPSPS sequence is joined to the 0.3-kb nopaline synthase 3' nontranslated sequence, NOS 3', from *Agrobacterium tumefaciens* (Fraley *et al.*, 1983) which provides the transcription termination and the mRNA polyadenylation signal.

An origin of replication sequence (*ori*) is also present in PV-ZMGT32 to allow for the replication of the plasmid in *E. coli* (Viera and Messing, 1987). Following the *ori* region is the sequence for the enzyme neomycin phosphotransferase type II (*nptII*). This enzyme confers resistance to aminoglycoside antibiotics (e.g., kanamycin and neomycin) and was used for selection of bacteria during the construction of the plasmid. The coding sequence for the *nptII* gene was derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982) and is present with its own bacterial promoter. Plasmid PV-ZMGT32 is shown in Figure 2.

The plasmid PV-ZMGT32 was amplified in *E. coli* and purified from bacterial lysates. The CP4 EPSPS gene expression linear DNA fragment was isolated from the plasmid prior to corn transformation experiments by digesting PV-ZMGT32 with the restriction enzyme *MluI*. The plasmid backbone (~2.6 kb) and the CP4 EPSPS expression cassettes (~6.7 kb) were separated by gel electrophoresis and the expression cassette fragment was electroeluted from a gel slice. The agarose gel-isolated *MluI* restriction fragment utilized in the transformation of Roundup Ready corn line NK603 was designated PV-ZMGT32L. The flowchart in Figure 4 illustrates the development of Roundup Ready corn line NK603.

Figure 4. Development of Roundup Ready corn line NK603.



V. Genetic Analysis and Agronomic Performance

A. Molecular characterization of Roundup Ready corn line NK603

Molecular analysis was performed to characterize the inserted DNA in Roundup Ready corn line NK603. Genomic DNA was analyzed using Southern blot analysis (Southern, 1975) to determine the insert number (number of integration sites within the corn genome), the copy number (the number of integrated linear DNA fragments used for transformation within one insertion site), the integrity of the inserted promoters, coding regions, and polyadenylation sequences, and the presence or absence of the plasmid backbone sequence. Polymerase chain reaction (PCR) (Saiki, 1990) was performed to verify the sequences at the 5' and 3' ends of the insert.

Data from the analyses support the following conclusions:

- (1) the genome of corn line NK603 contains a single DNA insertion;
- (2) within the single insert there is a single, complete copy of the DNA fragment, PV-ZMGT32L, used for transformation; the insertion also includes a non-functional, inversely linked 217-bp fragment of the enhancer region of the rice actin promoter at the 3' end;
- (3) both CP4 EPSPS gene cassettes within the single insert are intact; and,
- (4) the genome of corn line NK603 does not contain any detectable plasmid backbone DNA.

The 5' and 3' ends of the corn line NK603 insert were verified by PCR and DNA sequencing. All these data support the conclusion that only the full-length CP4 EPSPS protein should be encoded by the insert in line NK603. Expression of the full-length CP4 EPSPS protein in corn line NK603 has been confirmed by western blot analysis (Appendix).

1. Materials and methods

a. Test substance

The test substance for the molecular characterization study was the corn line NK603. Leaf tissue of the line was collected from plants grown under greenhouse conditions and treated with Roundup Ultra® (64 ounces/acre) at the V2-V3 stage (2-3 leaf collars).

b. Control substance

The control substance for this study was the non-transgenic corn

line LH82 x B73 (designated as B73). Leaf tissue of the line was collected from plants grown under greenhouse conditions.

c. Reference substances

The reference substances included the plasmid PV-ZMGT32 from which the DNA fragment used in the transformation of line NK603 was purified. DNA from the non-transgenic control line was mixed with the plasmid, digested, and separated by electrophoresis on agarose gels. The plasmid DNA served as a positive hybridization control and it was spiked into the non-transgenic control line genomic DNA at concentrations of approximately 0.5 and 1 copy of the plasmid DNA per copy of the genomic DNA to demonstrate the sensitivity of the Southern blotting method. Additional reference substances were MW size markers from Boehringer Mannheim (Indianapolis, IN) [MW Markers II (23.1-0.1 kb) and IX (1.4-0.072 kb)] and Gibco BRL (Gaithersburg, MD) [High MW DNA Marker (48.5-8.3 kb)].

d. DNA extraction

Corn leaf tissue (7.8-9.7 g) was ground to a fine powder using a pre-cooled mortar and pestle, and transferred to a 35-ml centrifuge tube. Sixteen milliliters of CTAB extraction buffer [1.5% (w/w) CTAB, 75 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 1.05 M NaCl, and 0.75% (w/w) PVP (MW 40,000)] was added to each tube and the tubes were incubated at 60°C for 60 min and then allowed to cool at room temperature for approximately 10 min. An equal volume (~16 ml) of chloroform:isoamyl alcohol (24:1) was added to each sample. The suspension was mixed by inversion of the tube several times and centrifuged for 10 min at approximately 8,800 x g at room temperature. The upper aqueous phase was transferred to a clean 35-ml centrifuge tube and the extraction with chloroform:isoamyl alcohol was repeated. The upper aqueous phase was transferred to a new tube, approximately 10 ml of isopropanol was added to each tube, and the contents of each tube were mixed by inversion. The samples were kept at approximately -20°C for at least 30 min. The samples were centrifuged at 14,000 x g for 20 min at 4°C to pellet the DNA and the supernatant was discarded. The pellet was re-dissolved in 2 ml of TE [10mM Tris-HCl pH 8.0, 1 mM EDTA] and transferred to a 13-ml tube. Approximately 20 µl of 10 mg/ml DNase-free RNase was added to each sample and the tubes were incubated at 37°C for 30 min. One milliliter of 7.5 M ammonium acetate was added to each tube and the contents were gently mixed. Approximately 2 volumes of 100% ethanol were added to each tube and the tubes were kept at -20°C for 2 h to overnight. The DNA was pelleted by centrifugation at 14,000 x g for 20 min at 4°C and subsequently washed with 70% ethanol, air dried, redissolved in 0.5 ml TE, pH 8.0, and stored at 4°C.

e. DNA quantitation and restriction enzyme digestion

Quantitation of the DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer (San Francisco, CA) with Boehringer Mannheim molecular size marker IX or plasmid pBR322 used as a calibration standard for quantitating genomic or plasmid DNA, respectively. Approximately 10 µg of genomic DNA from the line NK603 test and non-transgenic control lines was used for the restriction enzyme digests. Overnight digests were performed at 37°C in a total volume of 500 µl using 100 units of restriction enzyme. All restriction enzymes were purchased from Boehringer Mannheim. After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3 M NaOAc and 2 volumes (~1 ml relative to the original digest volume) of 100% ethanol, followed by incubation at -20°C for at least 1 h. The digested DNA was pelleted by centrifugation, washed with 75% ethanol, vacuum dried for approximately 10 min, and redissolved at room temperature in TE, pH 8.0.

f. Preparation of DNA probes

Plasmid DNA was isolated from *E. coli* cultures. DNA probe templates homologous to the full-length P-ract1/ract1 intron, the full-length e35S promoter, and the full-length NOS 3' polyadenylation sequence were prepared by PCR using plasmid PV-ZMGT32 as a template. The probe template for CTP2-CP4 EPSPS was prepared by PCR amplification of a CTP2-CP4 EPSPS fragment. The CTP2-CP4 EPSPS fragment was obtained by digestion of plasmid PV-ZMGT32 with restriction enzymes *EcoRI* and *NcoI* followed by gel purification. The probe template for the backbone sequence of plasmid PV-ZMGT32 was obtained by linearizing a plasmid which contains only this backbone sequence and subsequent agarose gel purification. This probe template was used to analyze corn line NK603 for the presence of any plasmid backbone sequence in the genome of the line. Linearized whole plasmid PV-ZMGT32 was also used to prepare the DNA probe for determining the insert number and copy number of line NK603. Approximately 25 ng of each probe template was labeled with ³²P using the random priming method (RadPrime DNA Labeling System, Gibco BRL). The NOS 3' polyadenylation sequence, however, was radioactively labeled by PCR amplification. The PCR reaction contained the following components in a total volume of 20 µl: 10 ng of template DNA, 10 mM Tris/HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.25 µM of each primer, 12.5 µM dCTP, dGTP and dTTP, 100 µCi (6000 Ci/mmol) of ³²P-dATP, and 2.5 units of *Taq* DNA polymerase. The cycling conditions were as follows: 1 cycle at 94°C for 3 min; 5 cycles at 94°C for 45 s, 52°C for 30 s, 72°C for 1.5 min; 1 cycle at 72°C for 10 min. The Hybaid Omn-E Thermal Cycler (Hybaid Limited, Middlesex, United Kingdom) was used for the PCR.

g. Southern blot analysis

Southern blot analyses were performed to characterize the DNA that was integrated into the corn genome. DNA samples digested with restriction enzymes were separated, based on size, using 0.6% agarose gel electrophoresis. A “long run” and a “short run” were performed for most gels. The “long run” samples (non-transgenic control and line NK603 test DNA) were loaded onto the gel and typically electrophoresed for 15 - 16 h at 35 V. High MW DNA Markers (400 ng) mixed with MW Marker II (1 µg) were the reference substance for the “long run”. The “short run” samples (non-transgenic control spiked with plasmid PV-ZMGT32 and line NK603 test DNA) and a reference substance [MW Marker II (1 µg) mixed with MW Marker IX (1 µg)] were then loaded onto the same gel and the gel was run for 5 - 6 additional h at 80 V. The long run allowed for greater separation of higher molecular weight DNAs while the short run allowed smaller molecular weight DNAs (<300 bp) to be retained on the gel. After photographing, the gel was placed in a depurination solution (0.125 N HCl) for approximately 10 min followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 min and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for ~30 min. The DNA from the agarose gels was transferred to Hybond-N™ nylon membranes (Amersham, Arlington Heights, IL) using a Turboblotter™ (Schleicher & Schuell, Keene, NH). The DNA was allowed to transfer for approximately 18 h (using 20X SSC as the transfer buffer) and covalently cross-linked to the membrane with a UV Stratalinker™ 1800 (Stratagene, La Jolla, CA) using the auto crosslink setting. The blots were pre-hybridized for at least 2 h in an aqueous solution containing 500 mM Na₂HPO₄·7H₂O, 7% SDS, and 0.1 mg/ml *Escherichia coli* (*E. coli*) tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 16-18 h at approximately 65°C. Membranes were washed in an aqueous solution of 0.1% SDS and 0.1X SSC for four ~20-min periods at approximately 65°C. Multiple exposures of blots were generated using Kodak Biomax MSTM film (Eastman Kodak, Rochester, NY) in conjunction with a Kodak Biomax MS™ intensifying screen.

h. Verification of the 5' and 3' flanking sequences

The sequences at the 5' and 3' ends of the line NK603 insert were verified with PCR using genomic DNA from corn line NK603 as a template. The PCR for the 5' junction was performed using one primer derived from the 5' genomic flanking sequence paired with a second primer located in the 5' end of the inserted DNA. This primer pair covered a 305-bp region. The PCR for the 3' junction was conducted using a primer derived from the 3' genomic flanking sequence paired with a second primer located in the NOS 3' polyadenylation sequence

at the 3' end of the insert. The amplified region was 299-bp long. The 5' and 3' PCR reactions contained the following components in a total volume of 50 μ l: 100 ng of genomic DNA, 10 mM Tris/HCl, 50 mM KCl, 1.25 mM MgCl₂, 200 μ M each dNTP, 0.2 μ M of each primer, and 2.5 units of *Taq* DNA polymerase. The cycling conditions were as follows: 1 cycle at 94°C for 3 min; 38 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1.5 min; 1 cycle at 72°C for 10 min, using a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA). A volume of 45 μ l of 5' and 3' PCR reactions was analyzed by 0.8% agarose gel electrophoresis with ethidium bromide staining. PCR products were purified from the gel, cloned into plasmid vector pCR[®]2.1-TOPO, using the TOPO[™] TA Cloning[®] Kit (Invitrogen, Carlsbad, CA), and transformed into TOP10 *E. coli* cells (Invitrogen). Plasmid DNAs were isolated from bacterial cultures, purified and subjected to DNA sequencing by the Monsanto Genomic Sequencing Center to further confirm the junction sequences.

