Study Title

Molecular Characterization of Roundup Ready® (CP4 EPSPS) Corn Line NK603

Authors

Ming Y. Deng
Ronald P. Lirette
Tracey A. Cavato
Ravinder S. Sidhu

Study Completed On

October 12, 1999

Performing Laboratory

Monsanto Company
Product Characterization Center
Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
St. Louis, MO 63198

Laboratory Project ID

Study 99-01-46-26
MSL-16214

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA.10(d)(l)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under provisions contained in FIFRA as amended, and thereby consent to use and disclosure of this material by EPA according to FIFRA. In submitting this material to the EPA according to method and format requirements contained in PR Notice 86-5, we do not waive any protection of rights involving this material that would have been claimed by the company if this material had not been submitted to the EPA."

Company: Monsanto Company

Company Agent: Kent Croon

Title: Regulatory Affairs Manager

Signature: _____ Date: 10/12/99

Statement of Compliance

This study meets the requirements under GLP as specified in 40 CFR Part 160 with the following exception:

Nucleotide sequence information used in this study was generated by the Monsanto Genomics Sequencing Center which is not a GLP facility. However, written procedures are in place for conduct of the sequencing experiments.

[signature] 10/12/99
Submitter Date

Ravinder S Sodhi 12 Oct-99
Sponsor Date

Tracey A Cavato 10/12/99
Study Director Date

QUALITY ASSURANCE UNIT STATEMENT

Study Title: Molecular Characterization of Roundup Ready® (CP4 EPSPS) Corn Line NK603

Study Number: 99-01-46-26

Reviews conducted by the QAU confirm that the final report reflects the raw data.

Following is a list of reviews conducted by the Monsanto AG Regulatory QAU on the study reported herein.

Dates Of Inspection / Audit	Phase	Date Reported To: Study Director	Management
April 30, 1999	Southern blot	August 16, 1999	August 16, 1999
May 6, 1999	Southern blot	May 26, 1999	May 26, 1999
October 7, 1999	Raw data audit	October 8, 1999	October 8, 1999
October 8, 1999	Final report draft	October 8, 1999	October 8, 1999

Joan M. Rejda-Heath

Joan M. Rejda-Heath, Ph.D.
Quality Assurance
Ag Regulatory, Monsanto Company

October 8, 1999

Date

Signatures of Approval

Study Number:	99-01-46-26
Title:	Molecular Characterization of Roundup Ready® (CP4 EPSPS) Corn line NK603
Facility:	Monsanto Company 700 Chesterfield Parkway North St. Louis, Missouri 63198
Sponsor:	Ravinder S. Sidhu
Study Director:	Tracey A. Cavato
Technical Center Leader:	Ronald P. Lirette
Contributor:	Ming Y. Deng
Study Initiation Date:	April 20, 1999
Study Completion Date:	October 12, 1999
Records Retention:	All study specific raw data, protocols, final reports and facility records will be retained at Monsanto, St. Louis.
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto, St. Louis.

Signatures of Approval:

Tracey A. Cavato — 10/12/99
Study Director — Date

Ravinder S Sidhu — 12 Oct-99
Sponsor — Date

Table of Contents

Table of Contents (cont'd)

Abbreviations

~	approximately
Ci, μCi	curie, microcurie
CP4 EPSPS	EPSPS from *Agrobacterium* sp. strain CP4
CTAB	cetyltrimethylammonium bromide
CTP	chloroplast transit peptide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EPSPS	enzyme 5-enolpyruvylshikimate-3-phosphate synthase
e35S	cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
kDa	kilodalton
HCl	hydrochloric acid
MW	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_2HPO_4	sodium phosphate dibasic
NOS 3'	nopaline synthase 3' polyadenylation sequence
PCR	polymerase chain reaction
P-ract1/ract1 intron	rice actin promoter and intron
PVP	polyvinylpyrolidine
RR	Roundup Ready®
SDS	sodium dodecyl sulfate
SSC	Saline-sodium citrate buffer. 20X SSC is 3 M sodium chloride, 0.3 M sodium citrate
Tris	tris(hydroxymethyl)aminomethane
tRNA	*Escherichia coli* transfer RNA
TE buffer	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
UV	ultraviolet
V	volts
*Zm*HSP70	maize (*Zea mays*) hsp70 gene (heat-shock protein)

I. SUMMARY

The purpose of this study was to characterize the DNA inserted into corn line NK603 containing the glyphosate tolerance trait. NK603 expresses a 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) which confers tolerance to glyphosate, the active ingredient in Roundup® herbicide. NK603 was produced by particle acceleration technology using the linear DNA fragment PV-ZMGT32L (Figure 1) derived from the plasmid vector PV-ZMGT32 (Figure 2). The DNA fragment used for corn transformation contained two gene expression cassettes: the first EPSPS gene cassette, containing the CP4 EPSPS coding sequence under the regulation of the rice actin promoter, a rice actin intron, a chloroplast transit peptide (CTP2) sequence, and a nopaline synthase (NOS) 3' polyadenylation sequence; and the second EPSPS gene cassette, also containing the CP4 EPSPS coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2, and the NOS 3' polyadenylation sequence (Table 1). Molecular analysis was performed to characterize the DNA integrated into the genome of corn line NK603. Specifically, the insert number (number of integration sites within the corn genome), the copy number (the number of integrated DNA fragments within one insertion site), the integrity of the inserted gene cassettes, and the absence of backbone sequences were assessed by Southern blot analysis. Data from the analyses support the following: (1) the genome of corn line NK603 contains a single DNA insertion; (2) there is a single copy of the DNA fragment used for transformation within that insertion; (3) both 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 NK603 insert were verified by PCR and DNA sequencing. All these data support the conclusion that only the full-length CTP2-CP4 EPSPS protein should be encoded by the insert in corn line NK603. The genetic stability of the transgene was also demonstrated by Southern blot analysis on genomic DNA from the F1 generation (progeny of R0 back-crossing) and the fifth generation of back-crossing (BC5) of NK603.

II. INTRODUCTION

A. Background. Monsanto Company has developed Roundup Ready® corn line NK603 which is tolerant to glyphosate (the active ingredient in Roundup™ herbicide) at the whole plant level. Corn line NK603 contains a 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). Corn plants that demonstrate commercial level tolerance to Roundup herbicide are called Roundup Ready® (RR). The coding region for CP4 EPSPS 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 protein is structurally and functionally similar to

plant EPSPS enzymes but has a much reduced affinity for glyphosate (Padgette et al., 1993). In non-transgenic plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby depriving plants of these essential nutrients (Steinrucken and Amrhein, 1980; Haslam, 1993). In RR plants, nutritional requirements for normal growth and development are met by the continued action of the tolerant CP4 EPSPS enzyme in the presence of glyphosate. A safety assessment of the CP4 EPSPS protein has been described in the literature (Harrison et al. 1996).

