Safety, Compositional and Nutritional Aspects of Roundup Ready® Corn Line NK603

Conclusion Based on Studies and Information Evaluated According to FDA's Policy on Foods from New Plant Varieties

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Abbreviations Used in this Summary of the Safety, Compositional and Nutritional Aspects of Roundup Ready Corn Line NK603

AACC	American Association of Cereal Chemists
ADF	Acid detergent fiber
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists Society
APHIS	Animal Plant and Health Inspection Service
~	Approximately
bp	Base pairs
CaMV	Cauliflower mosaic virus
CFR	Code of Federal Regulations
CP4 EPSPS	EPSPS derived from *Agrobacterium* sp. strain CP4
CTAB	Cetyltrimethylammonium bromide
CTP	Chloroplast transit peptide
DNA	Deoxyribonucleic Acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
df	Degrees of freedom
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
dw	Dry weight
E. coli	*Escherichia coli*
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
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FDA	Food and Drug Administration
FR	United States Federal Register
fw	Fresh weight
GenBank	A public domain database maintained by the National Center for Biotechnology Information
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IDENTITYSEARCH	Algorithm used to scan for 8 or more linearly contiguous amino acid identities between a pair of protein sequences
kb	Kilobases
LOD	Limit of detection
LOQ	Limit of quantitation
MW	Molecular weight

*Mlu*I	Restriction enzyme
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
Na_2HPO_4	Sodium phosphate dibasic
NDF	Neutral detergent fiber
NK603	Roundup Ready corn line number
NOS 3'	Nopaline synthase 3' polyadenylation sequence
nptII	The gene for the enzyme neomycin phosphotransferase type II.
OD	Optical density (sample absorbance)
OECD	Organization for Economic Co-operation and Development
ori	Origin of replication
P-ract1/ract1 intron	Rice actin promoter and intron
PCR	Polymerase chain reaction
PIR	Protein Information Resource
ppm	Part per million
PVP	Polyvinylpyrolidine
PV-ZMGT32	Plasmid vector
*Sca*I	Restriction enzyme
SDS	Sodium dodecyl sulfate
SOP	Standard operating procedure
sp.	Species
SSC	Saline-sodium citrate buffer. 20X SSC is 3 M sodium chloride, 0.3 M sodium citrate
SwissProt	Translated sequences from EMBL database
TE	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
TIU	Trypsin inhibitor units
TMB	(3,3',5,5' Tetramethylbenzidene) peroxidase substrate
Tris	Tris (hydroxymethyl)aminomethane
tRNA	*Escherichia coli* transfer RNA
U.S.C.	United States Code
USDA	United States Department of Agriculture
UV	Ultraviolet
*Xba*I	Restriction enzyme

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

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INFORMATION TO SUPPORT THE HUMAN FOOD/ANIMAL FEED SAFETY OF ROUNDUP READY CORN LINE NK603

I. Introduction

A. Subject of Request

Monsanto Company has developed, through molecular biological techniques, corn (*Zea mays* L.) plants which are commercially tolerant to glyphosate, the active ingredient in Roundup®[1] herbicide. The tissues of these plants produce a glyphosate-tolerant EPSPS from *Agrobacterium* sp. strain CP4 (CP4 EPSPS), the same tolerant EPSPS already used in Roundup Ready®[1] varieties of soybean, canola, beet and cotton. In nontransgenic plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby preventing production of these essential compounds. The CP4 EPSPS enzyme, however, has a reduced affinity for glyphosate when compared to the native corn EPSPS enzyme, which is inhibited by glyphosate (Padgette *et al.*, 1996). As a result, corn plants expressing the CP4 EPSPS protein, when treated with glyphosate, are unaffected, as the continued action of the tolerant enzyme provides the plant's need for aromatic amino acids.

Monsanto Company is filing this summary of the safety and nutritional assessment of Roundup Ready corn line NK603 with the Food and Drug Administration (FDA) based on scientific data and information evaluated according to FDA's policy on foods from new plant varieties (FDA, 1992) as well guidance provided in its "Guidance on Consultation Procedures - Foods Derived from New Plant Varieties", October 1997. Roundup Ready corn line NK603 may also be referred to as Roundup Ready corn line 603 in various technical and commercial communications. A detailed description of the Roundup Ready corn line NK603 follows in this summary and in the data as submitted and referenced.

B. Application of FDA Food Policy

In its May 29, 1992 statement of policy concerning "Foods Derived from New Plant Varieties," ("Food Policy" or the "Policy"), the Food and Drug Administration ("FDA") provided guidance for determining whether a new plant variety developed with the aid of new genetic techniques is as safe and nutritious as its parental variety (FDA, 1992). The Policy is based on decision trees that are designed to establish whether the new plant variety is materially different in

[1] Roundup® and Roundup Ready® are registered trademarks of Monsanto Company.

composition, safety or any relevant parameter from its parental variety. In the Policy, the agency noted that consultations on new plant varieties are appropriate forums for industry and the agency to discuss scientific and regulatory issues prior to market entry. The agency encouraged developers to consult early in the development phase of the product.

Monsanto Company initiated its consultation with the FDA by meeting with members of the Center for Food Safety and Applied Nutrition (CFSAN) and the Center for Veterinary Medicine (CVM) in March, 1995 to define and discuss the scientific data and information necessary to support the safety and nutritional assessment of glyphosate-tolerant corn varieties expressing the CP4 EPSPS protein. Since 1995, Monsanto has completed the consultation for five corn lines expressing the same CP4 EPSPS protein. (These lines were actually not developed commercially. A line expressing the modified maize EPSPS, for which the consultation with FDA was completed in 1998, has been commercialized since 1998.) In addition, from 1994 to 1998, Monsanto has completed the consultation process on a number of Roundup Ready crops expressing the same CP4 EPSPS protein for glyphosate tolerance including soybean, canola, cotton, and sugar beet.

Monsanto has followed the guidance in the Policy to assess whether corn line NK603, modified by the addition of a gene producing a CP4 EPSPS protein, is materially different from corn currently being marketed.

C. The Safety and Nutritional Assessment of Roundup Ready Corn Line NK603 Expressing the CP4 EPSPS Protein

The safety of the CP4 EPSPS protein and Roundup Ready corn line NK603 has been extensively investigated. This summary provides an assessment of the human health safety of the CP4 EPSPS protein based upon the characterization of the CP4 EPSPS protein and its comparability to EPSPS enzymes commonly found in a wide variety of food sources which have a long history of safe use. Further, the CP4 EPSPS protein is identical to the same protein found in Roundup Ready soybean and other Roundup Ready crops with a history of safe human and animal consumption. Additional studies were conducted and information gathered to support the safety of the CP4 EPSPS protein including the: (1) lack of acute toxicity of CP4 EPSPS protein as determined by a mouse gavage study, (2) rapid digestion of CP4 EPSPS protein in simulated gastric and intestinal fluids, (3) lack of homology of CP4 EPSPS with known protein toxins and (4) lack of allergenic potential of CP4 EPSPS protein. These data support the assessment of safety of the CP4 EPSPS protein and, when taken together with analyses performed on Roundup Ready corn line NK603 which demonstrate compositional equivalence to conventional corn

varieties, support the conclusion that corn line NK603 is as safe and nutritious as conventional corn currently being marketed.

The concepts and approaches Monsanto has employed are derived from, and consistent with, the principles outlined by independent international bodies such as the Organization for Economic Co-operation and Development (OECD), the United Nations World Health Organization (WHO) and the Food and Agriculture Organization (FAO) (OECD, 1992; WHO, 1995; 1996) and consistent with the guidance presented in the flow charts found in the FDA Food Policy (FDA, 1992). For each question, we have developed answers based on extensive studies or analyses. Our data and findings in every case have led us to the conclusion of "no concern", as described in the relevant sections of the following summary. Under these circumstances, following the Agency's Food Policy, the data have provided us with a basis for concluding that Roundup Ready corn line NK603 is as safe and nutritious as the corn varieties grown commercially today.

D. Coordination with Other U.S. Federal Agencies

Before commercializing Roundup Ready corn line NK603, Monsanto will or has taken the following actions in the United States:

1. 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. As a result of consultations on Roundup Ready corn with the FDA since March, 1995, Monsanto is providing this summary of the food and feed safety and nutritional assessment of Roundup Ready corn line NK603 to the Agency prior to commercial distribution.

2. Under regulations administered by the Animal and Plant Health Inspection Service (APHIS) in USDA (7 CFR 340), Roundup Ready corn line NK603 is considered a "regulated article". Monsanto has requested an extension of an existing determination of nonregulated status (97-099-01p) to Roundup Ready corn line NK603 and all progenies derived from crosses between this line and other corn hybrids.

3. Substances that are pesticides as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. section 136(u)), are subject to EPA's regulatory authority. The initial registration of Roundup Ultra®[1] herbicide (EPA Reg. No. 524-475) for use over-the-top of Roundup Ready corn was granted

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

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.

Pursuant to section 408(d) of the Federal Food Drug and Cosmetic Act (FFDCA), 21 U.S.C. 346 a(d), the EPA has previously reviewed and established an exemption from the requirement of a tolerance for CP4 EPSPS and the genetic material necessary for the production of this protein in or on all raw agricultural commodities (40 CFR 180.1174). This exemption, which included a toxicology assessment, was established in response to the use of the CP4 EPSPS in combination with expression of the Cry1Ab protein in corn for the control of certain lepidopteran insect pests.

II. Rationale for the Development of Roundup Ready Corn

Corn (*Zea mays* L.) is the largest crop in the United States in terms of planted acreage, total production, and crop value (National Corn Growers Association, 1999). United States production in 1998 was estimated at 9.76 billion bushels (248 million metric tons) produced on nearly 73 million acres (29 million hectares) with the majority of national production concentrated across what is known as the "Corn Belt" in the upper Midwest.

Effective weed control in corn protects against grain yield losses due to weed competition for light, water and nutrient resources (Olson and Sander, 1988). The control of weed species is also important to reduce contamination of corn grain and forage as certain weed species may be significant sources of toxins or antinutrients. Although Roundup herbicide has been used for over 20 years for non-selective weed control, the recent introduction of Roundup Ready genes into crops through the use of biotechnology has provided farmers with the option to use Roundup herbicide after crop and weed emergence for in-season weed control ([redacted]1996). No increase of the proportion of corn acreage treated with herbicides is expected since herbicides are currently used on the vast majority of corn acreage in the U.S. (USDA, 1999).

The use of Roundup in corn is significant as it enables the farmer to take advantage of the herbicide's favorable environmental properties. Roundup Ready corn benefits the farmer by providing (1) an additional broad-spectrum weed control option in corn, (2) a new 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 use of conservation 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 (Hebblethwaite, 1995; Reicosky *et al.*, 1995; Warburton and Klimstra, 1984; Edwards *et al.*, 1988).

Roundup herbicide has a very favorable environmental and health and safety profile. Glyphosate, the active ingredient in Roundup herbicide (1) has limited mobility as it binds rapidly and tightly to a wide variety of soils and sediments, (2) is non-persistent as it is readily metabolized, (3) has a low potential to move into surface or groundwater and (4) does not accumulate in and presents minimal risk to terrestrial and aquatic animals including birds, mammals, fish and invertebrates (Franz *et al.*, 1996; Ahrens, 1994). Based on the results of long-term feeding studies on glyphosate and a battery of negative genotoxicity studies, glyphosate is classified as Category E by the U.S. EPA, meaning that there is no evidence for carcinogenicity. Additionally, the World Health Organization stated in 1994 that glyphosate is not carcinogenic or mutagenic, as per Environmental Health Criteria 159.

Since 1994, Monsanto has initiated and completed the FDA consultation process on a number of crop varieties expressing the same CP4 EPSPS protein for glyphosate tolerance including soybean (*Glycine max*) (1994; file BNF 0001), canola (*Brassica napus*) (1995; file BNF 0020), cotton (*Gossypium hirsutum*) (1995; file BNF 0026), corn (Zea mays) (1996; file BNF0035) and sugar beet (*Beta vulgaris*) (1998; BNF0056). Specifically, in 1996, Monsanto completed its consultation with the FDA (Center for Veterinary Medicine and Center for Food Safety and Applied Nutrition) on a series of glyphosate-tolerant (MON 830, MON 831 and MON 832) and insect-protected/glyphosate-tolerant (MON 802 and MON 805) corn lines expressing the CP4 EPSPS protein. Monsanto also completed a consultation with the FDA in 1998 on an additional Roundup Ready corn line, GA21, expressing another form of glyphosate-tolerant EPSPS, modified maize EPSPS, for glyphosate tolerance (file BNF 000051).

III. Description of the Transformation System and Recipient Corn Material

An agarose gel-isolated *Mlu*I restriction fragment of plasmid DNA, designated as PV-ZMGT32L, was introduced into embryogenic corn cells using the particle acceleration method (Klein *et al.*, 1987; Gordon-Kamm *et al.*, 1990). Refer to Figures 1 and 2 and the detailed description given below for the construction of the restriction fragment and its parent plasmid vector PV-ZMGT32.

Using the particle acceleration method, DNA was precipitated onto microscopic gold particles using calcium chloride and spermidine. A drop of the coated particles was then placed onto a plastic macrocarrier, which is accelerated at a high velocity through a barrel by the discharge of compressed helium gas. The macrocarrier hits a metal screen which stops the flight of the macrocarrier but allows continued flight of the DNA-coated particles. The particles penetrate the target plant cells, where the DNA is deposited and incorporated into the cell chromosome. The introduced DNA contains a gene encoding for herbicide tolerance (e.g., the CP4 EPSPS gene) which, when expressed, allows transformed cells to survive on the medium and grow in the presence of glyphosate, the active ingredient in Roundup herbicide.

The corn plant tissue which was the recipient of the introduced DNA was embryogenic corn cells from an inbred line designated (AW x CW).

IV. Donor Genes and Regulatory Sequences

Roundup Ready corn line NK603 was generated using the particle acceleration transformation system described above and a gel-isolated *Mlu*I fragment, PV-ZMGT32L (Figure 1), containing the EPSPS gene from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). CP4 EPSPS encodes a tolerant form of EPSPS which confers glyphosate (Roundup) tolerance at the whole plant level (Figures 1 and 2).

The plasmid vector PV-ZMGT32 (Figure 2) contains two adjacent 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 and contains two adjacent 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 CP4 EPSPS plant gene expression cassettes and does NOT contain the *nptII* selectable marker gene or origin of replication (Figure 1).

In both plant gene expression cassettes, the CP4 EPSPS gene is fused to chloroplast transit peptide (CTP) sequences based on sequences isolated from *Arabidopsis thaliana* EPSPS, which targets the CP4 EPSPS protein to the chloroplast, which is 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).

In the first gene cassette, the CP4 EPSPS coding sequence is under the control of the 5' end of the rice actin 1 sequence (ract1) containing the promoter and first intron introduced upstream of the CTP sequence (McElroy *et al.*, 1990). The second cassette contains the CP4 EPSPS sequence under the control of the enhanced CaMV 35S promoter (e35S), which is approximately 0.6 kb in size (Kay *et al.*, 1985; Odell *et al.*, 1985). Located between the e35S promoter and the CP4 EPSPS sequence is the 0.8-kb intron from the maize *hsp*70 (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', which provides the mRNA polyadenylation signal (Fraley *et al.*, 1983).

An origin of replication sequence (*ori*) was present in the plasmid PV-ZMGT32 to allow for its replication 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 and is present under its own bacterial promoter (Beck *et al.*, 1982). The resulting plasmid was designated PV-ZMGT32 (Figure 2).

The plasmid PV-ZMGT32 was amplified in *E. coli* and purified from bacterial lysates. The CP4 EPSPS gene expression linear DNA sequence was isolated from the plasmid prior to corn transformation experiments by digesting PV-ZMGT32 with the restriction enzyme *Mlu*I. 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 *Mlu*I restriction fragment utilized in the transformation of Roundup Ready corn line NK603 was designated PV-ZMGT32L (Figure 1).

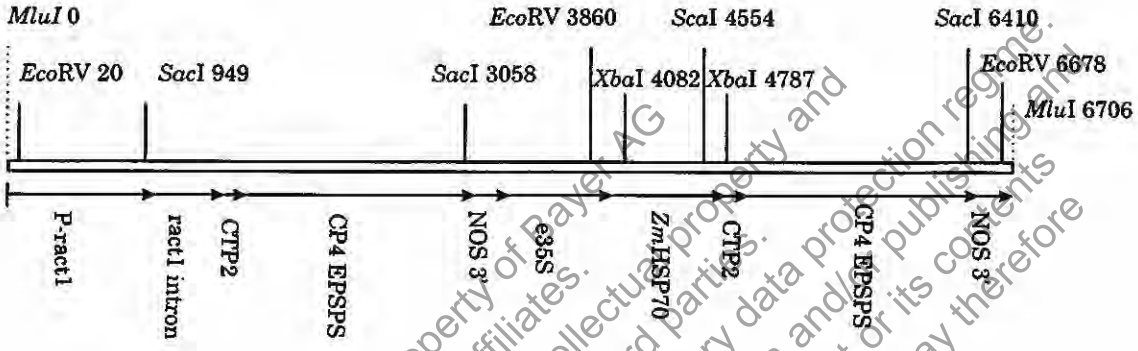

Figure 1. Linear map of PV-ZMGT32L. The DNA fragment PV-ZMGT32L was used to generate Roundup Ready corn line NK603 by particle acceleration technology. The dashed lines represent the remaining *Mlu*I sites following digestion of PV-ZMGT32.

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

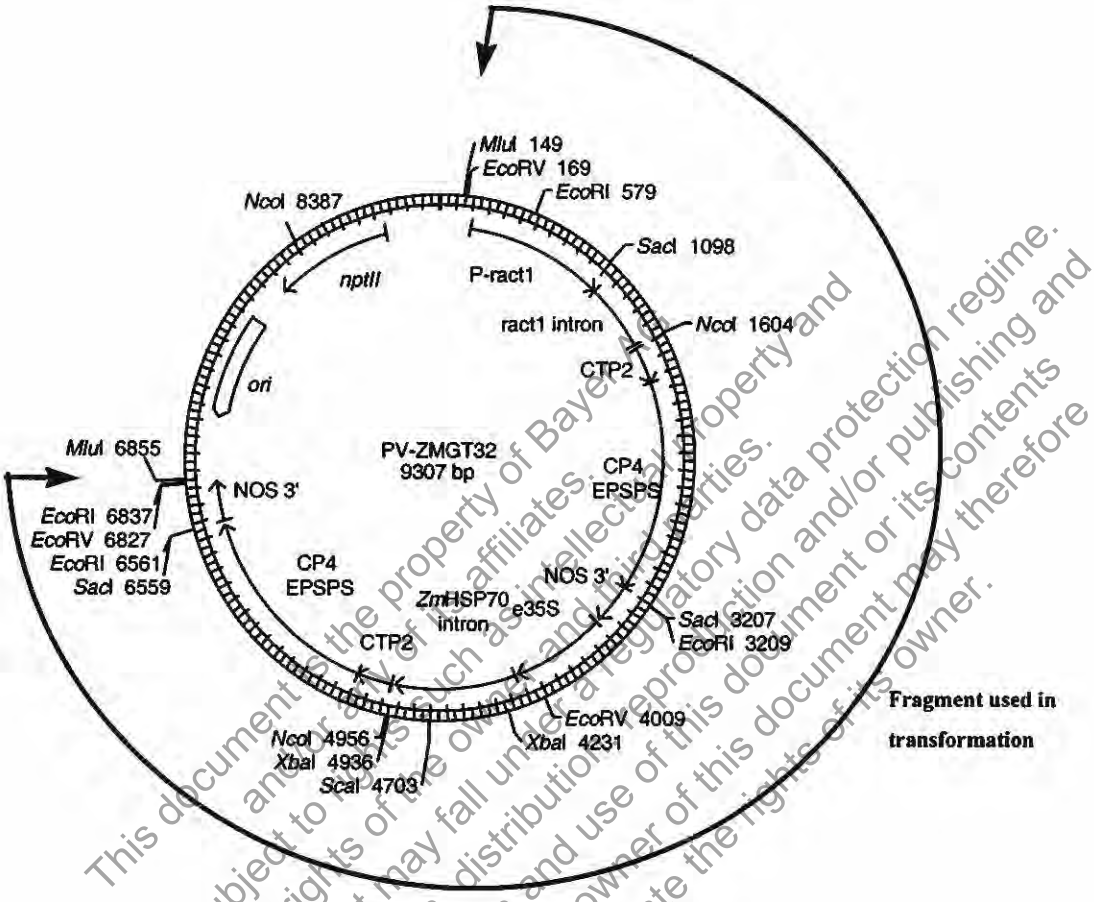

Figure 2. Plasmid map of PV-ZMGT32. The plasmid PV-ZMGT32 was used to prepare the *Mlu*I fragment used in the transformation of corn line NK603 by excision and discarding of the origin of replication (*ori*) and antibiotic resistance marker (*nptII*) sequences.