2. Results and discussion

The following is a discussion of the results of the molecular characterization of Roundup Ready corn line NK603.

a. Determination of insert number

Insert number, the number of integration sites of transgenic DNA in the corn genome, was determined using Southern blot analysis. The test (NK603) and control (B73) genomic DNAs were digested with the restriction enzyme *Stu*I, which does not cleave within the DNA fragment used for transformation and would cut within the plant genomic DNA. This digestion should generate a single fragment containing the inserted DNA and adjacent plant genomic DNA from line NK603 if there is a single insertion in the corn genome. Non-transgenic genomic DNA spiked with plasmid PV-ZMGT32 was digested with both *Stu*I and *Sca*I. Since *Stu*I does not cleave within PV-ZMGT32, a second restriction enzyme, *Sca*I, was necessary to linearize the plasmid. The plasmid was linearized to facilitate its migration through the gel so that it could serve as an accurate size standard. The blot was probed with ³²P-labeled whole plasmid PV-ZMGT32 DNA (Figure 2), the source plasmid for the linear DNA fragment used in the transformation. The results are shown in Figure 5. The control DNA alone (lane 1) did not produce bands, as expected. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lanes 3 and 4) produced the expected band at approximately 9.3 kb, which corresponds to the size of the whole plasmid PV-ZMGT32 (Figure 2). Line NK603 DNA (lanes 2 and 5) produced one band of approximately 23 kb not present in the control line. This result suggests that corn line NK603 contains one insertion of integrated DNA located within a

23-kb *StuI* restriction fragment. Due to the size of the *StuI* restriction fragment, it is possible for more than one hybridizing band to be located within this fragment. However, other data support the conclusion of a single insert. When NK603 genomic DNA is digested with *XbaI*, a restriction enzyme that cleaves only once within the transformation cassette, two border fragments are produced when probed with PV-ZMGT32 (see the following section, determination of copy number). If there were more than one insert located within the 23-kb *StuI* fragment, more than two border fragments would be detected. Therefore, it is concluded that the genome of corn line NK603 contains one insert located within a 23-kb *StuI* restriction fragment.

b. Determination of copy number

The number of copies of DNA fragments used for transformation inserted into one locus was determined. Line NK603 test DNA, non-transgenic control DNA, and non-transgenic control DNA spiked with plasmid PV-ZMGT32 DNA were digested with the restriction enzyme *XbaI* followed by Southern blotting. The blot was probed with ³²P-labeled PV-ZMGT32. The linear DNA fragment used for corn transformation contains two *XbaI* sites (nucleotides 4082 and 4787, Figure 1) but experimental data and sequence analysis on this fragment indicated that one of these restriction sites (at nucleotide 4082) is methylated (data not shown) and thus, resistant to cleavage with *XbaI*. Therefore, the enzyme *XbaI* cuts only once in the linear DNA fragment and digestion should produce two fragments containing both inserted and flanking genomic DNA if line NK603 contains only one copy of the DNA fragment used for corn transformation. The results are shown in Figure 6. The non-transgenic control DNA alone (lane 1) did not produce any hybridizing bands, as expected. Plasmid PV-ZMGT32 DNA mixed with non-transgenic control DNA (lanes 3 and 4) produced the expected band at approximately 9.3 kb, the size of the whole plasmid PV-ZMGT32 (Figure 2). Line NK603 test DNA (lanes 2 and 5) produced two bands at approximately 9.0 and 5.8 kb. The presence of two hybridizing bands establishes that corn line NK603 contains only one copy of the transformation cassette at the locus of DNA integration.

c. Integrity of inserted gene cassettes

The integrity of the inserted gene cassettes was determined by analyzing the components of the cassettes: the promoters, coding regions and the polyadenylation sequences.

i. P-ract1/ract1 intron

To assess the integrity of the P-ract1/ract1 intron sequence, line NK603 test DNA, non-transgenic control DNA, and non-transgenic control DNA mixed with plasmid PV-ZMGT32 were digested with *EcoRV* followed by Southern blotting. The blot was probed with a mixture of the full length P-ract1/ract1 intron sequence labeled with ^{32}P dCTP and a 175-bp fragment derived from the 5' end of the P-ract1/ract1 intron labeled with ^{32}P dATP. The results are shown in Figure 7. The non-transgenic control DNA alone (lane 1) showed no hybridization signals, as expected. Plasmid PV-ZMGT32 DNA mixed with the non-transgenic control DNA (lanes 3 and 4) produced the expected band at ~3.8 kb containing the sequence of P-ract1/ract1 intron (Figure 2). Line NK603 test DNA (lanes 2 and 5) also produced the band of ~3.8 kb confirming the presence of the P-ract1/ract1 intron sequence in the inserted DNA. A band of ~0.2 kb was detected in the "short run" line NK603 DNA (lane 5) indicating the presence of an additional fragment containing sequence from the P-ract1/ract1 intron. The ~0.2 kb band was not detected in lane 2 because it was not retained on the gel after the "long run". (See section V.A.1.g for an explanation of "short run" and "long run" Southern blot analysis.) Further experiments which determined the sequence of the ends of the integrated DNA in line NK603 revealed that a 217-bp fragment containing a portion of the enhancer region of the rice actin promoter was present in the reverse orientation proximal to the 3' end of the transformation cassette, and that this small fragment maintained an *EcoRV* site 20-bp away from its 3' end bordering corn genomic sequence (Figure 13). These findings confirmed and explained the results from this Southern blot analysis.

This 217-bp fragment includes a polylinker sequence (50 bp) and the first 167 bp of the enhancer region of the rice actin promoter, position -835 to -669 from the start of transcription as defined by McElroy *et al.* (1990). Neither the TATA box nor transcriptional initiation site is present within the fragment, which suggests that this fragment should not function as a promoter. This is supported by the work of Zhang *et al.* (1991) and Wang *et al.* (1992) in which the researchers clearly demonstrated that the region including -835 to -669 does not behave as a promoter. Therefore, the 217-bp fragment at the 3' end of the NK603 is highly unlikely to act as a promoter.

ii. CTP2-CP4 EPSPS sequence

To assess the integrity of the CTP2-CP4 EPSPS sequence, line NK603 test DNA, non-transgenic control DNA, and non-transgenic control DNA mixed with plasmid PV-ZMGT32 DNA were digested with *EcoRV*

followed by Southern blotting. The blot was probed with the full-length CTP2-CP4 EPSPS fragment. The results are shown in Figure 8. Non-transgenic control DNA alone (lane 1) showed no hybridization bands, as expected. Plasmid PV-ZMGT32 DNA mixed with non-transgenic control DNA (lanes 3 and 4) and line NK603 test DNA (lanes 2 and 5) all produced the expected bands of ~3.8 kb and ~2.8 kb, corresponding to the correct sizes of the two fragments generated by *EcoRV* digestion, each containing a full-length sequence of CTP2-CP4 EPSPS (Figure 2). No unexpected bands were detected, establishing that corn line NK603 does not contain any additional, detectable CTP2-CP4 EPSPS sequence other than those in the two inserted EPSPS gene cassettes.

iii. e35S promoter

To assess the integrity of the e35S promoter, line NK603 test DNA, non-transgenic control DNA, and non-transgenic control DNA mixed with plasmid PV-ZMGT32 DNA were digested with *EcoRV* followed by Southern blotting. The blot was probed with the full length e35S promoter. The results are presented in Figure 9. The non-transgenic control DNA alone (lane 1) showed no hybridization signal, as expected. Plasmid PV-ZMGT32 DNA mixed with the non-transgenic control DNA (lanes 3 and 4) and line NK603 test DNA (lanes 2 and 5) all showed the expected bands of ~3.8 kb and ~2.8 kb, corresponding to the correct sizes of the two fragments generated by *EcoRV* digestion, each containing a portion of e35S sequence (Figure 2). The ~2.8-kb fragment represents hybridization of the probe to a small portion (91 bp) of the e35S promoter cleaved from the rest of the e35S promoter by *EcoRV*, as evidenced by its weaker hybridization signal than that of the ~3.8-kb band. No unexpected bands were detected, establishing that corn line NK603 does not contain any additional, detectable e35S sequence other than that in the inserted DNA fragment.

iv. NOS 3' polyadenylation sequence

To assess the integrity of the NOS 3' polyadenylation sequence, line NK603 test DNA, non-transgenic control DNA, and non-transgenic control DNA mixed with plasmid PV-ZMGT32 DNA were digested with *EcoRV* followed by Southern blotting. The blot was probed with the full-length NOS 3' polyadenylation sequence. The results are presented in Figure 10. The non-transgenic control DNA alone (lane 1) showed no hybridization signal, as expected. Plasmid PV-ZMGT32 DNA mixed with the non-transgenic control DNA (lanes 3 and 4) and the line NK603 test DNA (lanes 2 and 5) all showed the expected bands of ~3.8 kb and ~2.8 kb, corresponding to the correct sizes of the two fragments generated by *EcoRV* digestion, each containing a full-length NOS 3' transcription termination and polyadenylation sequence

(Figure 2). No unexpected bands were detected, establishing that corn line NK603 does not contain any additional, detectable NOS 3' polyadenylation sequence other than those in the two inserted CP4 EPSPS gene cassettes.

The above results establish that the two inserted CP4 EPSPS gene cassettes are intact in line NK603 (see insert map, Figure 13). In addition, a 217-bp fragment containing a portion of the enhancer region of the rice actin promoter is inversely linked to the 3' end of the inserted CP4 EPSPS gene cassettes.

d. Analysis for backbone fragments

To assess for the presence of backbone sequence, line NK603 test DNA, non-transgenic control DNA, and non-transgenic control DNA mixed with plasmid PV-ZMGT32 were digested with *Sac*I followed by Southern blotting. The blot was probed with the entire backbone sequence. The results are presented in Figure 11. Plasmid PV-ZMGT32 DNA mixed with the non-transgenic control DNA (lanes 3 and 4) produced one band of ~3.8 kb, the expected size for the backbone-containing fragment (Figure 2). The non-transgenic DNA alone (lane 1) and the line NK603 test DNA (lanes 2 and 5) showed no hybridization signals. This result establishes that corn line NK603 does not contain any detectable plasmid backbone fragments including *ori* and the *nptII* coding sequences, as expected, since a purified linear DNA fragment without backbone sequence was used for the transformation.

e. Verification of sequences at the 5' and 3' ends of NK603 insert

PCR was performed on genomic DNA to verify the sequences at the 5' and 3' ends of the line NK603 insert. Results of these PCR reactions are shown in Figure 12. The negative controls of distilled water, non-transgenic control line B73, and an unrelated transgenic line did not yield a PCR product when either the 5' or 3' primer pair was used (lanes 1, 2, 3, and 6, 7, 8, respectively). This demonstrates the specificity of the primer pairs for line NK603. Corn line NK603 genomic DNA yielded products of expected size of 305 bp for the 5' PCR (lane 4) and 299 bp for the 3' PCR (lane 9). In addition, purified products from the 5' and 3' PCR reactions were cloned into plasmid vector pCR®2.1-TOPO, and the resulting recombinant plasmids were separately transformed into TOP10 *E. coli* cells. After culture growth, plasmid DNAs were purified and sequenced. Sequence data from the plasmid DNAs confirmed the DNA sequences of the PCR products and thus the sequences of the 5' and 3' ends of the insert in corn line NK603.

Based on the results obtained from Southern blot analysis and PCR analysis, a predicted restriction map of the insert in corn line NK603 is shown in Figure 13.

