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Appendix

Abbreviations

~	approximately
cfu	colony forming unit
$CoCl_2$	cobalt chloride
CP4 EPSPS	coding region for/ or the enzyme 5-enolpyruvylshikimate-3-phosphate synthase EPSPS isolated from *Agrobacterium sp.* strain CP4
CTAB	cetyltrimethylammonium bromide
CTP4	chloroplast transit peptide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
E35S	cauliflower mosaic virus (CaMV) 35S promoter containing a duplication of the -90 to -300 region
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EtOH	ethanol
g	gravity
HCl	hydrochloric acid
LB	Luria-Bertani medium
MOPS	4-morpholinepropanesulfonic acid
$MgCl_2$	magnesium chloride
MW	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_2HPO_4	disodium phosphate
NOS 3'	nopaline synthase 3' transcriptional termination element
nptII	coding region for neomycin phosphotransferase II
NPTII	neomycin phosphotransferase II protein
OD	optical density
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate

SSC	20X is 3 M sodium chloride, 0.3 M sodium citrate
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TE buffer	tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
UV	ultraviolet

I. SUMMARY

Soybean plants tolerant to glyphosate herbicide were produced by inserting an expression cassette encoding a native glyphosate-tolerant EPSPS (5-enol-pyruvylshikimate-3-phosphate synthase) isolated from *Agrobacterium sp.* strain CP4 (CP4 EPSPS) into the genome of soybean cultivar A5403. This resulted in the glyphosate tolerant, Roundup Ready® soybean event 40-3-2. Previous molecular characterization indicated that soybean event 40-3-2 contained a single DNA insertion from the plasmid PV-GMGT04, encoding a portion of the E35S promoter, a chloroplast transit peptide and CP4 EPSPS coding sequence, and the majority of the NOS 3' transcriptional termination element (Re *et al.*, 1993; Kolacz and Padgette, 1994; Padgette *et al.*, 1996). To develop event-specific detection assays for seed quality monitoring and detection, further analyses of Roundup Ready soybean event 40-3-2 were performed. This report describes the results of these recent experiments performed using more sensitive and precise methods, which extend the previous molecular characterization of soybean event 40-3-2.

Southern blot analyses of commercial varieties of soybean event 40-3-2 utilizing the restriction enzyme *Hin*d III were probed with the entire CP4 EPSPS coding sequence using more sensitive methods. These methods are approximately nine-fold more sensitive than the methods used in the original molecular characterization conducted in 1992 through 1994. In addition to the expected ~5.8 Kb *Hin*d III restriction fragment, autoradiograms developed using these more sensitive methods revealed the presence of a previously unobserved ~900 bp *Hin*d III restriction fragment, which weakly hybridized to the CP4 EPSPS probe. To more accurately delineate the portion of CP4 EPSPS DNA sequence that is present within the ~900 bp *Hin*d III restriction fragment, probe walking experiments were performed on Southern blots using portions of the CP4 EPSPS coding sequence as probes. Further experiments included cosmid library screening, nucleotide sequencing, and PCR analysis. The resulting data, based on Southern blot analysis and ultimately nucleotide sequencing, established that the second insert in soybean event 40-3-2 consists of 72 bp of the CP4 EPSPS sequence located on a 937 bp *Hin*d III restriction fragment. No other portion of the plasmid used in the transformation of soybean event 40-3-2 is present on the 937 bp *Hin*d III restriction fragment.

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Genome walking studies combined with nucleotide sequencing yielded the DNA sequences of the 5' and 3' ends of the primary, functional insert, as well as soybean genomic DNA flanking the 5' and 3' ends of the functional gene cassette in Roundup Ready soybean event 40-3-2. These sequences were generated to develop event-specific detection methods. Results of analysis of the 5' end confirmed the result reported in 1994 which demonstrated a deletion in the enhancer region of the E35S promoter. The remainder of the E35S promoter is functional. DNA sequencing of the 3' end of the functional insert revealed that the intact NOS 3' transcriptional termination element is present, rather than a portion of NOS as previously reported. In addition, 250 bp of a portion of the CP4 EPSPS element adjacent to the 3' end of the NOS 3' transcriptional termination element was observed. The restriction enzyme and probe combinations reported in the 1992-1994 molecular characterization studies did not detect this 250 bp CP4 EPSPS segment. DNA sequences of the flanking soybean genome were also obtained.

Several lines of evidence lead to the conclusion that no mRNA or protein is produced from either the 72 bp CP4 EPSPS segment that comprises the second insert or the 250 bp of the CP4 EPSPS element adjacent to the 3' end of NOS on the functional insert: 1) these segments do not contain a promoter or 3' transcriptional termination element, and are therefore not expected to be transcribed or translated, 2) northern blot analysis detected only the expected full-length mRNA species hybridizing to a CP4 EPSPS probe, and 3) western blot analyses performed with antibodies against CP4 EPSPS detected only the expected, full length CP4 EPSPS protein produced in soybean event 40-3-2 (Harrison *et al.*, 1993, Rogan *et al.*, 1999). Taken together, these results provide compelling evidence that these DNA segments are neither transcribed nor translated.

Comparative studies were performed to confirm the fact that these DNA segments were present in: 1) the soybeans tested in all Regulatory field trials, 2) the soybeans used in compositional analyses, and safety and nutrition assessments, and 3) the soybeans used as the common progenitor for all commercial varieties. Southern blot and PCR analyses, used to verify the presence of the 937 bp *Hin*d III restriction fragment and the 250 bp segment of CP4 EPSPS adjacent to the 3' end of NOS, clearly demonstrate that these CP4 EPSPS segments were constituents of the Roundup Ready soybeans which were tested in the comprehensive safety studies described in previous regulatory dossiers and scientific publications. Likewise, these segments are present in the progenitor used for all commercial Roundup Ready soybean varieties. Furthermore, these multi-generation studies on soybean event 40-3-2 demonstrate that the 72 bp CP4 EPSPS segment co-segregates with the primary insert.

In conclusion, the molecular characterization of Roundup Ready soybean event 40-3-2 has been extended using more sensitive and precise methods, including genome walking, higher sensitivity Southern blot analysis, genomic cloning, nucleotide sequencing, PCR, and northern blot analysis. These analyses identified: 1) a second insert comprised of a 72 bp segment of CP4 EPSPS, and 2) 250 bp of the CP4 EPSPS element adjacent to the 3' end of the complete NOS 3' transcriptional termination element in the primary, functional insert. These segments produce no detectable mRNA or protein based on: 1) northern blot analyses which demonstrate no detectable transcription of either CP4 EPSPS segment, and 2) western blot analysis in which only the predicted, full-length protein encoded by the functional CP4 EPSPS insert is produced. Lastly, the additional CP4 EPSPS segments were present in the material used to produce the regulatory data regarding CP4 EPSPS protein levels, environmental data, compositional analyses, animal feeding studies, and other well-documented safety assessments. Therefore, these results do not alter the conclusion of safety of Roundup Ready soybean event 40-3-2.

II. INTRODUCTION

A. Background.

Glyphosate, the active ingredient in Roundup® herbicide, controls weeds by inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS is an enzyme in the shikimate pathway for aromatic amino acid biosynthesis in plants. This amino acid synthetic pathway is not present in all mammalian, avian, or aquatic life forms, and hence glyphosate is only toxic to plants, fungi, and some microorganisms, but not to other living species.

Soybean plants tolerant to glyphosate herbicide were produced by inserting a gene expression cassette encoding a glyphosate-tolerant EPSPS coding region isolated from *Agrobacterium sp.* strain CP4 (CP4 EPSPS) into the genome of soybean cultivar A5403, resulting in the glyphosate tolerant, Roundup Ready® soybean event 40-3-2. Previous molecular characterization (Re *et al.*, 1993; Kolacz and Padgette, 1994, Padgette *et al.*, 1996) indicated that soybean event 40-3-2 contains a single DNA insertion from the plasmid PV-GMGT04 (Figure 1), containing a portion of the E35S promoter, a chloroplast transit peptide coding sequence, the CP4 EPSPS coding sequence, and the majority of the NOS 3' transcriptional termination element, located on an ~5.8 Kb *Hin*d III restriction fragment (Figure 2, Panel A).

During seed quality analysis of Roundup Ready soybean event 40-3-2 using a Southern blot method with higher sensitivity than that used in the initial characterizations (Re *et al.*,

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1993, Kolacz and Padgette, 1994, Padgette *et al.*, 1996), a second insert which weakly hybridizes to a full-length CP4 EPSPS probe was detected. This second insert is located on an ~900 bp *Hin*d III restriction fragment, which hybridizes to a full length CP4 EPSPS coding sequence probe with a much lower intensity hybridization signal when compared to the primary insertion, suggesting the CP4 EPSPS sequence present on the second insert is only a small portion of the full length CP4 EPSPS. This study was undertaken to thoroughly characterize this additional insert comprised of a portion of the CP4 EPSPS element.

B. Purpose.

The objective of this study was to characterize the ~900 base pair *Hin*d III restriction fragment which weakly hybridizes to a full-length CP4 EPSPS probe in Roundup Ready soybean event 40-3-2 by Southern blot analysis. Additional analyses were performed to obtain the sequences of soybean genomic DNA adjacent to this CP4 EPSPS sequence. DNA sequences at the 5' and 3' ends of the inserted DNA in the primary, functional insert and flanking soybean genomic DNA sequence adjacent to the primary insert were also obtained to facilitate the development of event-specific detection methods.

III. MATERIALS AND METHODS

A. Test Substances. The test substances for this study were the Roundup Ready soybean event 40-3-2 obtained from Regulatory field trial material (Study 92-01-30-03) and/or Resnick 40-3-2 BC1F2, a progenitor used for breeding purposes.

B. Control Substance. The control substance was the non-transgenic soybean event A5403.

C. Reference Substances. The reference substances include the plasmid PV-GMGT04 that was used in the transformation of soybean event 40-3-2. For Southern blot analyses of genomic soybean DNA, 15 pg of plasmid PV-GMGT04 (~0.3 genome copy equivalents) was mixed with DNA from the A5403 control event, digested, and separated by electrophoresis on agarose gels. For Southern blot analyses on cosmid DNA, the plasmid was digested alone prior to electrophoresis. Additionally, molecular size markers from Boehringer Mannheim [molecular size markers II (23.1 Kb-0.6 Kb) and IX (1.4 Kb-0.072 Kb), catalog #236 250 and #1449 460, respectively] and Gibco BRL [500 bp DNA Ladder (8.5 Kb-0.5 Kb), catalog # 10594-018] were used for size estimations on Southern blots, while Gibco BRL 500 bp DNA Ladder and 100 bp DNA Ladder (2.1 Kb-0.1 Kb), catalog #15628-050 were used for size estimations in PCR analyses.

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

E. DNA Isolation for Southern Blot and PCR Analyses. DNA extracted from leaf tissue was used for all analyses of plant DNA in this study. DNA extracted from leaf tissue in Study # 98-01-30-01 using the Nucleon™ PhytoPure™ DNA Extraction Kit from Amersham™ per the manufacturer's instructions was used in this study to generate Southern blots for probe walking experiments with portions of the CP4 EPSPS coding region, as well as to perform PCR analyses.