Corn line NK603 was produced by transformation of corn tissue with a 6.7-kb linear DNA fragment PV-ZMGT32L (Figure 1) derived from the plasmid vector PV-ZMGT32 (Figure 2), using particle acceleration technology. The DNA fragment used for corn transformation contained two gene expression cassettes: the first EPSPS gene cassette, containing the CP4 EPSPS coding sequence under the regulation of the rice actin promoter, a rice actin intron, a chloroplast transit peptide (CTP2) sequence, and a nopaline synthase (NOS) 3' polyadenylation sequence; and the second EPSPS gene cassette, containing the CP4 EPSPS coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2, and the NOS 3' polyadenylation sequence. A description of the elements in the linear DNA fragment PV-ZMGT32L is given in Table 1.

B. Purpose. The purpose of this study was to characterize the inserted DNA in corn line NK603. Genomic DNA was analyzed using Southern blot analysis (Southern, 1975) to determine the insert number (number of integration sites within the corn genome), the copy number (the number of integrated DNA fragments within one insertion site), the integrity of the inserted promoters, coding regions, and polyadenylation sequences, and the presence or absence of plasmid backbone sequence. Polymerase Chain Reaction (PCR, Saiki, 1990) was performed to verify the sequences at the 5' and 3' ends of the insert. In addition, Southern blot analysis fingerprints of genomic DNA extracted from the F1 generation (the progeny of R0 back-crossing) and the fifth generation of back-crossing (BC5) of corn line NK603 were compared to assess the genetic stability of the inserted DNA.

III. MATERIALS AND METHODS

A. Test substance. The test substance for this study was the corn line NK603. Leaf tissue of the corn line was collected from plants grown under greenhouse conditions and treated with Roundup Ultra™ (64 ounces/acre) at 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 (B73 for short in this report). Leaf tissue of the corn 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 corn 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 control line 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), catalog #236 250 and #1449 460, respectively] and Gibco BRL (Gaithersburg, MD) [High MW DNA Marker (48.5 - 8.3 kb), catalog #15618-010] used for size estimations.

D. Test system. There was no test system. This study used analytical methods to characterize the corn event.

Multiple plants for single plant?

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

F. DNA quantitation and restriction enzyme digestion. Quantitation of the DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer (San Francisco, CA)(SOP# BR-EQ-0065-01) 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 test and control lines was used for the restriction enzyme digests. Overnight digests were performed at 37°C according to SOP# GEN-PRO-010-01 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 re-dissolved at room temperature in TE, pH 8.0.

G. Preparation of DNA probes. Plasmid DNA was isolated from *E. coli* cultures. DNA probe templates homologous to P-ract1/ract1 intron, e35S promoter, and the 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 amplifying 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 NK603 for the presence of any plasmid backbone sequence in the genome of the corn line. Linearized whole plasmid PV-ZMGT32 was also used to prepare the DNA probe for determining the insert number and copy number of corn 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.

H. Southern blot analysis. Southern blot analyses were performed to characterize the DNA that was integrated into the corn genome. These analyses were done according to SOP# GEN-PRO-025-02. DNA samples digested with restriction enzymes were separated, based on size, using 0.6% agarose gel electrophoresis according to SOP# GEN-PRO-003-01. A "long run" and a "short run" were performed for most gels. The

"long run" samples were loaded onto the gel and typically electrophoresed for 15 - 16 h at 35 V. High MW DNA Markers (400 ng) mixed with MW Marker II (1 µg) were the reference substance for the "long run". The "short run" samples 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 $Na_2HPO_4 \bullet 7H_2O$, 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.

I. **Verification of the 5' and 3' flanking sequences.** The sequences at the 5' and 3' ends of the 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.

IV. RESULTS AND DISCUSSIONS

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. This digestion should generate a single fragment containing the inserted DNA and adjacent plant genomic DNA from NK603 if there is a single insertion of the DNA fragment used for transformation in the corn genome. Control 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 3. 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). NK603 DNA (lanes 2 and 5) produced one band of approximately 23 kb not present in the control line. This result suggests that event 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 insert 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 a 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. Test DNA, control DNA, and 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 experimental data and sequence analysis on this fragment indicated that one of these restriction sites (at nucleotide 4082) is methylated (data not shown) and thus, resistant to cleavage with *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 corn line NK603 contains only one copy of the DNA fragment used for corn transformation. The results are shown in Figure 4. The control DNA alone (lane 1) did not produce bands, as expected. Plasmid PV-ZMGT32 DNA mixed with 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). NK603 DNA (lanes 2 and 5) produced two bands at approximately 9.0 and 5.8 kb. This result establishes that NK603 contains only one copy of the transformation cassette at the locus of DNA integration.

C. Integrity of inserted gene cassettes: In order to determine the integrity of the inserted gene cassettes, components of the cassettes, i.e. the promoters, coding regions and the polyadenylation sequences, were analyzed.

1. P-ract1/ract1 intron. To assess the integrity of the P-ract1/ract1 intron sequence, test DNA, control DNA, and 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 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 5. The control DNA alone (lane 1) showed no hybridization signals, as expected. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lanes 3 and 4) produced the expected band at ~3.8 kb containing the sequence of P-ract1/ract1 intron (Figure 2). 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" test 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". Further experiments which determined the sequence of the ends of the integrated DNA in NK603 revealed that an additional 217-bp fragment containing a portion of the enhancer region of the rice actin promoter was present in the reverse orientation proximal to the 3' end of the transformation cassette, and that this small fragment maintained an *Eco*RV site 20-bp upstream from its 3' end bordering corn genomic sequence (Figure 12). These findings confirmed and explained the results from this Southern blot analysis.

The 217-bp fragment includes 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.

2. CTP2-CP4 EPSPS sequence. To assess the integrity of the CTP2-CP4 EPSPS sequence, test DNA, control DNA, and 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 6. Control DNA alone (lane 1) showed no hybridization bands, as expected. Plasmid PV-ZMGT32 DNA mixed with control DNA (lanes 3 and 4) and NK603 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 the corn line NK603 does not contain any additional, detectable CTP2-CP4 EPSPS sequence other than those in the two inserted EPSPS gene cassettes.

3. e35S promoter. To assess the integrity of the e35S promoter, test DNA, control DNA, and 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 7. The control DNA alone (lane 1) showed no hybridization signal, as expected. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lanes 3 and 4) and NK603 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 the corn line NK603 does not contain any additional, detectable e35S sequence other than that in the inserted DNA fragment.

4. NOS 3' polyadenylation sequence. To assess the integrity of the NOS 3' polyadenylation sequence, test DNA, control DNA, and 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 8. The control DNA alone (lane 1) showed no hybridization signal, as expected. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lanes 3 and 4) and the 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' polyadenylation sequence (Figure 12). No unexpected bands were detected establishing that the corn line NK603 does not contain any additional, detectable NOS 3' polyadenylation sequence other than those in the two inserted EPSPS gene cassettes.

The above results establish that the two inserted EPSPS gene cassettes are intact in corn line NK603 (see insert map, Figure 12). In addition, a 217-bp fragment containing a part of the enhancer region of the rice actin promoter is inversely linked to the 3' end of the inserted EPSPS gene cassettes.