3. Conclusions

The Roundup Ready (CP4 EPSPS) corn line NK603 was produced by particle acceleration technology using a linear DNA fragment, PV-ZMGT32L, which contains two CP4 EPSPS gene cassettes. Corn line NK603 contains one insertion of the integrated DNA located within a 23-kb *Stu*I restriction fragment. This insert contains one complete copy of the fragment used in transformation and 217 bp of the enhancer region of the rice actin promoter. The individual genetic components in each of the two CP4 EPSPS gene cassettes in the integrated DNA are intact. The extra 217-bp fragment of the enhancer region of the rice actin promoter inversely linked to the 3' end of the transformation cassette does not contain any defined elements required to promote gene expression and thus is highly unlikely to act as a promoter. The genome of line NK603 does not contain any detectable plasmid backbone DNA including *ori* or the *nptII* coding sequence. Sequences of the 5' and 3' ends of the insert were confirmed by PCR amplification. These data establish that only the expected full-length CTP2-CP4 EPSPS protein should be encoded by the insert in Roundup Ready corn line NK603.

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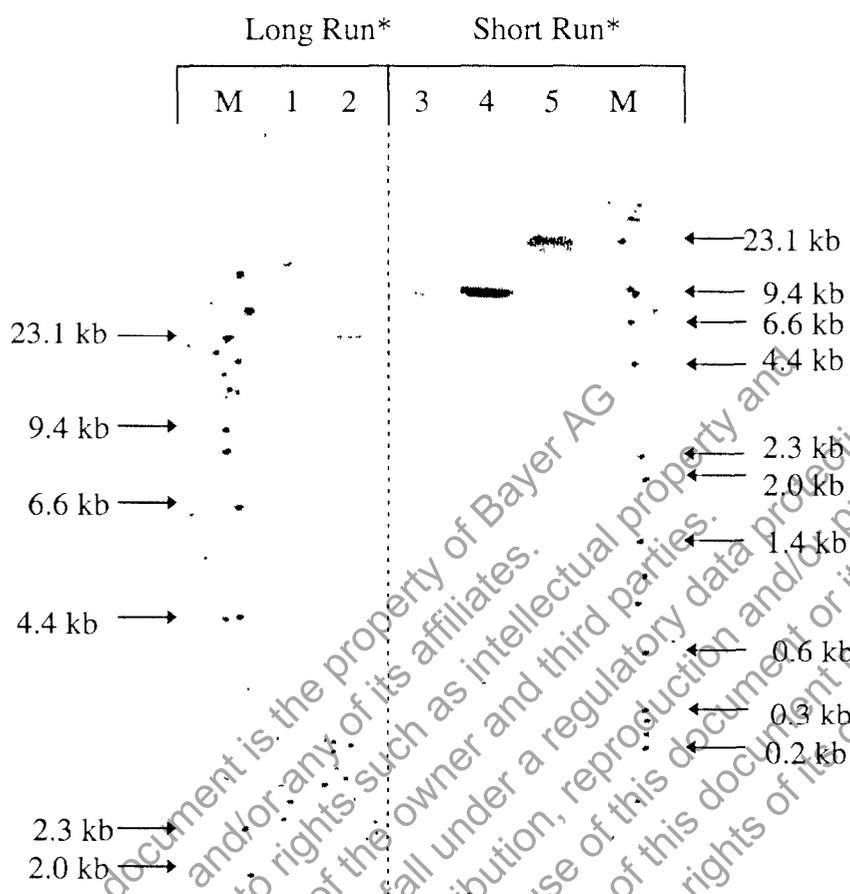


Figure 5. Southern blot analysis of Line NK603: determination of insert number.
 Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *StuI*. Plasmid PV-ZMGT32 DNA mixed with B73 DNA was digested with *StuI* and *ScaI*. DNA samples were separated by gel-electrophoresis, blotted and then probed with ³²P-labeled plasmid PV-ZMGT32. Lane designations are as follows:
 Lane 1: B73 DNA (Long Run)
 2: NK603 DNA (Long Run)
 3: B73 DNA spiked with 14.5 pg PV-ZMGT32 (Short Run)
 4: B73 DNA spiked with 29 pg PV-ZMGT32 (Short Run)
 5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel.
 M denotes MW marker lanes (see Section V.A.1.g. for details).

- See Section V.A.1.g. for explanation of “long run” and “short run” Southern blot analysis.

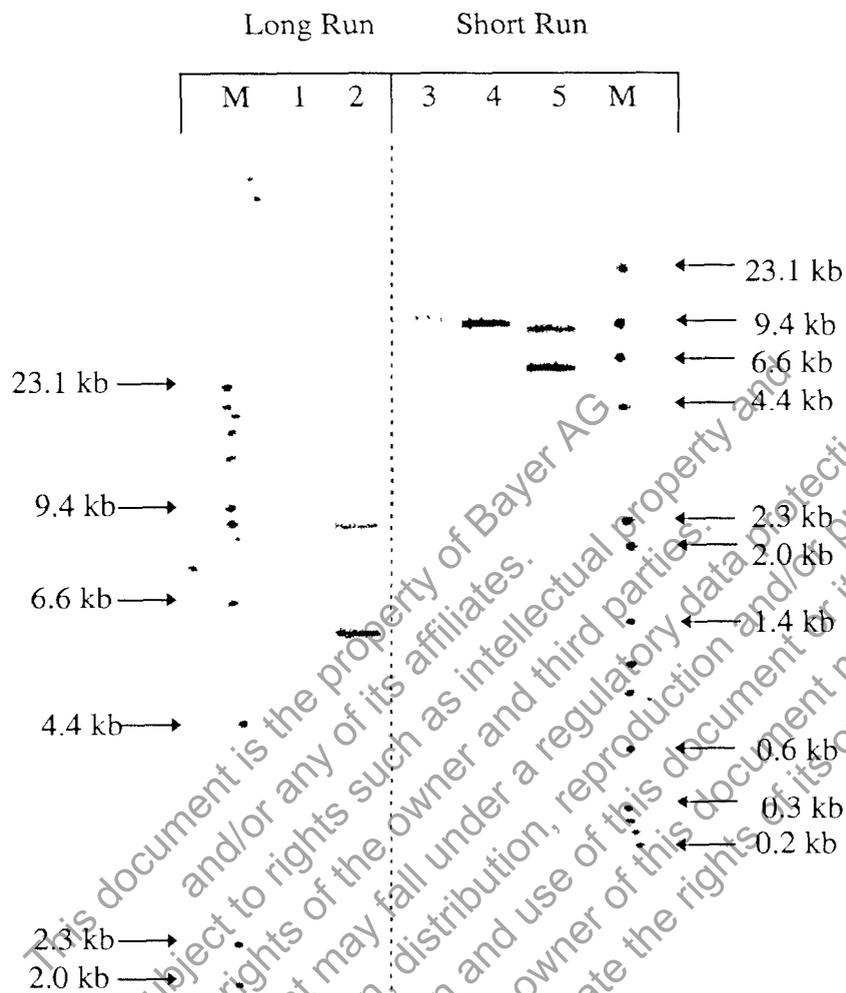


Figure 6. Southern blot analysis of Line NK603: determination of copy number.

Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Xba*I. The DNA samples were separated by gel-electrophoresis, blotted and then probed with ³²P-labeled plasmid PV-ZMGT32. Lane designations are as follows:

Lane 1: B73 DNA (Long Run)

2: NK603 DNA (Long Run)

3: B73 DNA spiked with 14.5 pg PV-ZMGT32 (Short Run)

4: B73 DNA spiked with 29 pg PV-ZMGT32 (Short Run)

5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel. M denotes MW marker lanes (see Section V.A.1.g. for details).

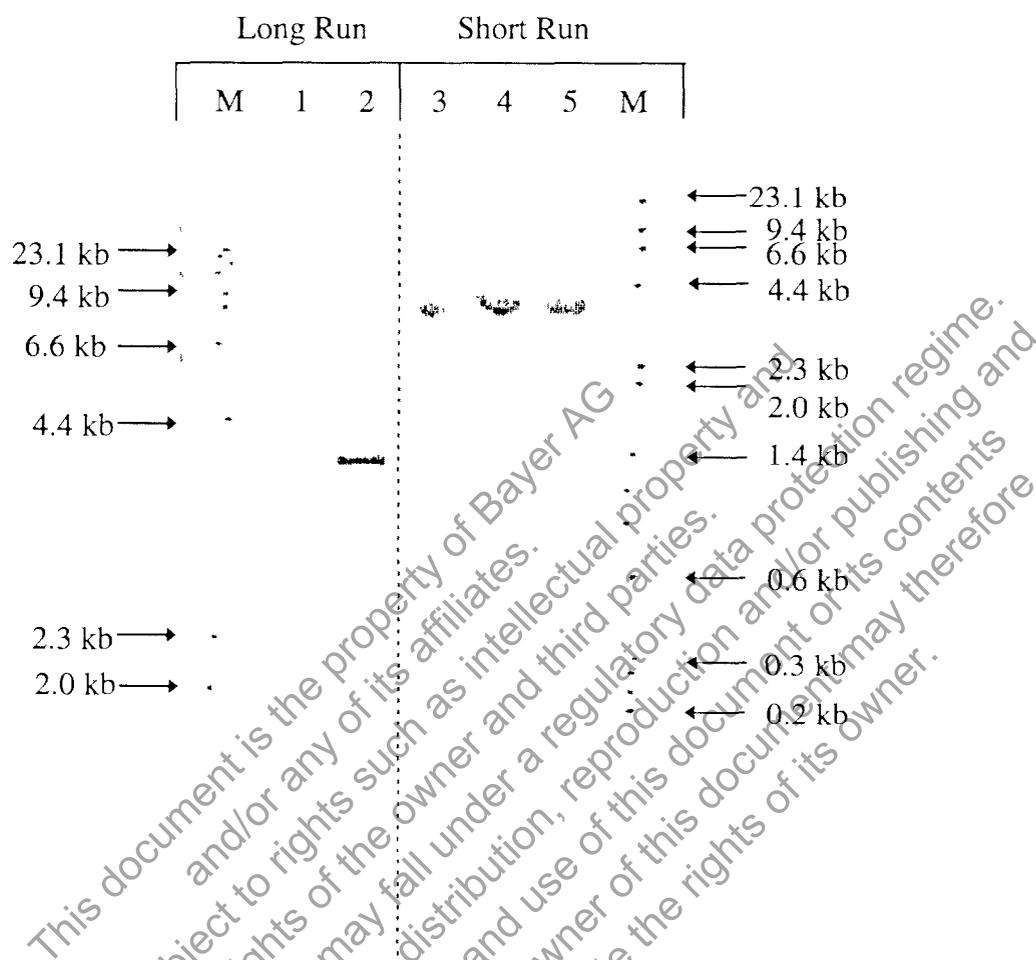


Figure 7. Southern blot analysis of Line NK603: P-ract1/ract1 intron. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *EcoRV*. DNA samples were separated by gel-electrophoresis, blotted and then hybridized with a mixture of two probes: the full-length P-ract1/ract1 intron labeled with ³²P-dCTP and a 175-bp fragment of the P-ract1/ract1 intron labeled with ³²P-dATP. Lane designations are as follows:

- Lane 1: B73 DNA (Long Run)
- 2: NK603 DNA (Long Run)
- 3: B73 DNA spiked with 14.5 pg PV-ZMGT32 (Short Run)
- 4: B73 DNA spiked with 29 pg PV-ZMGT32 (Short Run)
- 5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel. M denotes MW marker lanes (see Section V.A.1.g. for details).

