Updated Molecular Characterization and Safety Assessment of Roundup Ready® Soybean Event 40-3-2

Monsanto Company
700 Chesterfield Parkway North
St. Louis, MO, USA

Table of Contents

Abbreviations

RR	Roundup Ready
PCR	Polynerase chain reaction
CP4 EPSPS	5-enol-pyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium sp.* Strain CP4
NOS	Nopaline synthase
kDa	kilo-Daltons
CTP4	Chloroplast transit peptide 4
E35S	Cauliflower mosaic virus (CaMV) 35S promoter containing a duplication of the -90 to -300 bp region
bp	base pairs
Kbp	kilo-base pairs
Kb	Kilo-base
mRNA	messenger ribonucleic acid
ELISA	Ensyme linked immunosorbant assay
ORF	Open reading frame
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
FDA	United States Food and Drug Administration
FAO	Food and Agriculture Organization of the United Nations
WHO	World Health Organization of the United Nations

1.0 Summary

Roundup Ready® soybean event 40-3-2, the original transformation line (parent) of all Roundup Ready soybean (RR soybean) varieties available commercially today, was evaluated extensively in 1990-1994 in numerous food, feed and environmental studies prior to regulatory submissions and reviews. Additional molecular characterization has been completed recently as a result of seed quality control analyses and to facilitate the development of detection methods. The goals of this review are to briefly summarize the updated molecular characterization of RR soybean event 40-3-2 with data obtained from studies using more sensitive and precise methods and to assess the potential impact of these results on the food, feed and environmental safety of this product.

RR soybean event 40-3-2 initially was evaluated using techniques which included Southern blot analysis, PCR, western blot analysis and ELISA. Results using these methods led to the conclusion that RR soybean event 40-3-2 contained a single DNA insert which contains one functional EPSPS gene cassette: an E35S promoter, chloroplast transit peptide (CTP) and CP4 EPSPS coding sequences, and a NOS 3' transcription terminator. Expression of this gene cassette resulted in the production of the expected full-length 46 kDa CP4 EPSPS (5-enol-pyruvylshikimate-3-phosphate synthase) protein that is fully functional in the presence of glyphosate, the active ingredient of Roundup® herbicide, and represents the biochemical basis of the Roundup Ready trait.

Recently, additional characterization experiments have been completed using more sensitive and precise methods which include Southern blot analysis, PCR, genome walking, cosmid library construction, DNA sequencing, northern blot analysis and western blot analysis. These studies have provided additional details of the inserted DNA and flanking soybean genomic DNA sequences associated with the functional insert. At the 3' proximal region of the initially described functional insert, a 250 bp segment of CP4 EPSPS DNA is located adjacent to the NOS 3' transcriptional termination element of the functional CP4 EPSPS gene cassette. In addition, a second co-segregating insert comprising a 72 bp segment of CP4 EPSPS DNA was identified on a 937 bp *Hin*d III genomic DNA restriction fragment.

This information confirms the previous conclusion that a single functional gene cassette encoding CP4 EPSPS protein, which is responsible for glyphosate tolerance, is present in RR soybean event 40-3-2. Several lines of evidence establish that neither of the two CP4 EPSPS-derived segments produce mRNA transcripts or proteins. First, it is highly unlikely that either the 3' proximal CP4 EPSPS DNA segment or the secondary insertion of CP4 EPSPS DNA can be transcribed or translated because the segments do not contain the necessary genetic elements required for functional genes (i.e. there are no promoters or transcription termination elements defining an mRNA transcript and there are no translation start and stop codons defining a protein coding sequence). Second, northern

® Roundup and Roundup Ready are registered trademarks of Monsanto, St. Louis, MO.

blot analyses showed that only the CP4 EPSPS mRNA of the expected molecular weight for the full length CP4 EPSPS transcript was detected. Finally, western blot analysis, using polyclonal antibody methods at least ten fold more sensitive than previously used, confirm that only the full-length CP4 EPSPS protein was produced.