D. Analysis for backbone fragments. To assess for the presence of backbone sequence, test DNA, control DNA, and 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 9. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lanes 3 and 4) produced one band of ~3.8 kb, the expected size for the entire backbone (Figure 2). The control DNA alone (lane 1) and the 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 NK603 insert. Results of these PCR reactions are shown in Figure 10. The negative controls of distilled water, control corn line B73, and an unrelated transgenic corn 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 to NK603. 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.

F. Stability of the inserted DNA in NK603. Southern blot analysis was also conducted to assess the stability of the inserted DNA in 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 (BC5) of NK603 were digested with *Eco*RV, blotted and probed with the full-length CTP2-CP4 EPSPS fragment. The results are presented in Figure 11. The

control DNA (lane 1) showed no hybridization signals. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lane 2), NK603 F1 DNA (lane 3) and NK603 BC5 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 BC5 generation of corn line NK603. This demonstrates the stability of the inserted DNA in samples spanning five generations.

Based on the results obtained from Southern blot analysis and PCR analysis, a predicted restriction map of the insert in corn line NK603 is shown in Figure 12.

V. CONCLUSIONS

The Roundup Ready (CP4 EPSPS) corn line NK603 was produced by particle acceleration technology using a linear DNA fragment from plasmid PV-ZMGT32 containing two EPSPS gene cassettes. Corn line NK603 contains one insertion of the integrated DNA located within a 23-kb *Stu*I restriction fragment. This insert contains one complete copy of the fragment used in transformation and 217 bp of the enhancer region of the rice actin promoter. The individual genetic components in each of the two CP4 EPSPS gene cassettes in the integrated DNA are intact. The extra 217-bp fragment of the enhancer region of the rice actin promoter inversely linked to the 3' end of the transformation cassette does not contain any defined elements required to promote gene expression and thus is highly unlikely to act as a promoter. The genome of 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 corn line NK603. In addition, the genetic stability of the inserted DNA was demonstrated by Southern blot analysis on genomic DNA of the F1 and the BC5 generations of corn line NK603.

V. REFERENCES

Harrison, L., Bailey, M., Naylor, M., Ream, J., Hammond, B., Nida, D., Burnette, B., Nickson, T., Mitsky, T., Taylor, M., Fuchs, R., and Padgette, S. 1996. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. J. Nutr. 126, 728-740.

Haslam, E. 1993. Shikimic Acid: Metabolism and Metabolites. John Wiley and Sons, Chichester, England.

McElroy, D., Zhang, W., Cao, J. and Wu, R. 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell. 2, 163-171.

Padgette, S., Barry, G., Re, D., Eichholtz, D., Weldon, M., Kolacz, K., and Kishore, G. 1993. Purification, cloning, and characterization of highly glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4. Monsanto Technical Report MSL-12738, St. Louis.

Padgette, S., RE, D., Barry, G., Eichholtz, D., Delannay, X., Fuchs, R., Kishore, G., and Fraley, R. 1996. New weed control opportunities: development of soybeans with a Roundup Ready™ gene, p. 53-84. *In* S. O. Duke (ed.), Herbicide Resistant Crops. CRC Press, Boca Raton, Fl.

Saiki, R.K. 1990. Amplification of genomic DNA, p. 13-20. *In* M.A. Innis, D. Gelfand, J.J. Sninsky, and T.J. White (ed), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-17.

Steinrucken, H. and Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94, 1207-1212.

Wang, Y., Zhang, W., Cao, J., McElroy, D., and Wu, R. 1992. Characterization of *cis*-acting elements regulating transcription from the promoter of a constitutively active rice actin gene. Mol. Cell. Biol. 12, 3399-3406.

Zhang, W., McElroy, D., and Wu, R. 1991. Analysis of rice act1 5' region activity in transgenic rice plants. Plant Cell. 3, 1155-1165.

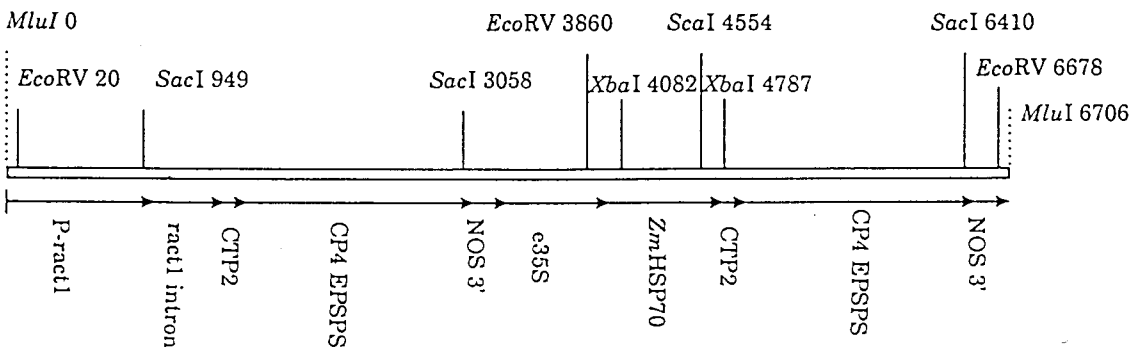

Figure 1. Linear map of PV-ZMGT32L. The DNA fragment PV-ZMGT32L was used to generate Roundup Ready corn line NK603 by particle acceleration technology. The dashed lines represent the remaining *Mlu*I sites following digestion of PV-ZMGT32. The *Xba*I restriction site at nucleotide 4082 is not active due to methylation (see section IV B).