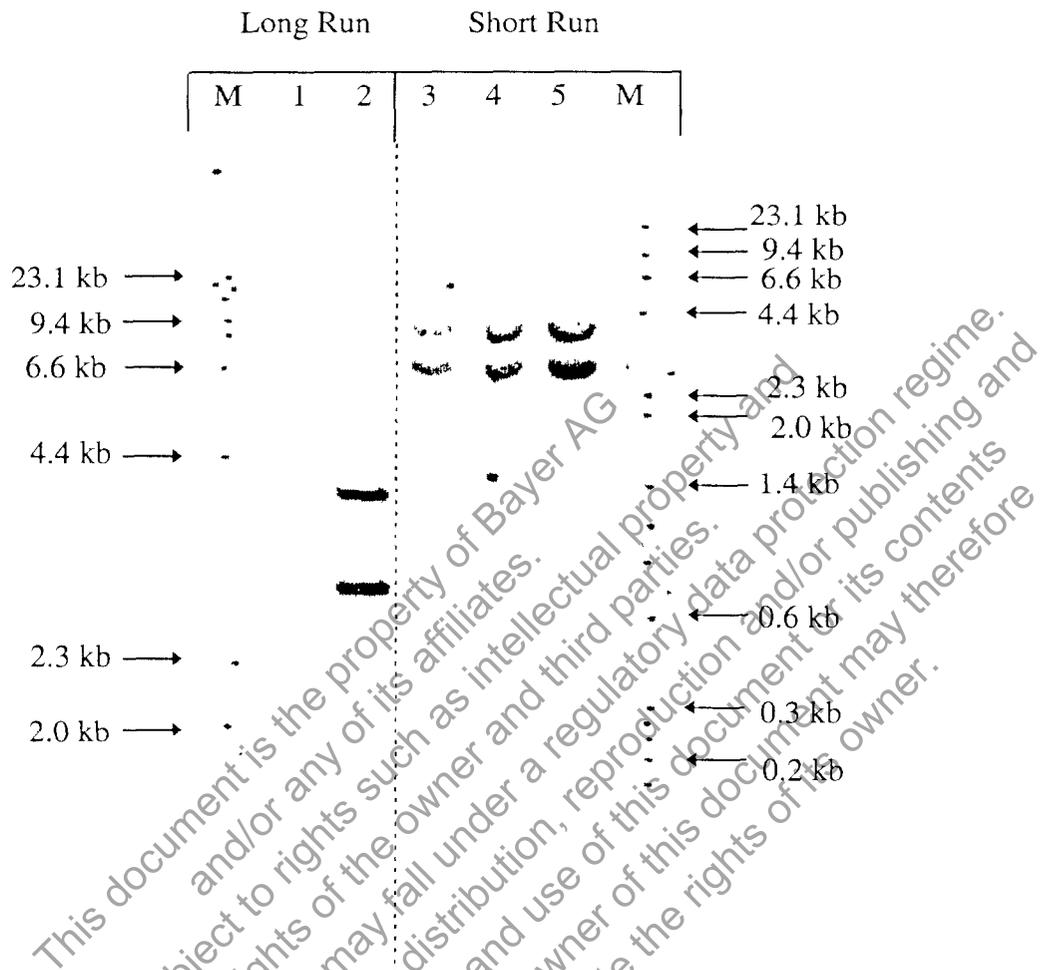


Figure 8. Southern blot analysis of Line NK603, CTP2-CP4 EPSPS sequence. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *EcoRV*. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ³²P labeled CTP2-CP4 EPSPS fragment. Lane designations are as follows:

- Lane 1: B73 DNA (Long Run)
- 2: NK603 DNA (Long Run)
- 3: B73 DNA spiked with 14.5 pg PV-ZMGT32 (Short Run)
- 4: B73 DNA spiked with 29 pg PV-ZMGT32 (Short Run)
- 5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel. M denotes MW marker lanes (see Section V.A.1.g. for details).

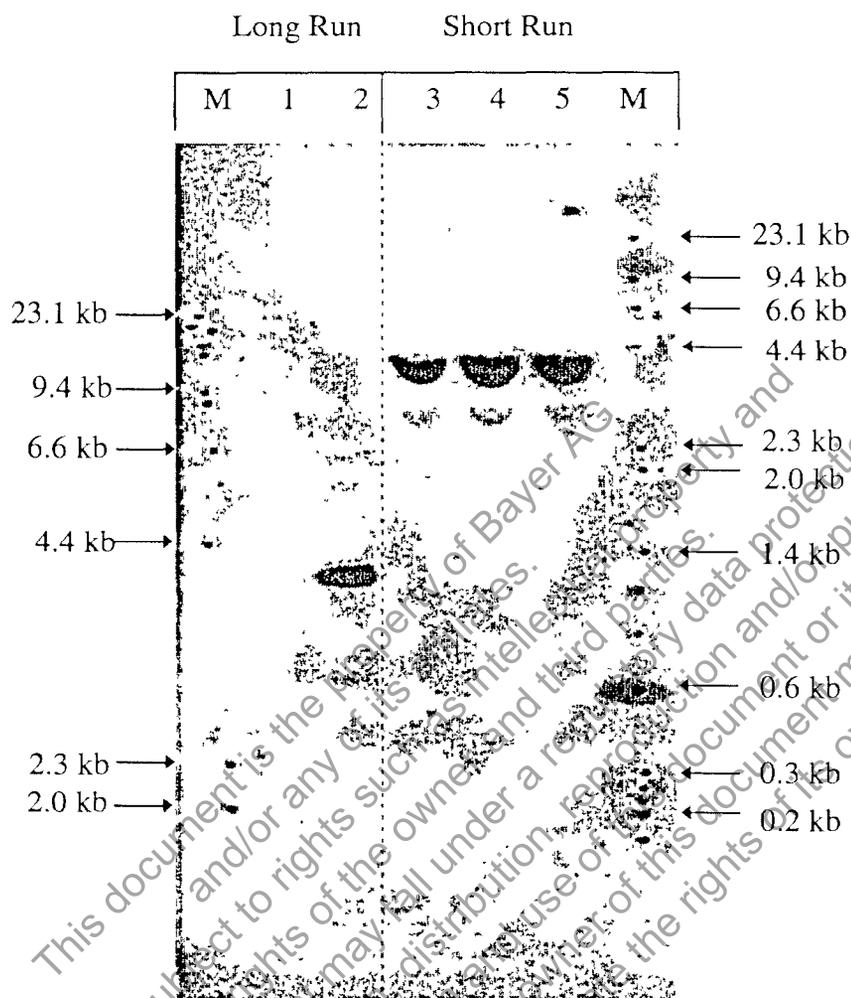


Figure 9. Southern blot analysis of Line NK603: e35S promoter. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *EcoRV*. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ^{32}P -labeled e35S fragment. Lane designations are as follows:

- Lane 1: B73 DNA (Long Run)
- 2: NK603 DNA (Long Run)
- 3: B73 DNA spiked with 14.5 μg PV-ZMGT32 (Short Run)
- 4: B73 DNA spiked with 29 μg PV-ZMGT32 (Short Run)
- 5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel.
M denotes MW marker lanes (see Section V.A.1.g. for details).

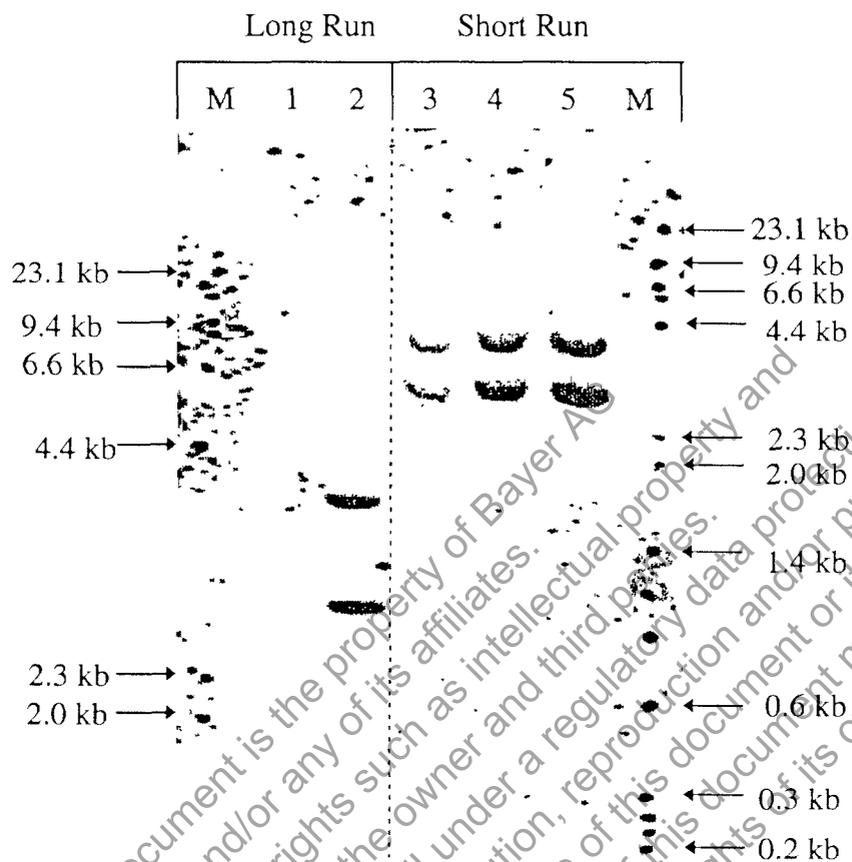


Figure 10. Southern blot analysis of Line NK603: NOS 3' polyadenylation sequence. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *EcoRV*. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ³²P-labeled NOS 3' polyadenylation sequence fragment. Lane designations are as follows:

- Lane 1: B73 DNA (Long Run)
- 2: NK603 DNA (Long Run)
- 3: B73 DNA spiked with 14.5 pg PV-ZMGT32 (Short Run)
- 4: B73 DNA spiked with 29 pg PV-ZMGT32 (Short Run)
- 5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel. M denotes MW marker lanes (see Section V.A.1.g. for details).

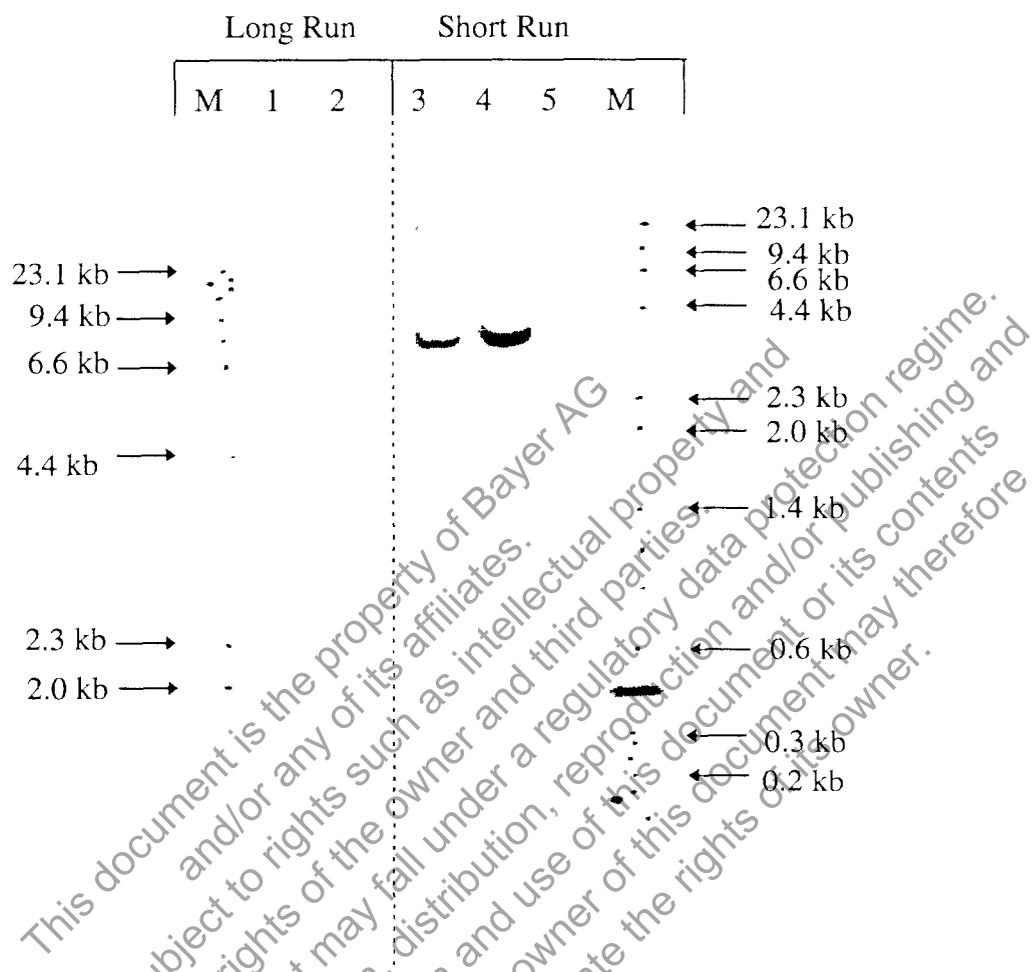


Figure 11. Southern blot analysis of Line NK603: backbone analysis. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Sac*I. The DNA samples were separated by gel-electrophoresis, blotted and then probed with ³²P-labeled entire backbone sequence consisting of the *ori* and *nptII* coding regions. Lane designations are as follows:

- Lane 1: B73 DNA (Long Run)
- 2: NK603 DNA (Long Run)
- 3: B73 DNA spiked with 14.5 µg PV-ZMGT32 (Short Run)
- 4: B73 DNA spiked with 29 µg PV-ZMGT32 (Short Run)
- 5: NK603 DNA (Short Run)

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel. M denotes MW marker lanes (see Section V.A.1.g. for details).

