

Study Title

**RT-PCR Analysis of the DNA Flanking the Two Partial CP4 EPSPS Segments in
Roundup Ready® Soybean Event 40-3-2**

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Abbreviations

~	approximately
CP4 EPSPS	coding region for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase isolated from <i>Agrobacterium sp.</i> strain CP4
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dNTP	deoxynucleotide triphosphate
Mg ²⁺	magnesium
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA
MW	molecular weight
NOS 3'	nopaline synthase 3' transcriptional termination element
Oligo(dT) ₁₅	primer for first strand cDNA synthesis containing oligothymidylic acid (15)
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR

SUMMARY

The molecular characterization of Roundup Ready® soybean event 40-3-2 was extended using more sensitive and precise methods (Lirette *et al.*, 2000). These analyses identified: 1) a second insert comprised of a 72 bp segment of CP4 EPSPS, and 2) 250 bp of the CP4 EPSPS sequence adjacent to the 3' end of the complete NOS 3' transcriptional termination element in the primary, functional insert. Previous northern blot analysis performed at a limit of detection of 10 ng of poly (A+) mRNA (Lirette *et al.* 2000), supported by western blot analyses (Harrison *et al.*, 1993; Rogan *et al.* 1999), established that transcription of these regions does not occur at detectable levels. In order to further demonstrate the lack of an RNA transcript encompassing either end of the 72 bp CP4 EPSPS segment or the 3' end of the 250 bp CP4 EPSPS segment adjacent to the NOS 3' transcriptional termination element, reverse transcriptase PCR (RT-PCR) has been performed with a limit of detection of 100 pg of cDNA, which represents at least a 100-fold increase in sensitivity compared to the previous northern blot analysis. No RT-PCR products were visible for either of the two partial CP4 EPSPS segments supporting and strengthening the previous conclusions that transcription of the two partial CP4 EPSPS DNA segments present in Roundup Ready soybean event 40-3-2 does not occur at detectable levels and both DNA segments are non-functional (Lirette *et al.* 2000).

II. INTRODUCTION

A. Background.

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.*, 1993, Kolacz and Padgett, 1994, Padgett *et al.*, 1996), a second insert which weakly hybridizes to a full-length CP4 EPSPS probe was detected. This second insert is comprised of 72 bp of the CP4 EPSPS sequence located on a 937 bp *Hind* III restriction fragment (Lirette *et al.*, 2000). In addition, 250 bp of the CP4 EPSPS sequence was identified adjacent to the 3' end of the complete NOS 3' transcriptional termination element in the primary, functional insert. Northern blot analysis, able to detect 10 ng of poly (A+) mRNA (Lirette *et al.* 2000), and western blot analyses (Harrison *et al.* 1993; Rogan *et al.* 1999) showed that only the expected CP4 EPSPS full-length transcript and protein are detected, respectively, supporting the conclusion that neither transcription nor translation of these CP4 EPSPS segments occurs at detectable levels.

B. Purpose.

The objective of this study was to perform RT-PCR analysis on Roundup Ready soybean event 40-3-2 to determine if either of the two partial CP4 EPSPS segments and their respective genomic flanking DNA produce a mRNA transcript.

III. MATERIALS AND METHODS

A. Test Substance. The test substance for this study was the Roundup Ready soybean event 40-3-2.

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

C. Reference Substance. The reference substance was the 100 bp DNA Ladder (2.1 Kb-0.1 Kb) molecular size marker from Gibco BRL (catalog #15628-019) used for size estimations in the PCR analysis.

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

E. RNA Isolation and Poly (A+) mRNA Selection. Total RNA was isolated and poly (A+) mRNA was selected from Roundup Ready soybean event 40-3-2 and non-transgenic soybean line A5403 in Study # 99-01-30-22. Details on the isolation and poly (A+) selection are located in Lirette *et al.* (2000).