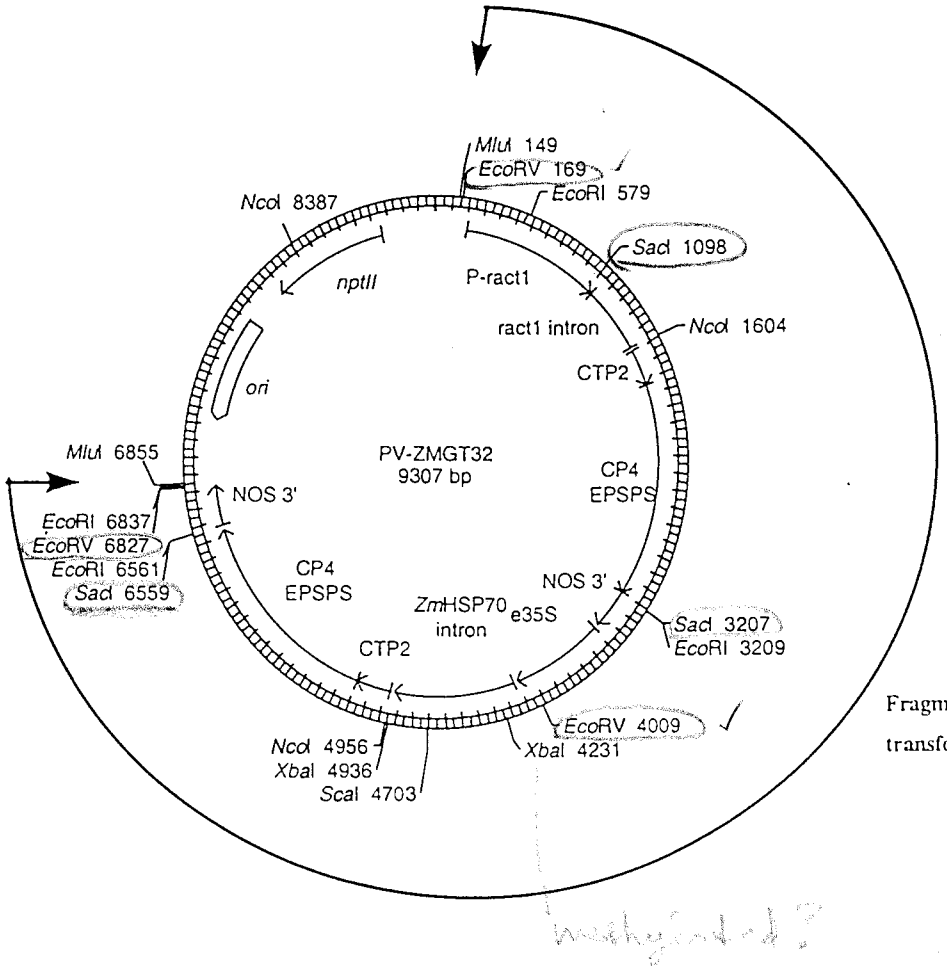

Figure 2. Plasmid map of PV-ZMGT32. The plasmid PV-ZMGT32 was used to prepare the *Mlu*I fragment used in the transformation of corn line NK603 by excision and discarding of the origin of replication (*ori*) and antibiotic resistance marker (*nptII*) sequences.

Table 1. Summary of genetic elements in linear DNA fragment PV-ZMGT32L used for transformation of corn line NK603

Genetic Element	Size (kb)	Function
The first EPSPS gene cassette:		
P-ract1/ ract1 intron	1.4	5' region of rice (*Oryzae sativa*) actin 1 gene containing the promoter, transcription start site and first intron.
CTP2	0.2	DNA sequence for chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS; transit peptide directs the CP4-EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
CP4 EPSPS	1.4	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sequence isolated from *Agrobacterium* sp. strain CP4 which imparts tolerance to glyphosate.
NOS 3'	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.
The second EPSPS gene cassette:		
e35S	0.6	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region.
*Zm*HSP70	0.8	Intron from the maize (*Zea mays*) *hsp*70 gene (heat-shock protein) present to stabilize the level of gene transcription.
CTP2	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.
CP4 EPSPS	1.4	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sequence isolated from *Agrobacterium* sp. strain CP4 which imparts tolerance to glyphosate.
NOS 3'	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.

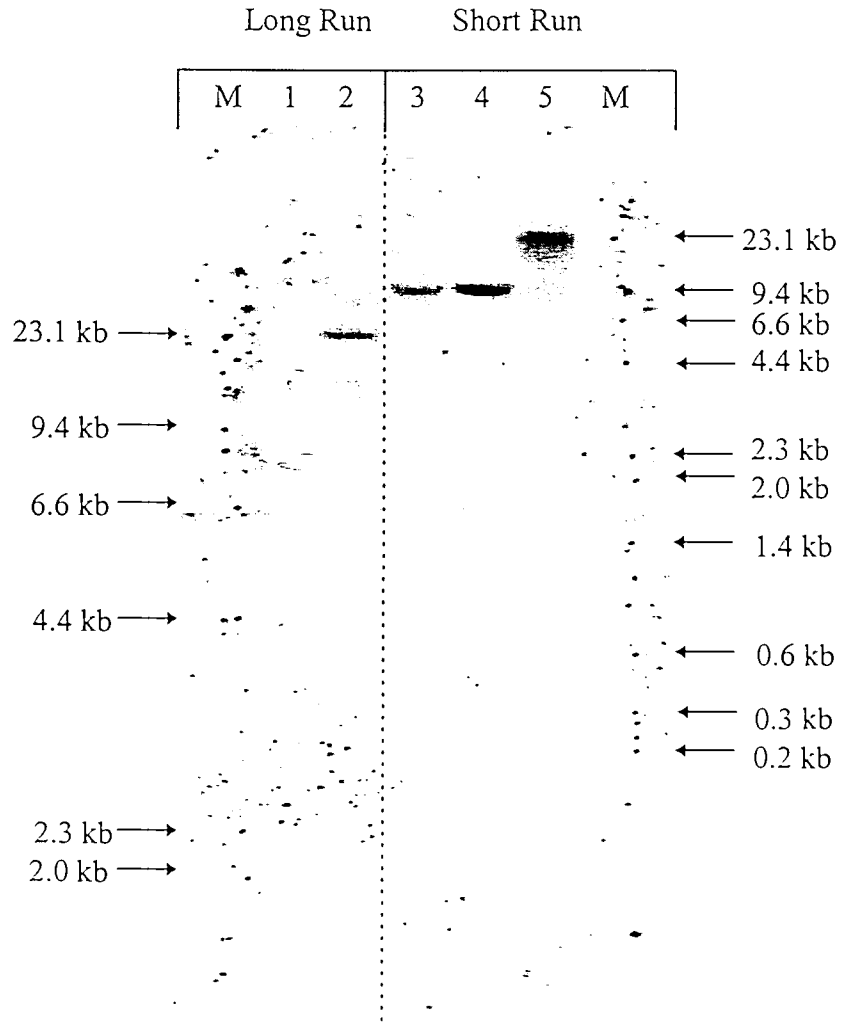

Figure 3. Southern blot analysis of NK603: determination of insert number. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Stu*I. Plasmid PV-ZMGT32 DNA mixed with B73 DNA was digested with *Stu*I and *Sca*I. DNA samples were then blotted and probed with ^{32}P-labeled 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 III H for details).