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

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

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 <u>not</u> present in the *Mlu*I restriction fragment (PV-ZGMT32L) used for transformation:			
ori	*Escherichia coli*	0.65	The origin of replication from the *E. coli* high copy plasmid pUC119 (Vieira and Messing, 1987).
nptII	*Transposon Tn5*	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 *et al.*, 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 ([redacted] 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 (Padgette *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; Padgette *et al.*, 1993; Padgette *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 that have already completed the FDA consultation process. These include corn as well as soybean, cotton, and sugar beet.

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).

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  1  MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51  LKKSGMTLIG SELRPLKVMS SVSTACMLHG ASSRPATARK SSGLSGTVRI
101  PGDKSISHRS FMFGGLASGE TRITGLLEGE DVINTGKAMQ AMGARIRKEG
151  DTWIIDGVGN GGLLAPEAPL DFGNAATGCR LTMGLVGVYD FDSTFIGDAS
201  LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP ITYRVPMASA
251  QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301  TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR
351  TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM
401  IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNGVDCDE
451  GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501  DATMIATSFP EFMDLMAGLG AKIELSDTKA A
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C. The chloroplast transit peptide (CTP2)

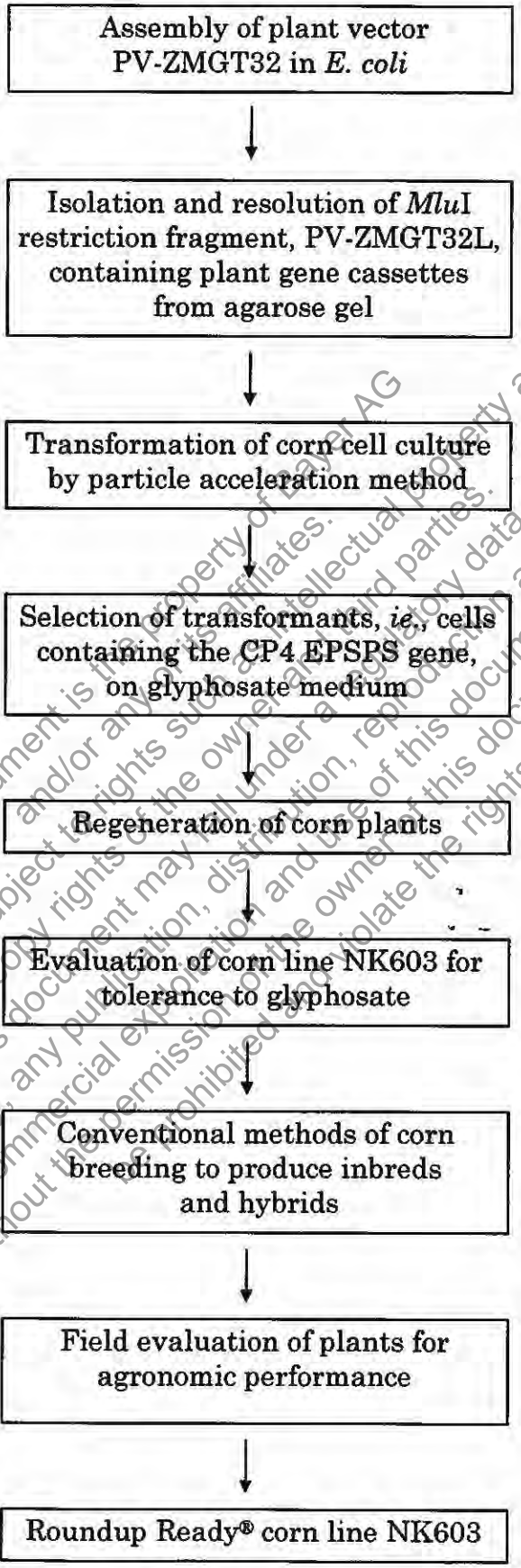
In both plant gene expression cassettes, the CP4 EPSPS coding sequence is fused to a chloroplast transit peptide (designated CTP2) whose sequence is based on the CTP isolated from *Arabidopsis thaliana* EPSPS (Klee *et. al.*, 1987). This CTP directs the CP4 EPSPS protein to the chloroplast, the location 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 CTP2-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, introduced upstream of the CTP sequence (McElroy *et al.*, 1990). The second cassette contains the CTP2-CP4 EPSPS coding sequence under the control of the enhanced CaMV 35S promoter (e35S), which is approximately 0.6 kb in size (Kay *et al.*, 1985; Odell *et al.*, 1985). Located between the e35S promoter and the CTP2-CP4 EPSPS sequence is the 0.8-kb intron from the corn *hsp*70 (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* which provides the transcription termination and the mRNA polyadenylation signal (Fraley *et al.*, 1983).

The enhanced 35S promoter from CaMV with an enhanced duplicator region, the corn *hsp70* intron, the chloroplast transit peptide from *Arabidopsis thaliana*, the CP4 EPSPS gene sequence and the NOS 3' sequence for termination of transcription and direction of polyadenylation as described in Table 1 were previously reviewed by the Agency during the consultation process for Roundup Ready/European corn borer protected lines MON 802 and MON 805 and Roundup Ready corn lines MON 830, MON 831 and MON 832 (1996). The rice actin 1 promoter and first intron (McElroy *et al.*, 1990) from the rice actin gene have been previously described in the transformation of Roundup Ready corn line GA21, which has already been assessed through consultation with the FDA (1998). No new DNA elements are included in Roundup Ready corn line NK603 which have not been reviewed by the Agency in previous consultations. The flow chart shown in Figure 4 illustrates the steps in the development of Roundup Ready corn line NK603.

Figure 4. Development of Roundup Ready corn line NK603.



V. Molecular Characterization of Roundup Ready Corn Line NK603

A. Molecular Analysis

Molecular analysis was performed to characterize the inserted DNA in Roundup Ready corn line NK603. Genomic DNA was analyzed using Southern blot analysis 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, introns, coding regions, and polyadenylation sequences, and the presence or absence of the plasmid backbone sequence (Southern, 1975). Polymerase chain reaction (PCR) was performed to verify the sequences at the 5' and 3' ends of the insert (Saiki, 1990).

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 I).

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)] were 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 *Eco*RI and *Nco*I, 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 ^{32}P 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 $MgCl_2$; 50 mM KCl; 0.25 µM of each primer; 12.5 µm dCTP, dGTP and dTTP; 100 µCi (6000 Ci/mmol) of ^{32}P-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 substances 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 $Na_2HPO_4{\cdot}7H_2O$, 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 MS™ 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 µl: 100 ng of genomic DNA, 10 mM Tris/HCl, 50 mM KCl, 1.25 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM 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 µl 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 ^{32}P-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 *Stu*I restriction fragment. Due to the size of the *Stu*I 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 *Xba*I, 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 *Stu*I 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 an approximately 23-kb *Stu*I 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 *Xba*I followed by Southern blotting. The blot was probed with ^{32}P-labeled PV-ZMGT32. The linear DNA fragment used for corn transformation contains two *Xba*I sites (nucleotides 4082 and 4787, Figure 1) but restriction digestion 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 *Xba*I. Therefore, the enzyme *Xba*I 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 *Eco*RV 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.) Sequence data 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 contained an *Eco*RV site 20-bp away from 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 *Eco*RV

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 *Eco*RV 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 *Eco*RV 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 *Eco*RV 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 *Eco*RV, 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 *Eco*RV 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 *Eco*RV 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, in aggregate, clearly 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 analyses and PCR analyses, 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 adjacent CP4 EPSPS gene cassettes. Corn line NK603 contains one insertion of the integrated DNA located within an approximately 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.

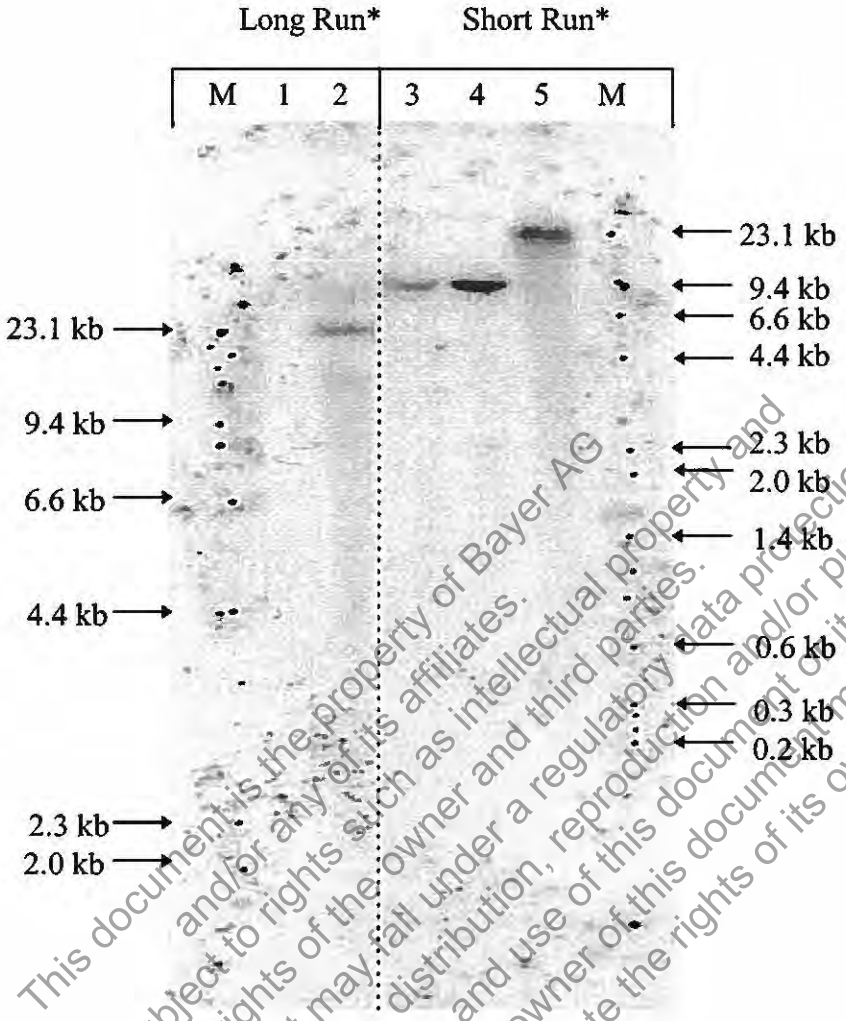


Figure 5. Southern blot analysis of NK603: determination of insert number. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Stu*I. Plasmid PV-ZMGT32 DNA mixed with B73 DNA was digested with *Stu*I and *Sca*I. DNA samples were separated by gel-electrophoresis, blotted and then probed with ^{32}P-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.

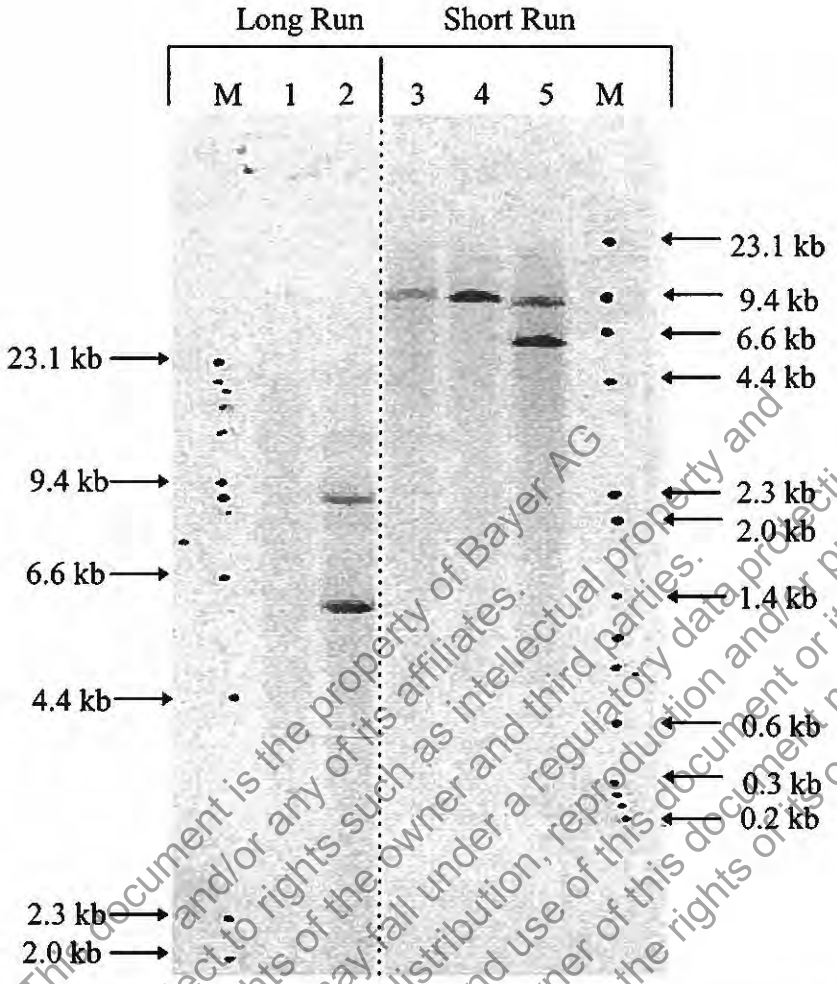


Figure 6. Southern blot analysis of NK603: determination of copy number. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Xba*I. The DNA samples were separated by gel-electrophoresis, blotted and then probed with ^{32}P-labeled plasmid PV-ZMGT32. Lane designations 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).

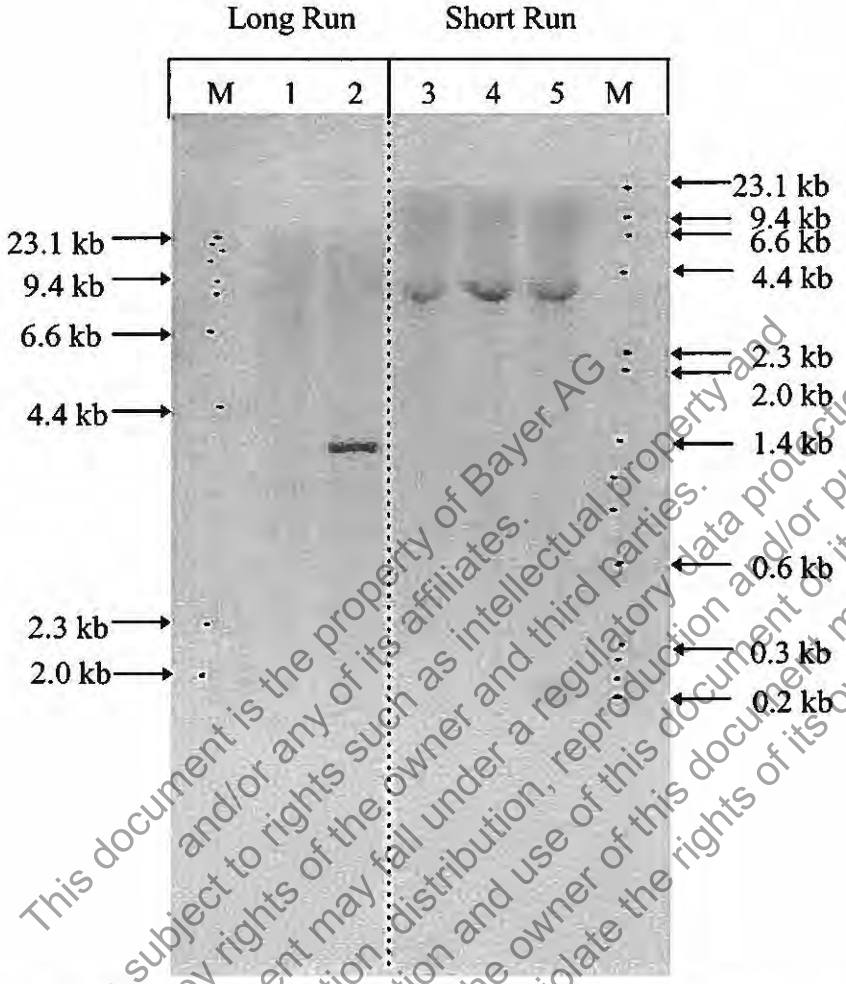


Figure 7. Southern blot analysis of NK603: P-ract1/ract1 intron. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. DNA samples were separated by gel-electrophoresis, blotted and then hybridized with a mixture of two probes: the full-length P-ract1/ract1 intron labeled with ^{32}P-dCTP and a 175-bp fragment of the P-ract1/ract1 intron labeled with ^{32}P-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).

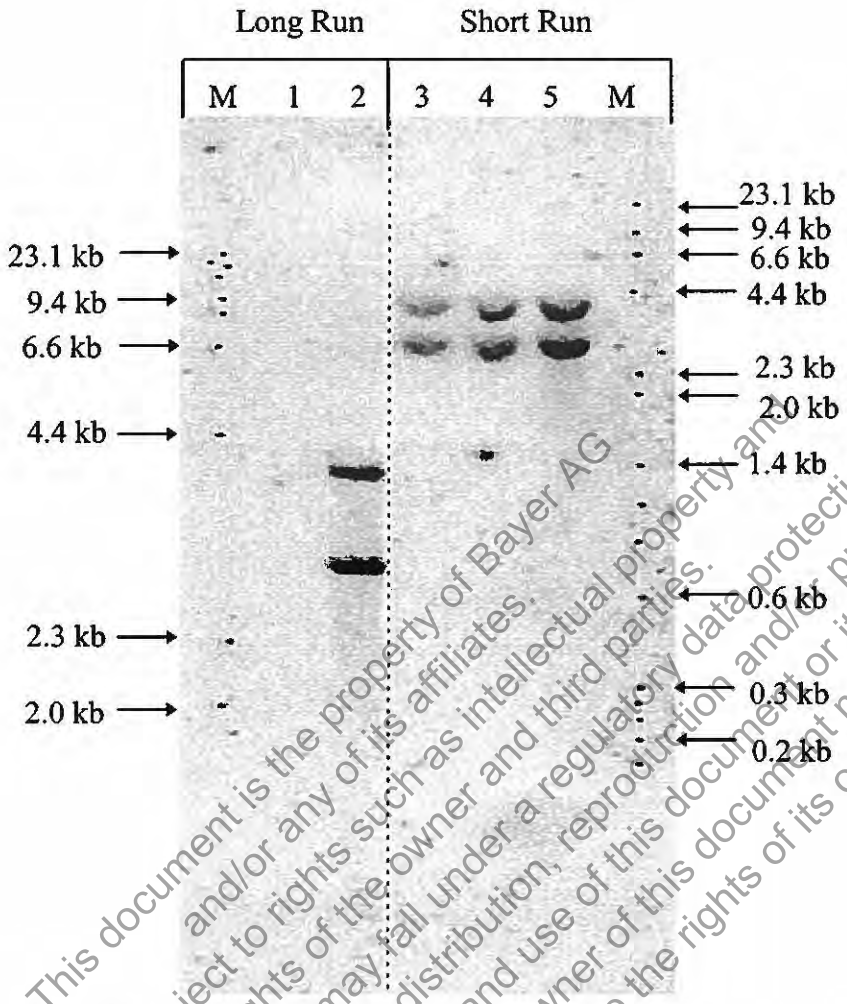


Figure 8. Southern blot analysis of NK603: CTP2-CP4 EPSPS sequence. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ^{32}P-labeled CTP2-CP4 EPSPS fragment. 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).

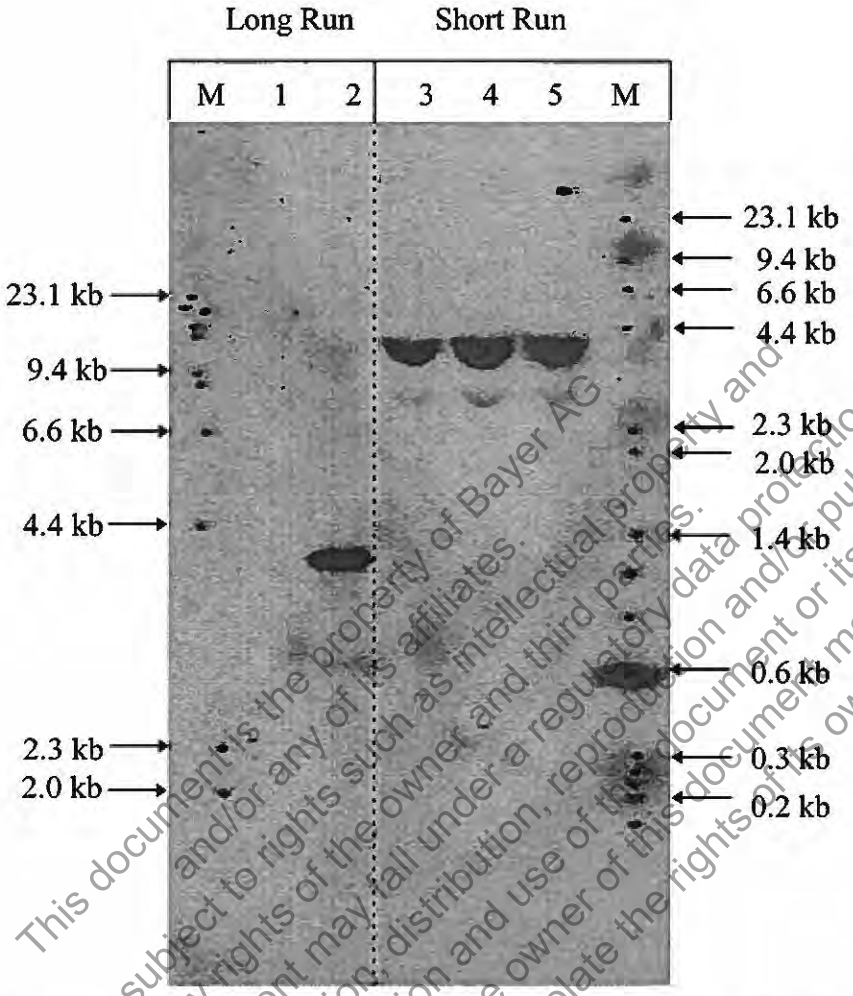


Figure 9. Southern blot analysis of NK603: e35S promoter. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ^{32}P-labeled e35S fragment. Lane designations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

Lane 1: B73 DNA (Long Run)
2: NK603 DNA (Long Run)
3: B73 DNA spiked with 14.5 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).