F. cDNA Synthesis. One microgram of poly (A+) selected mRNA from Roundup Ready soybean event 40-3-2 and non-transgenic soybean line A5403 was mixed individually with 0.5 µg of Oligo(dT)₁₅ in a total reaction volume of 15 µl and incubated at 70°C for 5 minutes. The mixes were placed immediately on ice, spun briefly in a microcentrifuge at maximum speed, and placed back on ice. The reverse transcription reactions were conducted in a 25 µl reaction volume containing a final concentration of 1X M-MLV reaction buffer, 0.5 mM each dNTP, 25 units of ribonuclease inhibitor, and 200 units of M-MLV reverse transcriptase. The reactions were prepared by adding the components in the order listed above to the mRNA/Oligo(dT)₁₅ mixes and incubating at 38°C for 1 hour. The reactions were then stored in a -20°C freezer.

G. mRNA and cDNA Quantitation. Quantitation of the mRNA and cDNA samples was performed using a Beckman DU640B spectrophotometer (Fullerton, CA) by measuring the absorbance at 260 nm.

H. RT-PCR Analysis of the DNA Flanking the Two Partial CP4 EPSPS Segments in Soybean Event 40-3-2.

1. Calibration of Cycle Number. The cycle number necessary to detect the full-length CP4 EPSPS cDNA in approximately 100 pg of total poly (A+) mRNA was determined using two primers specific to the CP4 EPSPS sequence. The calibration experiment was conducted using 100 pg of event 40-3-2 or line A5403 cDNA in a 50 μ l reaction volume containing a final concentration of 1.5 mM Mg^{2+} , 0.4 μ M of each primer, 200 μ M 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; 21, 22, 23, 23, 25, or 26 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. The RT-PCR products were separated using 2.0 % agarose gel electrophoresis at 120 V for 45 minutes and visualized by ethidium bromide staining.

2. RT-PCR Analysis. The RT-PCR analysis was performed using primers anchored in the two partial CP4 EPSPS segments paired with primers in the 3' flanking sequence of the primary insert and the 5' and 3' flanking sequences of the 72 bp insert. A positive control RT-PCR was also performed using the primers specific to the full-length CP4 EPSPS used in the calibration experiment outlined above. These primers were also used on line A5403 and event 40-3-2 mRNA, used to generate the cDNA, to evaluate for the presence of genomic DNA contamination. The RT-PCR analysis was conducted using 100 pg of event 40-3-2 or line A5403 cDNA with the same reaction conditions as were used for the calibration experiment and the following cycling conditions: 1 cycle at 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. Positive control PCR reactions were also performed to validate the primer sets using 25 ng of event 40-3-2 and line A5403 genomic DNA template with the same reaction and cycling conditions as the RT-PCR except that 35 cycles of PCR were performed. All reaction products were separated using 2.0 % agarose gel electrophoresis at 120 V for 45-50 minutes and visualized by ethidium bromide staining.

IV. RESULTS AND DISCUSSION

A. Calibration of Cycle Number for RT-PCR Analysis. A RT-PCR experiment was performed to calibrate the number of cycles necessary to detect the full-length CP4 EPSPS cDNA in 100 pg of poly (A+) mRNA (or cDNA). The detection of the full-length CP4 EPSPS transcript in 100 pg of poly (A+) mRNA will provide a level of sensitivity at least 100-fold greater than the northern blot performed in Lirette *et al.* (2000), which was able to detect 10 ng of poly (A+) mRNA. The experiment was performed on cDNA from Roundup Ready soybean event 40-3-2 and non-transgenic soybean line A5403 using Primers A and B located in the CP4 EPSPS sequence, and cycle numbers ranging from 21-26. The positions of the primers, as well as the results of the RT-PCR calibration experiment, are shown in Figure 1. The control reactions containing no template for all cycle numbers (Lanes 4, 7, 10, 13, 16, and 19), as well as