The PCR-based technique GenomeWalker™, used to obtain the DNA sequences at the 5' and 3' ends of the inserted DNA and the 5' and 3' genomic flanking sequences of both the primary and secondary inserts in event 40-3-2, was performed on leaf tissue that was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Approximately 1 g of the ground leaf tissue was transferred to a 13 ml centrifuge tube and 6 ml of extraction buffer [2.5 ml DNA extraction buffer (350 mM sorbitol, 100 mM Tris pH 7.5, 5 mM EDTA, 0.38% (w/v) sodium bisulfite), 2.5 ml Nuclei lysis buffer (200 mM Tris pH 7.5, 50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB), and 1 ml Sarkosyl (5% (w/v) solution)] was added. The samples were incubated at 65°C for approximately 30 minutes with intermittent mixing. Four and a half milliliters of a mixture of chloroform:isoamyl alcohol (24:1) at room temperature was added to the samples. The suspension was mixed for 2 to 3 minutes, and the two phases separated by centrifugation for 15 minutes at ~1,000 × *g* at 4°C. The aqueous (top) layer was removed using a transfer pipet and placed into a 13 ml centrifuge tube. Five milliliters of 100% isopropanol were added, and the tubes were mixed by inversion to precipitate the DNA. The precipitated DNA was pelleted by centrifuging at ~1,000 × *g* for 5 minutes at 4°C. The pellet was washed with approximately 1 ml of 70% ethanol and centrifuged for an additional 5 minutes at ~1,000 × *g* at 4°C. The DNA was allowed to dry at room temperature and was re-dissolved in TE buffer at 4°C overnight.

F. DNA Quantitation of Cosmid and Genomic DNA. Quantitation of the genomic and cosmid DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer (San Francisco, CA)(SOP BR-EQ-0065-01) using Boehringer Mannheim (Indianapolis, IN) molecular size marker IX as a DNA calibration standard.

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G. Restriction Enzyme Digestion of Genomic DNA. Approximately 10 µg of genomic DNA from each of the test and control lines were 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. Restriction enzymes were purchased from Boehringer Mannheim. After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3M NaOAc and 2 volumes (~1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a -20°C freezer for at least one hour. The digested DNA was pelleted at maximum speed in a microcentrifuge, washed with 75% ethanol, vacuum dried for approximately 10 minutes, and re-dissolved at room temperature in TE buffer.

H. DNA Probe Preparation for Soybean Event 40-3-2 Genomic DNA Southern Blots. All probe templates homologous to the CP4 EPSPS coding region were prepared by PCR using plasmid PV-GMGT05 as a template. PV-GMGT05 was used as a template for PCR instead of PV-GMGT04 since it contains only one copy of the CP4 EPSPS sequence. The probe template homologous to the NOS 3' polyadenylation sequence was generated by PCR using plasmid PV-ZMBK28, which is identical in its NOS 3' polyadenylation sequence to PV-GMGT04. Approximately 25 ng of each probe template (except the NOS 3' polyadenylation sequence) were labeled with ^{32}P-dCTP (6000 Ci/mmol) using a random priming method (RadPrime DNA Labeling System, Gibco BRL, Gaithersburg MD). The NOS 3' polyadenylation sequence was labeled using PCR with NOS 3' template (5 ng), NOS 3' specific primers (0.25 µM each), 1.5 mM $MgCl_2$, 3 µM dATP, dGTP, and dTTP, 100 µCi of ^{32}P-dCTP (6000 Ci/mmol), and 2.5 units of *Taq* DNA polymerase in a final volume of 20 µl. The cycling conditions were as follows: 1 cycle at 94°C for 3 minutes; 5 cycles at 94°C for 45 seconds, 52°C for 30 seconds, 72°C for 1.5 minutes; 1 cycle at 72°C for 10 minutes. All radiolabeled probes were purified using a Sephadex G-50 column (Boehringer Mannheim).

I. Southern Blot Analysis on Genomic Soybean DNA. Southern blot analyses (Southern, 1975) were performed according to SOP GEN-PRO-025-02. The samples of DNA digested with restriction enzymes were separated, based on size, using 0.8% agarose gel electrophoresis according to SOP GEN-PRO-003-01. The gels were electrophoresed at either 40 V overnight for ~17 hours or 35 V overnight for ~15 hours and then at 65 V the following day for ~3 hours.

After photographing, the gel was placed in a depurination solution (0.125 N HCl) for approximately 10 minutes followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for ~30 minutes. 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 16-26 hours (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 prehybridized for 6-6.5 hours at ~65°C in an aqueous solution of 500 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 16-17 hours at approximately 65°C. Membranes were washed in an aqueous solution of 0.1% SDS and 0.1× SSC for two ~15 minute periods followed by two ~20 minute periods at approximately 65°C using fresh solution for each of the four washes. Multiple exposures of blots were generated at approximately -80°C. Stripped blots were exposed to Kodak Biomax™ MS film in conjunction with one Kodak Biomax™ MS intensifying screen to ensure complete removal of previous ^{32}P-labeled probes.

J. High Molecular Weight DNA Extraction from Lyophilized Soybean Leaf Material.
High molecular weight DNA from Roundup Ready soybean event 40-3-2 was isolated by first lyophilizing leaf material overnight to remove the majority of the moisture from the tissue. Next, 1.2 g of plant material was divided into three ~400 mg aliquots and each was placed in a 50 ml conical tube with a sufficient number of glass beads (3 mm diameter) to fill between 5-7 ml of volume based on the gradations on the side of the tubes. The samples were mixed vigorously until the tissue was pulverized into a fine dust. Seventeen milliliters of CTAB extraction buffer [0.1 M Tris-HCl, pH 7.5, 0.7 M NaCl, 40 mM EDTA, 1.0% (w/v) CTAB] was added to each sample and then incubated at 55°C for ~2 hours with gentle rocking. Approximately 0.5 hours before the incubation at 55°C was completed, 40 µl of RNase A (5 mg/ml) was added to the samples. Following incubation, the aqueous phase from each sample was divided equally into two new 50 ml conical tubes and 8.5 ml of chloroform:isoamyl alcohol (24:1) was added to the glass beads. The tubes were inverted several times to wash the glass beads and the chloroform:isoamyl alcohol was then distributed evenly between the two 50 ml conical tubes containing the divided aqueous phase. Samples were mixed by gentle inversion for ~10 minutes at room temperature. The samples were centrifuged at room temperature for 20 minutes at 1430 × *g*. The aqueous layer was removed to a fresh 50 ml conical tube and the DNA was precipitated by adding 7 ml of isopropanol. Samples were gently mixed by inversion and the DNA spooled out of solution with a glass hook. The DNA was then washed by incubating the DNA on the hook in 5 ml of Wash Solution 1 (75% EtOH (v/v), 0.2 M NaOAc) for at least 20 minutes. The sample was transferred to 5 ml of Wash Solution 2 (75% EtOH (v/v), 10 mM ammonium acetate) for 5 minutes. The DNA was then dried by gently pressing it against a clean sheet of Whatman 3MM paper to remove excess ethanol. The resulting DNA from all three

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extractions was combined and resuspended in 12 ml of TE. The DNA was then further purified using a cesium chloride gradient according to SOP GEN-PRO-072-01. Following cesium chloride purification of the DNA, an aliquot was removed and the size examined by field inversion gel electrophoresis followed by ethidium bromide staining. The results of the electrophoresis revealed that the DNA was of suitable size for cosmid library construction (data not shown).

K. Cosmid Library Construction. A cosmid genomic library was constructed using high molecular weight DNA from Roundup Ready soybean event 40-3-2 and the pWEB::TNC cosmid cloning kit from Epicentre Technologies (Madison, WI). Ten individual colonies generated from the library were selected and grown overnight in 5 ml of LB (Luria-Bertani Medium) broth containing 100 µg/ml of carbenicillin. The use of carbenicillin was restricted to cosmid library construction and cloning only. Cosmid DNA was isolated using the QIAprep Spin Miniprep Kit from Qiagen (Valencia, CA) as per the manufacturer's protocol. DNA from these 10 colonies was digested with the *Eco*R I restriction endonuclease, separated on an agarose gel and visualized by ethidium bromide staining (data not shown). By assessing the banding patterns of the DNA from the individual preparations, it appeared that 80% of the samples contained inserts, and the inserts were approximately 40 Kb in size. Assuming the genome size of soybean is 1.1×10^9 bp, and that each cosmid clone contains approximately 40 Kb of DNA, then Equation 1 (Zilsel *et al*., 1992) can be used to calculate the percentage likelihood of cloning a given sequence of DNA from the soybean genome. In order to have an ~96% probability of identifying the target of interest, approximately 86,000 cfu were screened, representing a three-fold coverage of the soybean genome.

$$N = \frac{\ln(1-P)}{\ln(1-\frac{x}{y})}$$

Equation 1: Calculation for determining the number of colonies needed to be screened in order to have a certain probability of finding a particular sequence in a given genome size (Zilsel *et al.*1992). *N* is the number of colonies that need to be screened, *P* is the percentage likelihood of finding the sequence, *x* is the insert size of the library clones being screened and *y* is the genome size that the library is supposed to represent.

L. Cosmid Library Screening. The ~86,000 clones screened from the library were produced by infecting approximately 5 ml of *E. coli* strain EPI305 (OD_{600} = 0.8-1.0) per 500 µl of packaged phage extract prepared as per the manufacturer's protocol. Transfectants were diluted and plated at a density of ~4000 cfu per 182 mm LB agar plate containing 100 µg/ml of carbenicillin for selection. Colony lifts were prepared using

Hybond-XL membranes from Amersham Pharmacia Biotech (England) as per the manufacturer's protocol. The filters were prehybridized for 3.5-5 hours in an aqueous solution of 500 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml tRNA. Hybridization with the ^{32}P-dCTP (6000 Ci/mmol) random primer labeled CP4 EPSPS coding region was performed in fresh prehybridization solution overnight at approximately 65°C. The filters were washed in an aqueous solution of 0.1% SDS and 0.1× SSC for two ~15 minute periods followed by two ~20 minute periods at approximately 65°C using fresh solution for each of the four washes. The filters were incubated with gentle agitation in an orbital shaking incubator for all prehybridization, hybridization and wash steps. Multiple exposures of the filters were generated at approximately -80°C using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen. The positive signals observed on the autoradiograph films were aligned to the LB agar plate containing the colonies of interest.