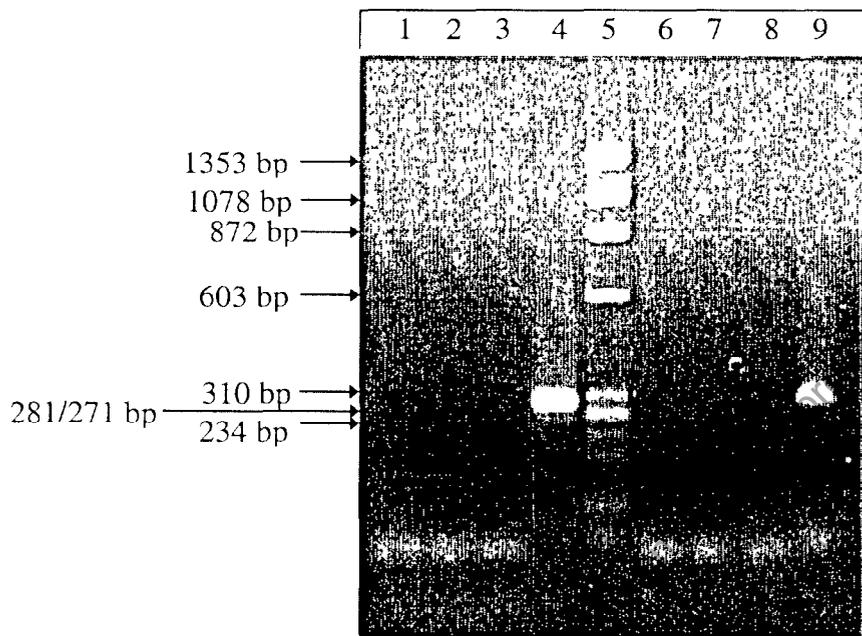


Figure 12. PCR verification of sequences at the 5' and 3' ends of Line NK603 insert.

PCR was performed using primers specific to the 5' and 3' flanking sequences for NK603 insert on genomic DNA extracted from lines B73 (non-transgenic control), an unrelated transgenic line, and NK603. The PCR primer pair for the 5' junction covered a 305-bp region and that for the 3' junction covered a 299-bp region. A volume of 13.5 μ l of reaction products was loaded in each lane. Lane designations are as follows:

- Lane 1: 5' PCR, no template control
- 2: 5' PCR, B73 (non-transgenic)
- 3: 5' PCR, an unrelated transgenic line
- 4: 5' PCR, NK603
- 5: MW Marker IX (500 ng)
- 6: 3' PCR, no template control
- 7: 3' PCR, B73 (non-transgenic)
- 8: 3' PCR, the unrelated transgenic line
- 9: 3' PCR, NK603

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel.

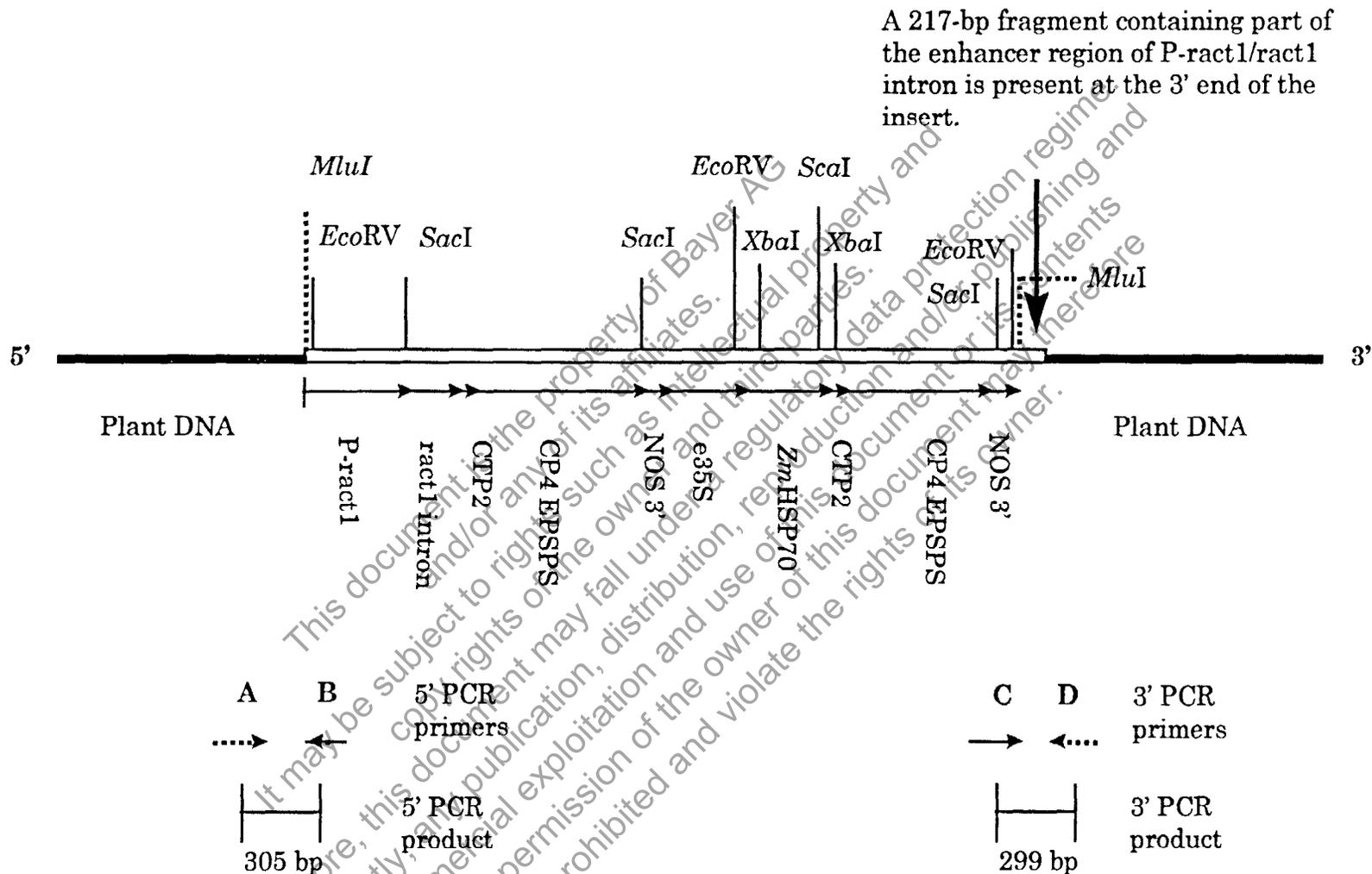


Figure 13. Schematic representation of the NK603 insert. This figure depicts the predicted insert in corn line NK603 based on data from Southern blot analysis and PCR confirming the sequences at the 5' and 3' ends of the insert. There is one complete copy of the PV-ZMGT32L fragment that was used in corn transformation to generate the line NK603.

B. Segregation data and stability of gene transfer of Roundup Ready corn line NK603

1. Segregation data and stability of corn line NK603

Segregation data for nine generations of line NK603 progeny are presented in Table 2. Data are presented for the BC0F1 generation (derived from crossing the R0 with the public inbred line "B73"), the BC1F1 generation (derived from crossing the BC0F1 plants with B73), the BC2F1, BC3F1, BC4F1, BC5F1, BC2F2 generation (derived from selfing individual BC2F1 plants), the BC2F3 generation (derived from selfing individual BC2F2 plants) and BC4F3 generation.

Statistical significance for the segregation data was determined using Chi square analysis. For these analyses a Chi square value (χ^2) was determined as follows: $\chi^2 = \sum [(|o-e|-0.5)^2/e]$ where o = observed frequencies for each class, e = expected frequencies for each class, and 0.5 = Yates correction factor for Chi square analysis with one degree of freedom (df) (Little and Hills, 1978). Yates correction factor was not used for Chi square analyses with two degrees of freedom. The calculated Chi square value was compared to a table of Chi square to determine whether the observed frequencies fit the expectation for a single insert at $p = 0.05$ and/or $p = 0.01$.

All generations segregated as expected for a single insertion site, except for the BC2F1 generation. The higher than predicted number of positive (CP4 gene containing) plants in the BC2F1 generation can be explained by gamete selection as a result of high application rates of glyphosate in the generation prior to the BC2F1 (i.e., BC1F1). Preferential selection for positive gametes has been documented in hemizygous herbicide-tolerant plants when the selective herbicide (e.g., glyphosate) is applied.

The Chi square analysis of the segregation results are consistent with a single active site of insertion of the CP4 EPSPS into the genomic DNA of Roundup Ready corn line NK603, segregating according to Mendelian genetics. These results are consistent with the genetic analysis described in Section V. The stability of the insert has been demonstrated through six generations of crossing and three generations of self pollination.

Table 2. Segregation data and analysis of progeny of Roundup Ready corn line NK603

Generation	Observed ¹			Expected			ChiSq
	Positive	Negative	Segregating	Positive	Negative	Segregating	
BC0F1	14	15		14.5	14.5		0.00 ^{ns}
BC1F1	32	23		27.5	27.5		1.16 ^{ns}
BC2F1	135	81		108.0	108.0		13.00 ^{**}
BC2F2	86	26		84.0	28.0		0.12 ^{ns}
BC2F3	9	16	24	12.3	12.3	24.5	2.02 [#]
BC3F1	44	45		44.5	44.5		0.00 ^{ns}
BC4F1	127	103		115.0	115.0		2.30 ^{ns}
BC4F3	12	5	17	8.5	8.5	17.0	2.88 [#]
BC5F1	26	35		30.5	30.5		1.05 ^{ns}

¹ Data expressed as number of positive and negative plants based on glyphosate sprays, except for the BC2F3 and BC4F3 generations where data are number of homozygous positive ear-rows, number of homozygous negative ear-rows and number of segregating ear-rows based on glyphosate sprays.

^{ns} not significant at p=0.05 (chi square = 3.84, 1 df).

[#] not significant at p=0.05 (chi square = 5.99, 2 df).

^{**} significant at p = 0.01 (chi square = 6.63, 1 df).