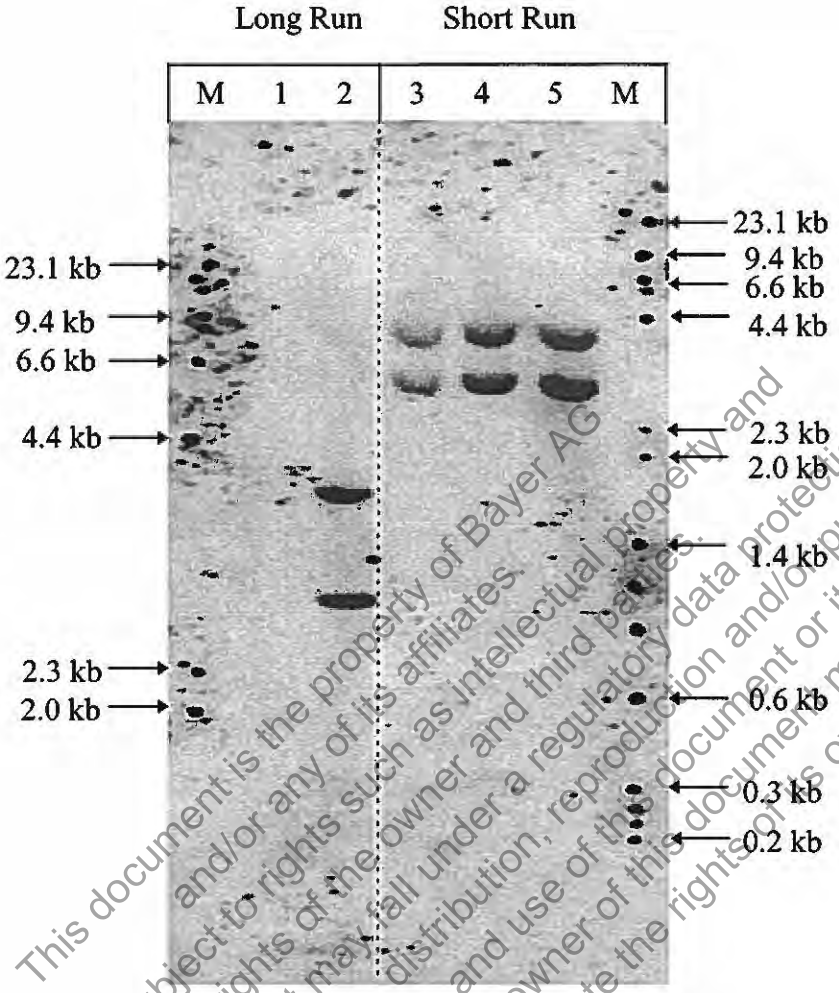


Figure 10. Southern blot analysis of NK603: NOS 3' polyadenylation sequence. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ^{32}P-labeled NOS 3' polyadenylation 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).

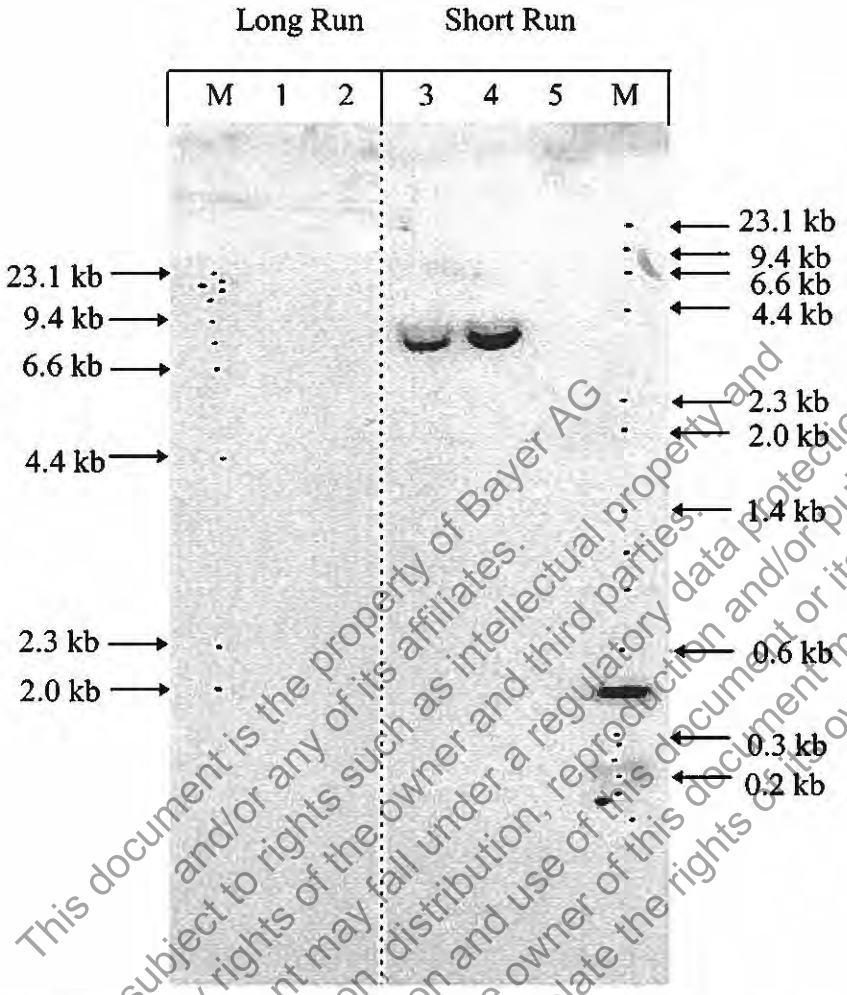


Figure 11. Southern blot analysis of NK603: backbone analysis. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Sac*I. The DNA samples were separated by gel-electrophoresis, blotted and then probed with ^{32}P-labeled entire backbone sequence consisting of the *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 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).

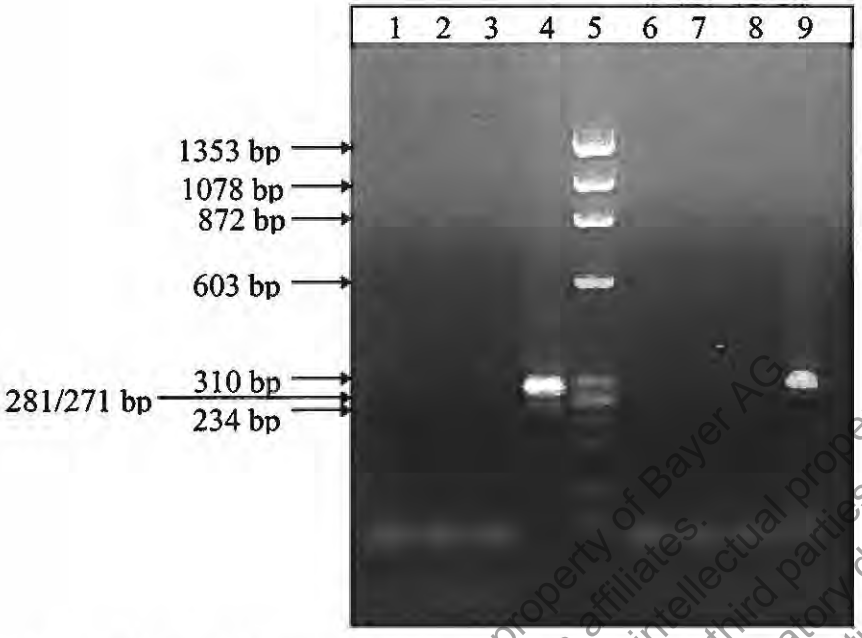


Figure 12. PCR verification of sequences at the 5' and 3' ends of NK603 insert. PCR was performed using primers specific to the 5' and 3' flanking sequences for NK603 insert on genomic DNA extracted from corn lines B73 (non-transgenic control), an unrelated transgenic corn line, and NK603. The PCR primer 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 µl 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 corn 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 corn line
9: 3' PCR, NK603

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

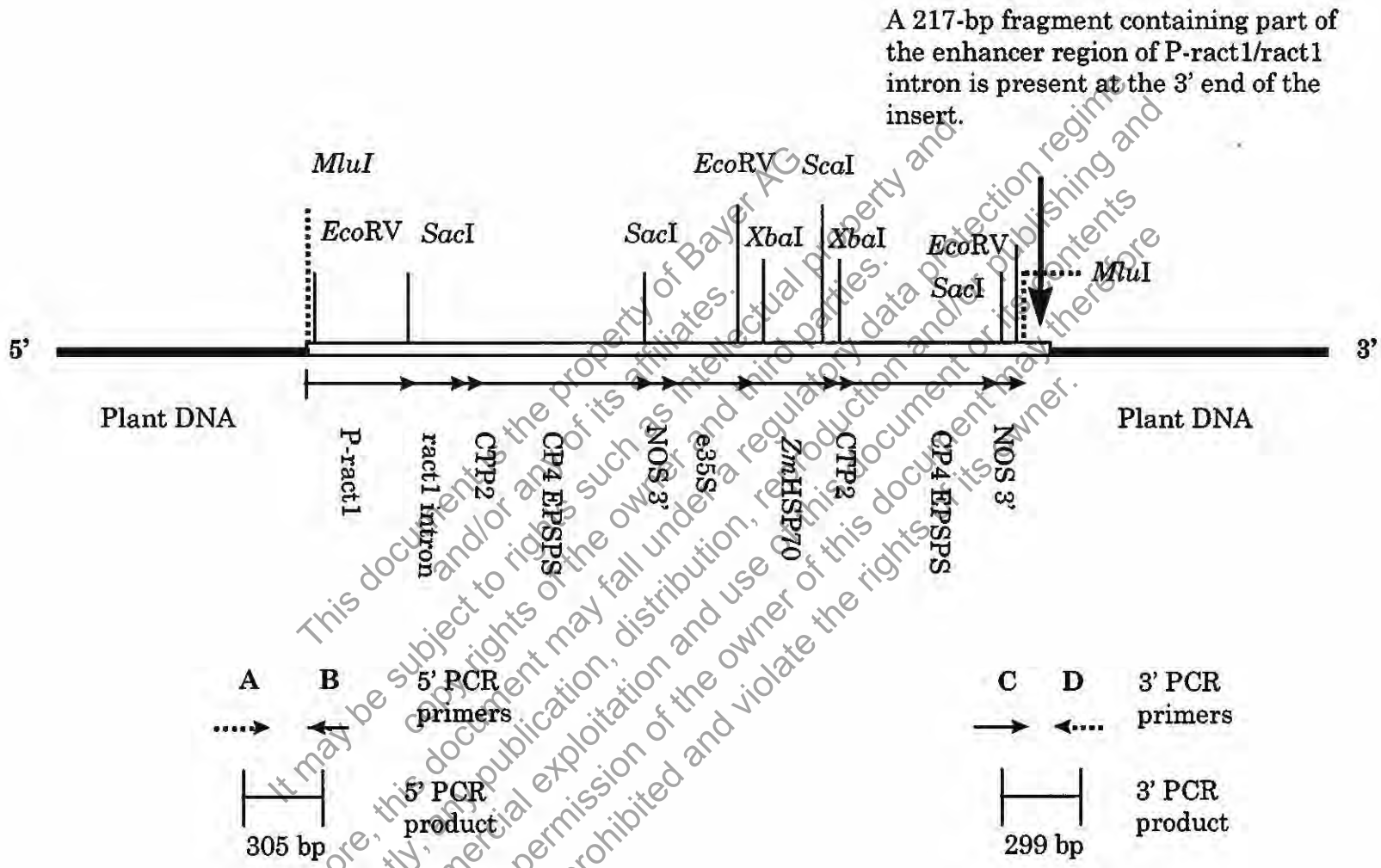


Figure 13. Schematic representation of the NK603 insert. This figure depicts the predicted insert in corn line NK603 based on data from Southern blot analysis and PCR confirming the sequences at the 5' and 3' ends of the insert. There is one complete copy of the PV-ZMGT32L fragment that was used in corn 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). The 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 plants when selective agents such as herbicides have been applied (Sari-Gorla et al., 1994; Touraev et al., 1995).

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.A. 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			
	Positive	Negative	Segregating	Positive	Negative	Segregating	ChiSq
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 *Eco*RV, 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.

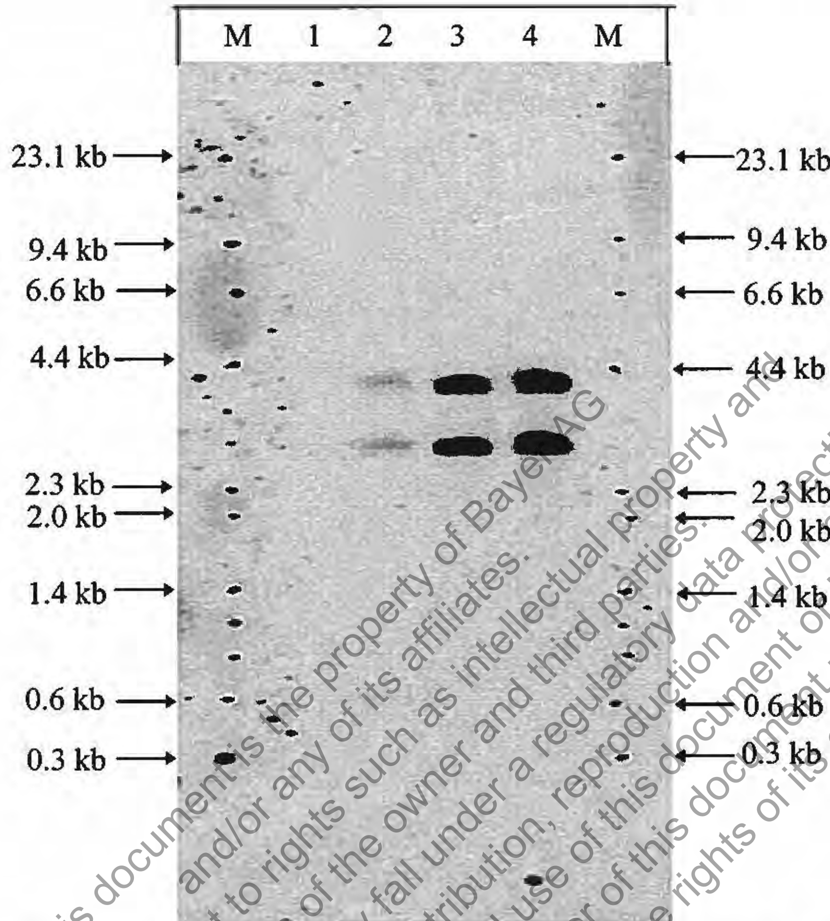


Figure 14. Southern blot analysis of NK603: stability of the inserted DNA. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were separated by gel-electrophoresis, blotted and then probed with the full-length ^{32}P-labeled CTP2-CP4 EPSPS fragment. Lane designations are as follows:

Lane 1: B73 DNA
2: B73 DNA spiked with 29 pg PV-ZMGT32
3: NK603 F1 DNA
4: NK603 BC5F1 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)].

VI. Safety of the New Corn Variety

The flow charts presented in the FDA Food Policy (FDA, 1992) were utilized to organize the following summary of the studies conducted and other information which demonstrate the substantial equivalence of Roundup ready Corn line NK603 to the nontransgenic parental control line and other corn varieties grown commercially. The pathway leading to "no concern" is highlighted with bold arrows in the flowcharts reproduced below.

A. Safety Assessment of New Varieties: the Host Plant, Corn

Corn, *Zea mays* L., the host plant, has a history of safe use. Corn grain and processed fractions are consumed in a multitude of human food and animal feed products. Corn forage is extensively consumed as an animal feed by ruminants. Corn does not contain known allergens or produce significant toxins or antinutrients warranting analytical or toxicological tests.

1. The History and Utilization of Modern Corn

Corn is one of the few major crop species indigenous to the Western Hemisphere and is grown in nearly all areas of the world (Hallauer *et al.*, 1988). The exact origin of modern corn has been debated among botanists for years although evidence exists to support a number of theories based upon teosinte and human involvement (Aldrich *et al.*, 1986; Galinat, 1988; Jugenheimer, 1976; Mangelsdorf, 1974). In the United States, corn is the largest crop in terms of planted acreage, total production, and crop value (National Corn Growers Association, 1999). United States production in 1998 was 248 million metric tons with the majority of national production concentrated across what is known as the "Corn Belt" in the upper Midwest (National Corn Growers Association, 1999). Corn is generally used as an animal feed to provide abundant, high-quality animal products and in a diverse range of human food and large volume industrial products. A detailed discussion of the history and utilization of modern corn has been previously provided as an appendix in prior Monsanto corn product summaries.

2. Corn as a Food Source in the United States

Although an ideal source of energy, little whole kernel or processed corn is consumed by humans worldwide when compared to corn-based food ingredients (Hodge, 1982; Watson, 1988). The low price and ready availability of corn has resulted in the development of large volume food and industrial uses. Corn is an excellent raw material for the

manufacture of starch, not only because of price and availability, but also because the starch is easily recovered in high yield and purity (Anderson and Watson, 1982). Nearly one fourth of corn starch is sold as starch products; more than three fourths of the starch is converted to a variety of sweetener and fermentation products including high fructose corn syrup and ethanol (Watson, 1988; National Corn Growers, 1999; Anderson and Watson, 1982; White and Pollak, 1995). Additionally, corn oil is commercially processed from the germ and accounts for approximately nine percent of domestic vegetable oil production (Orthoefer and Sinram, 1987). Each of these materials is a component of many foods including bakery and dairy goods, beverages, confections, and meat products. Corn does not contain any known allergens or produce significant toxins or antinutritional factors (Watson, 1982; White and Pollak, 1995). A detailed discussion of corn as a food source in the United States has been previously provided as an appendix in prior Monsanto corn product summaries. This information has been subsequently published by Drs. Pollak and White (White and Pollak, 1995; Pollak and White, 1995).

3. Corn and Animal Nutrition in the United States

Animal feeding is by far the largest use of corn in the United States with over one-half to two-thirds of annual production fed to cattle, chickens, and swine (Hodge, 1982; Perry, 1988; U.S. Grains Council, 1999; Watson, 1988). Of an approximately 200 million metric tons of grain, forty to fifty percent is fed to livestock directly as grain. Another 1.5 to 2 million metric tons of by-products of the wet and dry milling industries, primarily corn gluten meal and feed, are fed directly or in formulated feeds (Perry, 1988). In addition to corn grown for grain, approximately 10 to 12% of annual corn acreage is utilized as whole plant corn silage, with consumption confined almost entirely to ruminants (Watson, 1988; Perry, 1988). Corn is readily consumed by livestock and because of its high starch/low fiber content is one of the most concentrated sources of energy, containing more total digestible nutrients than any other feed grain. A detailed discussion of corn and animal nutrition in the United States has been previously provided as an appendix in prior Monsanto corn product summaries.

4. Compositional Analysis of Roundup Ready Corn Line NK603

Compositional analyses were conducted on key corn tissues produced in U.S. trials at two replicated sites in Illinois and Ohio and six non replicated sites in Iowa, Illinois, Indiana and Kansas. Grain and forage samples of line NK603 (treated with Roundup herbicide) and the nongenetically modified parental control line were collected from this range of sites across the U.S. Corn Belt. The nutritional value of

line NK603 was comparable to the control line and within the range ordinarily observed for corn.

a. Materials and methods

See Appendix II for a description of the compositional analytical methods used in the analysis of corn line NK603.

b. Compositional analyses of grain and forage from corn line NK603

The compositional analysis data and statistical evaluation are summarized in Appendix III. Statistical analyses of the data were conducted as described in Appendix II, section C. Component values are expressed as follows: amino acids as % total amino acids; proximates (except moisture), ADF, NDF, magnesium, calcium, phosphorus, potassium and phytic acid as % dry weight (dw); moisture as % fresh weight (fw); fatty acids as % total fatty acids; copper, iron, manganese and zinc as mg/kg dw; vitamin E as mg/g dw; and trypsin inhibitor in TIU (trypsin inhibitor units)/mg dw. The following components are not listed in Tables 1-7 of Appendix III since they had >50% of values below the LOD of the assay and hence were not used in the statistical analysis: sodium, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, and 20:4 arachidonic acid.