M. Positive Cosmid Clone Identification. Occasionally, due to the density of the colonies on the plate, it was not possible to localize the hybridization signal to a single colony. Therefore, the region where the signal was thought to be localized was cut from the agar plate and placed into 250 µl of LB broth. The blocks of agar containing the cosmid clones were mixed by vortexing for several seconds and a 50 µl aliquot was removed from each sample and used to inoculate individual 1 ml cultures of LB broth containing 100 µg/ml of carbenicillin for selection. In other instances, individual colonies were selected when the colony was well isolated and aligned directly with the signal detected on the autoradiogram. These colonies were also inoculated directly into individual 5 ml cultures of LB broth containing 100 µg/ml of carbenicillin for selection. These cultures were incubated at 37°C overnight while shaking. Cosmid DNA was extracted from these samples using the QIAprep Spin Miniprep Kit from Qiagen (Valencia, CA) as per the manufacturer's protocol. Isolated DNA from the cosmid clones was subjected to *Hin*d III restriction enzyme digestion followed by agarose gel electrophoresis and Southern blotting probed with ^{32}P-dCTP (6000 Ci/mmol) random primer labeled CP4 EPSPS Probe-3 (see diagram on the bottom of Figure 4). This portion of the CP4 EPSPS coding region is present in both the 5.8 Kb primary insert and the ~900 bp secondary insert. An ~900 bp band was observed to hybridize to the probe in two of the 34 samples represented on the Southern blot (data not shown). The positive hybridizing samples correspond to DNA isolated from two independent pools of clones identified as samples 4B and 6A. Based on this result, it was concluded that both the 4B and 6A samples could contain a cosmid clone which includes the ~900 bp *Hin*d III restriction fragment of DNA that is observed to hybridize to the CP4 EPSPS probe in Roundup Ready soybean event 40-3-2.

N. Purification of Individual Cosmid Clones. The samples 4B and 6A represent pools of cosmid clones and therefore are mixed populations. Three aliquots (1 µl, 5 µl and 10 µl) of the LB broth in which sample 4B was resuspended were added to 100 µl of SOC medium (Gibco BRL, Gaithersburg, MD) and plated out onto LB agar plates containing 100 µg/ml of carbenicillin for selection. The 4B sample had numerous viable cells giving rise to several colonies on the plates. These were screened by colony hybridization as described in Materials and Methods, Section M with ^{32}P-dCTP (6000 Ci/mmol) random primer labeled CP4 EPSPS Probe-3. In order to isolate a purified clone from the 6A pool, 1 µl of the cosmid DNA prepared from the pool was repackaged using MaxPlax® packaging extracts from Epicentre Technologies as per the manufacturer's protocol. Five hundred microliters of the phage from the repackaged cosmid DNA was used to transfect 5 ml of EPI305 cells. The resulting transfectants were plated onto LB medium containing 100 µg/ml of carbenicillin for selection. The resulting colonies were screened by colony hybridization as described in Materials and Methods, Section M with ^{32}P-dCTP random primed labeled CP4 EPSPS Probe-3. Several signals were observed on the autoradiogram from these experiments that aligned with well-isolated, individual colonies. Three positive hybridizing clones from both 4B and 6A were selected and grown overnight in 5 ml of LB broth containing 100 µg/ml of carbenicillin for selection. Cosmid DNA was isolated from 4 ml of the culture using the QIAprep Spin Miniprep Kit from Qiagen (Valencia, CA) as per the manufacturer's protocol. The DNA isolated from the individual cultures was digested with *Hin*d III and the restriction patterns compared following agarose gel electrophoresis and visualization by ethidium bromide staining. All three individual clones from both the 4B and 6A samples gave rise to identical restriction patterns, respectively. Additionally, the banding pattern between the 4B and 6A isolated clones was very similar, indicating the two individually isolated clones contain similar segments of the soybean genome.

O. Hybridization Using 3'-End Labeled Oligonucleotide Probes. Probes prepared in a 20 µl reaction volume containing 100 pmol of oligonucleotide, 1x reaction buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumin, pH 6.6), 5 mM $CoCl_2$, 0.05 mM DIG-dUTP and 2.5 units terminal transferase (Roche) were incubated at 37°C for 15 minutes. The reactions were then placed on ice and EDTA was added to a final concentration of 10 mM to stop the labeling reaction.

The Genius® digoxigenin-11-dUTP kit for nonradioactive hybridization and detection was used in conjunction with the digoxigenin-11-dUTP 3'-end labeled oligonucleotide probes. Probes were prepared as described above and hybridized to the filters as per the

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manufacturer's protocol. Detection of the oligonucleotide probes was performed as per the manufacturer's protocol using CSPD® luminescent substrate.

P. Identification and Verification of the 5' and 3' Ends of the Second Insert and the 5' and 3' Genomic Flanking Sequences. The sequences of the 5' and 3' ends of the second insert, as well as the genomic DNA flanking the 5' and 3' ends were determined by employing DNA sequencing using dye-terminator chemistry (Monsanto Genomic Sequencing Center) with template DNA isolated from cosmid clones 4B and 6A and primers designed to the 72 bp of CP4 EPSPS sequence constituting the second insert. The resulting 5' and 3' genomic flanking sequences were verified using one primer designed to either the 5' or 3' genomic flanking sequence, paired with a second primer in the 72 bp CP4 EPSPS sequence. The PCR products were separated using agarose gel electrophoresis and subjected to DNA sequencing to confirm the sequences. The 5' and 3' ends of the insert, as well as the 5' and 3' genomic flanking sequences were verified in the Resnick 40-3-2 BC1F2 material, the 1992 Regulatory field trial material, and cosmid clones 4B and 6A. The PCR analyses for verification were conducted using 25 ng of genomic DNA template or 0.5 ng of cosmid DNA template in a 50 µl reaction volume containing a final concentration of 1.5 mM Mg^{2+}, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase. The reactions were performed under the following cycling conditions: 1 cycle of 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1.5 minutes; 1 cycle of 72°C for 10 minutes. The PCR products were separated using 1.2 % agarose gel electrophoresis at 100 V for ~2 hours and visualized by ethidium bromide staining.

Q. Identification and Verification of the 5' and 3' Ends of the Primary Insert and the 5' and 3' Genomic Flanking Sequences. The DNA sequences at the 5' and 3' ends of the primary insert and the genomic DNA flanking the 5' and 3' ends were identified using the PCR-based Universal GenomeWalker Kit as per the manufacturer's protocol followed by nucleotide sequencing of the PCR products. The 5' and 3' genomic flanking sequences were verified using one primer designed to the 5' or 3' genomic flanking sequence paired with a second primer in the E35S promoter or NOS 3' transcriptional termination element, respectively. The PCR products were separated using agarose gel electrophoresis and subjected to DNA sequencing using dye-terminator chemistry (Monsanto Genomic Sequencing Center) to confirm the sequences. The 5' and 3' ends of the insert and the 5' and 3' genomic flanking sequences were verified in the Resnick 40-3-2 BC1F2 material as well as soybean event 40-3-2 material from the 1992 Regulatory field trials. The PCR analyses for verification were conducted using 50 ng of 40-3-2 genomic DNA template in a 50 µl reaction volume containing a final concentration of 1.5 mM Mg^{2+}, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase. The reactions were performed under the following cycling conditions: 1 cycle at 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes; 1 cycle at 72°C for 4

minutes. The PCR products were separated using 1.5 % agarose gel electrophoresis at 100 V for 2 hours and visualized by ethidium bromide staining.

R. Poly (A+) RNA Isolation: Young leaf tissue samples from Roundup Ready soybean event 40-3-2, non-transgenic control line A5403, and a non-commercial Roundup Ready soybean event were harvested, immediately placed on dry ice, and stored in a -80°C freezer. These samples were then ground to a fine powder using a mortar and pestle on dry ice. Total RNA was harvested by adding 1 ml of TRIzol® Reagent (Gibco BRL) per 0.1 gram of tissue, followed by homogenization for 30 seconds with a power homogenizer. The samples were then incubated for 5 minutes at room temperature. For every 1.0 ml of TRIzol, approximately 0.2 ml of chloroform:isoamyl alcohol (24:1) was then added. The tubes were shaken, incubated for 2-15 minutes at room temperature, and then centrifuged at 19,800 x *g* for 15 minutes at 4°C. The aqueous phase was transferred to a clean tube and ~0.2 ml of chloroform:isoamyl alcohol (24:1) for every 1.0 ml of TRIzol was again added to the tubes, mixed, and then centrifuged at 19,800 x *g* for 15 minutes at 4°C. The aqueous phase was transferred to a clean tube and the RNA was precipitated by adding 0.5 ml of 100% isopropanol per initial volume of TRIzol. The samples were incubated at room temperature for 10 minutes, followed by centrifugation at 19,800 x *g* for 15 minutes at 4°C to pellet the RNA. The resulting RNA pellets were washed by resuspending in cold 75% ethanol. The suspension was mixed thoroughly and the RNA was pelleted by centrifugation at 7,000 x *g* for 5 minutes at 4°C. The final RNA pellet was dried under vacuum, resuspended in RNase-free water and stored at approximately -20°C. Poly(A+) mRNA was isolated from approximately 1 mg of total RNA using Oligotex Isolation System (Qiagen), as per the manufacturer's protocol. All RNA was quantitated using a Beckman DU640B UV Spectrophotometer (Fullerton, CA). Yields of poly (A+) mRNA from 1 mg of total RNA were ~1.0 %.

S. Northern Blot Analysis. Ten micrograms of RNA molecular weight marker (Gibco BRL), 1 µl of a 1:1000 dilution of *in vitro* transcribed CP4 EPSPS RNA and various concentrations of Poly(A+) RNA were resuspended in RNA loading buffer (Sigma) and heated at 65-70°C for 15 minutes. These samples were then loaded onto an agarose gel (1% agarose, 1× MOPS, 2.2% formaldehyde) and electrophoresed at 90-100 V for 3-5 hours in 1× MOPS buffer (40 mM MOPS, 10 mM NaOAc, 1 mM EDTA). The RNA was then transferred from the gel onto a positively charged nylon membrane (Hybond N+, Amersham) using a Turboblotter™ overnight in 20× SSC. The mRNA was UV crosslinked to the membrane and placed in pre-hybridization buffer (500 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.05 mg/ml *E. coli* tRNA) at 65°C for 4 hours. Random primer labeled CP4 EPSPS DNA probes (^{32}P-dCTP) were added and allowed to hybridize

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overnight. The membranes were then washed for ~15 minutes a total of four times with wash buffer (0.1 × SSC, 0.1% SDS) at 65°C. The membranes were then exposed to Kodak Biomax film in conjunction with a Biomax Intensifying screen at approximately -80°C for detection.

IV. RESULTS AND DISCUSSION

A. Southern Blot Analysis of the ~900 bp *Hin*d III Restriction Fragment Which Weakly Hybridizes to the CP4 EPSPS Probe in Roundup Ready Soybean Event 40-3-2.