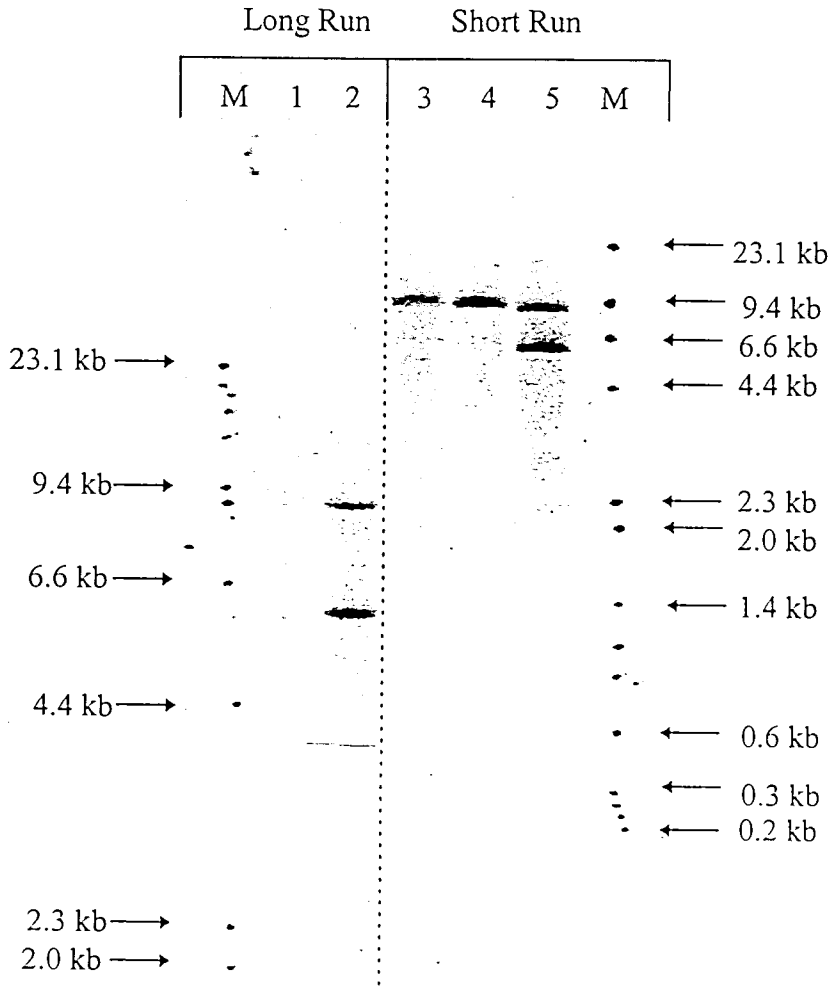

Figure 4. Southern blot analysis of NK603: determination of copy number. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Xba*I. The DNA samples were then blotted and probed with ^{32}P-labeled plasmid PV-ZMGT32. Lane designations are as follows:

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 III H for details).

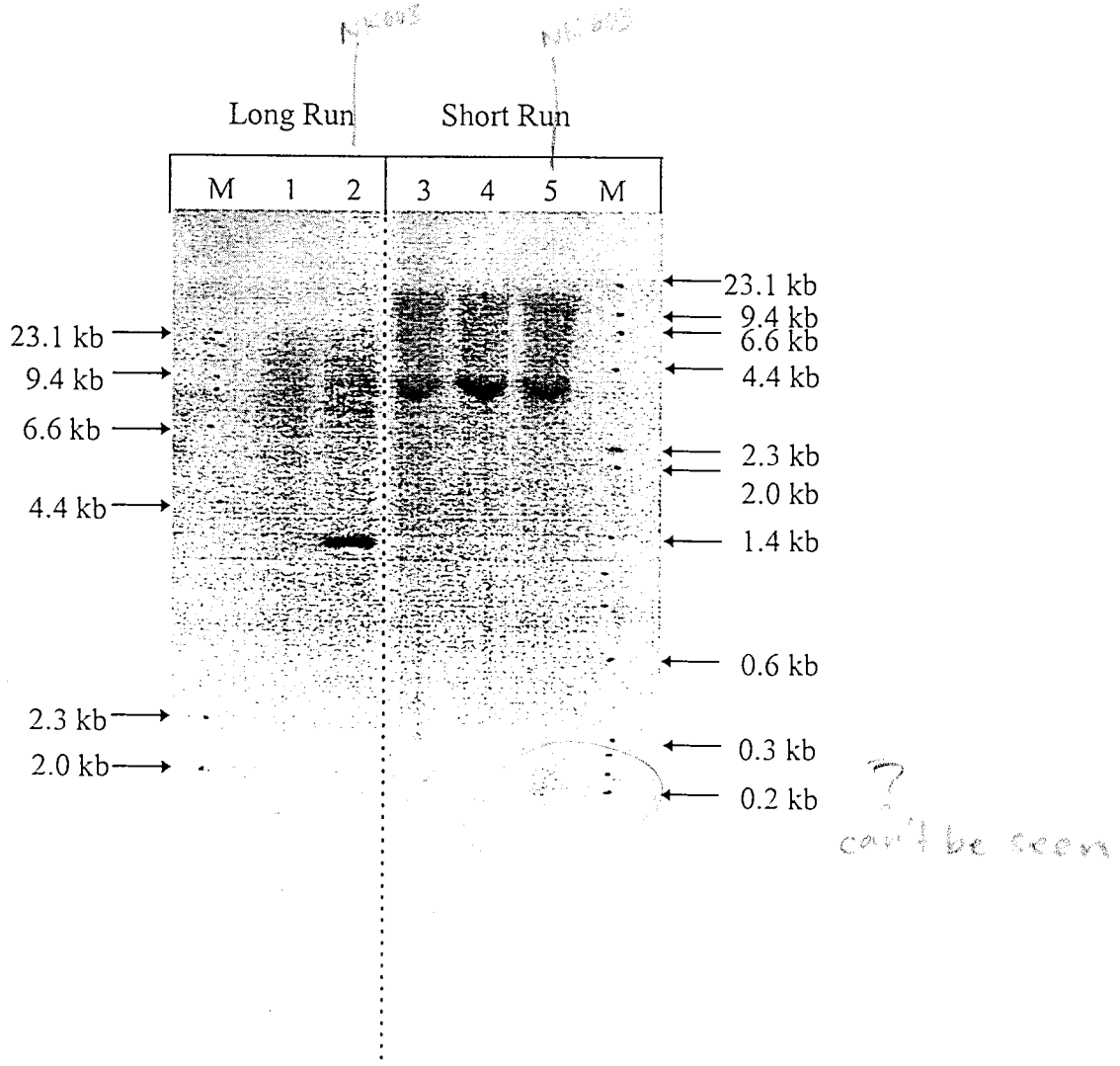

Figure 5. Southern blot analysis of NK603: P-ract1/ract1 intron. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. DNA samples were then blotted and hybridized with a mixture of two probes: the full-length P-ract1/ract1 intron labeled with ^{32}P-dCTP 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 III H for details).