Fifty-one different compositional components were evaluated for corn line NK 603 as part of the safety and nutritional assessment of this product. The values for all the compositional components assessed were either within the range observed for nontransgenic commercial corn lines, published literature ranges (Jugenheimer, 1976; Watson, 1982; Watson, 1987) or previously reported ranges for nontransgenic corn varieties ([redacted] 1995; [redacted] 1996a,b; 1997a,b). Data were developed and statistical analyses conducted for three sets of comparisons: analyses for each of two replicated trials and for a combination of trials at different field sites. Therefore, a total of 153 comparisons were made: 51 comparisons for each of these three statistical analyses.

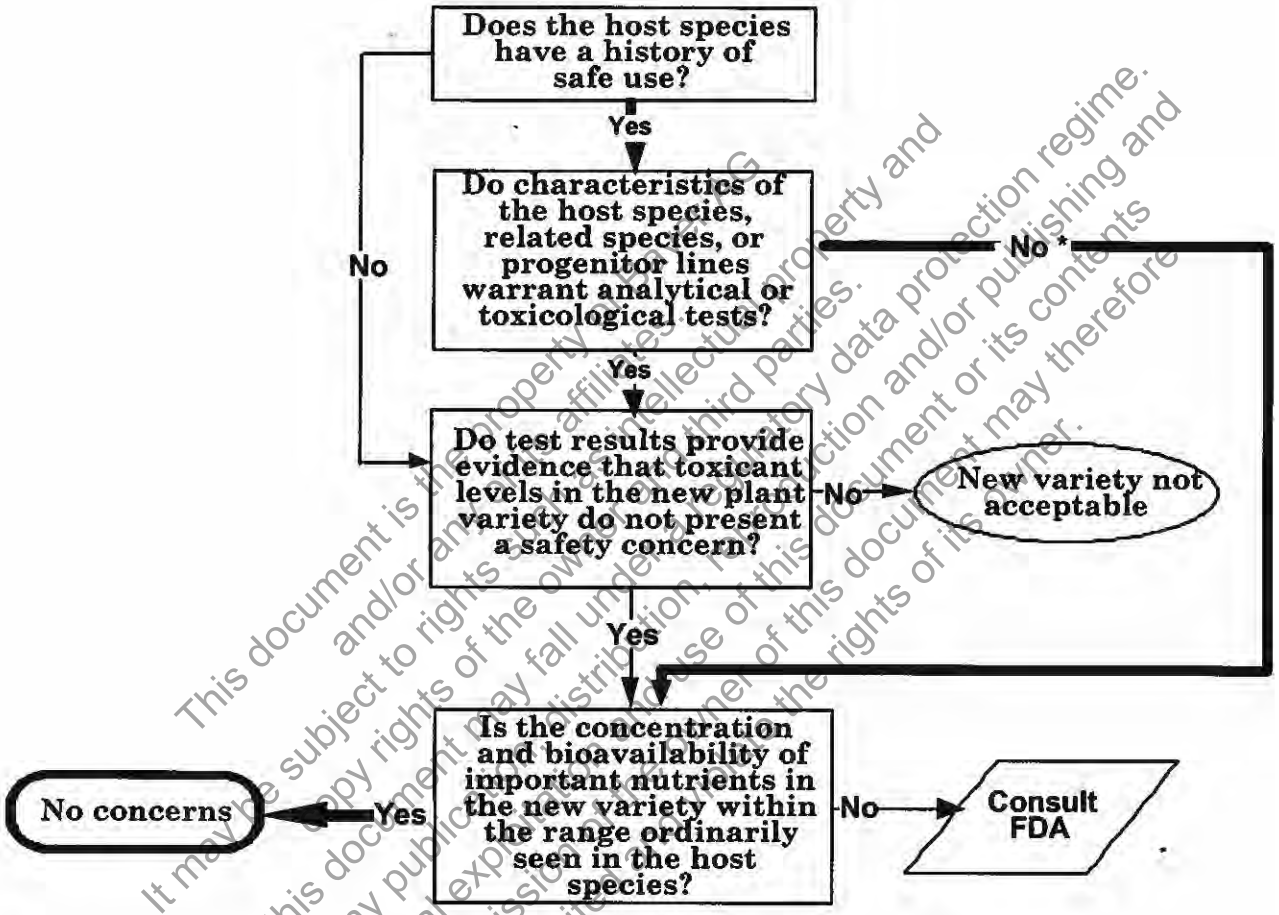
Statistical evaluation showed that there were no statistically significant differences in 132 of the 153 comparisons made between Roundup Ready corn line NK603 and the control line. Only one of the statistically significant differences was consistently observed for each of the two replicated trial comparisons and the comparison across

sites (Table 1 of Appendix III). Differences which were observed for only one or two of these comparisons, and not consistently across all three comparisons, are not considered biologically meaningful or relevant. Furthermore, all of these differences were well within reported ranges for corn. The only component for which statistically significant differences were observed across all three statistical evaluations was 18:0 stearic acid in grain. The absolute magnitude of the differences as a percent of the corresponding NK603 mean value for stearic acid ranged between 3.7-5.1%. These small differences are well within the range of natural variability and the published ranges for stearic acid in corn grain. Therefore, these minor differences are not considered biologically relevant and the grain from Roundup Ready corn line NK603 is considered compositionally equivalent to that of conventional corn grain.

5. Conclusions

The Food Policy recommends that key compositional components of genetically modified plant varieties be assessed prior to commercial introduction. Monsanto has performed extensive analytical studies to compare the composition of grain and forage of Roundup Ready corn line NK603 to the nontransgenic parental control. The compositional data demonstrates that the grain and forage from this line are substantially equivalent to the nontransgenic parental control corn and other corn varieties grown commercially. This point, together with the safe history of use of the host organism, corn, as a common source of animal feed and human food, leads to the conclusion of "no concern" in response to the question posed in Figure 15, "Safety Assessment of New Varieties: the Host Plant".

Figure 15. Safety Assessment of New Varieties: the Host Plant (taken from FDA Food Policy, Figure 2). The pathway leading to "no concerns" for Roundup Ready corn lines is highlighted with bold arrows.



* New corn varieties are not typically subjected to extensive analytical tests However, compositional analyses to verify levels of nutrients to ensure the wholesomeness of Roundup Ready Corn line NK603 were performed as discussed in the Food Policy.

B. Safety Assessment of New Varieties: the Donor(s)

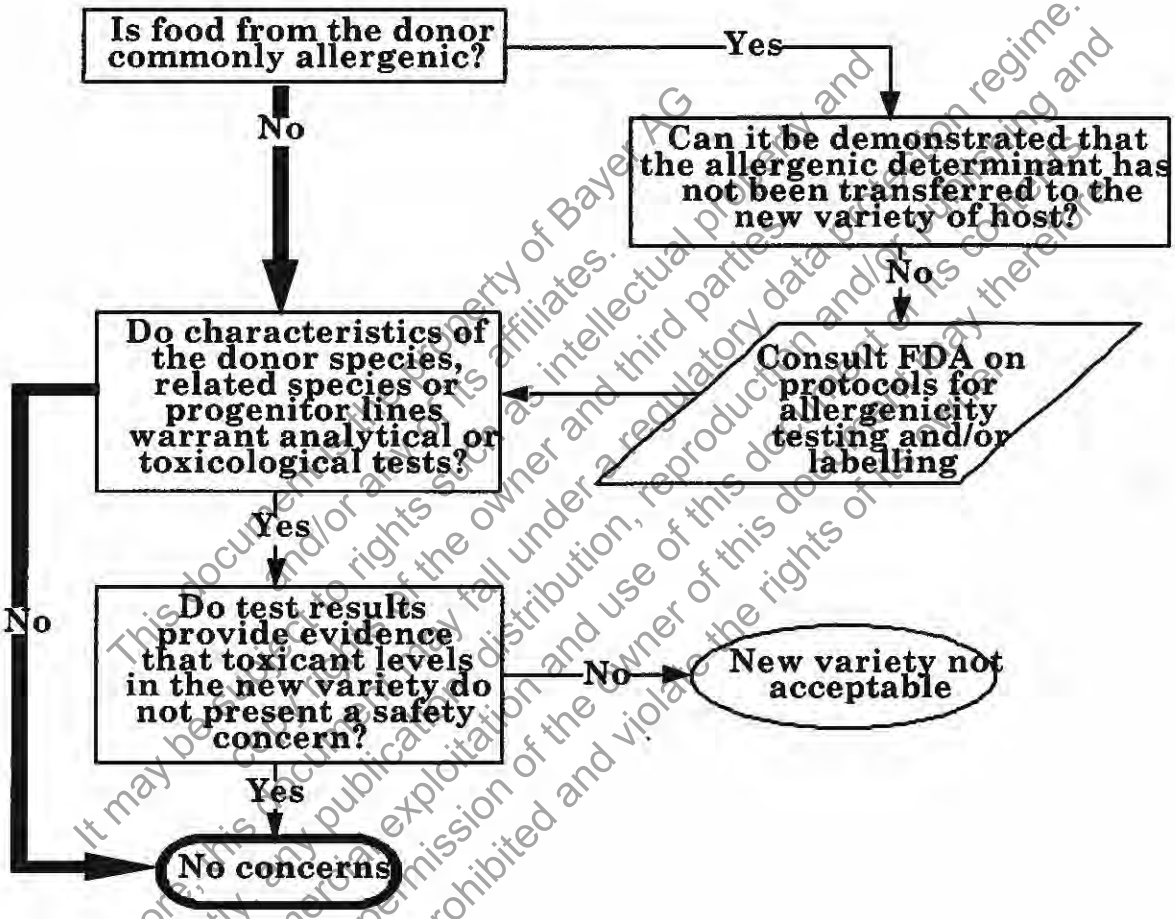
Donor organism: *Agrobacterium* sp. strain CP4

In response to the question posed in Figure 16, "Safety Assessment of New Varieties: the Donor(s)", the safety of the CP4 EPSPS gene was evaluated. *Agrobacterium* sp. strain CP4 was chosen as the donor organism due to the fact that this bacteria exhibited tolerance to glyphosate by expressing a naturally glyphosate tolerant EPSPS ([redacted] 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* is not known for human or animal pathogenicity, is not commonly allergenic and does not warrant analytical or toxicological tests. The CP4 and native corn EPSPS enzymes are functionally equivalent except for their affinity to glyphosate.

The EPSPS from *Agrobacterium* sp. strain CP4 is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most glyphosate tolerant EPSPSs (Barry *et al.*, 1992; [redacted] 1996). EPSPS is an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants (including corn) and micororganisms (Levin and Sprinson, 1964; Steinrüken and Amrhein, 1980); therefore, this enzyme and its activity are not novel in food derived from plant sources. Genes for numerous EPSPSs have been cloned (Padgette *et al.*, 1996), and active site domains are conserved among the known EPSPSs. Bacterial EPSPSs have been well characterized with respect to the 3-dimensional X-ray crystal structure (Stallings *et al.*, 1991) and detailed kinetic and chemical mechanism (Anderson and Johnson, 1990). CP4 EPSPS thus represents one of many different EPSPSs found in nature.

Agrobacterium sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding glyphosate tolerant soybean (*Glycine max*) (1994), canola (*Brassica napus*) (1995), cotton (*Gossypium hirsutum*) (1995), corn (*Zea mays*) (1996) and sugar beet (*Beta vulgaris*) (1998). Further, as a result of Monsanto-sponsored studies provided to the Environmental Protection Agency (EPA), the Agency established an exemption from the requirement of a tolerance for residues of the plant pesticide inert ingredient CP4 EPSPS and the genetic material necessary for its production in all plants (40 CFR 180.1174; 61 FR 40340, August 2, 1996). These points, taken together with the properties of the CP4 EPSPS protein discussed in section VI.C, lead to the conclusion of "no concerns" for the source of the donor gene as listed in Figure 16.

Figure 16. Safety Assessment of New Varieties: the Donor(s) (taken from FDA Food Policy, Figure 3). The pathway leading to "no concerns" for Roundup Ready corn line NK603 is highlighted with bold arrows.



C. Safety Assessment of new varieties: proteins introduced from donor(s)

The CP4 EPSPS protein expressed by Roundup Ready corn line NK603 confers tolerance to glyphosate, the active ingredient in the Roundup herbicide. To address the FDA flow chart "Safety Assessment of New Varieties: Proteins Introduced from Donor(s)" (Figure 17), the protein expression levels from the introduced gene are provided, as well as a demonstration of the safety of the expressed protein, CP4 EPSPS.

1. Expression of the CP4 EPSPS protein in Roundup Ready corn line NK603

a. Levels of the CP4 EPSPS protein expressed in line NK603

The newly introduced protein, CP4 EPSPS, is present in low concentrations in the grain and forage of Roundup Ready corn line NK603. Studies were conducted to characterize the expressed protein and determine the levels of expression in these food and feed components. Levels of the CP4 EPSPS protein were estimated in forage and grain samples collected from six non-replicated and two replicated field sites, representative of the major U.S. corn production region, 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) to estimate the levels of CP4 EPSPS protein present in these tissues (Harlow and Lane, 1988).

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'- tetramethylbenzidene), 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 [redacted]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 NK603 forage were comparable for the non-replicated sites (25.5 µg/g fw) and replicated sites (25.9 µg/g fw). CP4 EPSPS protein levels in control forage were, as expected, below the Limit of Quantitation (LOQ) of the assay (<0.05 µg/g fw). Mean CP4 EPSPS protein levels in NK603 grain were comparable for the non-replicated sites (11.0 µg/g fw) and replicated sites (10.6 µg/g fw). CP4 EPSPS protein levels in control grain were, as expected, below the LOQ of the assay (<0.09 µg/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 ELISA in tissues of NK603 corn plants (µg/g fresh weight)

Sites	Parameter	Forage[a,c] (µg/g fw)	Grain[b,c] (µg/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.
[a]LOQ = 0.05 µg/g fw.
[b]LOQ = 0.09 µg/g fw.
[c]Values for all non-transgenic control samples were below the LOQ of the assay.

b. Equivalence of the CP4 EPSPS protein expressed in corn line NK603 to *E. coli* and to the CP4 EPSPS protein produced in Roundup Ready soybeans

It was previously concluded that microbially-produced CP4 EPSPS was functionally and physicochemically equivalent to CP4 EPSPS produced in plants ([redacted] 1996). A western blot analysis (Appendix I) was conducted to assess the equivalence of CP4 EPSPS protein produced in *Escherichia coli* (*E. coli*) and in Roundup Ready corn line NK603. Western blot analytical methodology (immunoblotting) is a

highly specific and sensitive method for the comparison of apparent molecular weights and immunological properties of low abundance proteins contained in complex mixtures such as grain extracts. A protein extract prepared from a commercial variety of Roundup Ready soybean was included in the study for comparison. Regulatory approval for Roundup Ready soybean was obtained in the U.S. in 1995 and these soybean varieties were grown on 10.2 million hectares in 1998 (James, 1998). The CP4 EPSPS protein produced in Roundup Ready corn line NK603 was demonstrated to be equivalent to both the CP4 EPSPS protein expressed in and purified from *E. coli* for safety studies and CP4 EPSPS expressed by commercial Roundup Ready soybeans (Appendix I). Equivalence was visually confirmed by identical apparent molecular weights (~47 kDa) and immunological properties when detected using antibodies specific for CP4 EPSPS protein. The previously demonstrated functional and physicochemical equivalence of microbially produced and plant produced CP4 EPSPS protein in combination with the physicochemical equivalence reported for CP4 EPSPS produced in corn line NK603 enabled the conclusion that safety data generated using microbially produced CP4 EPSPS was valid for CP4 EPSPS produced in corn line NK603. This study also served to characterize corn line NK603 with respect to the CP4 EPSPS protein.

2. Human and animal safety of the CP4 EPSPS Protein

An assessment of the human and animal health safety of the CP4 EPSPS protein was conducted based upon the extensive characterization of the CP4 EPSPS protein and its comparability to EPSPS enzymes commonly found in a wide variety of food sources, which have a long history of safe use. Further, CP4 EPSPS itself has a history of safe human and animal consumption as based upon previous studies with Roundup Ready soybean and the extensive global adoption and consumption of this product expressing the same CP4 EPSPS protein. In addition to the history of safe use of both the EPSPS class of proteins and the host, corn, studies were conducted and information gathered which supports the safety of this CP4 EPSPS protein. This supportive material for the safety of the CP4 EPSPS protein includes the: (1) lack of acute toxicity as determined by a mouse gavage study, (2) rapid digestion in simulated gastric and intestinal fluids, (3) lack of homology with known protein toxins, and (4) lack of allergenic potential. Finally, compositional analyses of grain and forage demonstrate that Roundup Ready corn line NK603 is nutritionally equivalent to other corn.

a. Similarity of CP4 EPSPS to EPSPSs derived from food sources with a long history of safe consumption

The CP4 EPSPS protein utilized in Roundup Ready corn line NK603 is substantially similar to EPSPSs consumed in a variety of food and feed sources. The CP4 EPSPS gene has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids. The CP4 EPSPS protein is homologous to EPSPSs naturally present in all crops (e.g., soybean and corn) and in fungal and microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis* (Mountain, 1989) which have a history of safe human consumption [redacted] 1993; 1996; Harrison *et. al.*, 1996) as shown in the table below. The similarity of the CP4 EPSPS protein to EPSPSs in a variety of foods supports the lack of health concerns and extensive human consumption of the family of EPSPS proteins.

Table 4. Percent amino acid sequence identity and similarity of CP4 EPSPS with other EPSPSs

	soybean	corn	petunia	*E. coli*	*B. subtilis*	*S. cerevisiae*
CP4 EPSPS						
sequence identity	26	24	23	26	41	30
sequence similarity	51	49	50	52	59	54

b. CP4 EPSPS substrate specificity

The CP4 EPSPS protein has a well-characterized catalytic function in plants, bacteria and fungi. The expressed protein, 5-nolpyruvylshikimate-3-phosphate synthase (EPSPS), catalyses a non-rate limiting step in the shikimate pathway involved in aromatic amino acid biosynthesis in plants and microorganisms (Steinrüken and Amrhein, 1980). 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 [redacted] *et al.*, 1993; 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 structurally similar to the native corn EPSPS, but retains its catalytic activity in the presence of the inhibitor, glyphosate [redacted] 1993). The shikimate pathway is not present in mammals, which contributes to the selective toxicity of glyphosate to plants.

c. Human and animal consumption of the CP4 EPSPS protein

The CP4 EPSPS protein as expressed in Roundup Ready corn line NK603 has a history of safe human and animal consumption due to the previous approval and commercialization of Roundup Ready soybean expressing the same 47.6 kDa protein for glyphosate tolerance. Animal feeding studies with rats, broiler chickens, catfish and dairy cows were conducted to compare the feeding value (wholesomeness) of Roundup Ready soybean to their parental cultivars. In all studies, measured variables were similar for animals fed both CP4 EPSPS-containing lines and the parental lines, indicating that the feeding value was comparable and confirming the safety of the CP4 EPSPS introduced protein (Hammond *et al.*, 1996). Commercially, Roundup Ready soybean has been rapidly adopted by growers with 400,000 (1% of U.S. soybean area) hectares grown in 1996, 3.6 million hectares grown in 1997 (13% of U.S. soybean area) and 10.2 million hectares grown in 1998 (36% of U.S. soybean area) (James, 1998). Globally, of the 67 million hectares of soybean planted, Roundup Ready soybean expressing the CP4 EPSPS protein was produced on 14.5 million hectares in 1998 including almost 4.3 million hectares in Argentina and 40,000 hectares in Canada. Although CP4 EPSPS is expressed at low levels in oilseed, which is further reduced in soybean processing, a safe history of human and animal dietary exposure by ingestion of protein products derived from soybean exists today. The CP4 EPSPS protein as expressed in corn line NK603 is also equivalent to the CP4 EPSPS protein expressed in Roundup Ready canola, cotton and sugar beet, from which processed food and feed fractions have a history of safe consumption.

d. Safety of CP4 EPSPS protein demonstrated by mouse acute gavage

In addition to the history of safe use of both the EPSPS class of proteins and the host, corn, studies were conducted and information gathered which supports the safety of the CP4 EPSPS protein. In an acute oral toxicity study, CP4 EPSPS was administered to mice as a single high dose to confirm its safety ([redacted] 1996). Results from this study demonstrated, as expected, that the CP4 EPSPS protein is not toxic. The CP4 EPSPS protein was over-produced ([redacted] 1993) and purified ([redacted] 1993) from *Escherichia coli*, characterized ([redacted] 1993), demonstrated to be equivalent to the corn-produced CP4 EPSPS ([redacted] 1999) and administered by gavage to mice to assess acute toxicity. Acute administration was considered appropriate to assess the safety of CP4 EPSPS, since proteins that are toxic act via acute mechanisms (Sjoblad *et al.*, 1992; Pariza and Foster, 1983; Jones and Maryanski, 1991). The no effect level (NOEL) for oral toxicity in mice was 572 mg/kg, the highest dose tested ([redacted] 1993; [redacted] 1996). This NOEL

represents a safety margin of approximately 260,000-fold based on mean U.S. daily corn consumption and mean average protein expression in Roundup Ready corn grain (assuming no loss of CP4 EPSPS due to processing). There were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and CP4 EPSPS protein-treated groups. This result was expected as the great majority of proteins are not toxic (Pariza and Foster, 1983), the CP4 EPSPS protein was demonstrated to be digested readily in gastric and intestinal fluid *in vitro* [redacted] 1993) and similar EPSPS proteins with a history of safe use are already present in the diet.

e. Digestion of the CP4 EPSPS protein in simulated gastric and intestinal fluids

Factors which increase the likelihood of allergic oral sensitization to proteins include level of food consumption, the relative quantity of the protein in the food, and stability of the protein to gastro-intestinal digestion. Protein allergens tend to be stable to the peptic and acidic conditions of the digestive systems if they are to reach and pass through the intestinal mucosa to elicit an allergenic response (Kimber *et al.*, 1999; Astwood *et al.*, 1996b; Metcalfe *et al.*, 1996). Simulated mammalian gastric and intestinal fluids were prepared and used to assess the susceptibility of the CP4 EPSPS protein to proteolytic digestion *in vitro*. The method of preparation of the simulated mammalian gastric and intestinal digestive solutions used is described in the United States Pharmacopeia (1989). A similar model has also been used to examine the stability of milk allergens (Asselin *et al.*, 1989). *In vitro* studies with simulated digestive solutions are widely used as models of animal digestion. These models have been used to investigate the digestibility of plant proteins (Nielson, 1988; Marquez and Lajolo, 1981), animal proteins (Zikakis *et al.*, 1977) and food additives (Tilch and Elias, 1984), to assess protein quality (Akeson and Stahmann, 1964), to study digestion in pigs and poultry, to measure tablet dissolution rates to monitor biodegradation for pharmaceutical applications (Akeson and Stahmann, 1964), and to investigate the controlled-release of experimental pharmaceuticals (Doherty *et al.*, 1991).