1. **Southern Blot Analysis of Samples Used to Generate 1993 Regulatory Data and a R3 Pre-Commercial Sample.** To demonstrate that the ~900 bp *Hin*d III restriction fragment which weakly hybridizes to a full length CP4 EPSPS probe was present in the 1992 Regulatory sample used to conduct the safety assessment of Roundup Ready soybean event 40-3-2 and in the R3 (Resnick BC1F2) generation used to develop all commercial varieties, Southern blot analysis was performed as follows. DNA from both sources was digested with the restriction enzyme *Hin*d III and subjected to Southern blot hybridization analysis using a full length CP4 EPSPS coding sequence probe. A5403 control DNA and A5403 control DNA spiked with plasmid PV-GMGT04 DNA were also digested with *Hin*d III and used as controls. The results are shown in Figure 3. A5403 control DNA (Lane 2) showed no hybridization bands, as expected, while A5403 control DNA spiked with plasmid PV-GMGT04 DNA (Lane 3) produced two bands at ~2.5 Kb and ~8.0 Kb as predicted from the plasmid map in Figure 1. Resnick 40-3-2 BC1F2 DNA (Lane 4) and soybean event 40-3-2 DNA extracted from Regulatory field trial material (92-01-30-03, Lane 5) produced the expected size band at approximately 5.8 Kb, which represents the primary, functional insert described in the 1993 dossier, as well as a band at approximately 900 bp. There is a slight difference in the migration of the ~900 bp band between the two samples due to variations in DNA quality. This result establishes that the CP4 EPSPS segment located on an ~900 bp *Hin*d III restriction fragment in soybean event 40-3-2 was present in the Regulatory field trial material from Study 92-01-30-03 used to conduct the safety assessment of event 40-3-2, as well as in Resnick 40-3-2 BC1F2 material, which served as the progenitor for all commercial Roundup Ready soybean variety development. Furthermore, this second insert co-segregates with the primary insert, and has been a constituent of soybean event 40-3-2 throughout the numerous studies performed to demonstrate the safety of this product.

2. **Southern Blot Analysis to Define the CP4 EPSPS Segment Present on the ~900 bp *Hin*d III Restriction Fragment.** To more clearly define the region of CP4 EPSPS present on the ~900 base pair *Hin*d III restriction fragment, genomic DNA extracted from both Regulatory field trial material (92-01-30-03) and Resnick 40-3-2 BC1F2 material was analyzed by Southern blot hybridization with sequential portions of the CP4 EPSPS coding sequence and the NOS 3' transcriptional termination element (see diagram at bottom of Figure 4). A5403 control DNA, A5403 control DNA spiked with plasmid PV-GMGT04 DNA, Resnick 40-3-2 BC1F2 DNA, and 40-3-2 DNA extracted from the 1992 Regulatory field trial material were digested with *Hin*d III and included on all Southern blots. Southern blot analyses on the Resnick 40-3-2 BC1F2 and the 1992 Regulatory field trial 40-3-2 DNA samples performed using the NOS 3' probe and three CP4 EPSPS probes (Probe-1, Probe-2, and Probe-4) generated only the expected band at ~5.8 Kb representing the primary, functional insert in soybean event 40-3-2 (data not shown). The only Southern blot on which the ~900 bp *Hin*d III restriction fragment was observed in the Resnick 40-3-2 BC1F2 DNA and the 1992 Regulatory field trial 40-3-2 DNA samples is shown in Figure 4, Panel A. This blot was probed with CP4 EPSPS Probe-3 (see diagram at bottom of Figure 4). The blot in Figure 3 was stripped and reprobed to generate this result, therefore the size of the ~900 bp *Hin*d III restriction fragment is again slightly shifted between the two soybean event 40-3-2 samples. Probes designed to overlap the 5' and 3' ends of CP4 EPSPS Probe-3 did not hybridize to the ~900 bp *Hind* III fragment (Figure 4, Panels B and C). These results indicate that the NOS 3' transcriptional termination element is not present on the ~900 bp *Hin*d III restriction fragment, and that the portion of the CP4 EPSPS coding region contained within the ~900 bp *Hin*d III restriction fragment is less than 200 bp in length.

3. **Oligonucleotide Probe Hybridization Against Cosmid Clones Containing the ~900 bp *Hin*d III Restriction Fragment.** To further delineate the CP4 EPSPS sequence present on the ~900 bp *Hin*d III restriction fragment, cosmid DNA isolated from pool 6A (see Materials and Methods, Section N), which contained the ~900 bp *Hin*d III restriction fragment, was digested with *Hin*d III, separated by agarose gel electrophoresis and transferred to a nylon membrane. The plasmid vector PV-GMGT04 was used as a positive hybridization control and should result in the visualization of two bands at ~8.0 kb and ~2.5 kb based on the plasmid map (Figure 1). Several identical blots were hybridized separately with oligonucleotide probes 3'-end labeled with digoxigenin-11-dUTP (see diagram at bottom of Figure 5). Hybridization of

the cosmid DNA prepared from the 6A pool of clones was not observed with the oligonucleotide probes Oligo-1, Oligo-2, Oligo-3, Oligo-4, Oligo-8 and Oligo-9, although the probes did hybridize to the PV-GMGT04 plasmid positive control, indicating that the conditions employed were conducive for hybridization (data not shown). However, oligonucleotide probes Oligo-5 and Oligo-6 did hybridize to the ~900 bp *Hin*d III restriction fragment in the DNA extracted from the 6A cosmid pool (data not shown).

The pool of cosmid clones represented in sample 6A was further screened to isolate single colonies that contained the ~900 bp *Hin*d III restriction fragment. The purified cosmid clone 6A was digested with *Hin*d III, separated by agarose gel electrophoresis, and transferred to a nylon membrane. The controls were identical to those used in the experiment on cosmid pool 6A. Hybridization was observed between the oligonucleotide probes Oligo-5 and Oligo-6 with the ~900 bp *Hin*d III restriction fragment as was previously observed with DNA prepared from the 6A pool. However, oligonucleotide probe Oligo-7 located immediately 3' of the Oligo-6 probe did not hybridize to the ~900 bp *Hin*d III restriction fragment in the cosmid DNA prepared from clone 6A. Oligonucleotide probe Oligo-4, located immediately 5' of Oligo-5 probe and used on a pool of 6A cosmid DNA, also did not hybridize to the ~900 bp *Hin*d III restriction fragment (Figure 5). The two oligonucleotide probes, Oligo-5 and Oligo-6, which did hybridize to the ~900 bp *Hind* III restriction fragment in the DNA from cosmid clone 6A, are contiguous in the CP4 EPSPS coding region and represents a minimum of 53 bp of the maximum 200 bp region expected to be present from previous probe walking experiments on soybean event 40-3-2 genomic DNA (Figure 4). In conclusion, the oligonucleotide probe hybridization to the cosmid clones allowed the portion of the CP4 EPSPS sequence present on the ~900 bp *Hin*d III restriction fragment to be defined as ~53 bp consisting of sequence which hybridized to the Oligo-5 and Oligo-6 probes (Figure 5).

B. Delineation of the CP4 EPSPS Sequence Present as the Second Insert in Roundup Ready Soybean Event 40-3-2 and Soybean Genomic Sequences Flanking the 5' and 3' Ends of This Insert.

1. **Determination and Verification of the Inserted CP4 EPSPS Sequence and the 5' and 3' Soybean Genomic Flanking Sequences.** Oligo-5 and Oligo-6 (Figure 5) were used as primers to generate DNA sequence directly from purified cosmid clones 6A and 4B (a second cosmid clone shown to contain a similar insert, see Materials and Methods, Section N) in both the 5' and 3' directions. Multiple primers were then designed to the resulting potential 5'

and 3' flanking sequences and paired with Oligo-5 and Oligo-6 primers. PCR products were obtained and subsequently sequenced. The combination of DNA sequence data revealed that 72 bp of CP4 EPSPS (base pairs 855-926, Figure 1) are located on a 937 bp *Hind* III restriction fragment. No other sequences derived from plasmid PV-GMGT04 (Figure 1) used in the transformation of soybean event 40-3-2 were identified on the 937 bp *Hin*d III restriction fragment. A schematic of the additional insert is shown in Figure 2, Panel B. The observation that only 72 bp of the CP4 EPSPS sequence are present on the 937 bp *Hin*d III restriction fragment explains the low hybridization intensity of this band when compared to the ~5.8 kb *Hin*d III restriction fragment containing the primary, functional insert when probed with a full-length CP4 EPSPS probe (Figure 3, Lanes 4 and 5), as well as why the additional CP4 EPSPS segment was not reported using less sensitive methods in the molecular characterization reported in 1993 (Re *et al.*, 1993).

2. **PCR and DNA Sequence Verification of the 5' and 3' Genomic Flanking Sequences of the Second Insert.** PCR analyses were performed on DNA extracted from Resnick 40-3-2 BC1F2 material and soybean event 40-3-2 1992 Regulatory field trial material, as well as isolated cosmid clones 4B and 6A, to demonstrate that the 5' and 3' genomic flanking sequences of the 72 bp CP4 EPSPS segment were consistent in all samples. Three different PCR analyses were performed, including one PCR verifying the 5' genomic flanking sequence using Primers A and B, a second PCR verifying the 3' genomic flanking sequence using Primers A' and C, and a third PCR amplifying from the 5' genomic flanking sequence to the 3' genomic flanking sequence using Primers B and C. The positions of all primers as well as the results of all PCR analyses are shown in Figure 6. The control reactions without template (Lanes 7, 13, and 19) and A5403 non-transgenic negative control DNA (Lanes 6, 12, and 18) did not generate a PCR product in any of the analyses. The Resnick 40-3-2 BC1F2 DNA samples (Lanes 2, 8, and 14), the 40-3-2 1992 Regulatory field trial DNA samples (Lanes 3, 9, and 15), cosmid clone 4B (Lanes 5, 11, and 17) and cosmid clone 6A (Lanes 4, 10, and 16) generated the expected specific size PCR products of 532 bp for the 5' flanking sequence, 599 bp for the 3' flanking sequence, and 1103 bp for the 5' to 3' flanking sequence (see diagram at bottom of Figure 6). The PCR products from similar reactions were subjected to DNA sequencing. The results revealed that the genomic flanking sequence present in cosmid clones 4B and 6A is consistent with the genomic flanking sequence in Resnick 40-3-2 BC1F2 material and the 40-3-2 material from the 1992 Regulatory field trials. These results: 1) further establish the validity of the cosmid clones used in this analysis; 2) establish that the second insert in Roundup Ready soybean event 40-3-2 consists of 72 bp of

the CP4 EPSPS element (base pairs 855-926 of PV-GMGT04, Figure 1) located on a 937 bp *Hin*d III restriction fragment with no other sequences from plasmid PV-GMGT04 used in the transformation of the event; and (3) further establish that the second insert in soybean event 40-3-2 was present in the Regulatory field trial material from Study 92-01-30-03 used to conduct the safety assessment of event 40-3-2, as well as in Resnick 40-3-2 BC1F2 material which served as the progenitor for all commercial Roundup Ready soybean variety development.