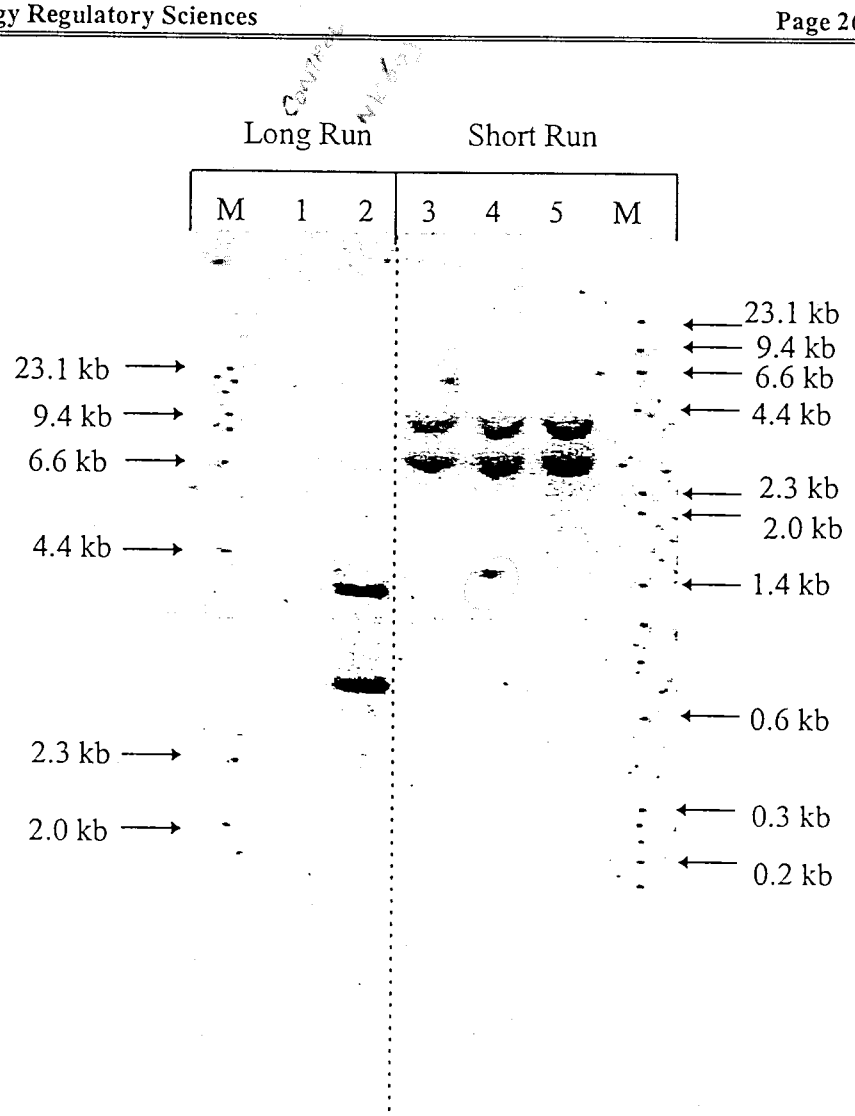

Figure 6. Southern blot analysis of NK603: CTP2-CP4 EPSPS sequence. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were then blotted and probed with the full-length ^{32}P-labeled CTP2-CP4 EPSPS fragment. Lane designations are as follows:

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 III H for details).

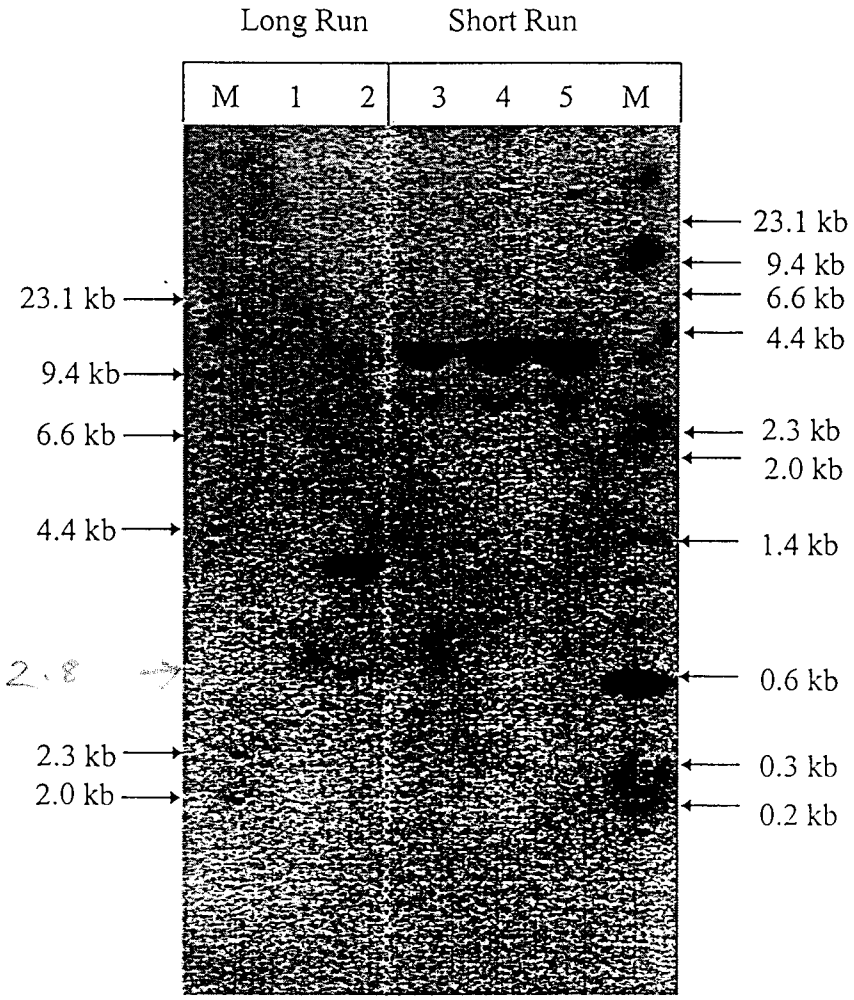

Figure 7. Southern blot analysis of NK603: e35S promoter. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were then blotted and probed with the full-length ^{32}P-labeled e35S fragment. Lane designations are as follows:

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 III H for details).