The CP4 EPSPS protein was shown to be rapidly degraded *in vitro* using simulated digestive fluids [redacted] 1996; [redacted] 1993). The data demonstrated a half-life for CP4 EPSPS protein of less than 15 seconds in the gastric system and less than 10 minutes in the intestinal system, based on western blot analysis. If any of the CP4 EPSPS protein did survive the gastric system, it would be rapidly degraded in the intestine. To put the rapid degradation of this protein in the simulated gastric system into perspective, solid food has been

estimated to empty from the human stomach by about 50% in two hours, while liquid empties by 50% in approximately 25 minutes (Sleisenger and Fordtran, 1989). Based on these results, CP4 EPSPS protein is expected to be readily digested in the mammalian digestive tract.

f. Lack of structural homology of CP4 EPSPS to known toxins

A database of 4,677 protein sequences associated with toxicity was assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt). The amino acid sequence of the CP4 EPSPS protein was compared to protein sequences in the toxin database using the FASTA[1] sequence alignment tool. In addition, the amino acid sequence of the CP4 EPSPS protein was compared to all protein sequences in the publicly-available genetic databases to screen for structural similarity to other known proteins, including pharmacologically active proteins. As expected, CP4 EPSPS shared sequence similarities to homologous EPSPS proteins. These EPSPS proteins have not been described as toxins relevant to human health. No other significant structural homology was observed ([redacted] 1999b).

The safety of the introduced CP4 EPSPS protein to humans was established based on the following considerations: (1) characterization of the CP4 EPSPS protein and its similarity to plant and microbial EPSPSs with a history of safe human consumption; (2) confirmation of safety in an acute mouse gavage study, (3) rapid digestion in simulated digestive systems and (4) a lack of homology to known protein toxins. These facts support the conclusion that the CP4 EPSPS protein is not reported to be toxic and is considered safe for human consumption.

g. Assessment of the allergenic potential of CP4 EPSPS Protein

A comparison of the amino acid sequence of an introduced protein with the amino acid sequences of known allergens is a useful indicator of allergenic potential. The amino acid sequences of most major allergens, including food allergens, have been reported (Astwood *et al.*, 1996a). The important IgE binding epitopes of many allergenic proteins have been mapped (Elsayed and Apold, 1983; Elsayed *et al.*, 1991; Zhang *et al.*, 1992). The optimal peptide length for binding is between 8 and 12 amino acids (Rothbard and Gefter, 1991). T-cell epitopes of allergenic proteins and peptide fragments appear to be least eight amino acids in length (O'Hehir *et al.*, 1991). Exact conservation of epitope sequences is observed in homologous allergens of disparate species (Astwood *et*

[1]FASTA is based on the algorithms of Needleman and Wunsch (1970) and of Smith and Waterman (1981), which consider all possible alignments between a query sequence and a database sequence.

al., 1995). Indeed, conservative substitutions introduced by site-directed mutagenesis reduce epitope efficacy (Smith and Chapman, 1995). Based on this information, an immunologically relevant sequence comparison test for similarity between the amino acid sequence of the introduced protein and known allergens is defined as a match of at least eight contiguous identical amino acids.

A database of 567 protein sequences associated with allergy and coeliac disease was assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt) and from current literature. The amino acid sequence of the CP4 EPSPS protein was compared to these sequences using the sequence alignment tool FASTA (Pearson and Lipman, 1988). CP4 EPSPS shared no structurally significant sequence similarity to sequences within the allergen database ([redacted] 1999a). In addition, the amino acid sequence of the CP4 EPSPS protein was compared to the allergen database[1] using an algorithm that scans for a window of eight linearly contiguous identical amino acids. The CP4 EPSPS protein sequence does not share eight linearly contiguous amino acid identities to any sequence in the allergen database. These data establish that the CP4 EPSPS protein does not share any immunologically significant sequence similarity to proteins considered as allergens or gliadins (*i.e.*, a sequence derived from a common ancestor gene and/or containing a potential allergenic epitope).

The biochemical profile of the CP4 EPSPS enzyme also provides a basis for allergenic assessment when compared with known protein allergens. Protein allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response (Kimber *et al*, 1999; Astwood *et al.*, 1996b; Metcalfe *et al.*, 1996). The CP4 EPSPS protein was shown to be very labile to digestion by proteases present in the mammalian digestive system (see above), minimizing any potential for it to be absorbed by the intestinal mucosa.

Another significant factor contributing to the allergenicity of proteins is their high concentration in foods that elicit an allergenic response (Taylor, 1992; Taylor *et al.*, 1987; and Taylor *et al.*, 1992). Most allergens are present as major protein components in the specific food, typically ranging between 1 and 80% of total protein (Astwood and Fuchs, 1996). This is true for the allergens in egg (Yunginger, 1991), milk (Baldo, 1984; Lebenthal, 1975; Taylor, 1986; Taylor *et al.*, 1987), soybean (Shibasaki *et al.*, 1980; Burks *et al.*, 1988; Pedersen and Djurtoft, 1989), and peanuts (Barnett *et al.*, 1983; Sachs *et al.*, 1981; Barnett and Howden, 1986; Kemp *et al.*, 1985). In contrast, CP4 EPSPS

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[1] A linear pairwise comparison was made using "IDENTITYSEARCH' (A program developed at Monsanto) to identify potential short (<7 amino acids) regions of sequence identity.

is present at approximately 0.01% of the total protein found in the grain of Roundup Ready corn. The low levels of the CP4 EPSPS protein in corn grain, combined with the digestive liability of this protein relative to that for known food allergens, establishes an extremely low probability of the CP4 EPSPS protein being absorbed via the intestinal mucosa during consumption and therefore would not be expected to trigger production of antibodies, including the IgE antibodies responsible for allergenicity.

The data and analyses described above and summarized in Table 5 support the conclusion that the CP4 EPSPS protein does not pose a significant allergenic risk as it is not derived from an allergenic source, does not possess immunologically relevant sequence similarity with known allergens and does not possess the characteristics of known protein allergens. Furthermore, this protein is homologous to EPSPSs from a variety of commonly consumed plant and microbial sources which have a long history of safe consumption and is, in fact, identical to the CP4 EPSPS protein with a history of safe consumption in Roundup Ready soybean and other Roundup Ready crops.

Table 5. Characteristics of known allergenic proteins[a]

Characteristic	Allergens	CP4 EPSPS
Allergenic source of gene	yes	no
Stable to digestion	yes	no
Similar sequence to allergens	yes	no
Prevalent protein in food	yes	no

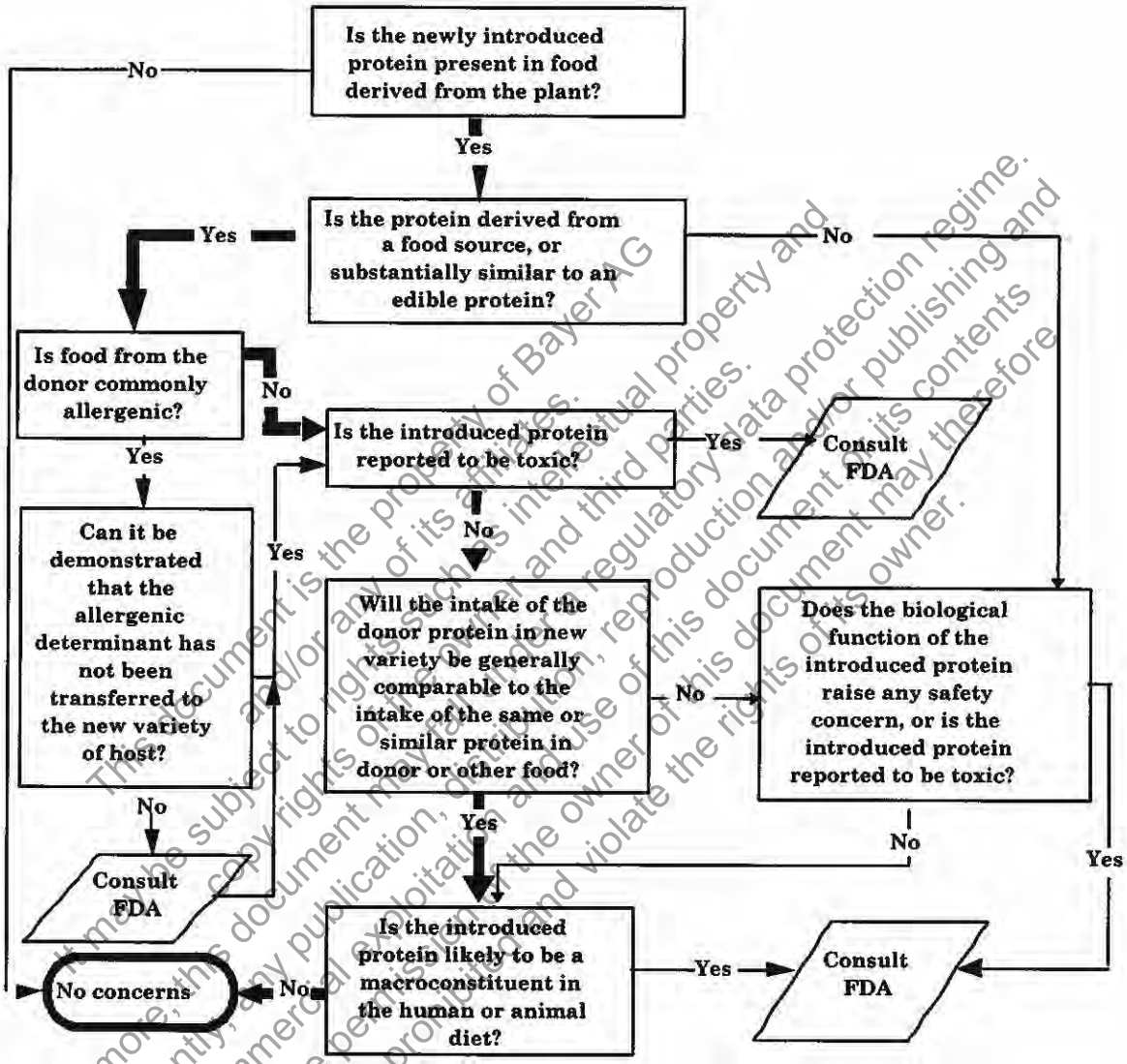
[a] As described in Taylor (1992) and Taylor *et al.*, (1987)

3. Conclusions

In summary, the human and animal health safety of the CP4 EPSPS protein is based upon the extensive characterisation of the CP4 EPSPS protein and its comparability to EPSPS enzymes commonly found in a wide variety of food sources which have a long history of safe use. Furthermore, the CP4 EPSPS protein is identical to the same protein found in Roundup Ready soybean and other Roundup Ready crops with a history of safe human and animal consumption. The safety of the CP4 EPSPS protein is further supported by studies and information including the: (1) lack of acute toxicity of CP4 EPSPS protein as determined by a mouse gavage study, (2) rapid digestion of CP4 EPSPS protein in simulated gastric and intestinal fluids, (3) lack of homology of CP4 EPSPS with known protein toxins and (4) lack of allergenic potential of CP4 EPSPS protein.

These facts support the conclusion that the CP4 EPSPS protein is considered safe for human consumption and based upon these data and information, we have reached the conclusion of "No Concerns" as listed on Figure 17.

Figure 17. Safety assessment of new varieties: proteins introduced from donor(s) (taken from FDA Food Policy Figure 4). The pathway leading to "no concerns" for Roundup Ready corn line NK603 is highlighted with bold arrows.



VII. Conclusion for the Safety Assessment of Roundup Ready Corn Line NK603

Monsanto Company has developed, through molecular biological techniques, corn (*Zea mays* L.) plants which are commercially tolerant to glyphosate, the active ingredient in Roundup® herbicide. The tissues of these plants produce a glyphosate-tolerant EPSPS from *Agrobacterium* sp. strain CP4 (CP4 EPSPS), the same tolerant EPSPS protein already expressed in Roundup Ready® varieties of soybean, canola, beet and cotton. The CP4 EPSPS enzyme has a reduced affinity for glyphosate when compared to the native corn EPSPS enzyme, which is inhibited by glyphosate. As a result, corn plants expressing the CP4 EPSPS protein are unaffected when treated with glyphosate, as the continued action of the tolerant enzyme provides the plant's need for aromatic amino acids.

The safety of the EPSPS protein and CP4 EPSPS-expressing corn have been extensively investigated. This summary provides an assessment of the human health safety of the CP4 EPSPS protein based upon the characterization of the CP4 EPSPS protein and its comparability to EPSPS enzymes commonly found in a wide variety of food sources which have a long history of safe use. Further, the CP4 EPSPS protein is identical to the same protein found in Roundup Ready soybean and other Roundup Ready crops with a history of safe human and animal consumption. Additional studies were conducted and information gathered which supports the safety of the CP4 EPSPS protein including the: (1) lack of acute toxicity of CP4 EPSPS protein as determined by a mouse gavage study, (2) rapid digestion of CP4 EPSPS protein in simulated gastric and intestinal fluids, (3) lack of homology of CP4 EPSPS with known protein toxins and (4) lack of allergenic potential of CP4 EPSPS protein. These data support the assessment of safety of the CP4 EPSPS protein and, when taken together with analyses performed on Roundup Ready corn line NK603 demonstrating compositional equivalence to conventional corn varieties, support the conclusion that corn line NK603 is as safe and nutritious as conventional corn currently being marketed.

These data lead to a conclusion of "no concerns" for every criterion in the flow charts outlined in the FDA's Food Policy. Corn line NK603 modified to be tolerant to Roundup herbicide is not materially different in composition, safety, or any relevant parameter from corn now grown, marketed, and consumed. Sales and consumption of corn grain derived from Roundup Ready corn line NK603 would be fully consistent with the Agency's Food Policy, the Federal Food, Drug, and Cosmetic Act, and current practices for the development and introduction of new corn varieties.

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Appendix I Equivalence of the CP4 EPSPS Protein

I. Introduction

Western blot analysis was conducted to assess the equivalence of CP4 EPSPS protein produced in *E. coli*, in Roundup Ready corn line NK603, and in 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 by 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 plant-produced protein to the protein expressed in *E. coli* has been confirmed. This demonstration of equivalence justifies the application of the safety data generated using the *E. coli* produced protein to 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 for 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 presence of the CP4 EPSPS gene in the Polymerase Chain Reaction (PCR).

B. Control materials. There were two control materials for this study. One control material was a non-transgenic parental line (LH82 x B73) which does not contain the genetic material to encode CP4 EPSPS. Grain for the control line was collected from field grown plants as specified 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 absence of the CP4 EPSPS gene in the PCR.

The second control material was non-transgenic soybean line A5403, which does not contain the genetic material to encode CP4 EPSPS. The control grain sample was obtained from plants produced according to Study Protocol # 92-01-30-02 [redacted]1993). Control soybean grain was stored at approximately -20 °C or below.

C. Reference materials. The first reference material was CP4 EPSPS protein produced by fermentation (100 L) of *E. coli* strain GB100 ([redacted] 1993), transformed with plasmid pMON21104 ([redacted] 1993). The protein was purified to greater than 90% purity ([redacted] *et al.*, 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 ([redacted] 1993b). This CP4 EPSPS standard 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 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 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.

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 ([redacted] 1996; Padgette *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 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.

B. Preparation of Protein extracts for western blot analysis. All extraction procedures were conducted on ice or at ~ 4 °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 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 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 ~100 °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 was determined by the method of Bradford (Bradford, 1976).

C. Electrophoresis. Extracts prepared as above for electrophoresis were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on 4-20% gradient gels using a mini gel system. 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, full-range color markers were analyzed on the gel so that effective transfer to PVDF membrane could be confirmed. Finally, biotinylated MW markers were loaded on the gel so that blots could be calibrated.

D. Western blot analysis (immunoblotting). Immunoblotting was conducted as follows: Proteins separated by SDS-PAGE were electrophoretically transferred to a PVDF membrane (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 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. Unbound NeutrAvidin-HRP was rinsed away with TBST washes. Immunoreactive bands were visualized on X-ray film using the enhanced chemiluminescence kits according the instructions provided by the manufacturer. Films were developed using an automatic film processor.

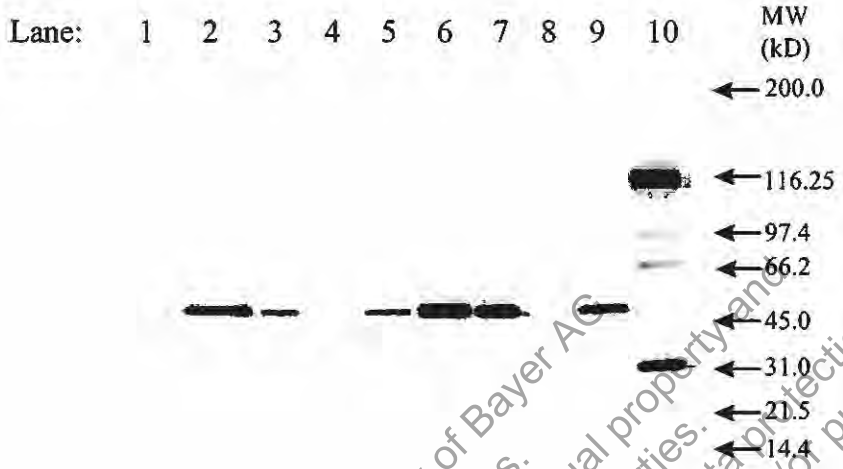
IV. Results and Discussion

The *E. coli*-produced CP4 EPSPS used for safety studies, the CP4 EPSPS expressed by Roundup Ready soybeans and the CP4 EPSPS expressed by 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 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. Immunoreactive 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 the CP4 EPSPS 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 Roundup Ready 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 serves to characterize Roundup Ready corn line NK603 with respect to the CP4 EPSPS protein.

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



Lane	Description	Amount
1	Full range color MW markers	7.5µL, 1.125 µg/band
2	*E. coli* CP4 EPSPS standard	10 µL, 5 ng
3	*E. coli* 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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Appendix II

Summary of Compositional Analytical Methods

Compositional analyses were conducted on key corn tissues produced in Roundup Ready corn grown in 1998 U.S. field trials at two replicated sites in Illinois and Ohio (IL2, OH) and at six non-replicated sites in Iowa, Illinois, Indiana and Kansas, U.S.A. (IA1, IA2, IA3, IL1, IN and KS) following treatment with Roundup herbicide. Forage and grain samples collected from Roundup Ready corn line NK603 and the nontransgenic parental control line (LH82 x B73) were analyzed. The control line has the same genetic background as that of the test line but lacks the gene encoding CP4 EPSPS protein. Compositional analyses were conducted to measure proximate (protein, fat, ash, carbohydrate, moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acid, fatty acid, vitamin E, mineral (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), phytic acid and trypsin inhibitor content of grain; as well as proximate, ADF and NDF content of forage. Statistical analyses were conducted for each of the replicated trials and for all trials combined, using SAS® software to determine statistically significant differences ($p < 0.05$). Details of the statistical analyses are reported in Appendix III.