C. Isolation of the 5' and 3' DNA Sequences of the Primary Insert and PCR Verification Across Multiple Generations.

1. **Determination and Verification of the DNA Sequences of the 5' and 3' Ends of the Inserted DNA and of the 5' and 3' Genomic Flanking Sequences.** The PCR-based technique GenomeWalker was used to generate PCR products containing DNA at the 5' and 3' ends of the inserted DNA, as well as the DNA flanking the 5' and 3' ends of the primary insert in soybean event 40-3-2. The PCR products were subjected to DNA sequencing and multiple primers designed to the flanking sequences were paired with insert specific primers located: 1) in the E35S promoter, to validate the sequence at the 5' end of the inserted DNA and the 5' flanking genomic sequence, and 2) in the NOS 3' transcriptional termination element, to validate the DNA sequence at the 3' end of the inserted DNA and the sequence of the 3' flanking genomic DNA. PCR products were obtained and sequenced. The resulting sequences are shown in Figures7A and 7B. Figure 7A contains the 5' DNA sequence which shows that the first 354 bp of the E35S promoter are missing with the insert beginning at base pair 2347 of PV-GMGT04 (Figure 1). This deletion removes a duplicated portion of the E35S enhancer region and is not likely to have a significant effect on the functionality of the promoter since the region necessary for transcriptional initiation remains intact (Odell *et al*., 1985). These results are consistent with previous results (Kolacz and Padgette, 1994; Padgette et al., 1996). In addition to the 105 bp of E35S which were sequenced, 186 bp of the soybean genomic DNA adjacent to the 5' end of the inserted DNA is shown in Figure 7A.

   Figure 7B contains the 3' DNA sequence, which demonstrates that the entire NOS 3' transcriptional termination element is present [rather than the partial NOS sequence reported previously (Kolacz and Padgette, 1994)] in soybean event 40-3-2with the inserted DNA ending at base pair 160 of PV-GMGT04. Adjacent to the inserted DNA ending at base pair 160 of PV-GMGT04 (Figure 1), a previously unobserved 250 bp portion of the CP4 EPSPS element was

identified which consists of base pairs 195-444 in Figure 7B. This sequence corresponds to base pairs 1490-1739 of PV-GMGT04 in Figure 1. Figure 7B also shows the sequence of 416 bp of flanking soybean genomic DNA. The restriction enzyme and probe combinations and the sensitivity of methods used in the molecular characterization reported in 1993 (Re *et al.,* 1993) were not able to detect this additional 250 bp CP4 EPSPS segment.

A schematic representation of the primary insert containing this additional CP4 EPSPS segment is shown in Figure 2, Panel B. This CP4 EPSPS segment (base pairs 1490-1739 of PV-GMGT04, Figure 1) does not contain a promoter or 3' transcriptional termination element. Therefore transcription and subsequent translation of this region is highly unlikely. A northern blot was conducted and is described below (Section IV.D and Figure 9) which establishes that no mRNA is detected other than the full-length mRNA. Furthermore, in the highly unlikely event that this region would have been transcribed and translated as a fusion to the full length CP4 EPSPS protein, western blot analysis using antisera to CP4 EPSPS would have resulted in a higher molecular weight protein species being detected. No protein other than the full-length CP4 EPSPS was observed (Rogan *et al*., 1999), strongly suggesting that this DNA sequence is not transcribed or translated as a fusion protein.

2. **PCR Analysis and DNA Sequence Confirmation of the 5' and 3' DNA Sequences on Multiple Generations of Soybean Event 40-3-2.** PCR analyses were performed on DNA extracted from Resnick 40-3-2 BC1F2 material and soybean event 40-3-2 material produced during the 1992 Regulatory field trial to demonstrate that the 5' and 3' ends of the insert, as well as the 5' and 3' genomic flanking sequences of the primary insert are consistently present. Three different PCR analyses were performed, including one PCR verifying the 5' genomic flanking sequence using Primers D and E, a second PCR verifying the 3' genomic flanking sequence using Primers F and G, and a third PCR amplifying from the 5' genomic flanking sequence to the 3' genomic flanking sequence using Primers H and G. The positions of all primers as well as the results of all PCR analyses are shown in Figure 8. The control reactions without template (Lanes 5, 9, and 13) and A5403 non-transgenic negative control DNA (Lanes 4, 8, and 12) did not generate a specific PCR product in any of the analyses. The Resnick 40-3-2 BC1F2 DNA samples (Lanes 2, 6, and 10) and the 1992 Regulatory field trial DNA samples (Lanes 3, 7, and 11) generated the expected size PCR products of ~400 bp for the 5' flanking sequence, ~1350 bp for the 3' flanking sequence, and ~3500 bp for the 5' to 3' flanking sequence (see diagram at bottom of Figure 8). The

PCR products from similar reactions were subjected to DNA sequencing. The combined DNA sequences establish that the 250 bp CP4 EPSPS segment adjacent to the 3' NOS transcriptional termination element was present in the Regulatory field trial material from Study 92-01-30-03 used to conduct the safety assessment of event 40-3-2, as well as in Resnick 40-3-2 BC1F2 material which served as the progenitor for all commercial Roundup Ready soybean variety development.

D. Northern Blot Analysis of Poly (A+) RNA Isolated from Roundup Ready Soybean Event 40-3-2. Northern blot analysis was performed to determine whether or not detectable transcription of the 72 bp CP4 EPSPS segment or the 250 bp CP4 EPSPS segment adjacent to the 3' end of the NOS 3' transcriptional termination element occurs. Poly (A+) RNA, isolated from A5403 (non-transgenic, negative control), a non-commercial transgenic positive control encoding CP4 EPSPS, and from Roundup Ready soybean event 40-3-2 was subjected to northern blot analysis. CP4 EPSPS Probe-3 was used as the probe (see schematic, Figure 4). Results of the analysis are shown in Figure 9. Lane 2 contains 1 μg of A5403 poly (A+) RNA, and showed no detectable hybridization to the probe, as expected. Lane 3 contains 1 μg of poly (A+) RNA isolated from a non-commercial transgenic soybean event that encodes CP4 EPSPS. A strong signal was clearly observed at the expected size of the full length CP4 EPSPS mRNA (~1.5 Kb) for the positive control, indicating that the probe and hybridization conditions were suitable for detection of expressed CP4 EPSPS mRNA. Lanes 4 to 6 contain 1.0 μg, 0.1 μg, and 0.01 μg of poly (A+) RNA from Roundup Ready soybean event 40-3-2, respectively. Only the expected ~1.5 Kb band is visible in all three samples. Although the signal was faint at the lowest concentration of RNA (0.01 μg), this result established the limit of detection in this experiment as 0.01 μg of poly (A+) RNA (Lane 6). Although minor diffuse hybridization is visible in both the positive control RNA and in soybean event 40-3-2 RNA, no unexpected bands are evident. This result shows that a transcript derived from either the 72 bp region of CP4 EPSPS present on a second insert or the 250 bp CP4 EPSPS segment adjacent to the 3' end of the functional CP4 EPSPS insert is not present at detectable levels. This conclusion is strengthened by the observation that if a transcript derived from these regions was present at a level 100-fold less than the CP4 EPSPS mRNA produced from the primary insert, it would have been detected. It is unlikely that the CP4 EPSPS transcript from the functional insert would obscure other transcripts of identical size hybridizing to the CP4 EPSPS probe, although this cannot be ruled out. The results of the northern analysis, along with the fact that the 72 bp CP4 EPSPS segment and the 250 bp CP4 EPSPS segment adjacent to the 3' end of the NOS 3' transcriptional termination element do not contain identifiable promoter elements or 3' transcriptional termination elements, establishes that

transcription of these regions does not occur at detectable levels and that both DNA segments are non-functional.

Note that because of the large amount of CP4 EPSPS mRNA produced in both the positive control event and in soybean event 40-3-2, longer exposures of this autoradiogram yielded unacceptably high levels of diffuse non-specific binding, and were not useable. In addition, Lane 1 of Figure 9 contained a degraded, *in vitro* transcribed control RNA, so data from that lane was not considered in the analysis.

V. CONCLUSIONS

The original molecular characterization studies indicated that the DNA inserted in Roundup Ready soybean event 40-3-2 consisted of a single cassette encoding a portion of the E35S promoter sequence, a chloroplast transit peptide and the CP4 EPSPS coding sequence, and the majority of the NOS 3' transcriptional termination element. As a result of data generated during seed quality testing on commercial Roundup Ready soybean varieties using methods with an approximately nine-fold increase in sensitivity, additional molecular characterization has been performed. Results of these recent experiments, including Southern blotting, genomic cloning, PCR, and nucleotide sequencing, show that soybean event 40-3-2 contains a second insert consisting of 72 bp of CP4 EPSPS sequence. Additionally, sequencing of soybean DNA flanking the functional CP4 EPSPS insert, performed to develop event-specific detection methods, confirmed a deletion in the E35S enhancer region. The region known to be critical for proper transcriptional initiation was not disturbed. Sequencing of the NOS 3' transcriptional termination element and the flanking plant DNA revealed that the NOS sequence is intact, and not a partial element, as previously reported. An additional, previously unobserved 250 bp segment of the CP4 EPSPS element adjacent to the 3' end of the NOS 3' transcriptional termination element was shown to be present. Since neither a promoter nor a 3' transcriptional termination element is evident within either of the newly detected CP4 EPSPS segments, it is extremely unlikely that these regions would be transcribed. Furthermore, northern blot and western blot data show that only the expected CP4 EPSPS full-length transcript and protein are detected, respectively. These data support the conclusion that neither transcription nor translation of these CP4 EPSPS DNA segments occurs. Lastly, bridging studies show that both the 250 bp CP4 EPSPS segment adjacent to the NOS 3' transcriptional termination element and the 72 bp CP4 EPSPS segment which comprises the second insert, have been constituents of the Roundup Ready soybean event 40-3-2 throughout the comprehensive safety studies performed on this product, were present in the common progenitor of all commercial varieties, and co-segregate with the primary insert described in the 1993 submission. Therefore, these results do not alter the conclusion of safety of Roundup Ready soybean event 40-3-2.

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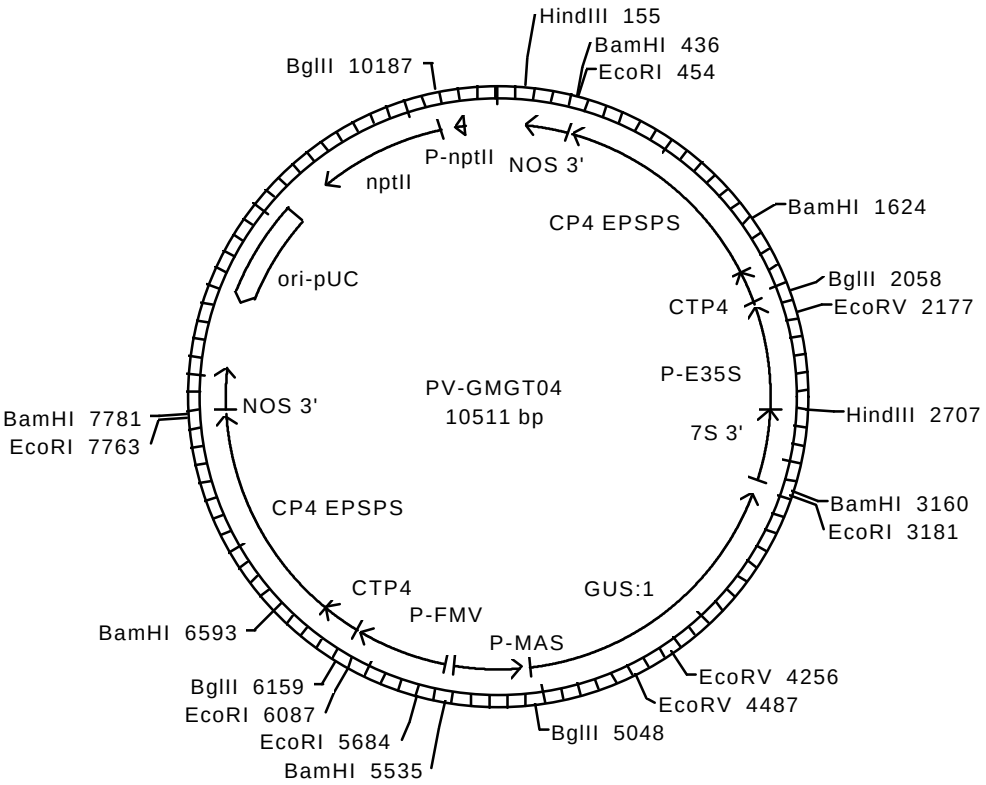

Figure 1. Plasmid Map of PV-GMGT04. Plasmid PV-GMGT04 was used to generate Roundup Ready soybean event 40-3-2.