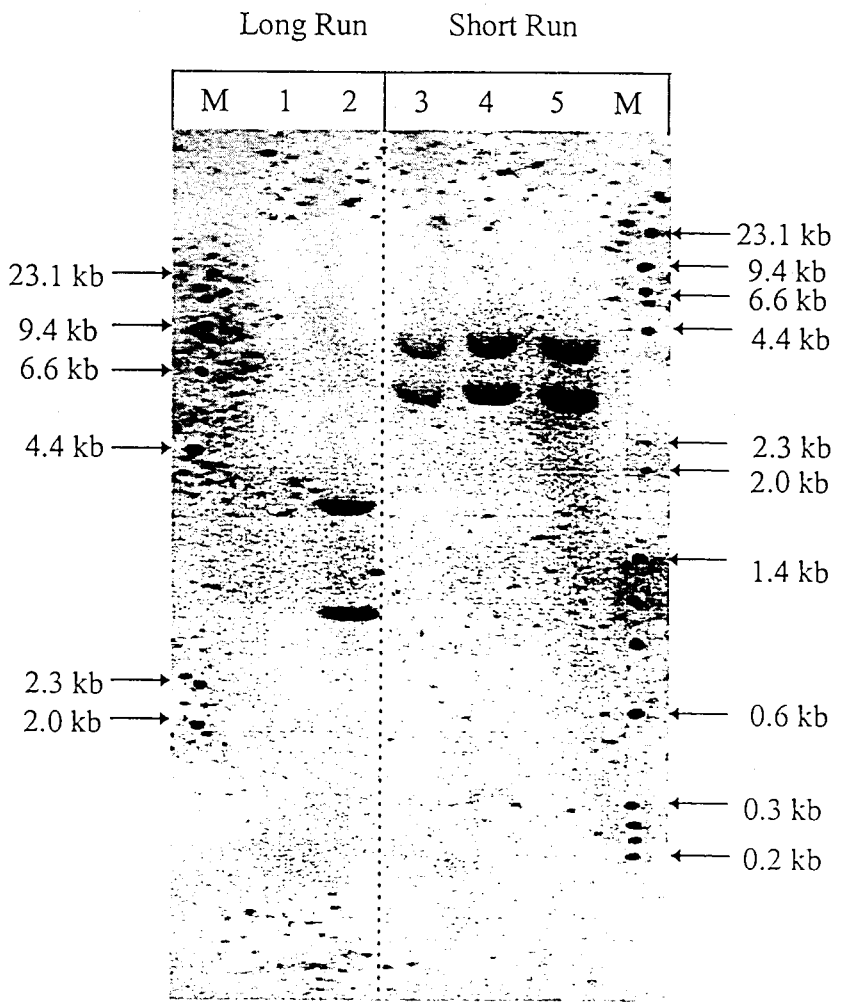

Figure 8. Southern blot analysis of NK603: NOS 3' polyadenylation sequence. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were then blotted and probed with the full-length ^{32}P-labeled NOS 3' polyadenylation sequence fragment. Lane 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 III H for details).

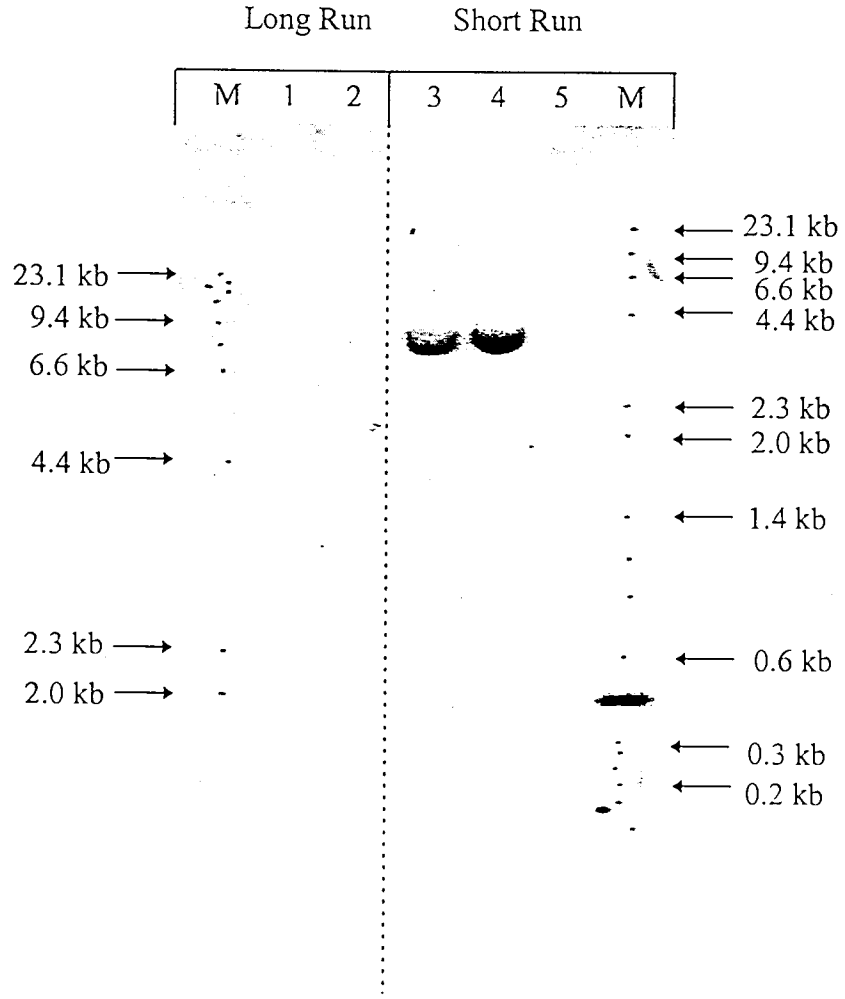

Figure 9. Southern blot analysis of NK603: backbone analysis. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *SacI*. The DNA samples were then blotted and probed with ^{32}P-labeled entire backbone sequence consisting of the *ori* and *nptII* coding regions. 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 III H for details).

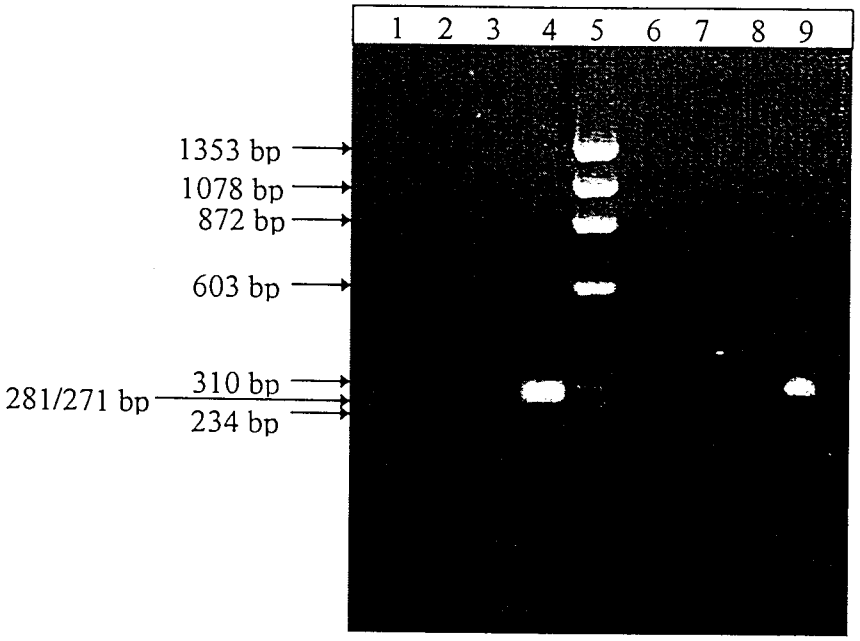

Figure 10. PCR verification of sequences at the 5' and 3' ends of NK603 insert. PCR was performed using primers specific to the 5' and 3' flanking sequences for NK603 insert on genomic DNA extracted from corn lines B73 (non-transgenic control), an unrelated transgenic corn line, and NK603. The PCR primer 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.