2. Corn line NK603 generation stability: Southern blot analysis

Southern blot analysis was also conducted to assess the stability of the inserted DNA in line NK603. Genomic DNA extracted from leaf tissues of the F1 generation (the progeny of the R0 back-crossing) and the fifth generation of back-crossing (BC5F1) of line NK603 were digested with *EcoRV*, blotted and probed with the full-length CTP2-CP4 EPSPS fragment. The results are presented in Figure 14. The non-transgenic control DNA (lane 1) showed no hybridization signals. Plasmid PV-ZMGT32 DNA mixed with the non-transgenic control DNA (lane 2), line NK603 F1 DNA (lane 3) and line NK603 BC5F1 DNA (lane 4) all produced the expected bands of ~3.8 kb and ~2.8 kb, each carrying the sequence of CTP2-CP4 EPSPS. No significant differences in banding pattern were observed between DNA extracted from the F1 generation and that from the BC5F1 generation of line NK603. This demonstrates the stability of the inserted DNA in samples spanning five generations.

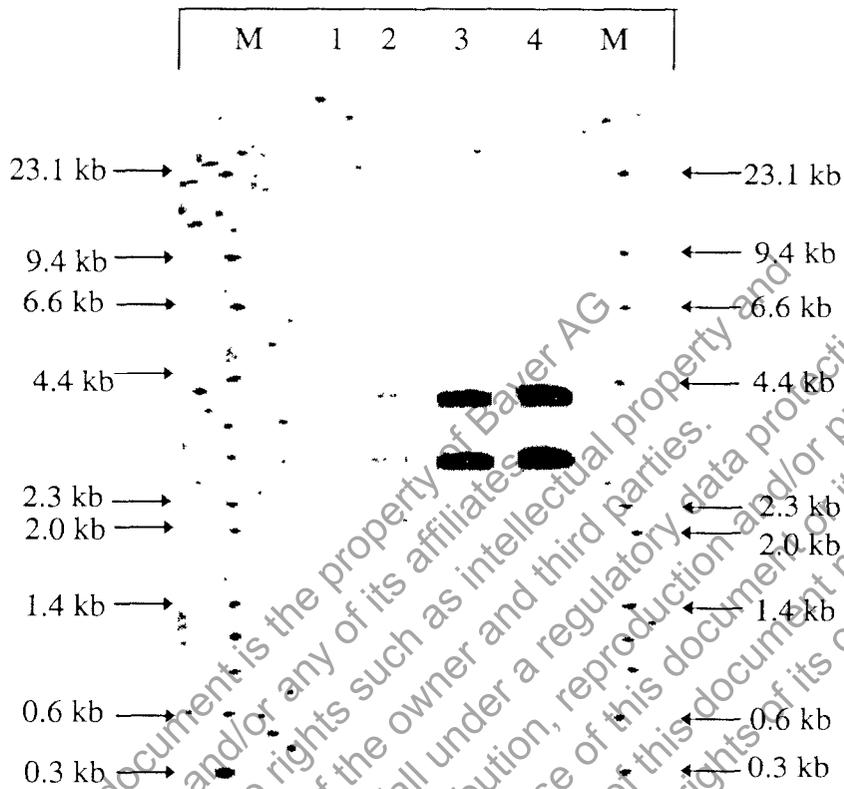


Figure 14. Southern blot analysis of Line NK603: stability of the inserted DNA. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *EcoRV*. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ³²P-labeled CTP2-CP4 EPSPS fragment. Lane designations are as follows:
 Lane 1: B73 DNA
 2: B73 DNA spiked with 29 µg PV-ZMGT32
 3: NK603 F1 DNA
 4: NK603 BC5 DNA

→ Symbol denotes sizes obtained from MW markers on ethidium bromide-stained gel. M represents MW marker lanes [MW Marker II (1 µg) mixed with MW Marker IX (1 µg)].

C. Expression of the inserted CP4 EPSPS gene

1. Introduction

A western blot analysis (Appendix) was conducted to assess the equivalence of CP4 EPSPS protein produced by Roundup Ready corn line NK603 with CP4 EPSPS protein produced in *E. coli* and in commercial Roundup Ready Soybeans. This western blot showed that the CP4 EPSPS protein produced in corn line NK603, and in *E. coli* and in commercial Roundup Ready Soybeans are equivalent to each other and that only one immuno-reactive protein of the expected apparent molecular weight is found in crude extracts of Roundup Ready corn line NK603 tissue. This equivalence justified the use of the *E. coli*-produced protein as a reference standard in the ELISA (Enzyme Linked Immuno-Sorbent Assay) assay used to estimate the expression levels of the CP4 EPSPS in line NK603, as described in the following section.

2. Expression levels of the CP4EPSPS protein in Roundup Ready corn line NK603

Levels of the CP4 EPSPS protein were estimated in forage and grain samples collected from six non-replicated and two replicated field sites during the 1998 growing season. The six non-replicated trials were conducted at the following locations: Richland, Iowa; Webster City, Iowa; Bagley, Iowa; Carlyle, Illinois; Indianapolis, Indiana; and Wichita, Kansas. Two replicated trials, in Jerseyville, Illinois, and New Holland, Ohio, were utilized in the assessment of CP4 EPSPS expression. Forage and grain samples collected from line NK603 and a nontransgenic parental control line (LH82 x B73) were analyzed using an enzyme-linked immunosorbent assay (ELISA) (Harlow and Lane, 1988) to estimate the levels of CP4 EPSPS protein present in these tissues.

CP4 EPSPS protein levels in forage and grain extracts were estimated using a double antibody sandwich ELISA consisting of a monoclonal anti-CP4 EPSPS antibody as the capture antibody and a polyclonal anti-CP4 EPSPS conjugated to horseradish peroxidase (HRP) as the detection antibody. A horseradish peroxidase substrate, TMB (3,3',5,5' tetramethylbenzidine), was added for color development. The CP4 EPSPS protein levels in plant tissue extracts were quantified by comparison of the sample absorbance (OD) to the absorbance produced by a range of concentrations of the *E. coli*-produced CP4 EPSPS reference standard. The CP4 EPSPS protein standard was purified from an *E. coli* strain expressing the *Agrobacterium* sp. strain CP4 EPSPS gene. The protein standard has been previously characterized (Harrison *et al.*, 1993).

The CP4 EPSPS protein levels (corrected for assay bias) estimated in corn forage and grain samples for Roundup Ready corn line NK603 are summarized in Table 3. Mean CP4 EPSPS protein levels in line NK603 forage were comparable for the non-replicated sites (25.5 $\mu\text{g/g}$ fw) and replicated sites (25.9 $\mu\text{g/g}$ fw). CP4 EPSPS protein levels in control forage were, as expected, below the Limit of Quantitation of the assay (<0.05 $\mu\text{g/g}$ fw). Mean CP4 EPSPS protein levels in line NK603 grain were comparable for the non-replicated sites (11.0 $\mu\text{g/g}$ fw) and replicated sites (10.6 $\mu\text{g/g}$ fw). CP4 EPSPS protein levels in control grain were, as expected, below the Limit of Quantitation of the assay (<0.09 $\mu\text{g/g}$ fw). Therefore, it is concluded that the CP4 EPSPS protein introduced into Roundup Ready corn line NK603 is expressed at approximately the same levels within site or across geographically dispersed sites. This low level of CP4 EPSPS protein expression in line NK603 is sufficient to confer tolerance to glyphosate, the active ingredient in Roundup herbicide.

Table 3. Summary of CP4 EPSPS protein levels measured by the ELISA in tissues of line NK603 corn plants ($\mu\text{g/g}$ fresh weight)

Sites	Parameter	Forage ^{a,c} ($\mu\text{g/g}$ fw)	Grain ^{b,c} ($\mu\text{g/g}$ fw)
Non-replicated	mean	25.5	11.0
	range	18.0 - 31.2	6.9 - 15.6
	SD	4.5	3.2
Replicated	mean	25.9	10.6
	range	25.7 - 26.1	9.8 - 11.3
	SD	0.3	1.0
All sites	mean	25.6	10.9
	range	18.0 - 31.2	6.9 - 15.6
	SD	3.8	2.6

SD = Standard Deviation.

^aLimit of Quantitation = 0.05 $\mu\text{g/g}$ fw.

^bLimit of Quantitation = 0.09 $\mu\text{g/g}$ fw.

^cValues for all control samples below the Limit of Quantitation of the assay.

D. CP4 EPSPS protein specificity and homology to EPSPSs derived from a variety of plant and microbial sources.

The CP4 EPSPS gene was sequenced from the naturally occurring glyphosate-degrading bacterium identified as *Agrobacterium* sp. strain CP4 and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids. CP4 EPSPS catalyses a non-rate limiting step in the shikimate pathway involved in aromatic amino acid biosynthesis in plants. The enzymatic activity of EPSPSs from a variety of glyphosate tolerant and sensitive plant and microbial sources has been extensively characterized. It has been established that CP4 EPSPS is highly specific for its natural substrates, shikimate-3-phosphate and phosphoenolpyruvate (██████████, 1996; Gruys and Sikorski, 1999). This characterization included an examination of the three-dimensional folding patterns and active site homology. The EPSPS derived from *Agrobacterium* sp. strain CP4 produced in Roundup Ready corn is functionally and similar to the native corn EPSPS, but retains its catalytic activity in the presence of the inhibitor, glyphosate (Padgett *et al.*, 1993). The shikimate pathway is not present in mammals, which contributes to the favorable toxicology profile for glyphosate.

EPSPSs are ubiquitous in plants and microbes and not considered to be a protein component that confers a plant pest property. The CP4 EPSPS protein is homologous to EPSPSs naturally present in soybean and corn, which are planted in large acreages in the U.S., as well as in ubiquitous fungal and microbial sources such as *E. coli*, Baker's yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis* (Mountain, 1989), as shown in the table below (Padgett *et al.*, 1996). The ubiquitous presence of homologous EPSPS enzymes in food crops and common microbes establishes that EPSPS proteins and their enzyme activity pose no hazards for human consumption or to the environment. Crops containing CP4 EPSPS should be determined to be as safe as crops containing EPSPS generally and as safe as crops which already contain CP4 EPSPS, including Roundup Ready soybean, canola, sugar beet and cotton.

Comparison of the deduced amino acid sequence of CP4 EPSPS to that of other EPSPSs

	soybean	corn	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. cerevisiae</i>	Roundup Ready Crops*
CP4 EPSPS						
% sequence identity	26	24	26	41	30	100
% sequence similarity	51	49	52	59	54	100

*Roundup Ready soybean, cotton, and sugar beet.

E. Disease and pest susceptibilities

Roundup Ready corn line NK603 has been field tested in the United States since 1997, under the USDA notifications listed in Table 4.

Field data reports have been submitted to the attention of Mrs. Dianne Hatmaker of APHIS for field trials conducted in 1997 and 1998 as directed in the USDA guidance. Final reports have not been submitted for field trials conducted in 1999 or for the field trials which are currently ongoing for the 1999-2000 winter nursery season.

Monitoring for the disease and insect susceptibilities of the Roundup Ready corn line NK603 when compared to nontransgenic plants was performed one or more times per season at each of these locations. No differences in disease severity or insect infestations were detected between Roundup Ready corn line NK603 and non-genetically modified control plants. Insects observed in the field included european corn borer (*Ostrinia nubilalis*), thrips (*Anaphothrips and Frankliniella* sp.), corn earworm (*Heliothis zea*), corn rootworm beetles (*Diabrotica* sp.), beet armyworm (*Spodoptera* sp.), fall armyworm (*Spodoptera frugiperda*), southwestern corn borer (*Diatraea* sp.), grasshoppers (*Melanoplus* sp.) and aphids (*Rhopalosiphum maidis*). Diseases observed in the field included smut (*Ustilago* sp), maize mosaic virus (MMV), gray leaf spot disease (*Cercospora zea-maydis*), Stewart's leaf blight (*Erwinia stewartii*), and Southern Rust (*Puccinia polysora*).