the A5403 non-transgenic cDNA (Lanes 2, 5, 8, 11, 14, and 17) did not generate a RT-PCR product, as expected. RT-PCR using primers specific for the CP4 EPSPS on Roundup Ready soybean event 40-3-2 cDNA (Lanes 3, 6, 9, 12, 15, and 18) generated the expected size product of 702 bp, representing a portion of the full-length CP4 EPSPS cDNA, at all six cycle numbers tested. Therefore, the cDNA encoding the full-length CP4 EPSPS protein can be detected in 100 pg of total cellular cDNA at 21 cycles. However, the products generated at cycle numbers 21-23 (Lanes 3, 6, and 9) are relatively faint in intensity. In order to ensure at least a 100-fold increase in sensitivity as compared to the northern blot in Lirette *et al.* (2000), in which the mRNA encoding the full-length CP4 EPSPS protein was detectable in 10 ng of poly (A+) mRNA, 25 cycles (Lane 15) were chosen for use in the RT-PCR analysis to determine if a mRNA transcript is produced from either of the two partial CP4 EPSPS segments in Roundup Ready soybean event 40-3-2.

B. RT-PCR Analysis. RT-PCR was performed on cDNA from Roundup Ready soybean event 40-3-2 using 25 cycles, thus allowing for the detection of the cDNA encoding the full-length CP4 EPSPS protein in 100 pg of poly (A+) mRNA (or cDNA) and providing at least a 100-fold increase in sensitivity compared to the previous northern blot analysis performed by Lirette *et al.* (2000). Four different primer sets were used in the analyses, three of which were used to assess the two partial CP4 EPSPS segments and one used as a positive control. Primers A and B, also used in the RT-PCR calibration experiment, reside in the full-length CP4 EPSPS cDNA and served as a positive control for the RT-PCR analysis. Primers C and D were used to determine if a mRNA transcript is produced from the partial CP4 EPSPS segment located at the 3' end of the primary, functional insert, while Primers E and F and Primers G and H were used to assess the possibility of stable transcription from the 5' and 3' ends, respectively, of the 72 bp insert consisting of CP4 EPSPS. In addition to cDNA template, all four primer sets were also used on event 40-3-2 and line A5403 genomic DNA to validate their functionality. The positions of all primers as well as the results of the RT-PCR analysis are shown in Figure 2. The control reactions containing no template for all primer sets (Lanes 4, 9, 14, and 19), as well as the A5403 non-transgenic cDNA (Lanes 2, 7, 12, and 17) and genomic DNA (Lanes 5, 10, 15, and 20) did not generate a PCR product, as expected. In addition, PCR on the 40-3-2 mRNA and A5403 mRNA, used to synthesize the cDNA, did not produce a product in the absence of reverse transcriptase using Primers A and B (Lanes 22 and 23, respectively). The absence of a visible product demonstrates that the mRNA was not contaminated with genomic DNA. The positive control reactions using 40-3-2 genomic DNA to validate the primer sets all yielded the correct size PCR products of 96 bp for Primers C and D (Lane 6), 243 bp for Primers E and F (Lane 11), 102 bp for Primers G and H (Lane 16), and 702 bp for Primers A and B (Lane 21). Primers C and D also generated a secondary product at ~1.7 Kb (Lane 6) due to Primer C also being present in the full-length CP4 EPSPS. The presence of the correct size PCR products using 40-3-2 genomic DNA as a template demonstrates that the primer