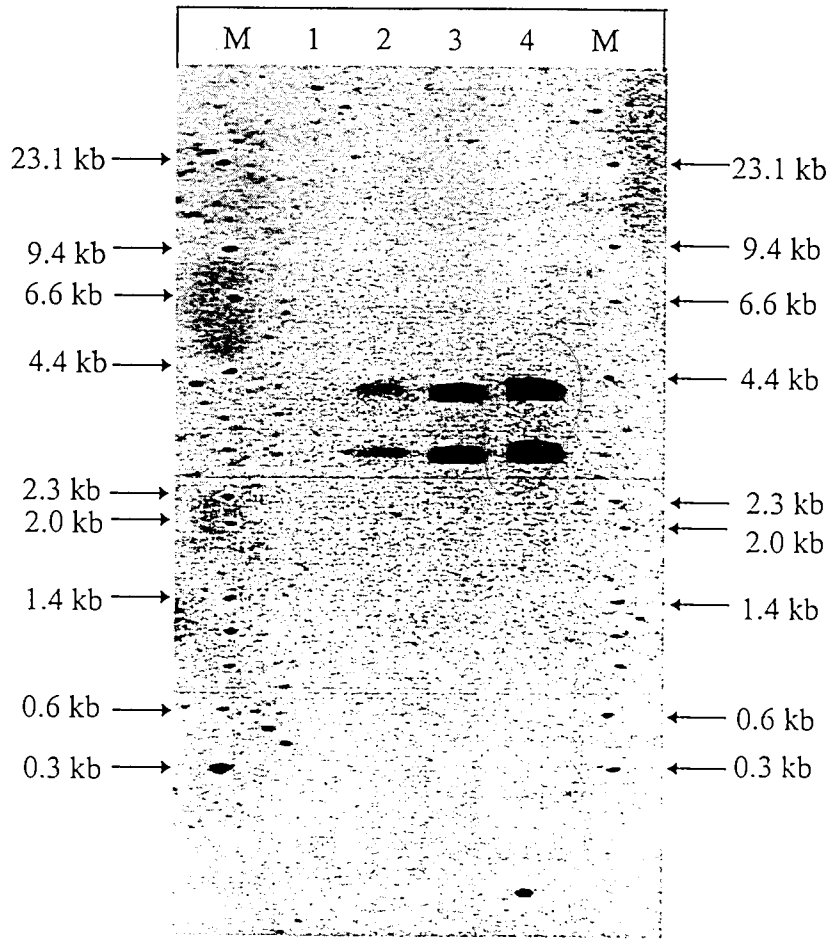

Figure 11. Southern blot analysis of NK603: stability of the inserted DNA. Ten micrograms of B73 and NK603 genomic DNA extracted from leaf tissues were digested with *Eco*RV. The DNA samples were then blotted and probed with the full-length ^{32}P-labeled CTP2-CP4 EPSPS fragment. Lane designations are as follows:

Lane 1: B73 DNA
2: B73 DNA spiked with 29 pg PV-ZMGT32
3: NK603 F1 DNA
4: NK603 BC5 DNA

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

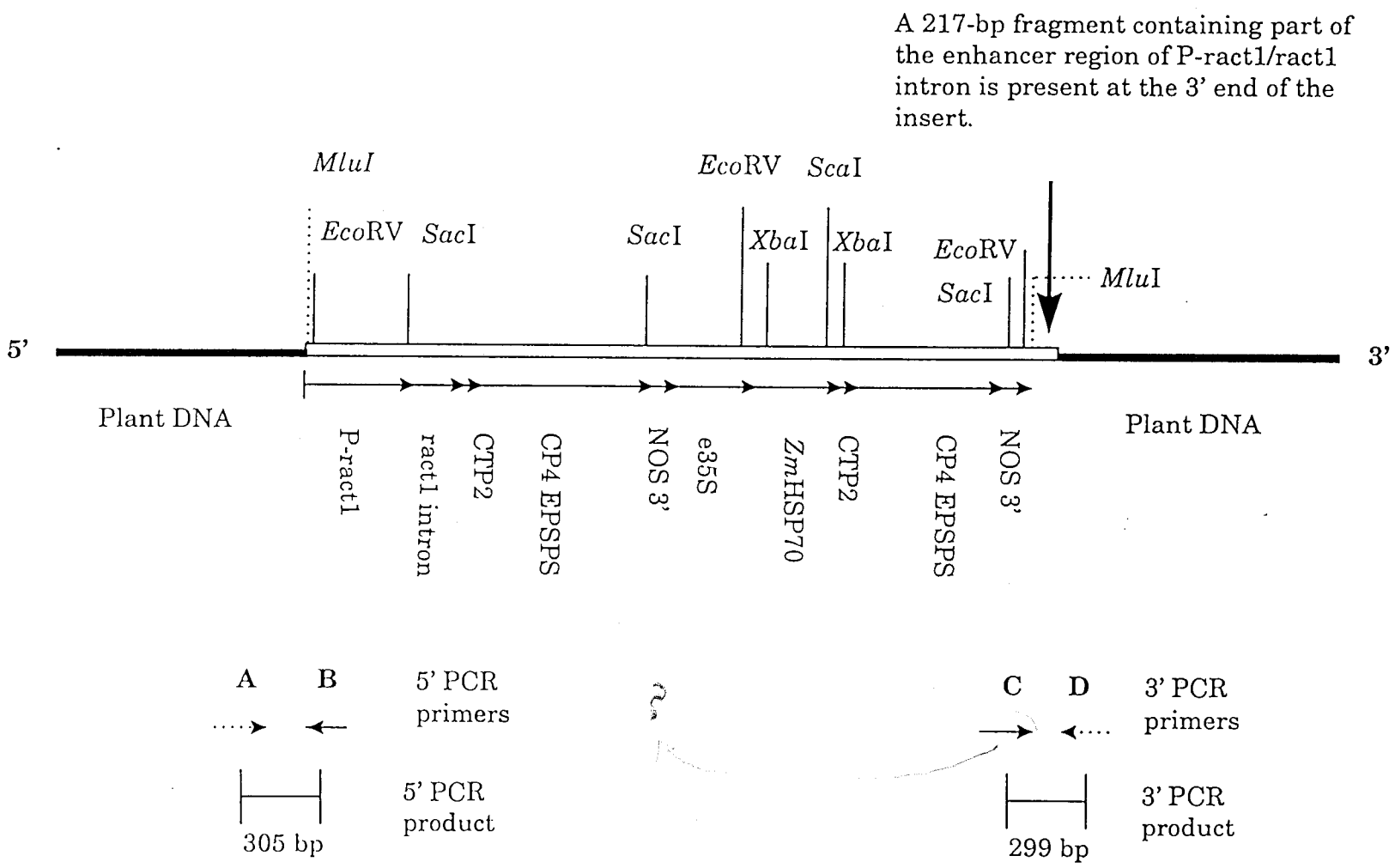

Figure 12. Schematic representation of the NK603 insert. This figure depicts the predicted insert in corn line NK603 based on data from Southern blot analysis and PCR confirming the sequences at the 5' and 3' ends of the insert. There is one complete copy of the PV-ZMGT32L fragment that was used in corn transformation to generate the line NK603.

Appendix 1

Standard Operating Procedures

BR-EQ-0065-01	DyNA Quant 200 Fluorometer
GEN-PRO-010-01	Procedure for Restriction Enzyme Digestion of DNA
GEN-PRO-003-01	Procedure for Agarose Gel Electrophoresis
GEN-PRO-025-02	Procedure for Southern Blot Analysis

ADDITIONAL DATA REQUIREMENTS

1. Sequence 5' + 3' to insert required
2. Is corn line LH82 x 573 (B73) isogenic?
3. Why doesn't primer C give a PCR product?
   How many primers A and B dedicated?
   (given that they are in the plant genome twice)

MSL-12902

Insert