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Table 4. USDA notifications relevant to the field testing of Roundup Ready corn line NK603.

Year	USDA Reference No.	Effective Date	Release Sites (by State) Covered by Notification
1997 Field Trials			
	97-099-07n*	05/08/1997	HI, PR
	97-099-08n*	05/09/1997	AL, CT, IA, IL
1998 Field Trials			
	98-036-05n*	03/07/1998	CT, HI, IL
	98-040-03n*	03/17/1998	HI, IA, PR
	98-049-05n*	03/23/1998	IL, PR
	98-085-29n*	05/04/1998	IA, IL, IN, KS, MO, OH, TX
1998/1999 Winter Trial			
	98-252-06n	10/09/1998	PR
1999 Field Trials			
	98-294-06n	11/20/1998	IA, PR
	98-294-07n	11/20/1998	HI, IA, IL, IN, PR
	98-294-08n	11/20/1998	HI, IL
	98-300-01n	12/07/1998	IA, IL, MN, PR
	98-300-02n	11/26/1998	HI, IA
	98-300-03n	11/20/1998	IL, IN, PR
	98-300-04n	11/26/1998	HI, NE
	98-300-05n	11/26/1998	HI, IL, NE, OH, WI
	98-310-05n	12/06/1998	PR
	98-310-06n	12/06/1998	IA, IL, PR
	98-328-01n	01/12/1999	HI, IN
	98-350-02n	01/15/1999	HI, PR
	99-033-04n	03/04/1999	HI, IA
	99-069-13n	04/09/1999	IA, IL
	99-071-33n	04/11/1999	OH
	99-071-34n	04/19/1999	IA, IL, KS, MO, NE
	99-071-35n	04/29/1999	IN, MD, OH, PA
	99-126-22n	05/12/1999	IA, NE
1999/2000 Winter Trial			
	99-215-06n	09/07/1999	FL, HI, PR, TN
1999/2000 Winter Trials - for Seed Partners			
	99-221-05n	09/08/1999	HI, IL
	99-239-04n	09/26/1999	TX
	99-252-02n	10/12/1999	PR
	99-252-03n	10/12/1999	HI, IA
	99-252-04n	10/07/1999	HI, PR
	99-252-05n	10/12/1999	HI, NE
	99-252-06n	10/15/1999	IA, IL, MN, PR
	99-267-01n	10/25/1999	HI, IL, IN, PR
	99-272-04n	10/29/1999	HI, IA
	99-280-06n	11/06/1999	HI, IN
	99-312-02n	12/08/1999	IL, WI
	99-312-03n	12/09/1999	HI, IA, IN, MN

*Final field reports submitted to Dianne Hatmaker, USDA-APHIS.

F. Lack of toxicants in corn

Corn (*Zea mays* L.) has a long history of safety in terms of production in the environment as well as food and feed use. Toxicants are not considered a significant component of sound corn (White and Pollak, 1995; Watson, 1987). Compositional analysis of nutritional fractions of Roundup Ready corn line NK603 is being provided to the FDA as per FDA Food Policy (1992) which recommends that key compositional components of genetically modified plant varieties be assessed prior to commercial introduction. Monsanto has performed extensive analytical studies which demonstrated that the grain and forage of Roundup Ready corn line NK603 are compositionally equivalent to the parental variety and other corn hybrids grown commercially. Compositional analyses included proximates (protein, fat, ash, carbohydrates and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids, fatty acids, vitamin E and mineral content, and certain anti-nutritional factors in line NK603 grain. Line 603 forage was also analyzed for proximate, ADF and NDF content.

G. Agronomic characteristics of Roundup Ready corn line NK603

In-crop postemergence application of Roundup Ultra® at 24 to 32 ounces/acre provides control of a broad range of monocot and dicot weed species including foxtail (*Setaria* sp.), panicum (*Panicum* sp.), velvetleaf (*Abutilon theophrasti*) pigweed (*Amaranthus* sp.) and morningglory (*Ipomoea* sp.). Both the antecedent line, GA21, and Roundup Ready corn line NK603 have shown excellent crop safety at these rates in side-by-side comparisons. Further, both lines remained susceptible to labeled rates of a number of alternative herbicides that are labeled for the control of volunteer corn.

In 1999, a range of agronomic criteria were used to compare Roundup Ready corn line NK603 to a non-genetically modified control line. Field trials were conducted at 17 locations in IA, IL, KS, IN, NE, MD, OH and MO under USDA Notifications 99-071-34n and 99-071-35n. A randomized complete block design with four replicated plots/line was used at each location. Plot sizes averaged 200-250 sq. ft (10 x 20-25') with four rows planted per plot. Preemergence herbicide applications were made at planting to maintain all plots weed free and avoid any weed competition within the crop. Evaluations made through the growing season included: early plant stand count (middle two rows); days from planting to 50% pollination; days from planting to 50% silk; ear height; plant height after tasseling; stay green rating; number of dropped ears at harvest; grain moisture at harvest; grain test weight at harvest; and yield. Statistical evaluation of the data was conducted using established methods (SAS, 1996) and statistically significant

differences between Roundup Ready corn line NK603 and the non-transgenic control line determined at the 5% level of significance ($p < 0.05$).

The results of these trials show that Roundup Ready corn line NK603 was equivalent to the control line except for ear height and days to 50% silking (Table 5). Corn line NK603 was found to be statistically significantly lower than the control line for ear height (38.8 vs. 40.3) and higher than the control line for days to 50% silking (61.8 vs. 60.2). However, due to the use of the available B73 BC2F3 corn material, the small differences are unlikely to be of biological significance as they are within the range of natural variability.

Table 5. Agronomic evaluation of Roundup Ready corn line NK603

Parameter	No. of *Trials	NK603	Control	Difference	P Value
Early stand count	12	69.4	69.0	0.4	0.685
Days to 50% pollen shed	12	62.1	61.7	0.4	0.373
Days to 50% silking	12	61.8	60.2	1.6	0.048
Ear height (in)	13	38.8	40.3	-1.5	0.037
Plant height (in)	13	85.8	85.1	0.7	0.437
Stay green rating**	9	4.9	5.1	-0.2	0.328
# Dropped ears	11	1.7	1.4	0.3	0.436
% Grain moisture	16	19.8	19.4	0.4	0.066
Test weight (lb/bu)	10	57.4	56.9	0.5	0.074
Yield (bu/a)	17	145.3	141.9	3.4	0.197

*Data on all agronomic parameters not available from all trials.

**Rating is according to a scale of 1 to 9.

H. Conclusion

Roundup Ready corn line NK603 is tolerant to Roundup through the expression of the CP4 EPSPS protein. Information included in this section demonstrates that, other than the expression of Roundup tolerance, there are no phenotypic or agronomic differences between Roundup Ready corn line NK603 and nonmodified control corn. This is the same conclusion drawn for antecedent organism, GA21.

VI. Environmental Consequences of Introduction of Roundup Ready Corn Line NK603

There are no significant changes from Section VI of the previously approved petition submission (97-099-01p) in terms of the description of the herbicide glyphosate, current uses of corn herbicides, weediness potential of Roundup Ready Corn, cross pollination to wild and cultivated related species and transfer of genetic material to species to which corn cannot interbreed (e.g. "horizontal transfer"). To address the issue of the appearance of glyphosate-resistant weeds, the following section has been updated and included in this request for extension of determination. There is no expectation that cultivation of Roundup Ready corn line NK603 would have environmental effects different from the cultivation of line GA21, which has already been deregulated by USDA-APHIS.

Appearance of glyphosate-resistant weeds

Today, some 109 herbicide-resistant weed biotypes have been identified; over half of them are resistant to the triazine family of herbicides (Holt and Le Baron, 1990; Le Baron, 1991; Shaner, 1995). Resistance has usually developed because of the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action, long residual activity of the herbicide with the capacity to control weeds year-long, and frequent applications of the same herbicide without rotation to the other herbicides or cultural control practices. Using these criteria, and based on current use data, glyphosate is considered to be a herbicide with a low risk for weed resistance (Benbrook, 1991). Nonetheless, a question has been raised as to whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds resistant to that particular herbicide. This concern is based on the assumptions that the use of the herbicide will increase significantly, and possibly that it will be used repeatedly in the same location. However, other increases in glyphosate use over the previous years have been more significant than the projected increase associated with the introduction of Roundup Ready crops. Although it cannot be stated that evolution of resistance to glyphosate will not occur, the development of weed resistance to glyphosate is expected to be a very rare event because:

1. weeds and crops are inherently not tolerant to glyphosate, and the long history of extensive use of glyphosate has resulted in few instances of resistant weeds (Bradshaw *et al.*, 1997);
2. glyphosate has many unique properties, such as its mode of action, chemical structure, limited metabolism in plants, and lack of residual activity in soil, which make the development of resistance unlikely;

3. selection for glyphosate resistance using whole plant and cell/tissue culture techniques was unsuccessful, and would, therefore, be expected to occur rarely in nature under normal field conditions.

In 1996 in Australia, it was reported that a biotype of annual rye-grass (*Lolium rigidum*) was surviving application of label recommended rates of glyphosate. (Pratley *et al.*, 1996). A relationship was established with Charles Sturt University to develop an agronomic understanding of the biotype. To date, after examination of thousands of samples, only three locations have been confirmed as having the resistance population, indicating that the phenomenon is not wide spread. A large body of biochemical and molecular biology experiments to determine the cause of observed weed control differences between Australian rye-grass biotypes resistant and susceptible to glyphosate indicate that the observed resistance is due to a combination of factors. Conclusions drawn to date are that the resistant biotype is easily controlled by conventional practices (tillage, other herbicides), and is caused by a complex inheritance pattern, unlikely to occur across a wide range of other species. Results of these studies were presented by Dr. Jim Pratley, Professor of Weed Science at Charles Sturt University, at the Weed Science Society of America meeting in February, 1999.

Monsanto is also working in cooperation with the University of California to investigate two additional reports of resistant ryegrass in northern California. Similar to the Australian locations, these fields are small and isolated. Again, the use of mowing and other herbicides have been very effective in controlling the ryegrass. Research continues in an effort to better understand the resistance mechanism. Most recently, a population of *Elusine indica* (goosegrass) was reported to survive labeled rates of glyphosate in Malaysia. Monsanto obtained seed and found that the resistant goosegrass has a modified EPSPS that is 2-4 times less sensitive to glyphosate than in more sensitive biotypes. Research is underway to investigate the resistance mechanism.

VII. Adverse Consequences of Introduction

Monsanto Company knows of no unfavorable grounds associated with Roundup Ready corn line NK603, and no adverse consequences of its introduction are expected. Therefore, on the basis of the substantial benefits to the grower and the environment, Monsanto requests that this line no longer be regulated under 7 CFR part 340.

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Appendix. Expression of the CP4 EPSPS Protein

I. Introduction

Western blot analysis was conducted to assess the equivalence of CP4 EPSPS protein produced in *E. coli* and in Roundup Ready corn line NK603 and commercially available Roundup Ready Soybeans. This equivalence assessment was based on demonstration of comparable electrophoretic mobilities (i.e., comparable apparent MW) and immunological properties based on the results obtained immunoblotting. The CP4 EPSPS protein as expressed by a commercially available soybean line was included for comparison because it represents a plant biotechnology product for which the full equivalence of the *E. coli* and plant-produced protein has been confirmed. Demonstration of equivalence justified the application of the safety data generated using the *E. coli* produced protein for the CP4 EPSPS protein produced in Roundup Ready corn line NK603. Additionally, the results serve to characterize the Roundup Ready corn line NK603 with respect to the CP4 EPSPS protein.

II. Materials

A. Test materials. The test material was Roundup Ready corn line NK603 which endogenously expresses the CP4 EPSPS protein. Grain from this test line was collected from field grown plants grown under Monsanto Production Plan 98-01-46-01. All grain was stored at approximately -20 °C or below. The identity of the test line was established by use of the polymerase chain reaction (PCR, Study # 99-01-46-38).