A. Compositional Analytical Methods

Forage and grain samples were processed by grinding to a fine powder on dry ice in a blender or a vertical cutter mixer and shipped to Covance Laboratories, Inc., Madison, Wisconsin for compositional analyses. Grain samples were analyzed for proximate (protein, fat, ash, moisture), ADF, NDF, amino acid, fatty acid, vitamin E, mineral (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc) phytic acid and trypsin inhibitor content. Forage samples were analyzed for proximate, ADF and NDF content. Carbohydrate values in forage and grain were estimated by calculation. The same methods were used for the proximate analyses of forage and grain except for the analysis of fat as described below.

Acid detergent fiber (ADF). The method used was a modified version of the method described in USDA Agricultural Handbook No. 379.8 (1970). The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash removed the fats and pigments. The lignocellulose fraction was collected on the frit and the weight was determined gravimetrically. The limit of detection of the

method for this study was 0.1% fresh weight (fw)[1] . There was no analytical reference substance for this analysis.

Amino acid composition (TAAP). The method used was a modified version of AOAC method 982.30 (1995) which estimates the levels of 18 amino acids in the sample: alanine, arginine, aspartic acid (including asparagine), cystine, glutamic acid (including glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct hydrolysis with hydrochloric acid. The individual amino acids were then quantitated using an automated amino acid analyzer. The limit of detection of the method for this study was 0.1 mg/g. The reference standards were: Beckman K18, 2.5 µmol/mL per constituent except cystine (1.25 µmol/mL), lot no. S901670; Aldrich L-tryptophan, 99%, lot no. 12729HS; Sigma L-cysteic acid hydrate, 99.4%, lot no. 65H2658; Sigma L-methionine sulfone, 100%, lot no. 12H3349.

Ash (ASHM). The method used was a modified version of AOAC method 923.03 (1995). The sample was placed in an electric furnace at 550 °C and ignited to drive off volatile organics. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The limit of detection of the method for this study was 0.1% fw. There was no analytical reference substance for this analysis.

Carbohydrates (CHO). This method is described in USDA Agricultural Handbook No. 74, p. 2-11 (1973). Carbohydrate values were calculated by difference using the fresh weight-derived data and the following equation:

% carbohydrates = 100% - (% protein + % fat + % ash + % moisture)

Fat-acid hydrolysis (FAAH). The method used was a modified version of AOAC methods 922.06 and 954.02 (1995). The forage sample was hydrolyzed with hydrochloric acid at elevated temperature. The fat was extracted using ether and hexane. The extracts were washed with a dilute alkali solution and filtered through a sodium sulfate column. The extract was then evaporated, dried and weighed. The limit of detection of this method for this study was 0.1% fw. There was no analytical reference substance for this analysis.

[1] % fw = g/g x 100 fw

Fat-soxhlet extraction (FSOX). The method used was a modified version of AOAC method 960.39 (1995). The grain sample was weighed into a cellulose thimble containing sand or sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was evaporated, dried and weighed. This method was used for the grain sample analysis. The limit of detection of the method for this study was 0.1% fw.

Fatty acids (FAPM). The method used was a modified version of AOCS method Ce 1-62 (1981) which estimates the levels of 22 fatty acids in the sample: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:0 palmitic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic, 18:3 gamma linolenic acid, 20:0 arachidic acid, 20:1 eicosenoic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, and 22:0 behenic acid. The lipid in grain samples was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% (v/v) boron trifluoride:methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The limit of detection of this method for this study was 0.004% fw. The analytical reference standards (purity 100%) were: Nu Chek Prep Hazelton special prep nos 1 (lot no. JA10-I), 2 (lot no. JA10-H), 3 (lot no. F23-J), and 4 (lot no. JY30-I); and Nu Chek Prep methyl gamma linolenate (lot no. U-63M-F25-J).

Minerals/ICP emission spectrometry (ICPS). The method used was a modified version of AOAC methods 984.27 and 985.01 (1995) and a literature method (Dahlquist *et al.*, 1978). This method estimates the levels of nine minerals in the sample: calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc The sample was dried, precharred, and ashed overnight at 500° ± 50°C. The ashed sample was treated with hydrochloric acid, taken to dryness, and put into a solution of 5% (v/v) hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured by the inductively coupled plasma, with the emission of the standard solutions described as follows:

Mineral	Lot Numbers	Concentration (ppm)	Limit of Detection (ppm, fw)
Calcium	J5-111CA	10,000	20.0
Copper	6-137CU	1,000	0.5
Iron	6-172FE	1,000	2.00
Magnesium	K5-67MG	10,000	20.0
Manganese	6-55MN	1,000	0.3
Phosphorus	15-75P	10,000	20.0
Potassium	L5-149K	10,000	100.0
Sodium	L5-80NA	10,000	100.0
Zinc	6-171ZN	1,000	0.4

Moisture (M100). The method used was a modified version of AOAC methods 926.08 and 925.09 (1995). The sample was dried in a vacuum oven at 100°C to a constant weight. The moisture loss was determined and converted to percent moisture. The limit of detection of this method for this study was 0.1% fw. There was no analytical reference substance for this analysis.

Neutral detergent fiber, enzyme method (NDFE). The method used was a modified version of AACC method 32.20 (1983) and USDA Agricultural Handbook Method No. 379 (1970). The sample was placed in a fritted vessel and washed with a boiling detergent solution that dissolved the protein, carbohydrate, enzyme and ash. An acetone wash removed the fats and pigments. The hemicellulose, cellulose and lignin fractions were collected on the frit and determined gravimetrically. The limit of detection of this method for this study was 0.1% fw. There was no analytical reference substance for this analysis.

Phytic acid (VCXX). The method used was a modification of two literature methods (Lehrfeld 1989, 1994). The sample was extracted using ultrasonication. Purification and concentration were performed on a silica-based anion exchange (SAX) column. Quantitation was performed on a macroporous polymer HPLC column PRP-1, 5µm (150 x 4.1) and a refractive index detector. The limit of quantitation for this study was between 0.05 and 0.08% fw. The reference substance for this assay was Aldrich phytic acid, dodecasodium salt hydrate, 99%, lot no. 13529MS.

Protein (PGEN). The method used was a modified version of AOAC methods 955.04 and 979.09 (1995) and literature methods (Bradstreet, 1965; Kalthoff and Sandell, 1948). Protein and other nitrogenous compounds in the sample were reduced to ammonia by digesting the sample with sulfuric acid containing a mercury catalyst mixture. The acid digest was made alkaline, and the ammonia was distilled and

titrated with a standard acid. The percent nitrogen was determined and converted to protein using the factor 6.25. The limit of detection of this method for this study was 0.1% fw. There was no analytical reference substance for this analysis.

Trypsin inhibitor (MIXX). The method used was a modified version of AOCS method Ba 12-75 (1997). Trypsin inhibitor activity in the sample was determined by suspending the ground, defatted sample in dilute sodium hydroxide solution. An appropriate dilution of the suspension was made, and an increasing series of aliquots of the diluted suspension was mixed with trypsin and benzoyl-DL-arginine-p-nitroanilide. After 10 minutes, the action of the trypsin was stopped by the addition of acetic acid. The diluted suspension mixture was filtered or centrifuged and the absorbance of each filtered solution was measured at 410 nm. Trypsin inhibitor activity was calculated from the change in absorbance values due to the aliquot volume. The limit of detection for this study was 1.0 TIU/mg fw.

Vitamin E (EFD2). The method used was a modification of a literature method (Cort *et al.*, 1983). The sample was saponified to break down any fat and release any vitamin E. The saponified mixture was extracted with ethyl ether and then quantitated directly by high-performance liquid chromatography on a silica column. The limit of quantitation for this study was between 0.001 and 0.002 mg/g fw. The reference substance for this assay was USP alpha tocopherol, 100%, lot number L1.

B. Control of Bias

Corn tissues were ground thoroughly and mixed to minimize tissue bias. Samples were analyzed in a nonsystematic manner to minimize assay bias.

C. Data Reduction and Statistical Analysis

Statistical analyses of the composition data were conducted by Certus International, Inc., Chesterfield, MO. Analytes that had >50% of values at or below the LOD of the assay were excluded from statistical analysis.

Statistical analyses were conducted using a mixed model analysis of variance for three sets of comparisons: analyses for each of the two replicated trials and for a combination of trials at different field sites. Individual replicated trial analyses used the model:

$$Y_{ij} = U + T_i + B_j + e_{ij},$$

where Y_{ij} = the individual component value, U = overall mean, T_i = line effect, B_j = random block effect, and e_{ij} = residual error.

Combined trial analyses used the model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk},$$

where Y_{ijk} = the individual component value, U = overall mean, T_i = line effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by line interaction effect, and e_{ijk} = residual error. In these analyses, corn line NK603 was compared to the nontransgenic control line LH82 x B73.

SAS® software was used to generate all summary statistics and perform all analyses (SAS Institute, 1989, 1990, 1996). Report tables present p-values as either <0.001 or the actual value truncated to three decimal places.

Statistical analysis results are reported in Appendix III.

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Appendix III

1998 Composition Data and Statistical Analysis Results of Roundup Ready Corn Line NK603

Tables

Table 1. Summary of Statistically Significant Differences

Tissue/	$p < 0.05$[b]			Mean NK603[b]			Mean Control[b]			Mean difference[b] (NK603 minus control)			Mean difference[c] % of NK603 value		
Component[a]	IL2	OH	All trials	IL2	OH	All trials	IL2	OH	All trials	IL2	OH	All trials	IL2	OH	All trials
Forage															
Carbohydrate	-	0.049	-	-	87.94	-	-	89.25	-	-	-1.31	-	-	-1.49	-
Protein	-	0.047	-	-	7.86	-	-	6.46	-	-	1.4	-	-	17.81	-
Moisture[d]	0.011	-	-												
Grain															
Arginine	0.037	-	-	4.31	-	-	4.55	-	-	-0.24	-	-	-5.57	-	-
Cystine	0.016	-	-	2.14	-	-	1.88	-	-	0.26	-	-	12.15	-	-
Phenylalanine	-	0.033	0.008	-	5.43	5.34	-	5.31	5.26	-	0.11	0.078	-	2.03	1.46
16:0 Palmitic acid	-	0.007	<0.001	-	8.94	9.16	-	8.6	8.92	-	0.34	0.24	-	3.80	2.62
18:0 Stearic acid	0.027	0.001	<0.001	2.03	1.95	1.95	1.95	1.85	1.86	0.076	0.1	0.093	3.74	5.13	4.77
18:1 Oleic acid	0.003	-	0.001	22.45	-	22.46	23.38	-	23.08	-0.93	-	-0.62	-4.14	-	-2.76
20:1 Eicosenoic acid	-	0.049	0.036	-	0.3	0.29	-	0.33	0.3	-	-0.026	-0.013	-	-8.67	-4.48
Calcium	<0.001	-	-	0.004	-	-	0.0034	-	-	0.0006	-	-	14.75	-	-
Magnesium	0.028	-	-	0.11	-	-	0.011	-	-	0.0058	-	-	5.27	-	-
Phosphorus	0.007	-	-	0.35	-	-	0.33	-	-	0.019	-	-	5.43	-	-
Moisture[d]	0.005	-	0.037												

[a]carbohydrate, protein, calcium, potassium and magnesium as % dw; amino acids as % of total; fatty acids as % of total.

[b]Data obtained from Tables 2-7.

[c]Calculated values.

[d]only p values listed as moisture differences not considered nutritionally significant. The differences can be found in Tables 2 to 7.

Table 2. Replicated Trial (Illinois): Fiber and Proximate Content of Forage and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Commercial[e] (Range)	Reported[f] (Range)
Ash (% dw)	4.49 ± 0.28 (3.59 - 4.88)	4.62 ± 0.28 (3.85 - 5.15)	-0.13 ± 0.40 (-0.27 - 0.12)	0.744	-1.00, 0.73	(2.03 - 7.49)	(2.9 - 5.1)
Carbohydrates (% dw)	83.85 ± 0.70 (82.68 - 85.15)	84.56 ± 0.70 (83.71 - 86.10)	-0.71 ± 0.94 (-1.57 - -0.15)	0.465	-2.77, 1.35	(81.5 - 88.9)	(84.6 - 89.1)
ADF (% dw)	28.74 ± 0.91 (26.48 - 33.52)	27.65 ± 0.91 (25.55 - 29.01)	1.09 ± 1.28 (-1.33 - 4.51)	0.412	-1.71, 3.89	(17.6 - 34.5)	(21.4 - 29.2)
NDF (% dw)	41.94 ± 1.09 (39.39 - 46.00)	39.51 ± 1.09 (35.44 - 42.34)	2.42 ± 1.54 (-1.00 - 10.56)	0.140	-0.93, 5.77	(29.6 - 50.7)	(39.9 - 46.6)
Moisture (% fw)	74.40 ± 0.39 (73.60 - 75.00)	72.75 ± 0.39 (71.70 - 73.70)	1.65 ± 0.56 (1.30 - 2.00)	0.011	0.43, 2.87	(47.0 - 78.8)	(68.7 - 73.5)
Protein (% dw)	8.80 ± 0.34 (8.56 - 8.98)	8.21 ± 0.34 (7.87 - 8.69)	0.59 ± 0.42 (0.29 - 0.81)	0.182	-0.32, 1.50	(4.9 - 11.0)	(4.8 - 8.4)
Total fat (% dw)	2.86 ± 0.27 (2.58 - 3.50)	2.60 ± 0.27 (2.10 - 3.42)	0.25 ± 0.38 (-0.77 - 1.41)	0.510	-0.56, 1.07	(0.79 - 3.64)	(1.4 - 2.1)

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber; dw = dry wt.; fw = fresh wt.
[b]The mean of four replicate values.
[c]S.E. = standard error of the mean.
[d]C.I. = confidence interval.
[e]The range of sample values across commercial lines grown in 1998 ([redacted] 1999).
[f]Range for two control lines analysed in Monsanto Company trials conducted in 1994 and 1995 ([redacted] 1996b; 1997a).

Table 3. Replicated Trial (Illinois): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Amino acids (% of total)								
Alanine	7.90 ± 0.037 (7.85 - 7.98)	7.98 ± 0.037 (7.90 - 8.05)	-0.077 ± 0.048 (-0.13 - -0.042)	0.137	-0.18, 0.028	(7.1 - 8.2)	(6.4-9.9)	(7.3-8.8)
Arginine	4.31 ± 0.073 (4.24 - 4.47)	4.55 ± 0.073 (4.35 - 4.63)	-0.24 ± 0.10 (-0.39 - 0.13)	0.037	-0.47, -0.016	(4.0 - 5.5)	(2.9-5.9)	(3.6-5.0)
Aspartic acid	6.41 ± 0.040 (6.29 - 6.48)	6.40 ± 0.040 (6.29 - 6.51)	0.011 ± 0.057 (-0.17 - 0.18)	0.844	-0.11, 0.14	(6.3 - 7.4)	(5.8-7.2)	(6.3-7.5)
Cystine	2.14 ± 0.071 (2.05 - 2.27)	1.88 ± 0.071 (1.63 - 2.01)	0.26 ± 0.094 (0.094 - 0.52)	0.016	0.057, 0.47	(1.8 - 2.9)	(1.2-1.6)	(1.8-2.7)
Glutamic acid	19.48 ± 0.073 (19.16 - 19.67)	19.32 ± 0.073 (19.19 - 19.43)	0.16 ± 0.10 (-0.19 - 0.34)	0.149	-0.066, 0.38	(17.4 - 20.1)	(12.4-19.6)	(19.5-22.8)
Glycine	3.64 ± 0.053 (3.52 - 3.74)	3.74 ± 0.053 (3.61 - 3.86)	-0.10 ± 0.075 (-0.26 - 0.13)	0.206	-0.26, 0.063	(3.4 - 4.6)	(2.6-4.7)	(3.2-4.2)
Histidine	2.77 ± 0.029 (2.72 - 2.81)	2.79 ± 0.029 (2.76 - 2.88)	-0.022 ± 0.041 (-0.094 - 0.049)	0.597	-0.11, 0.067	(2.6 - 3.4)	(2.0-2.8)	(2.8-3.3)
Isoleucine	3.86 ± 0.043 (3.81 - 3.91)	3.81 ± 0.043 (3.73 - 3.91)	0.051 ± 0.061 (-0.053 - 0.099)	0.415	-0.082, 0.18	(3.0 - 4.1)	(2.6-4.0)	(3.2-4.3)
Leucine	13.90 ± 0.094 (13.63 - 14.05)	13.75 ± 0.094 (13.59 - 13.91)	0.15 ± 0.13 (-0.28 - 0.46)	0.295	-0.14, 0.44	(11.3 - 14.4)	(7.8-15.2)	(12.6-15.8)

(continued over)

Table 3. Replicated Trial (Illinois): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Lysine	2.79 ± 0.059 (2.64 - 2.96)	2.86 ± 0.059 (2.72 - 3.00)	-0.068 ± 0.083 (-0.22 - 0.24)	0.427	-0.25, 0.11	(2.6 - 3.9)	(2.0-3.8)	(2.6-3.5)
Methionine	2.13 ± 0.056 (2.08 - 2.16)	1.97 ± 0.056 (1.74 - 2.14)	0.15 ± 0.078 (-0.0037 - 0.42)	0.075	-0.018, 0.32	(1.6 - 2.9)	(1.0-2.1)	(1.3-2.6)
Phenylalanine	5.28 ± 0.026 (5.20 - 5.33)	5.22 ± 0.026 (5.15 - 5.27)	0.056 ± 0.034 (-0.037 - 0.13)	0.120	-0.017, 0.13	(4.7 - 5.5)	(2.9-5.7)	(5.0-6.1)
Proline	8.93 ± 0.067 (8.60 - 9.10)	8.90 ± 0.067 (8.84 - 8.96)	0.030 ± 0.095 (-0.35 - 0.25)	0.755	-0.18, 0.24	(8.0 - 9.9)	(6.6-10.3)	(8.7-10.1)
Serine	4.77 ± 0.034 (4.72 - 4.84)	4.87 ± 0.034 (4.84 - 4.91)	-0.098 ± 0.046 (-0.18 - -0.040)	0.056	-0.20, 0.0031	(3.5 - 5.5)	(4.2-5.5)	(4.9-6.0)
Threonine	3.40 ± 0.026 (3.36 - 3.46)	3.38 ± 0.026 (3.29 - 3.47)	0.025 ± 0.035 (-0.11 - 0.099)	0.494	-0.051, 0.10	(3.1 - 4.0)	(2.9-3.9)	(3.3-4.2)
Tryptophan	0.54 ± 0.016 (0.47 - 0.56)	0.58 ± 0.016 (0.56 - 0.59)	-0.042 ± 0.022 (-0.11 - 0.0014)	0.087	-0.090, 0.0072	(0.4 - 0.8)	(0.5-1.2)	(0.4-1.0)
Tyrosine	2.94 ± 0.25 (2.46 - 3.40)	3.18 ± 0.25 (2.43 - 3.49)	-0.24 ± 0.35 (-0.98 - 0.086)	0.495	-1.00, 0.51	(2.1 - 4.0)	(2.9-4.7)	(3.7-4.3)
Valine	4.82 ± 0.042 (4.76 - 4.85)	4.82 ± 0.042 (4.73 - 4.94)	-0.00096 ± 0.059 (-0.094 - 0.092)	0.987	-0.13, 0.13	(3.9 - 5.5)	(2.1-5.2)	(4.2-5.3)

(continued over)

Table 3. Replicated Trial (Illinois): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Fatty acids (% of total)								
16:0 palmitic acid	9.50 ± 0.067 (9.42 - 9.57)	9.32 ± 0.067 (9.23 - 9.44)	0.18 ± 0.094 (-0.022 - 0.34)	0.085	-0.029, 0.38	(8.8 - 13.8)	(7-19)	(9.9-12.0)
18:0 stearic acid	2.03 ± 0.021 (1.97 - 2.06)	1.95 ± 0.021 (1.92 - 1.98)	0.076 ± 0.030 (0.032 - 0.14)	0.027	0.010, 0.14	(1.4 - 2.6)	(1-3)	(1.4-2.2)
18:1 oleic acid	22.45 ± 0.18 (22.04 - 22.62)	23.38 ± 0.18 (23.09 - 23.78)	-0.93 ± 0.25 (-1.23 - -0.47)	0.003	-1.48, -0.38	(20.7 - 37.7)	(20-46)	(20.6-27.5)
18:2 linoleic acid	64.09 ± 0.24 (63.81 - 64.64)	63.41 ± 0.24 (63.07 - 63.74)	0.67 ± 0.33 (0.070 - 1.13)	0.066	-0.052, 1.40	(48.0 - 66.1)	(35-70)	(55.9-66.1)
18:3 linolenic acid	1.09 ± 0.011 (1.08 - 1.11)	1.09 ± 0.011 (1.07 - 1.11)	0.0019 ± 0.015 (-0.026 - 0.018)	0.901	-0.031, 0.035	(0.9 - 1.5)	(0.8-2)	(0.8-1.1)
20:0 arachidic acid	0.38 ± 0.0057 (0.38 - 0.39)	0.38 ± 0.0057 (0.38 - 0.40)	-0.00059 ± 0.0080 (-0.013 - 0.016)	0.942	-0.018, 0.017	(0.3 - 0.6)	(0.1-2)	(0.3-0.5)
20:1 eicosenoic acid	0.29 ± 0.0062 (0.28 - 0.29)	0.29 ± 0.0062 (0.27 - 0.30)	0.00007 ± 0.0088 (-0.020 - 0.019)	0.993	-0.019, 0.019	(0.2 - 0.4)	(na)	(0.2-0.3)
22:0 behenic acid	0.17 ± 0.0053 (0.16 - 0.17)	0.17 ± 0.0053 (0.16 - 0.18)	0.00004 ± 0.0076 (-0.010 - 0.011)	0.996	-0.016, 0.017	(0.1 - 0.3)	(na)	(0.1-0.3)