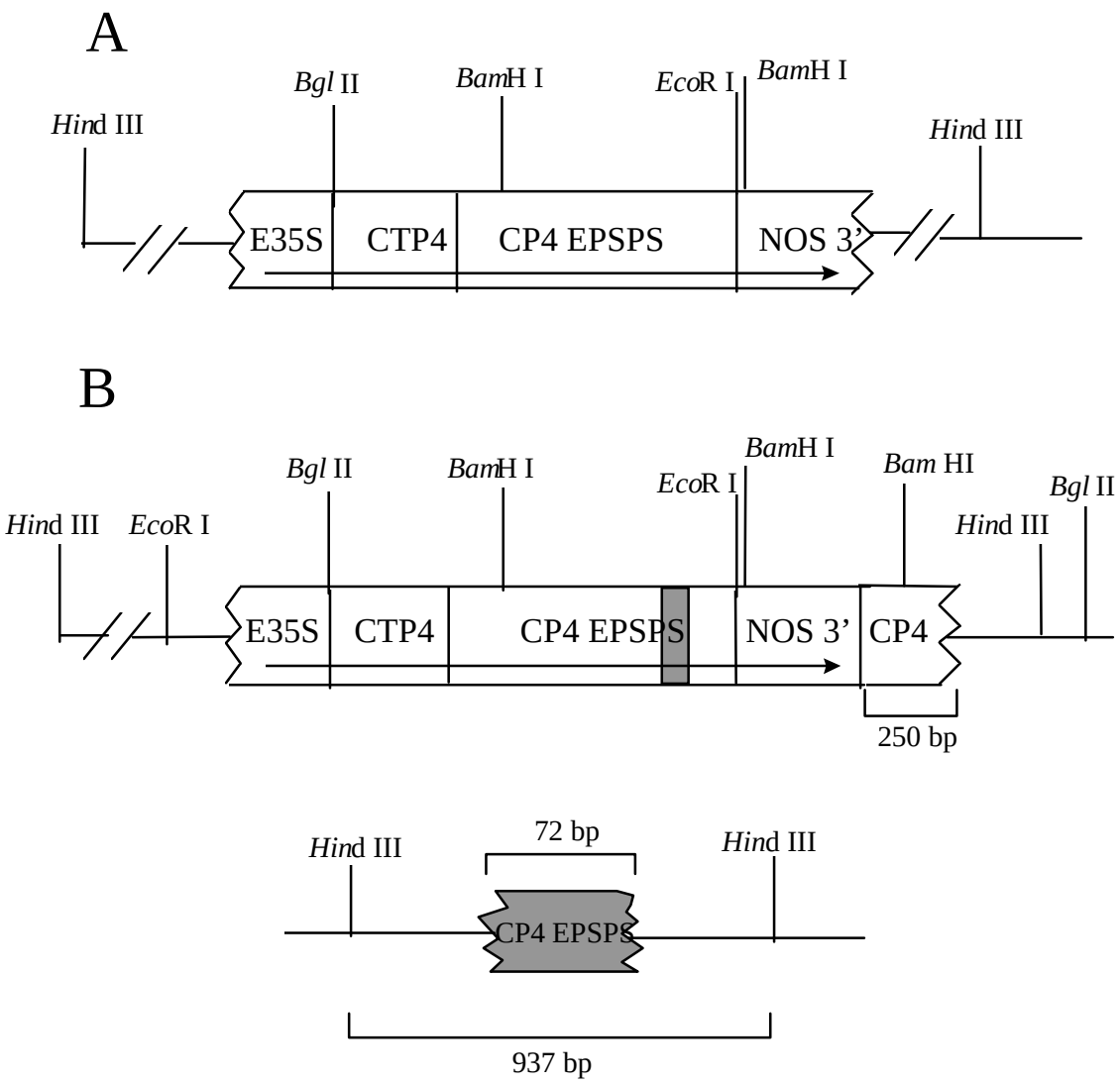

Figure 2. Schematic Representations of the Insert DNA in Soybean Event 40-3-2. Panel A represents the predicted DNA insert in soybean event 40-3-2located on an ~5.8 Kb *Hin*d III restriction fragment as described in the dossiers (Re *et al.*, 1993; Kolacz and Padgette, 1994, Padgette *et al.*, 1996). Panel B represents the predicted DNA inserts in soybean event 40-3-2based on genome walking, higher sensitivity Southern blot analysis, genomic cloning, nucleotide sequencing and PCR. There is an additional 250 bp segment of the CP4 EPSPS sequence immediately adjacent to the NOS 3' transcriptional termination element on the primary insert and an additional insert located on a 937 bp *Hin*d III restriction fragment consisting of 72 bp of the CP4 EPSPS sequence. The

shaded region in the CP4 EPSPS sequence in the functional primary insert represents the 72 bp present in the second insert.

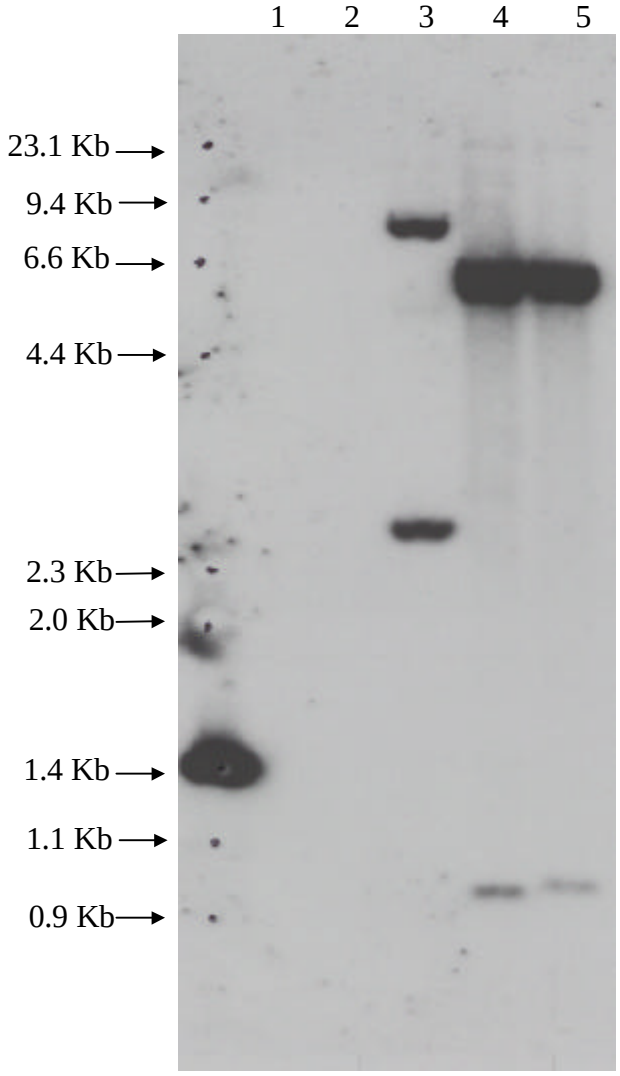

Figure 3. Southern Blot Analysis of Soybean Event 40-3-2. Ten micrograms of genomic DNA extracted from leaf tissue of A5403 control (lane 2), A5403 control spiked with ~15 pg PV-GMGT04 plasmid DNA (lane 3), Resnick 40-3-2 BC1F2 (lane 4), and 40-3-2 from 1992 Regulatory Field Trial material (lane 5) were digested with *Hin*d III. Lane 1 was left blank. The blot was probed with the ^{32}P-labeled full length CP4 EPSPS coding region.

⟶ Symbol denotes sizes obtained from MW markers on ethidium stained gel.