B. Control materials. There were two control materials for this study. One control material was non-transgenic line (LH82 x B73) which does not contain the genetic material for CP4 EPSPS. Grain from the control line was collected from field grown plants as specified by under Monsanto Production Plan 98-01-46-01. All grain for line (LH82 x B73) was stored at approximately -20 °C or below. The identity of the control line was established by use of the polymerase chain reaction (PCR, Study # 99-01-46-38).

The second control material was non-transgenic soybean line A5403 which does not contain the genetic material for CP4 EPSPS. The control grain sample was obtained from plants produced according to Study Protocol # 92-01-30-02 (██████████ 1993). Control soybean grain was stored at approximately -20 °C or below. This second control material (A5403 soybeans) was added by amendment to the study protocol.

C. Reference materials. The first reference material was CP4 EPSPS protein produced by fermentation (100 L) of *E. coli* strain GB100 (██████████ 1992), transformed with plasmid pMON21104 (██████████ 1993). The protein was purified to greater than 90% purity (██████████, 1993) by a combination of cell extraction, ammonium sulfate precipitation, hydrophobic and anion exchange chromatography. The *E. coli*-expressed CP4 EPSPS protein has been characterized (██████████ 1993b). This CP4 EPSPS standard (lot # 5192245) was stored in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM DTT and 50% (v/v) glycerol at approximately 3.96 mg/mL total protein. A comprehensive safety assessment of the CP4 EPSPS protein has been conducted (Harrison *et al.*, 1996). The stock solution of CP4 EPSPS protein was stored at -20 °C or below. Prior to the initiation of this study, the purity and immunoreactivity of CP4 EPSPS (lot #5192245) was reassessed and its suitability as a reference standard for immunoblotting confirmed. The purity of the CP4 EPSPS by image analysis of a Colloidal Brilliant Blue G-stained SDS-PAGE gel was estimated to be approximately 83%. Only one immunoreactive band was observed at the expected MW when 5 ng of total protein was analyzed.¹

The second reference substance was CP4 EPSPS protein endogenously expressed by Roundup Ready soybean line AG3701, obtained from Asgrow (Stonington, Illinois). Roundup Ready soybeans have been approved by regulatory agencies in several countries including the U.S., Europe and Japan. The safety of CP4 EPSPS protein and Roundup Ready soybeans has been confirmed (██████████, 1996; Padgett *et al.*, 1996). Chain of custody records were used to confirm the identity of line AG3701 soybeans. The soybeans were stored at room temperature or below.

III. Methods

A. Summary of experimental design. Corn and soybean grain extracts were prepared in an appropriate buffer solution and the extracts clarified by centrifugation. Corn extracts were subsequently concentrated approximately 2-fold. The total protein concentration of each sample extract was determined according to the method of Bradford (Bradford, 1976). Laemmli extracts were analyzed by Western blot methodology (following the appropriate SOPs) to assess the equivalence of CP4 EPSPS protein expressed in *E. coli*, soybeans and in Roundup Ready corn line NK603 based on apparent MW (electrophoretic mobility) and immunological response when detected using specific antibodies.

¹Data for the re-analysis of CP4 EPSPS purity and immunoreactivity was recorded in Monsanto red notebook pages 6458796-6458800 and 661430-6614307, respectively.

B. Preparation of Protein extracts for western blot analysis.

All extraction procedures were conducted on ice or at $\sim 4^{\circ}\text{C}$.

Approximately 1 g of the corn and soybean samples was homogenized in ~ 6 mLs of a buffer solution containing: 100 mM Tris-Cl, pH 8.0, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM PMSF, 1% (w/v) PVPP, 1 mM benzamidine-HCl and 2 mM glutathione (reduced form).

Homogenization was accomplished by use of a hand held tissue homogenizer (Omni 2000, Waterbury, CT) for 30 sec at speed setting of 4. After homogenization, extracts were centrifuged for 20 min at 12,000 rpm (20,800 x g) using an SA-600 rotor to clarify. The supernatant solutions for all corn protein extracts were concentrated approximately 2-fold using Ultrafree-4 (Millipore, Cat. No. UFV4BCC25) centrifugal concentration devices according to the manufacturer's instructions. An aliquot of each extract was mixed with an equal amount of 2X Laemmli buffer (Laemmli, 1970) and heated at $\sim 100^{\circ}\text{C}$ for 5 min. Samples were stored at -80°C until analyzed using the western blot analytical procedure. Before subsequent analysis by SDS-PAGE and immunoblotting, the total protein concentration of an aliquot of each extract (not in 1X Laemmli buffer) was determined by the method of Bradford (Bradford, 1976) according to SOP GEN-PRO-015-00.

C. Electrophoresis.

Extracts prepared as above for electrophoresis were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on 4-20% gradient gels according to the current version of SOP PB-EQP-005 using the mini gel system of NOVEX (San Diego, CA). Electrophoresis was conducted at constant voltage (approximately 150V) until the dye front reached the bottom of the gel. The extract of line NK603 was analyzed on a gel with an appropriate set of reference and control material extracts. Additionally, Amersham full-range color markers (RPN 800) were analyzed on the gel so that effective transfer to PVDF membrane could be confirmed. Finally, Bio-Rad biotinylated MW markers were loaded on the gel so that blots could be calibrated.

D. Western blot analysis (immunoblotting).

Immunoblotting was conducted according to the current version of SOP GEN-PRO-002. Proteins separated by SDS-PAGE were electrophoretically transferred to a PVDF membrane (Immobilon-P, 0.45 μM) and non-specific sites on blots were blocked using 5% non-fat dry milk in 1X TBST [25 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20]. Blots were probed for the presence of CP4 EPSPS using a 1:1000 dilution of antisera raised in goats (DR2, bleed 5) against *E. coli*-produced CP4 EPSPS in 1X TBST with 1% (w/v) NFDM. Unbound polyclonal antibody was rinsed away with TBST washes. Polyclonal antibody bound to the membrane was probed with a 1:2000 dilution of biotinylated protein G

(Pierce, P/N 29988) in 1X TBST with 1% (w/v) NFDM. Unbound biotinylated protein G was rinsed away with TBST washes.

Biotinylated protein G bound to blots was detected using a 1:10,000 dilution of NeutrAvidin-HRP conjugate (Pierce, P/N 31001). Unbound NeutrAvidin-HRP was rinsed away with TBST washes.

Immunoreactive bands were visualized on X-ray film using the Enhanced Chemiluminescence kits of Amersham (RPN 2106) according to the instructions provided by the manufacturer. Films were developed using an automatic film processor (Konica SRX-101).

IV. Results and Discussion

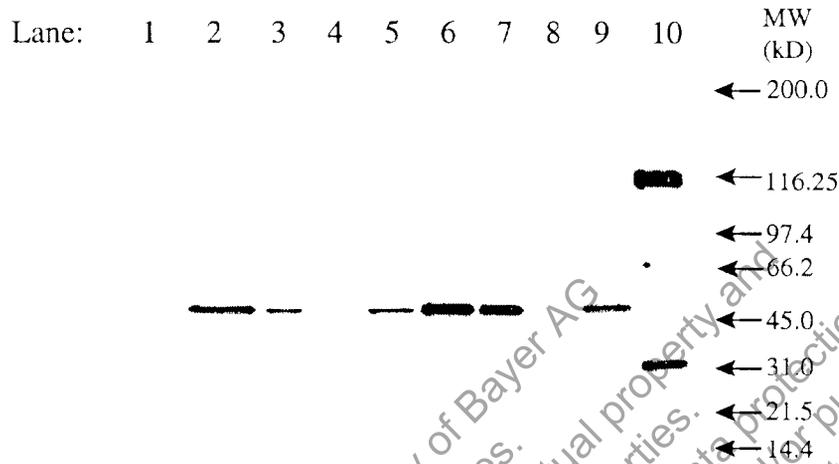
The *E. coli*-produced CP4 EPSPS used for safety studies, the CP4 EPSPS expressed by Roundup Ready soybeans and Roundup Ready corn line NK603 were found to be equivalent based on identical electrophoretic mobilities and detection using specific antibodies as established by the western blot analytical method. Results for Roundup Ready corn line NK603 are shown in Figure 1. For the blot, the *E. coli* standard (lot # 5192245) was loaded at two concentrations (1 ng and 5 ng) in lanes 2 and 3, respectively. Additionally, *E. coli* CP4 EPSPS was spiked at 1 ng in both non-transgenic control corn (lane 5) and control soybean (lane 9) matrix to account for any possible bias associated with the relative mobility of CP4 EPSPS in plant extract. The protein extract prepared from the Roundup Ready Corn line (NK603) was loaded in lane 6, and the protein extract prepared from Roundup Ready soybeans (line AG3701) was loaded in lane 7 of the gel. Stained bands at the expected apparent MW (~47 kDa) were observed for the *E. coli*-produced CP4 EPSPS (whether alone or in plant matrix), the CP4 EPSPS in Roundup Ready corn line NK603 and in Roundup Ready soybeans (line AG3701). No bands were detected in the control corn or soybean extracts, confirming the specificity of the antibodies. No other immuno-reactive bands were detected on the blot film. CP4 EPSPS has been expressed as a nuclear-encoded CP4 EPSPS with an amino-terminal fusion to a chloroplast transit peptide (CTP2). The CTP is necessary for transport of the protein into the chloroplast, which is followed by cleavage of the CTP to yield the "mature" form of the protein. These results provide indirect evidence that the chloroplast transit peptide (CTP2) is properly processed upon chloroplast import yielding the "mature" CP4 EPSPS of the same size as that expressed in Roundup Ready soybeans. As expected, these data clearly establish that the CP4 EPSPS expressed in Roundup Ready corn line NK603 is equivalent to the *E. coli* CP4 EPSPS protein used for safety assessment studies and is also equivalent to the CP4 EPSPS protein expressed in the commercial Roundup Ready soybean variety AG3701.

V. Conclusions

CP4 EPSPS protein produced in Roundup Ready corn line NK603 was demonstrated to be equivalent to both the *E. coli*-expressed CP4 EPSPS protein used for safety studies and CP4 EPSPS expressed by a commercial RR soybean variety. Equivalence was based on visually equivalent apparent molecular weights and immunological properties when detected using antibodies specific for CP4 EPSPS protein. This demonstration of equivalence justifies the application of the safety data generated using the *E. coli* produced protein for the CP4 EPSPS protein produced in Roundup Ready corn line NK603. This study also served to characterize Roundup Ready corn line NK603 with respect to the CP4 EPSPS protein.

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Figure 1. Western Blot Showing the Equivalence of CP4 EPSPS Protein Expressed by *E. coli*, Roundup Ready® Soybean and Roundup Ready® Corn Line NK603



<u>Lane</u>	<u>Description</u>	<u>Amount</u>
1	Full range color MW markers	7.5 µL, 1,125 µg/band
2	<i>E. coli</i> CP4 EPSPS standard	10 µL, 5 ng
3	<i>E. coli</i> CP4 EPSPS standard	5 µL, 1 ng
4	Control corn extract (LH82 x B73)	8.4 µL, 10 µg total protein
5	Control corn extract (LH82 x B73) spiked with CP4 EPSPS	8.4 µL control extract and 2 µL (1 ng) standard
6	NK603 corn extract	12.8 µL, 10 µg total protein
7	AG3701 soybean extract	2 µL, 2.6 µg total protein
8	A5403 control soybean extract	2 µL, 2.2 µg total protein
9	Control soybean extract (A5403) spiked with CP4 EPSPS	2 µL control extract and 2 µL (1 ng) of standard
10	Biotinylated MW markers	7.5 µL

The soybean 1X Laemmli samples were diluted 10-fold before analysis because of the high expression level of CP4 EPSPS in Roundup Ready® soybean line AG3701.

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