(continued over)

Table 3. Replicated Trial (Illinois): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Minerals								
Calcium (%)	0.0040 ± 0.00009 (0.0037 - 0.0043)	0.0034 ± 0.00009 (0.0033 - 0.0036)	0.00059 ± 0.00011 (0.00034 - 0.00091)	<0.001	0.00034, 0.00083	(0.003 - 0.009)	(0.01-0.1)	(0.003-0.006)
Copper (mg/kg dw)	2.20 ± 0.097 (2.06 - 2.37)	2.22 ± 0.097 (2.01 - 2.33)	-0.014 ± 0.14 (-0.24 - 0.36)	0.922	-0.31, 0.28	(0.9 - 2.8)	(0.9-10)	(na)
Iron (mg/kg dw)	25.68 ± 0.48 (25.39 - 25.94)	24.91 ± 0.48 (23.59 - 26.62)	0.77 ± 0.68 (-1.23 - 2.18)	0.281	-0.72, 2.25	(11 - 49)	(1-100)	(na)
Magnesium (%)	0.11 ± 0.0018 (0.11 - 0.12)	0.11 ± 0.0018 (0.11 - 0.11)	0.0058 ± 0.0023 (0.0033 - 0.0074)	0.028	0.00074, 0.011	(0.08 - 0.2)	(0.09-1.0)	(na)
Manganese (mg/kg dw)	5.34 ± 0.091 (5.03 - 5.77)	5.53 ± 0.091 (5.38 - 5.63)	-0.19 ± 0.13 (-0.56 - 0.25)	0.173	-0.47, 0.094	(2.6 - 7.8)	(0.7-54)	(na)
Phosphorus (%)	0.35 ± 0.0041 (0.34 - 0.36)	0.33 ± 0.0041 (0.32 - 0.34)	0.019 ± 0.0058 (0.010 - 0.025)	0.007	0.0062, 0.032	(0.24 - 0.43)	(0.26-0.75)	(0.31-0.36)
Potassium (%)	0.36 ± 0.0041 (0.35 - 0.36)	0.35 ± 0.0041 (0.34 - 0.36)	0.011 ± 0.0058 (0.0055 - 0.016)	0.086	-0.0018, 0.024	(0.29 - 0.53)	(0.32-0.72)	(na)
Zinc (mg/kg dw)	30.78 ± 0.72 (29.18 - 32.07)	29.96 ± 0.72 (27.93 - 31.37)	0.81 ± 1.01 (-1.24 - 4.14)	0.439	-1.40, 3.02	(15 - 33)	(12-30)	(na)

(continued over)

Table 3. Replicated Trial (Illinois): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Fiber and Proximates								
Ash (% dw)	1.40 ± 0.035 (1.29 - 1.47)	1.42 ± 0.035 (1.32 - 1.51)	-0.020 ± 0.050 (-0.15 - 0.14)	0.700	-0.13, 0.089	(0.8 - 1.8)	(1.1-3.9)	(1.2-1.8)
Carbohydrates . (% dw)	83.84 ± 0.16 (83.63 - 84.06)	83.46 ± 0.16 (83.22 - 83.70)	0.38 ± 0.23 (-0.074 - 0.84)	0.117	-0.11, 0.87	(83.1 - 89.6)	(na)	(na)
ADF (% dw)	3.93 ± 0.20 (3.48 - 4.22)	3.98 ± 0.20 (3.78 - 4.19)	-0.053 ± 0.27 (-0.71 - 0.31)	0.846	-0.64, 0.53	(2.3 - 5.7)	(3.3 - 4.3)	(3.1 - 5.3)
NDF (% dw)	10.30 ± 1.15 (9.34 - 12.06)	10.13 ± 1.15 (9.06 - 11.38)	0.18 ± 1.23 (-1.83 - 2.09)	0.888	-2.50, 2.85	(8.2 - 16.1)	(8.3-11.9)	(9.6 - 15.3)
Moisture (% fw)	12.28 ± 0.37 (11.00 - 13.30)	14.00 ± 0.37 (13.40 - 14.80)	-1.73 ± 0.52 (-2.60 - -0.90)	0.005	-2.85, -0.60	(6.1 - 15.6)	(7-23)	(9.4 - 15.8)
Total fat (%)	3.63 ± 0.11 (3.39 - 3.87)	3.91 ± 0.11 (3.81 - 4.13)	-0.28 ± 0.16 (-0.42 - 0.046)	0.115	-0.63, 0.079	(1.74 - 4.31)	(3.1-5.7, 2.9-6.1)	(2.4-4.2)
Protein (% dw)	11.13 ± 0.13 (10.84 - 11.37)	11.21 ± 0.13 (11.02 - 11.60)	-0.084 ± 0.18 (-0.34 - 0.35)	0.655	-0.48, 0.32	(6.7 - 13.4)	(6.0 - 12.0, 9.7 - 16.1)	(9.0 - 13.6)

(continued over)

Table 3. Replicated Trial (Illinois): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Miscellaneous								
Phytic Acid (%)	0.90 ± 0.047 (0.70 - 1.05)	0.92 ± 0.047 (0.83 - 1.02)	-0.022 ± 0.058 (-0.23 - 0.15)	0.715	-0.15, 0.11	(0.5 - 1.3)	(to 0.9%)	(na)
Trypsin Inhibitor (TIU/mg dw)	3.76 ± 0.55 (2.66 - 5.08)	3.29 ± 0.55 (2.43 - 5.14)	0.47 ± 0.78 (-2.15 - 2.07)	0.559	-1.24, 2.18	(3.40 - 7.18)	(na)	(na)
Vitamin E (mg/g dw)	0.0095 ± 0.00027 (0.0089 - 0.010)	0.0093 ± 0.00027 (0.0087 - 0.010)	0.00019 ± 0.00035 (-0.00083 - 0.0013)	0.592	-0.00057, 0.00095	(0.006 - 0.022)	(0.017-0.047)	(0.008-0.012)

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber; dw = dry wt.; fw = fresh wt; TIU = trypsin inhibitor units.
[b]The mean of four replicate values.
[c]S.E. = standard error of the mean.
[d]C.I. = confidence interval.
[e]Comm. = commercial. The range of sample values for commercial lines grown in 1998 ([redacted]1999).
[f]Lit. = literature. For amino and fatty acids, Watson, 1982; for all other components, Watson, 1987; protein and fat second values from Jugenheimer, 1976.
[g]Rpt. = reported. For amino and fatty acids, range for five control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted] 1996a,b; 1997a,b).
[h]For ash, moisture and total fat, range for five control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted]1996a,b; 1997a,b).
[i]For ADF and NDF, range for three control lines analysed in Monsanto trials conducted between 1994 and 1995 ([redacted]1996b; 1997a,b).
[j]For calcium and phosphorus, range for three control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted]1996a; 1997b).

Table 4. Replicated Trial (Ohio): Fiber and Proximate Content of Forage and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Commercial[e] (Range)	Reported[f] (Range)
Ash (% dw)	3.36 ± 0.19 (2.88 - 3.67)	3.29 ± 0.19 (2.89 - 3.66)	0.062 ± 0.26 (-0.46 - 0.40)	0.814	-0.51, 0.63	(2.03 - 7.49)	(2.9 - 5.1)
Carbohydrates (% dw)	87.94 ± 0.43 (87.23 - 89.18)	89.25 ± 0.43 (87.95 - 89.97)	-1.31 ± 0.60 (-2.41 - -0.093)	0.049	-2.61, -0.0052	(81.5 - 88.9)	(84.6 - 89.1)
ADF (% dw)	28.91 ± 0.84 (27.45 - 29.48)	28.10 ± 0.84 (25.07 - 30.58)	0.81 ± 1.19 (-3.13 - 4.20)	0.509	-1.78, 3.39	(17.6 - 34.5)	(21.4 - 29.2)
NDF (% dw)	47.43 ± 1.36 (46.53 - 48.14)	48.48 ± 1.36 (44.88 - 51.10)	-1.05 ± 1.71 (-3.13 - 3.14)	0.549	-4.77, 2.67	(29.6 - 50.7)	(39.9 - 46.6)
Moisture (% fw)	65.88 ± 0.88 (63.20 - 67.80)	63.88 ± 0.88 (61.90 - 66.10)	2.00 ± 1.25 (-0.70 - 5.20)	0.134	-0.72, 4.72	(47.0 - 78.8)	(68.7 - 73.5)
Protein (% dw)	7.86 ± 0.49 (6.04 - 8.51)	6.46 ± 0.49 (5.66 - 7.64)	1.40 ± 0.63 (0.38 - 2.20)	0.047	0.020, 2.78	(4.9 - 11.0)	(4.8 - 8.4)
Total fat (% dw)	0.84 ± 0.12 (0.69 - 1.11)	0.99 ± 0.12 (0.61 - 1.52)	-0.15 ± 0.17 (-0.77 - 0.21)	0.387	-0.52, 0.22	(0.79 - 3.64)	(1.4 - 2.1)

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber; dw = dry wt.; fw = fresh wt.
[b]The mean of four replicate values.
[c]S.E. = standard error of the mean.
[d]C.I. = confidence interval.
[e]The range of sample values across commercial lines grown in 1998 ([redacted]1999).
[f]Range for two control lines analyzed in Monsanto Company trials conducted in 1994 and 1995 ([redacted]996b; 1997a).

Table 5. Replicated Trial (Ohio): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Amino acids (% of total)								
Alanine	8.17 ± 0.046 (8.07 - 8.22)	8.09 ± 0.046 (8.01 - 8.17)	0.082 ± 0.065 (0.039 - 0.17)	0.234	-0.061, 0.22	(7.1 - 8.2)	(6.4-9.9)	(7.3-8.8)
Arginine	3.88 ± 0.086 (3.80 - 3.94)	3.96 ± 0.086 (3.90 - 4.04)	-0.081 ± 0.12 (-0.099 - -0.051)	0.517	-0.35, 0.18	(4.0 - 5.5)	(2.9-5.9)	(3.6-5.0)
Aspartic acid	6.49 ± 0.068 (6.43 - 6.62)	6.43 ± 0.068 (6.34 - 6.56)	0.055 ± 0.096 (-0.033 - 0.10)	0.580	-0.15, 0.26	(6.3 - 7.4)	(5.8-7.2)	(6.3-7.5)
Cystine	1.75 ± 0.058 (1.69 - 1.79)	1.87 ± 0.058 (1.68 - 2.15)	-0.12 ± 0.074 (-0.38 - 0.088)	0.127	-0.28, 0.040	(1.8 - 2.9)	(1.2-1.6)	(1.8-2.7)
Glutamic acid	20.27 ± 0.10 (20.15 - 20.47)	20.23 ± 0.10 (20.11 - 20.41)	0.043 ± 0.15 (-0.085 - 0.10)	0.776	-0.28, 0.36	(17.4 - 20.1)	(12.4-19.6)	(19.5-22.8)
Glycine	3.29 ± 0.065 (3.22 - 3.37)	3.37 ± 0.065 (3.30 - 3.51)	-0.079 ± 0.092 (-0.14 - 0.033)	0.402	-0.28, 0.12	(3.4 - 4.6)	(2.6-4.7)	(3.2-4.2)
Histidine	2.55 ± 0.033 (2.45 - 2.62)	2.62 ± 0.033 (2.56 - 2.70)	-0.071 ± 0.041 (-0.11 - -0.00035)	0.107	-0.16, 0.018	(2.6 - 3.4)	(2.0-2.8)	(2.8-3.3)
Isoleucine	3.93 ± 0.047 (3.84 - 4.06)	3.86 ± 0.047 (3.81 - 3.93)	0.070 ± 0.066 (-0.019 - 0.13)	0.312	-0.074, 0.21	(3.0 - 4.1)	(2.6-4.0)	(3.2-4.3)
Leucine	14.69 ± 0.13 (14.58 - 14.79)	14.45 ± 0.13 (14.39 - 14.50)	0.24 ± 0.19 (0.19 - 0.29)	0.227	-0.17, 0.64	(11.3 - 14.4)	(7.8-15.2)	(12.6-15.8)

(continued over)

Table 5. Replicated Trial (Ohio): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Lysine	2.49 ± 0.079 (2.42 - 2.55)	2.49 ± 0.079 (2.41 - 2.65)	-0.0013 ± 0.11 (-0.11 - 0.093)	0.990	-0.25, 0.24	(2.6 - 3.9)	(2.0-3.8)	(2.6-3.5)
Methionine	1.83 ± 0.050 (1.76 - 1.86)	1.96 ± 0.050 (1.83 - 2.08)	-0.13 ± 0.062 (-0.22 - -0.015)	0.056	-0.27, 0.0045	(1.6 - 2.9)	(1.0-2.1)	(1.3-2.6)
Phenylalanine	5.43 ± 0.034 (5.35 - 5.52)	5.31 ± 0.034 (5.28 - 5.36)	0.11 ± 0.047 (0.044 - 0.16)	0.033	0.010, 0.22	(4.7 - 5.5)	(2.9-5.7)	(5.0-6.1)
Proline	8.58 ± 0.077 (8.44 - 8.69)	8.68 ± 0.077 (8.59 - 8.78)	-0.097 ± 0.11 (-0.14 - -0.022)	0.393	-0.33, 0.14	(8.0 - 9.9)	(6.6-10.3)	(8.7-10.1)
Serine	4.90 ± 0.083 (4.83 - 4.97)	4.88 ± 0.083 (4.68 - 4.99)	0.017 ± 0.12 (-0.16 - 0.22)	0.888	-0.24, 0.27	(3.5 - 5.5)	(4.2-5.5)	(4.9-6.0)
Threonine	3.35 ± 0.040 (3.33 - 3.40)	3.38 ± 0.040 (3.31 - 3.50)	-0.029 ± 0.057 (-0.16 - 0.045)	0.616	-0.15, 0.095	(3.1 - 4.0)	(2.9-3.9)	(3.3-4.2)
Tryptophan	0.50 ± 0.012 (0.48 - 0.52)	0.51 ± 0.012 (0.49 - 0.53)	-0.0040 ± 0.012 (-0.032 - 0.030)	0.744	-0.030, 0.022	(0.4 - 0.8)	(0.5-1.2)	(0.4-1.0)
Tyrosine	3.17 ± 0.26 (2.36 - 3.73)	3.20 ± 0.26 (2.46 - 3.64)	-0.024 ± 0.36 (-0.51 - 0.42)	0.949	-0.82, 0.77	(2.1 - 4.0)	(2.9-4.7)	(3.7-4.3)
Valine	4.71 ± 0.046 (4.63 - 4.83)	4.69 ± 0.046 (4.62 - 4.76)	0.023 ± 0.065 (-0.060 - 0.10)	0.727	-0.12, 0.17	(3.9 - 5.5)	(2.1-5.2)	(4.2-5.3)

(continued over)

Table 5. Replicated Trial (Ohio): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Fatty acids (% of total)								
16:0 palmitic acid	8.94 ± 0.076 (8.67 - 9.11)	8.60 ± 0.076 (8.41 - 8.74)	0.34 ± 0.11 (-0.068 - 0.64)	0.007	0.11, 0.57	(8.8 - 13.8)	(7-19)	(9.9-12.0)
18:0 stearic acid	1.95 ± 0.018 (1.92 - 1.98)	1.85 ± 0.018 (1.79 - 1.91)	0.10 ± 0.025 (0.052 - 0.19)	0.001	0.049, 0.16	(1.4 - 2.6)	(1-3)	(1.4-2.2)
18:1 oleic acid	22.70 ± 0.22 (22.52 - 23.12)	22.79 ± 0.22 (22.15 - 23.40)	-0.083 ± 0.28 (-0.84 - 0.46)	0.774	-0.70, 0.53	(20.7 - 37.7)	(20-46)	(20.6-27.5)
18:2 linoleic acid	64.44 ± 0.27 (64.02 - 64.97)	64.75 ± 0.27 (64.16 - 65.65)	-0.31 ± 0.34 (-1.23 - 0.49)	0.380	-1.05, 0.43	(48.0 - 66.1)	(35-70)	(55.9-66.1)
18:3 linolenic acid	1.10 ± 0.020 (1.07 - 1.17)	1.12 ± 0.020 (1.07 - 1.20)	-0.015 ± 0.028 (-0.13 - 0.060)	0.606	-0.075, 0.046	(0.9 - 1.5)	(0.8-2)	(0.8-1.1)
20:0 arachidic acid	0.38 ± 0.0045 (0.37 - 0.39)	0.39 ± 0.0045 (0.38 - 0.39)	-0.0082 ± 0.0062 (-0.019 - 0.0057)	0.211	-0.022, 0.0054	(0.3 - 0.6)	(0.1-2)	(0.3-0.5)
20:1 eicosenoic acid	0.30 ± 0.0084 (0.29 - 0.32)	0.33 ± 0.0084 (0.31 - 0.34)	-0.026 ± 0.012 (-0.038 - 0.0061)	0.049	-0.052, -0.00012	(0.2 - 0.4)	(na)	(0.2-0.3)
22:0 behenic acid	0.18 ± 0.0043 (0.17 - 0.19)	0.18 ± 0.0043 (0.18 - 0.19)	-0.0016 ± 0.0061 (-0.0083 - 0.0078)	0.790	-0.015, 0.012	(0.1 - 0.3)	(na)	(0.1-0.3)

(continued over)

Table 5. Replicated Trial (Ohio): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Minerals								
Calcium (%)	0.0051 ± 0.00023 (0.0047 - 0.0056)	0.0049 ± 0.00023 (0.0046 - 0.0051)	0.00020 ± 0.00027 (-0.00021 -0.00057)	0.460	-0.00038, 0.00079	(0.003 - 0.009)	(0.01-0.1)	(0.003-0.006)
Copper (mg/kg dw)	1.48 ± 0.14 (1.19 - 1.83)	1.68 ± 0.14 (1.50 - 1.82)	-0.21 ± 0.19 (-0.63 - 0.23)	0.308	-0.63, 0.22	(0.9 - 2.8)	(0.9-10)	(na)
Iron (mg/kg dw)	19.99 ± 0.81 (19.08 - 21.27)	20.86 ± 0.81 (18.77 - 25.69)	-0.86 ± 1.14 (-4.42 - 0.54)	0.462	-3.35, 1.62	(11 - 49)	(1-100)	(na)
Magnesium (%)	0.13 ± 0.0027 (0.12 - 0.13)	0.13 ± 0.0027 (0.12 - 0.13)	0.0010 ± 0.0038 (-0.0098 - 0.010)	0.795	-0.0072, 0.0092	(0.08 - 0.2)	(0.09-1.0)	(na)
Manganese (mg/kg dw)	6.65 ± 0.29 (5.74 - 7.25)	6.07 ± 0.29 (5.66 - 6.90)	0.58 ± 0.40 (0.0057 - 1.34)	0.169	-0.29, 1.46	(2.6 - 7.8)	(0.7-54)	(na)
Phosphorus (%)	0.37 ± 0.0076 (0.34 - 0.39)	0.37 ± 0.0076 (0.35 - 0.38)	-0.0031 ± 0.011 (-0.040 - 0.025)	0.774	-0.027, 0.020	(0.24 - 0.43)	(0.26-0.75)	(0.31-0.36)
Potassium (%)	0.38 ± 0.0075 (0.37 - 0.39)	0.38 ± 0.0075 (0.36 - 0.41)	-0.0026 ± 0.011 (-0.014 - 0.022)	0.813	-0.026, 0.021	(0.29 - 0.53)	(0.32-0.72)	(na)
Zinc (mg/kg dw)	30.87 ± 1.02 (28.31 - 33.17)	30.92 ± 1.02 (29.53 - 33.26)	-0.048 ± 1.45 (-4.95 - 3.06)	0.973	-3.20, 3.10	(15 - 33)	(12-30)	(na)

(continued over)