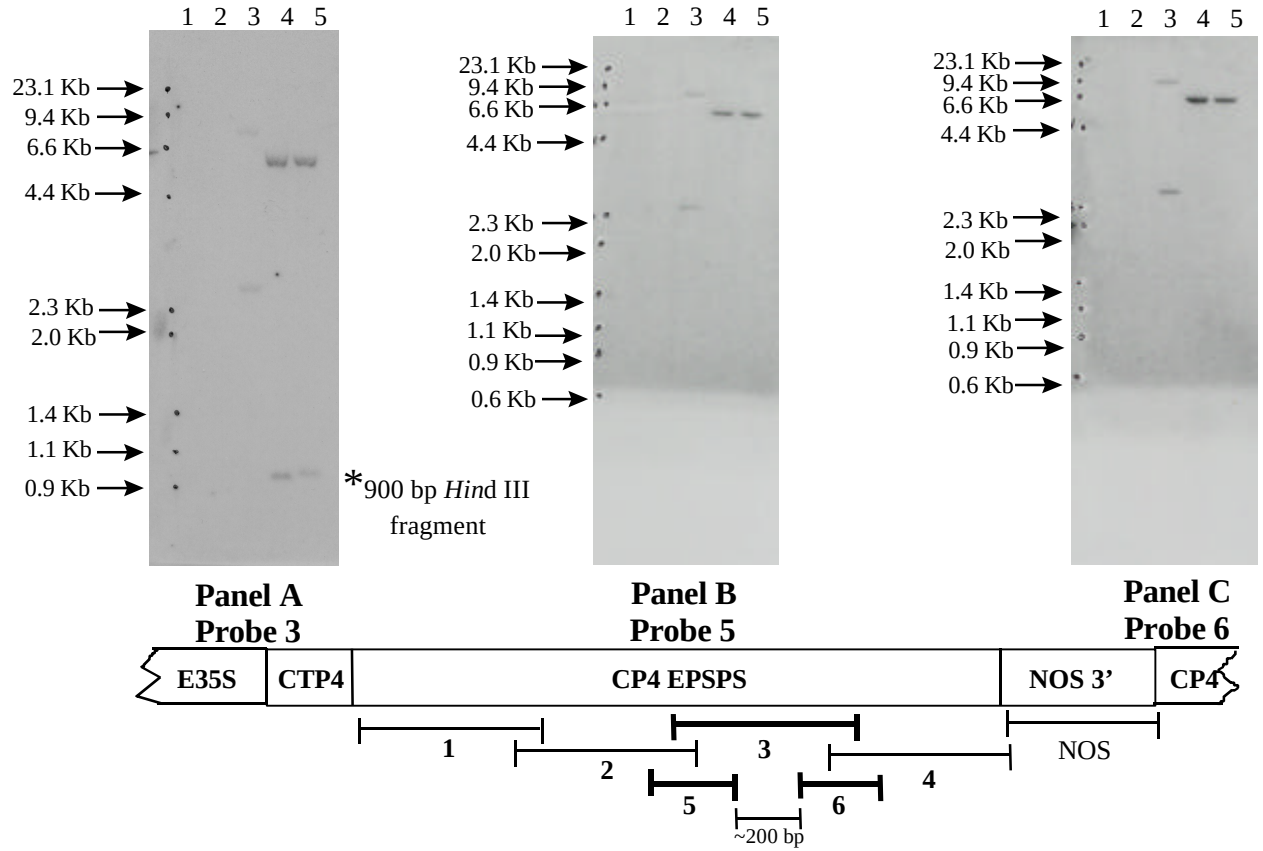

Figure 4. Southern Blot Analysis Using Overlapping CP4 EPSPS Probes. Ten micrograms of genomic DNA extracted from leaf tissue of A5403 control (Lane 2), A5403 control spiked with ~15 pg PV-GMGT04 plasmid DNA (Lane 3), Resnick 40-3-2 BC1F2 (Lane 4), and 40-3-2 from 1992 Regulatory field trial material (Lane 5) were digested with *Hin*d III. Lane 1 is blank in all Panels. Panel A was probed with CP4 EPSPS Probe-3, Panel B with CP4 EPSPS Probe-5, and Panel C with CP4 EPSPS Probe-6. The blot in Panel A is the result of stripping and reprobing of the blot in Figure 3. The positions of the probes with respect to the CP4 EPSPS coding sequence and NOS are illustrated on the linear map below the panels with the probes used in Panels A, B, and C in bold print.

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

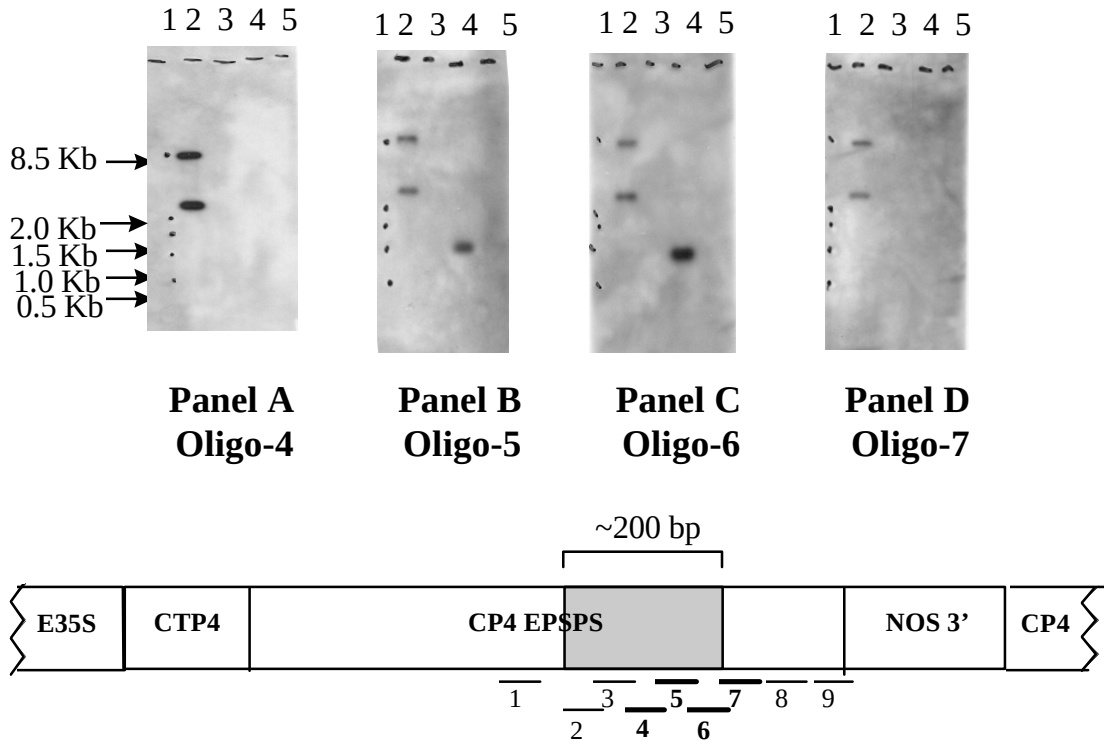

Figure 5. Southern Blot Analysis with Various Oligonucleotide Probes of Cosmid DNA Prepared from the Isolated Cosmid Clone 6A. Oligonucleotide probes were 3'-end labeled with digoxigenin-11-dUTP and probed against individual Southern blots of DNA from the purified cosmid clone 6A digested with *Hin*d III (Lane 4, 4 ng per lane except for Panel A where 900 pg of DNA from a pool of cosmid clone 6A was used). Molecular weight marker DNA was loaded in Lane 1 of each panel for size estimation of the bands being observed. The same molecular weight marker was used for each panel. Plasmid PV-GMGT04 digested with the *Hin*d III served as a positive control (Lane 2, 1 ng per lane). Lanes 3 and 5 of each panel were blank. The positions of the oligonucleotide probes with respect to the CP4 EPSPS coding sequence are illustrated on the linear map below the panels with the probes used in Panels A-D in bold print. The shaded ~200 bp region represents the maximum region delineated to be present on the ~900 bp *Hin*d III fragment of DNA from soybean event 40-3-2 that was observed to hybridize with CP4 EPSPS Probe-3 (See Section IV.A.2).

⟶ Symbol denotes sized obtained from MW markers on ethidium bromide stained gel.

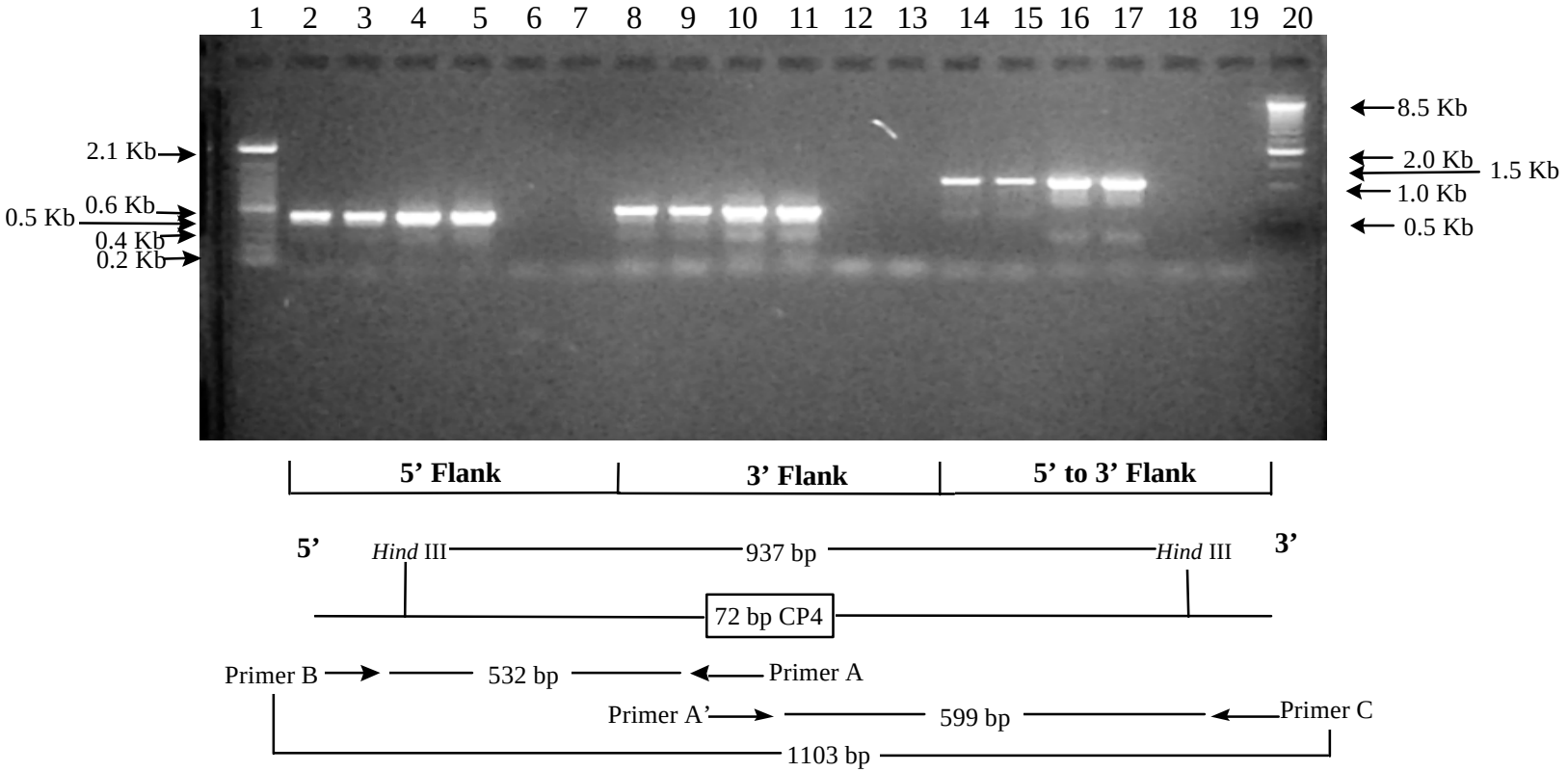

Figure 6. PCR Analyses of Second Insert. PCR analyses were performed using Primers A and B to confirm the 5' flanking sequence, Primers A' and C to confirm the 3' flanking sequence, and Primers B and C to perform PCR from the 5' to 3' flank on DNA extracted from leaf tissue of Resnick 40-3-2 BC1F2 (Lanes 2, 8, and 14) and 1992 Regulatory Field Trial 40-3-2 material (Lanes 3, 9, and 15), as well as cosmid clones 6A (Lanes 4, 10, and 16) and 4B (Lanes 5, 11, and 17) DNA. Lanes 1 and 20 contain Gibco BRL 100 bp DNA Ladder and 500 bp DNA Ladder, respectively. Lanes 6, 12, and 18 contain A5403 non-transgenic DNA PCR reactions and Lanes 7, 13, and 19 were no template control PCR reactions. Ten microliters of each PCR reaction was loaded on the gel.