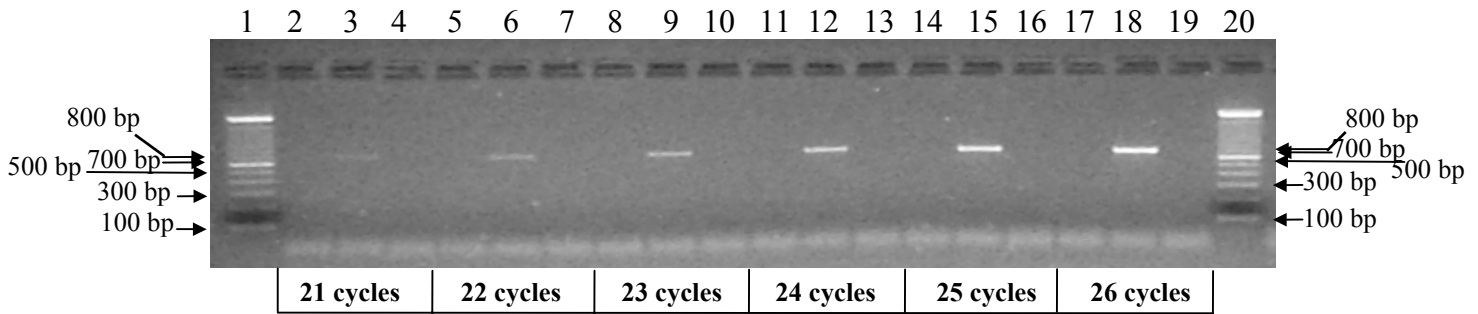
sets function as expected. None of the three reactions containing 40-3-2 cDNA using flanking sequence primers paired with primers in the two partial CP4 EPSPS segments generated a RT-PCR product (Lanes 3, 8, and 13), while the positive control Primers A and B, specific to the full-length CP4 EPSPS cDNA, did generate the expected 702 bp product using 40-3-2 cDNA as a template (Lane 18). These results indicate that transcription of the 72 bp CP4 EPSPS segment and the 250 bp CP4 EPSPS segment adjacent to the NOS 3' transcriptional termination element in the functional insert does not occur at detectable levels, further strengthening the conclusions drawn in Lirette *et al.* (2000).

V. CONCLUSIONS

Previous northern blot analysis on Roundup Ready soybean event 40-3-2 performed at a limit of detection of 10 ng of poly (A+) mRNA (Lirette *et al.* 2000) established that transcription of the two partial CP4 EPSPS segments does not occur at detectable levels. To further support that observation, RT-PCR has been performed at a limit of detection representing at least a 100-fold increase in sensitivity compared to the previous northern blot analysis. No RT-PCR products were visible for either of the two partial CP4 EPSPS segments, supporting and strengthening the previous conclusions in Lirette *et al.* (2000) that transcription of the two partial CP4 EPSPS DNA segments present in Roundup Ready soybean event 40-3-2 does not occur at detectable levels and that the two DNA segments are non-functional.

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Primary, Functional Insert

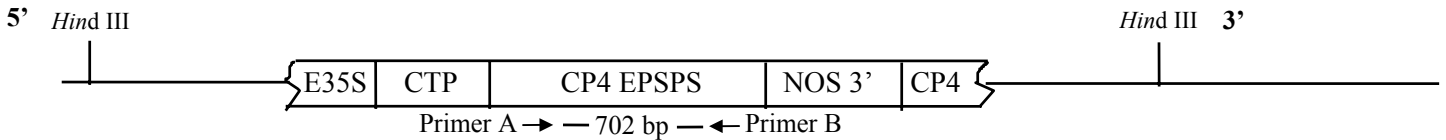
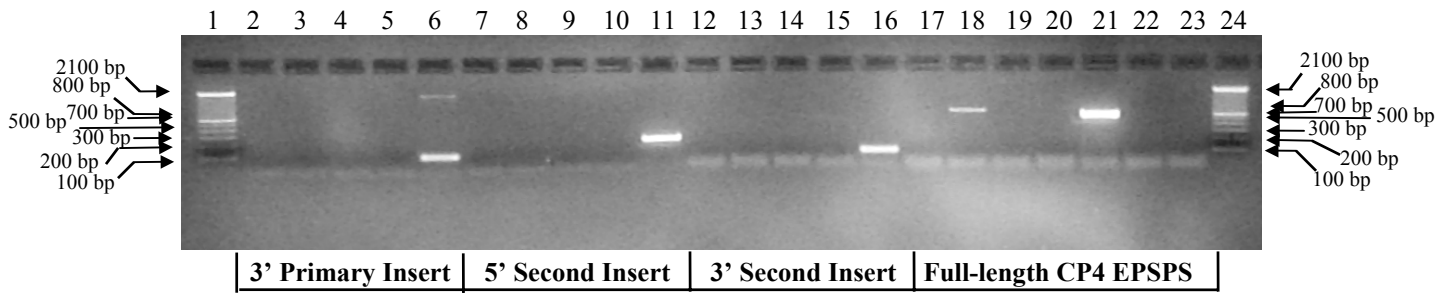
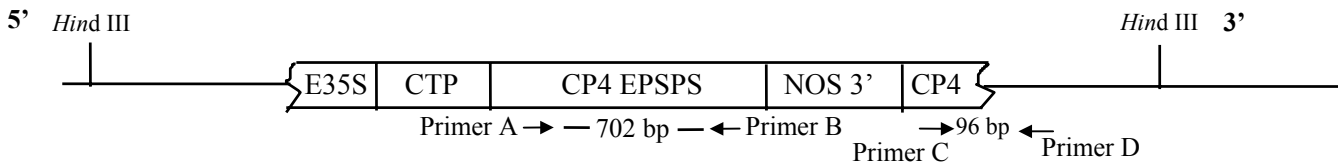