Table 5. Replicated Trial (Ohio): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Fiber and Proximates								
Ash (% dw)	1.49 ± 0.069 (1.37 - 1.62)	1.56 ± 0.069 (1.41 - 1.75)	-0.067 ± 0.097 (-0.29 - 0.21)	0.503	-0.28, 0.14	(0.8 - 1.8)	(1.1-3.9)	(1.2-1.8)
Carbohydrates (% dw)	80.95 ± 0.19 (80.71 - 81.25)	81.11 ± 0.19 (80.23 - 81.52)	-0.15 ± 0.26 (-0.76 - 0.49)	0.560	-0.72, 0.41	(83.1 - 89.6)	(na)	(na)
ADF (% dw)	3.73 ± 0.26 (3.14 - 4.23)	3.86 ± 0.26 (3.07 - 4.28)	-0.13 ± 0.37 (-0.50 - 0.071)	0.727	-0.94, 0.68	(2.3 - 5.7)	(3.3 - 4.3)	(3.1 - 5.3)
NDF (% dw)	11.69 ± 1.50 (10.95 - 12.53)	12.04 ± 1.50 (8.68 - 15.42)	-0.35 ± 2.09 (-3.72 - 2.89)	0.868	-4.91, 4.21	(8.2 - 16.1)	(8.3-11.9)	(9.6 - 15.3)
Moisture (% fw)	9.86 ± 0.36 (9.22 - 11.10)	9.73 ± 0.36 (8.56 - 10.70)	0.13 ± 0.51 (-1.48 - 2.54)	0.805	-0.98, 1.23	(6.1 - 15.6)	(7-23)	(9.4 - 15.8)
Total fat (%)	3.30 ± 0.14 (2.92 - 3.79)	3.18 ± 0.14 (2.88 - 3.61)	0.12 ± 0.19 (-0.69 - 0.90)	0.547	-0.30, 0.54	(1.7 - 4.3)	(3.1-5.7, 2.9-6.1)	(2.4-4.2)
Protein (% dw)	14.25 ± 0.19 (13.95 - 14.77)	14.15 ± 0.19 (13.55 - 14.84)	0.10 ± 0.21 (-0.16 - 0.66)	0.640	-0.36, 0.57	(6.7 - 13.4)	(6.0 - 12.0, 9.7 - 16.1)	(9.0 - 13.6)

(continued over)

Table 5. Replicated Trial (Ohio): Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Miscellaneous								
Phytic Acid (%)	0.96 ± 0.041 (0.91 - 0.99)	0.86 ± 0.041 (0.81 - 0.95)	0.097 ± 0.058 (0.020 - 0.18)	0.122	-0.030, 0.22	(0.5 - 1.3)	(to 0.9%)	(na)
Trypsin Inhibitor (TIU/mg dw)	3.30 ± 0.31 (3.00 - 3.90)	3.58 ± 0.31 (3.08 - 4.24)	-0.28 ± 0.44 (-0.95 - 0.50)	0.541	-1.25, 0.69	(3.40 - 7.18)	(na)	(na)
Vitamin E (mg/g dw)	0.0092 ± 0.00034 (0.0084 - 0.010)	0.0098 ± 0.00034 (0.0091 - 0.011)	-0.00060 ± 0.00048 (-0.0024 - 0.00093)	0.231	-0.0016, 0.00044	(0.006 - 0.022)	(0.017-0.047)	(0.008-0.012)

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber; dw = dry wt.; fw = fresh wt; TIU = trypsin inhibitor units.
[b]The mean of four replicate values.
[c]S.E. = standard error of the mean.
[d]C.I. = confidence interval.
[e]Comm. = commercial. The range of sample values for commercial lines grown in 1998 ([redacted]1999).
[f]Lit. = literature. For amino and fatty acids, Watson, 1982; for all other components, Watson, 1987; protein and fat second values from Jugenheimer, 1976.
[g]Rpt. = reported. For amino and fatty acids, range for five control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted] 1996a,b; 1997a,b).
[h]For ash, moisture and total fat, range for five control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted]1996a,b; 1997a,b).
[i]For ADF and NDF, range for three control lines analysed in Monsanto trials conducted between 1994 and 1995 ([redacted]1996b; 1997a,b).
[j]For calcium and phosphorus, range for three control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted]1996a; 1997b).

Table 6. All Trials: Fiber and Proximate Content of Forage and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Commercial[e] (Range)	Reported[f] (Range)
Ash (% dw)	3.84 ± 0.30 (2.36 - 6.80)	4.05 ± 0.30 (2.46 - 6.28)	-0.21 ± 0.18 (-0.99 - 0.51)	0.252	-0.56, 0.15	(2.03 - 7.49)	(2.9 - 5.1)
Carbohydrates (% dw)	86.39 ± 0.60 (82.68 - 90.32)	86.83 ± 0.60 (83.71 - 90.03)	-0.44 ± 0.41 (-2.41 - 2.72)	0.296	-1.26, 0.39	(81.5 - 88.9)	(84.6 - 89.1)
ADF (% dw)	26.98 ± 0.98 (17.01 - 33.52)	26.09 ± 0.98 (19.53 - 31.83)	0.89 ± 0.91 (-4.15 - 8.05)	0.334	-0.94, 2.71	(17.6 - 34.5)	(21.4 - 29.2)
NDF (% dw)	43.23 ± 1.36 (36.39 - 49.03)	43.33 ± 1.36 (35.44 - 53.24)	-0.10 ± 1.04 (-4.21 - 10.56)	0.920	-2.19, 1.98	(29.6 - 50.7)	(39.9 - 46.6)
Moisture (% fw)	68.24 ± 1.40 (60.30 - 75.00)	67.43 ± 1.40 (61.00 - 73.70)	0.81 ± 0.58 (-2.30 - 5.20)	0.164	-0.34, 1.97	(47.0 - 78.8)	(68.7 - 73.5)
Protein (% dw)	7.61 ± 0.35 (5.57 - 8.98)	7.15 ± 0.35 (5.49 - 8.69)	0.46 ± 0.28 (-1.61 - 2.20)	0.113	-0.11, 1.03	(4.9 - 11.0)	(4.8 - 8.4)
Total fat (% dw)	2.16 ± 0.25 (0.69 - 3.64)	1.97 ± 0.25 (0.61 - 3.42)	0.18 ± 0.17 (-0.77 - 1.53)	0.290	-0.16, 0.53	(0.79 - 3.64)	(1.4 - 2.1)

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber; dw = dry wt.; fw = fresh wt.
[b]The mean of all values.
[c]S.E. = standard error of the mean.
[d]C.I. = confidence interval.
[e]The range of sample values across commercial lines grown in 1998 ([redacted]1999).
[f]Range for two control lines analyzed in Monsanto Company trials conducted in 1994 and 1995 ([redacted]1996b; 1997a).

Table 7. All Trials: Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Amino acids (% of total)								
Alanine	7.99 ± 0.047 (7.78 - 8.22)	7.96 ± 0.047 (7.65 - 8.17)	0.034 ± 0.036 (-0.13 - 0.28)	0.355	-0.039, 0.11	(7.1 - 8.2)	(6.4-9.9)	(7.3-8.8)
Arginine	4.13 ± 0.079 (3.79 - 4.49)	4.22 ± 0.079 (3.90 - 4.63)	-0.094 ± 0.073 (-0.46 - 0.27)	0.205	-0.24, 0.053	(4.0 - 5.5)	(2.9-5.9)	(3.6-5.0)
Aspartic acid	6.45 ± 0.032 (6.29 - 6.62)	6.39 ± 0.032 (6.18 - 6.56)	0.057 ± 0.040 (-0.17 - 0.19)	0.164	-0.024, 0.14	(6.3 - 7.4)	(5.8-7.2)	(6.3-7.5)
Cystine	1.97 ± 0.052 (1.69 - 2.27)	1.95 ± 0.052 (1.63 - 2.22)	0.021 ± 0.049 (-0.38 - 0.52)	0.669	-0.078, 0.12	(1.8 - 2.9)	(1.2-1.6)	(1.8-2.7)
Glutamic acid	19.86 ± 0.12 (19.16 - 20.47)	19.80 ± 0.12 (19.19 - 20.41)	0.060 ± 0.095 (-0.44 - 0.54)	0.530	-0.13, 0.25	(17.4 - 20.1)	(12.4-19.6)	(19.5-22.8)
Glycine	3.47 ± 0.058 (3.22 - 3.74)	3.51 ± 0.058 (3.22 - 3.86)	-0.036 ± 0.052 (-0.35 - 0.24)	0.489	-0.14, 0.068	(3.4 - 4.6)	(2.6-4.7)	(3.2-4.2)
Histidine	2.69 ± 0.035 (2.45 - 2.81)	2.71 ± 0.035 (2.56 - 2.88)	-0.018 ± 0.024 (-0.13 - 0.10)	0.457	-0.065, 0.030	(2.6 - 3.4)	(2.0-2.8)	(2.8-3.3)
Isoleucine	3.88 ± 0.028 (3.59 - 4.06)	3.81 ± 0.028 (3.65 - 3.93)	0.065 ± 0.034 (-0.060 - 0.19)	0.063	-0.0039, 0.13	(3.0 - 4.1)	(2.6-4.0)	(3.2-4.3)
Leucine	14.25 ± 0.13 (13.63 - 14.79)	14.11 ± 0.13 (13.59 - 14.60)	0.14 ± 0.11 (-0.52 - 0.99)	0.217	-0.087, 0.37	(11.3 - 14.4)	(7.8-15.2)	(12.6-15.8)

(continued over)

Table 7. All Trials: Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Lysine	2.66 ± 0.060 (2.42 - 2.96)	2.65 ± 0.060 (2.35 - 3.00)	0.012 ± 0.060 (-0.36 - 0.30)	0.839	-0.11, 0.13	(2.6 - 3.9)	(2.0-3.8)	(2.6-3.5)
Methionine	1.95 ± 0.042 (1.76 - 2.16)	2.01 ± 0.042 (1.74 - 2.21)	-0.060 ± 0.048 (-0.41 - 0.42)	0.217	-0.16, 0.037	(1.6 - 2.9)	(1.0-2.1)	(1.3-2.6)
Phenylalanine	5.34 ± 0.032 (5.18 - 5.52)	5.26 ± 0.032 (5.09 - 5.36)	0.078 ± 0.029 (-0.10 - 0.21)	0.008	0.020, 0.14	(4.7 - 5.5)	(2.9-5.7)	(5.0-6.1)
Proline	8.82 ± 0.062 (8.44 - 9.10)	8.90 ± 0.062 (8.59 - 9.26)	-0.076 ± 0.049 (-0.35 - 0.25)	0.130	-0.17, 0.023	(8.0 - 9.9)	(6.6-10.3)	(8.7-10.1)
Serine	4.86 ± 0.036 (4.72 - 5.09)	4.86 ± 0.036 (4.68 - 4.99)	0.0039 ± 0.046 (-0.18 - 0.25)	0.931	-0.088, 0.096	(3.5 - 5.5)	(4.2-5.5)	(4.9-6.0)
Threonine	3.37 ± 0.021 (3.26 - 3.46)	3.34 ± 0.021 (3.19 - 3.50)	0.029 ± 0.027 (-0.16 - 0.14)	0.290	-0.025, 0.083	(3.1 - 4.0)	(2.9-3.9)	(3.3-4.2)
Tryptophan	0.52 ± 0.011 (0.44 - 0.58)	0.54 ± 0.011 (0.48 - 0.60)	-0.017 ± 0.012 (-0.11 - 0.072)	0.156	-0.041, 0.0068	(0.4 - 0.8)	(0.5-1.2)	(0.4-1.0)
Tyrosine	3.03 ± 0.13 (2.36 - 3.73)	3.26 ± 0.13 (2.43 - 3.64)	-0.23 ± 0.17 (-1.12 - 0.42)	0.195	-0.58, 0.12	(2.1 - 4.0)	(2.9-4.7)	(3.7-4.3)
Valine	4.75 ± 0.027 (4.59 - 4.85)	4.72 ± 0.027 (4.62 - 4.94)	0.026 ± 0.035 (-0.094 - 0.16)	0.462	-0.045, 0.097	(3.9 - 5.5)	(2.1-5.2)	(4.2-5.3)

(continued over)

Table 7. All Trials: Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Fatty acids (% of total)								
16:0 palmitic acid	9.16 ± 0.077 (8.67 - 9.57)	8.92 ± 0.077 (8.41 - 9.44)	0.24 ± 0.053 (-0.068 - 0.64)	<0.001	0.13, 0.34	(8.8 - 13.8)	(7-19)	(9.9-12.0)
18:0 stearic acid	1.95 ± 0.028 (1.80 - 2.06)	1.86 ± 0.028 (1.67 - 1.98)	0.093 ± 0.020 (-0.066 - 0.19)	<0.001	0.052, 0.13	(1.4 - 2.6)	(1-3)	(1.4-2.2)
18:1 oleic acid	22.46 ± 0.16 (21.37 - 23.12)	23.08 ± 0.16 (22.15 - 24.14)	-0.62 ± 0.18 (-2.27 - 0.46)	0.001	-0.99, -0.25	(20.7 - 37.7)	(20-46)	(20.6-27.5)
18:2 linoleic acid	64.49 ± 0.22 (63.79 - 65.80)	64.18 ± 0.22 (63.07 - 65.65)	0.31 ± 0.21 (-1.23 - 2.23)	0.152	-0.12, 0.73	(48.0 - 66.1)	(35-70)	(55.9-66.1)
18:3 linolenic acid	1.10 ± 0.0096 (1.07 - 1.17)	1.11 ± 0.0096 (1.07 - 1.20)	-0.00062 ± 0.013 (-0.13 - 0.060)	0.963	-0.028, 0.026	(0.9 - 1.5)	(0.8-2)	(0.8-1.1)
20:0 arachidic acid	0.37 ± 0.0057 (0.34 - 0.39)	0.37 ± 0.0057 (0.33 - 0.40)	-0.0030 ± 0.0041 (-0.019 - 0.016)	0.466	-0.011, 0.0052	(0.3 - 0.6)	(0.1-2)	(0.3-0.5)
20:1 eicosenoic acid	0.29 ± 0.0062 (0.28 - 0.32)	0.30 ± 0.0062 (0.27 - 0.34)	-0.013 ± 0.0062 (-0.038 - 0.019)	0.036	-0.026, -0.00083	(0.2 - 0.4)	(na)	(0.2-0.3)
22:0 behenic acid	0.17 ± 0.0036 (0.14 - 0.19)	0.17 ± 0.0036 (0.14 - 0.19)	-0.0019 ± 0.0034 (-0.010 - 0.011)	0.580	-0.0088, 0.0050	(0.1 - 0.3)	(na)	(0.1-0.3)

(continued over)

Table 7. All Trials: Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Minerals								
Calcium (%)	0.0047 ± 0.00021 (0.0037 - 0.0056)	0.0044 ± 0.00021 (0.0033 - 0.0058)	0.00024 ± 0.00013 (-0.00050 -0.00091)	0.073	-0.00002, 0.00050	(0.003 - 0.009)	(0.01-0.1)	(0.003-0.006)
Copper (mg/kg dw)	1.81 ± 0.090 (1.19 - 2.37)	1.92 ± 0.090 (1.50 - 2.33)	-0.11 ± 0.11 (-0.63 - 0.36)	0.311	-0.33, 0.11	(0.9 - 2.8)	(0.9-10)	(na)
Iron (mg/kg dw)	22.69 ± 0.76 (19.08 - 25.94)	22.93 ± 0.76 (18.77 - 26.62)	-0.24 ± 0.49 (-4.42 - 2.18)	0.629	-1.22, 0.74	(11 - 49)	(1-100)	(na)
Magnesium (%)	0.12 ± 0.0021 (0.11 - 0.13)	0.12 ± 0.0021 (0.11 - 0.13)	0.00089 ± 0.0020 (-0.016 - 0.010)	0.657	-0.0031, 0.0049	(0.08 - 0.2)	(0.09-1.0)	(na)
Manganese (mg/kg dw)	6.26 ± 0.32 (4.64 - 9.63)	6.25 ± 0.32 (4.96 - 8.83)	0.015 ± 0.21 (-0.88 - 1.34)	0.943	-0.41, 0.44	(2.6 - 7.8)	(0.7-54)	(na)
Phosphorus (%)	0.36 ± 0.0046 (0.32 - 0.39)	0.36 ± 0.0046 (0.32 - 0.39)	-0.00084 ± 0.0054 (-0.042 - 0.025)	0.875	-0.012, 0.0099	(0.24 - 0.43)	(0.26-0.75)	(0.31-0.36)
Potassium (%)	0.37 ± 0.0057 (0.35 - 0.39)	0.37 ± 0.0057 (0.34 - 0.41)	0.00004 ± 0.0058 (-0.039 - 0.022)	0.993	-0.012, 0.012	(0.29 - 0.53)	(0.32-0.72)	(na)
Zinc (mg/kg dw)	29.28 ± 0.88 (20.23 - 33.17)	29.66 ± 0.88 (23.47 - 33.26)	-0.37 ± 0.66 (-4.95 - 4.14)	0.575	-1.69, 0.95	(15 - 33)	(12-30)	(na)

(continued over)

Table 7. All Trials: Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Fiber and Proximates								
Ash (% dw)	1.44 ± 0.032 (1.28 - 1.62)	1.49 ± 0.032 (1.32 - 1.75)	-0.044 ± 0.042 (-0.29 - 0.21)	0.302	-0.13, 0.041	(0.8 - 1.8)	(1.1-3.9)	(1.2-1.8)
Carbohydrates (% dw)	82.59 ± 0.39 (80.71 - 84.33)	82.26 ± 0.39 (80.23 - 83.70)	0.33 ± 0.20 (-1.60 - 2.01)	0.105	-0.072, 0.73	(83.1 - 89.6)	(na)	(na)
ADF (% dw)	3.79 ± 0.16 (3.14 - 5.17)	3.70 ± 0.16 (2.79 - 4.28)	0.095 ± 0.19 (-0.71 - 1.48)	0.628	-0.30, 0.49	(2.3 - 5.7)	(3.3 - 4.3)	(3.1 - 5.3)
NDF (% dw)	10.38 ± 0.67 (7.89 - 12.53)	10.32 ± 0.67 (8.25 - 15.42)	0.057 ± 0.76 (-3.72 - 2.89)	0.940	-1.47, 1.59	(8.2 - 16.1)	(8.3-11.9)	(9.6 - 15.3)
Moisture (% fw)	11.08 ± 0.45 (9.01 - 13.30)	11.76 ± 0.45 (8.56 - 14.80)	-0.68 ± 0.32 (-2.60 - 2.54)	0.037	-1.32, -0.042	(6.1 - 15.6)	(7-23)	(9.4 - 15.8)
Total fat (%)	3.54 ± 0.095 (2.92 - 3.94)	3.59 ± 0.095 (2.88 - 4.13)	-0.058 ± 0.093 (-0.69 - 0.90)	0.532	-0.24, 0.13	(1.7 - 4.3)	(3.1-5.7, 2.9-6.1)	(2.4-4.2)
Protein (% dw)	12.43 ± 0.44 (10.30 - 14.77)	12.66 ± 0.44 (11.02 - 14.84)	-0.23 ± 0.20 (-1.62 - 1.42)	0.257	-0.63, 0.17	(6.7 - 13.4)	(6.0 - 12.0, 9.7 - 16.1)	(9.0 - 13.6)

(continued over)

Table 7. All Trials: Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Phytic Acid, Trypsin Inhibitor and Vitamin E Content of Grain and Statistical Summary

Component[a]	NK603 Mean[b] ± S.E.[c] (Range)	Control Mean[b] ± S.E.[c] (Range)	Difference (NK603 minus Control) Mean ± S.E.[c] (Range)	p-value	95% C.I.[d] (Lower, Upper)	Comm.[e] (Range)	Lit.[f] (Range)	Rpt.[g,h,i,j] (Range)
Miscellaneous								
Phytic Acid (%)	0.95 ± 0.028 (0.70 - 1.06)	0.97 ± 0.028 (0.81 - 1.21)	-0.020 ± 0.037 (-0.29 - 0.18)	0.580	-0.094, 0.053	(0.5 - 1.3)	(to 0.9%)	(na)
Trypsin Inhibitor (TIU/mg dw)	3.41 ± 0.27 (2.34 - 5.08)	2.91 ± 0.27 (1.39 - 5.14)	0.49 ± 0.35 (-2.15 - 2.84)	0.168	-0.22, 1.20	(3.40 - 7.18)	(na)	(na)
Vitamin E (mg/g dw)	0.0090 ± 0.00026 (0.0070 - 0.010)	0.0092 ± 0.00026 (0.0064 - 0.011)	-0.00015 ± 0.00026 (-0.0024 - 0.0013)	0.546	-0.00067, 0.00036	(0.006 - 0.022)	(0.017-0.047)	(0.008-0.012)

[a]ADF = acid detergent fiber; NDF = neutral detergent fiber; dw = dry wt.; fw = fresh wt; TIU = trypsin inhibitor units.
[b]The mean of all values.
[c]S.E. = standard error of the mean.
[d]C.I. = confidence interval.
[e]Comm. = commercial. The range of sample values for commercial lines grown in 1998 ([redacted]1999).
[f]Lit. = literature. For amino and fatty acids, Watson, 1982; for all other components, Watson, 1987; protein and fat second values from Jugenheimer, 1976.
[g]Rpt. = reported. For amino and fatty acids, range for five control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted] 1996a,b; 1997a,b).
[h]For ash, moisture and total fat, range for five control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted]1996a,b; 1997a,b).
[i]For ADF and NDF, range for three control lines analysed in Monsanto trials conducted between 1994 and 1995 ([redacted]1996b; 1997a,b).
[j]For calcium and phosphorus, range for three control lines analysed in Monsanto trials conducted between 1993 and 1995 ([redacted]1995; [redacted]1996a; 1997b).

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