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

```
   1 CGTGGGTGGG GTCCATCTTT GGGACCCTGT CGGCAGAGGC ATCTTCAACG
  51 ATGGCCTTTC CTTTATCGCA ATGATGGCAT TTGTAGGAGC CACCTTCCTT
 101 TTCCATTTGG GTTCCCTATG TTTATTTTAA CCTGTATGTA TGATCTTATT
 151 TTGAATGAAA TGCAATAAGT TATTTCTAGT AAAAAAAAAT AAACATTTGA
 201 TAGAAACAAA TTAAAGCATG CAAAAATAAC TCATTAGCAT CGGTTAAATT
 251 GAAGGGTTTG AATAATTTGC ACAAGGTTCT GAATTCAAAT C
```

Figure 7A. 5' Flanking Sequence of Primary Insert in Soybean Event 40-3-2. The underlined base pairs 1-105 (corresponding to base pairs 2241-2347 of PV-GMGT04, Figure 1) represent a portion of the E35S promoter. Base pairs 106-291 represent flanking soybean genomic DNA.

```
   1 TTTCTGTTGA ATACGTTAAG CATGTAATAA TTAACATGTA ATGCATGACG
  51 TTATTTATGA GATGGGTTTT TATGATTAGA GTCCCGCAAT TATACATTTA
 101 ATACGCGATA GAAAACAAAA TATAGCCGCG CAAACTAGGA TAAATTATCG
 151 CGCGCGGTGT CATCTATGTT ACTAGATCGG GGATCGATCC CCCACCGGTC
 201 CTTCATGTTC GGCGGTCTCG CGAGCGGTGA AACGCGCATC ACCGGCCTTC
 251 TGGAAGGCGA GGACGTCATC AATACGGGCA AGGCCATGCA GGCCATGGGC
 301 GCCAGGATCC GTAAGGAAGG CGACACCTGG ATCATCGATG GCGTCGGCAA
 351 TGGCGGCCTC CTGGCGCCTG AGGCGCCGCT CGATTTCGGC AATGCCGCCA
 401 CGGGCTGCCG CCTGACCATG GGCCTCGTCG GGGTCTACGA TTTCAAGCGC
 451 ATCATGCTGG GAAATTTTAG CGAGATTATA AGTATCTTCC TGGGGATCTC
 501 TGCTGTTACT GGTGAATAGT GAGACAGAGT CTTCTGAGCT CATAGGATAA
 551 AATAAATTAT AATTAGTAAA TTTTTTAATT AAATAAATCA ATTACTTCAT
 601 AAATAATTTT TTTTATAGAA TATGTTGACA TTCTAGCTGG ATATAGAACT
 651 AATATAAAGA AACCTTAAAA ATTTTGTTTG GAAGAATATG TTATTGAAAG
 701 ACAAATCTAA TTAAGTTTAT CAGGGTCATT TGTTGAAGAT AGGAAACCTT
 751 CAGCAATTTG AATATTAAGT AACTGCTTCT CCCAGAATGA TCGGAGTTTC
 801 TCCTCCTGCT ATTACATGAA AAAAAATAAA AAATAAAAAA AAGATAAGAT
 851 TAAGCTTCAA
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Figure 7B. 3' Flanking Sequence of Primary Insert in Soybean Event 40-3-2. The underlined base pairs 1-194 (corresponding to base pairs 160-353 of PV-GMGT04, Figure 1) represent the 3' portion of the NOS 3' transcriptional termination element present within the functional insert, along with 16 base pairs of plasmid PV-GMGT04 (italics) immediately adjacent to NOS. The double underlined region at base pairs 195-444 (corresponding to base pairs 1490-1739 of PV-GMGT04, Figure 1) delineates 250 bp of the CP4 EPSPS coding region. Base pairs 445-860 represent flanking soybean genomic DNA with a *Hin*d III site indicated in bold letters beginning at base pair 852.

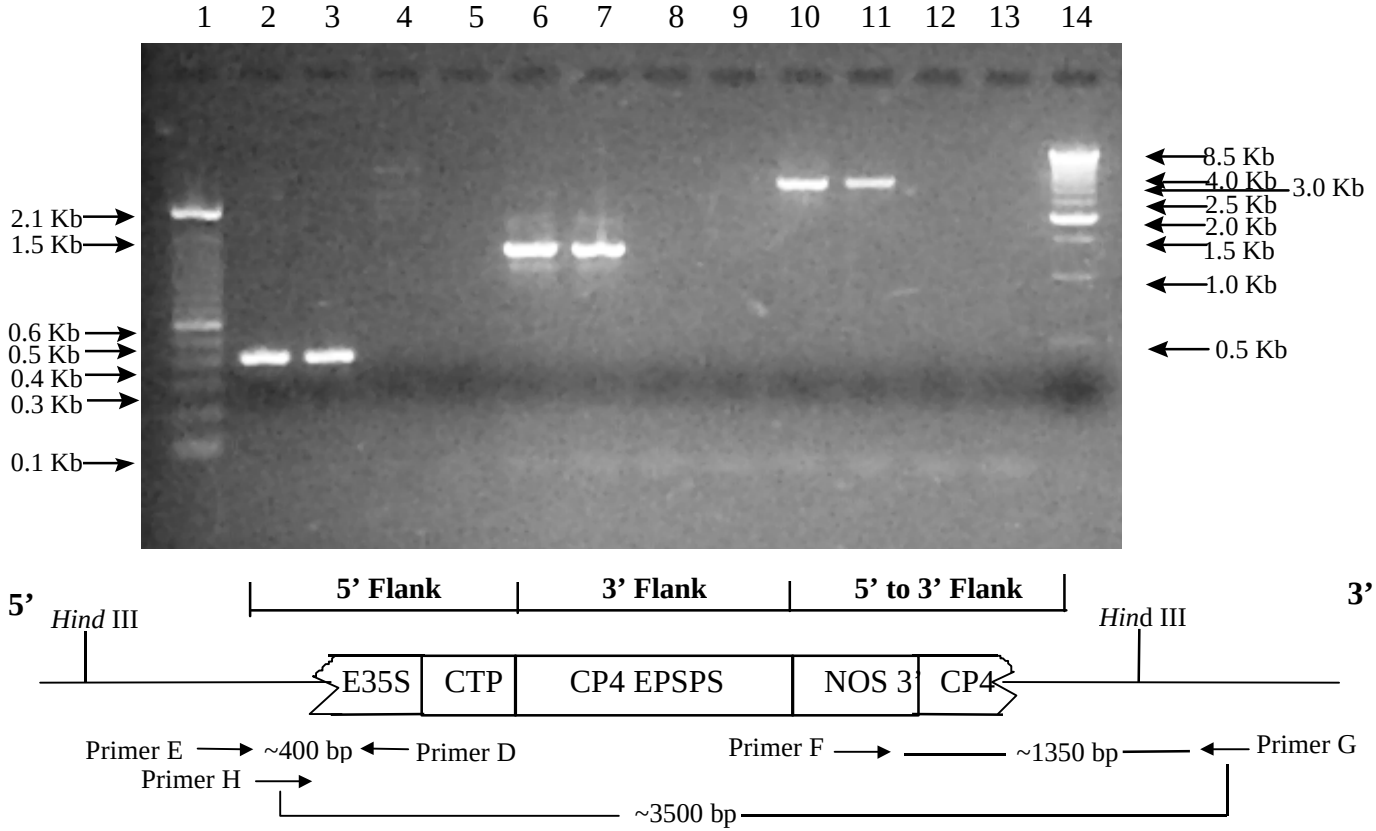

Figure 8. PCR Analyses of Primary Insert. PCR analyses were performed using Primers D and E to confirm the 5' flanking sequence, Primers F and G to confirm the 3' flanking sequence and Primers E and G to perform PCR from the 5' to the 3' flank on DNA extracted from leaf tissue of Resnick 40-3-2 BC1F2 (Lanes 2, 6, and 10) and 1992 Regulatory Field Trial 40-3-2 material (Lanes 3, 7, and 11). Lanes 1 and 14 contain Gibco BRL 100 bp DNA Ladder and 500 bp DNA Ladder, respectively. Lanes 4, 8 and 12 contain A5403 non-transgenic control DNA PCR reactions while Lanes 5, 9, and 13 contain no template control PCR reactions. Nine microliters of each PCR reaction was loaded on the gel.
⟶ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

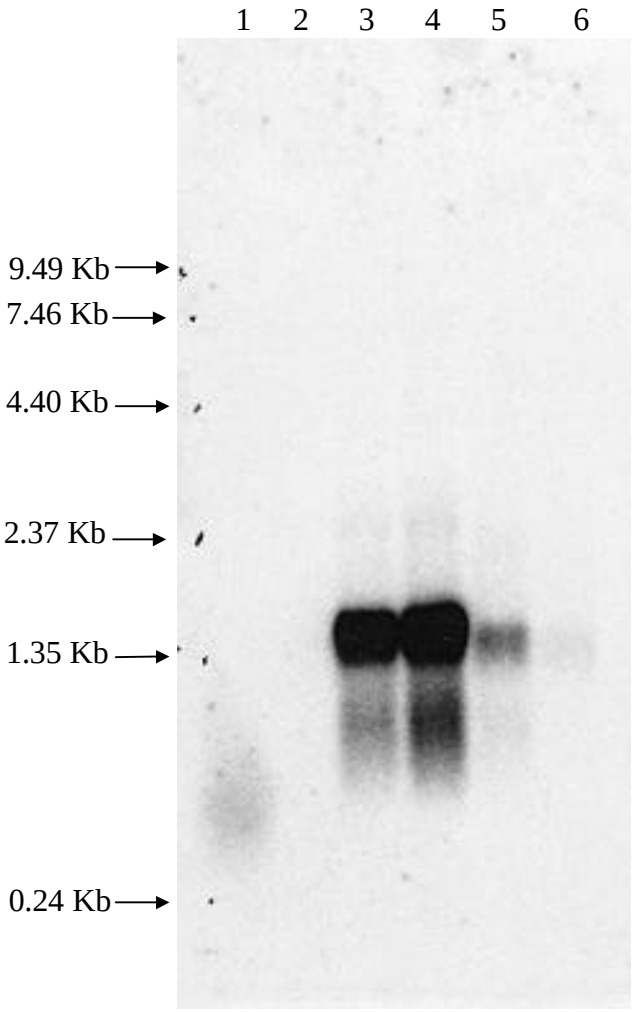

Figure 9. Northern Blot Analysis of Soybean Event 40-3-2. A northern blot was performed using poly (A+) RNA isolated from Roundup Ready soybean event 40-3-2, A5403 non-transgenic soybean, and a non-commercial transgenic soybean event which expresses CP4 EPSPS, and probed with CP4 EPSPS Probe-3 (bp 713-1104 on PV-GMGT04, Figure 1). Lanes 2 and 3 contain 1 µg of poly (A+) RNA from A5403 and a non-commercial transgenic soybean event, respectively. Lanes 4, 5, and 6 contain 1 µg, 0.1 µg, and 0.01 µg of soybean event 40-3-2poly (A+) RNA, respectively. Lane 1 contains an *in vitro* transcribed control RNA.

→ Symbol denotes MW markers on ethidium bromide stained gel.

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
GEN-PRO-072-01	Isolation of Genomic DNA from Plant Tissue with Subsequent Purification on a Cesium Chloride Gradient