Figure 1. RT-PCR Calibration of Cycle Number Experiment. RT-PCR was performed using Primers A and B specific to the full-length CP4 EPSPS sequence at cycle numbers ranging from 21-26. The RT-PCR was performed on cDNA from both event 40-3-2 (Lanes 3, 6, 9, 12, 15, and 18), as well as cDNA from non-transgenic soybean line A5403 (Lanes 2, 5, 8, 11, 14, and 17). Lanes 1 and 20 contain Gibco BRL 100 bp DNA Ladder. Lanes 4, 7, 10, 13, 16, and 19 were no template control RT-PCR reactions. Ten microliters of each RT-PCR reaction were analyzed on the gel.

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



Primary, Functional Insert



Second, Non-Functional Insert

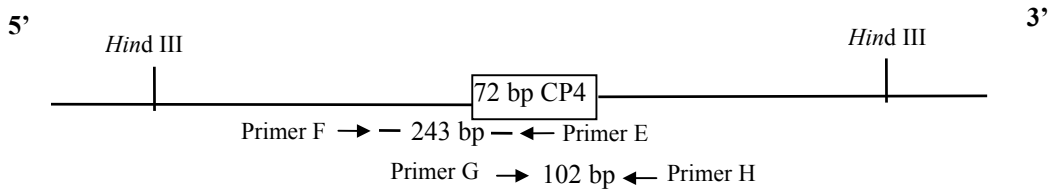


Figure 2. RT-PCR Analysis of the Two Partial CP4 EPSPS Segments in Roundup Ready Soybean Event 40-3-2. RT-PCR was performed on event 40-3-2 using four primer sets. Primers A and B, specific to the full-length CP4 EPSPS sequence, served as a RT-PCR positive control while Primers C and D were used to determine if a mRNA transcript is produced from the partial CP4 EPSPS segment located at the 3' end of the primary, functional insert. Primers E and F and Primers G and H were used to assess the possibility of stable transcription from the 5' and 3' ends, respectively, of the 72 bp insert consisting of CP4 EPSPS. The RT-PCR was performed on cDNA from both event 40-3-2 (Lanes 3, 8, 13, and 18), as well as cDNA from non-transgenic soybean line A5403 (Lanes 2, 7, 12, and 17). Both the A5403 and 40-3-2 mRNA were also tested for the presence of genomic DNA using Primers A and B (Lanes 22 and 23, respectively). In addition, the functionality of the primer sets was tested on genomic DNA extracted from leaf tissue from event 40-3-2 (Lanes 6, 11, 16, and 21) and line A5403 (Lanes 5, 10, 15, and 20). Lanes 1 and 24 contain Gibco BRL 100 bp DNA Ladder. Lanes 4, 9, 14, and 19 were no template control RT-PCR reactions. Ten microliters of each RT-PCR or PCR reaction were analyzed on the gel.

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

Appendix 1

Protocol