Both CP4 EPSPS DNA segments are present in all RR soybeans, including the soybeans tested in extensive regulatory field trials, composition, nutrition, safety and allergenicity studies considered during the risk assessment and subsequent reviews and approvals by regulatory agencies. These soybeans were previously established as safe based on: the safety of the genetic elements contained on the transformation vector used to produce RR soybean event 40-3-2; the history of safe use of the EPSPS family of proteins present in all plants, fungi and bacteria; the functionality and safety assessment of the CP4 EPSPS protein; the assessment of compositional and nutritional equivalence of event 40-3-2 (comparing the key nutrients, anti-nutrients and allergens to the parental event and conventional soybeans); a comparison of crop agronomic characteristics of event 40-3-2 to the parental and conventional soybeans; and a comparison of the safety and nutritional properties of event 40-3-2 to parental and conventional soybean varieties in animal feeding studies.

This review of the safety assessment, which accounts for the new molecular information obtained using more sensitive methods, confirms the conclusions reached previously that: 1) RR soybean event 40-3-2 contains one functional CP4 EPSPS gene cassette; 2) RR soybeans are as safe and as nutritious as conventional soybean varieties and; 3) RR soybeans do not pose a plant pest risk or otherwise pose a risk to the environment. Any risks associated with the production and consumption of RR soybean event 40-3-2 to human health or the environment are no different than those associated with conventional soybean varieties.

2.0 Introduction

Glyphosate, the active ingredient in Roundup herbicide (1), 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 mammalian, avian, or aquatic life forms, and hence glyphosate is only toxic to plants, fungi, and microorganisms, but not to other living species.

Roundup Ready soybean event 40-3-2 was produced by particle-acceleration transformation of non-transgenic soybean line A5403 (often used as a negative control in characterization and safety evaluations) utilizing DNA derived from plasmid PV-GMGT04 (Figure 1). The primary insertion of DNA derived from the transformation plasmid PV-GMGT04 includes a single CP4 EPSPS gene cassette: the E35S promoter, the chloroplast transit peptide and CP4 EPSPS coding sequences, and the NOS 3' transcriptional terminator. The CP4 EPSPS coding sequence encodes 456 amino acids, includes

translation start and stop codons, and results in the synthesis of full length and functional 46 kDa CP4 EPSP synthase (5-enol-pyruvylshikimate-3-phosphate synthase) protein in RR soybean event 40-3-2 as confirmed by western blotting, ELISA and EPSPS activity assays (2-7).

As part of Monsanto's seed quality program, a commercial RR soybean variety was analyzed by Southern blot analysis to confirm the genetic identity and purity for the 40-3-2 insertion event. The original non-transgenic parent line, A5403, was included as a negative control. The complete CP4 EPSPS gene was used as a probe in Southern blot analysis using a method with an approximately nine-fold increased sensitivity compared to the original Southern blot method (8). In addition to the expected 5.8 kbp *Hin*d III hybridizing DNA band containing the CP4 EPSPS gene cassette, a previously unobserved 937 bp *Hin*d III restriction fragment weakly hybridized to the CP4 EPSPS probe. Subsequently, it was determined that this 937 bp genomic DNA restriction fragment contains a second insert comprising a 72 bp segment of the CP4 EPSPS DNA corresponding to nucleotides 855-926[1] of the EPSPS coding sequence and that this second insert co-segregates with the primary functional insert (8).

In an effort to both characterize the 937 bp *Hin*d III fragment and to develop event-specific PCR-based detection methods, genome walking experiments were performed. These analyses yielded DNA sequence information at the 5 ' and 3' ends of the functional insert as well as from soybean genomic DNA flanking the 5' and 3' ends. The results of DNA sequencing confirm deletion of a portion of the E35S promoter at the 5' end of the insert, as previously described (2). Through DNA sequencing of the 3' end of the insert, a 250 bp segment (bp 1490-1739, Figure 1) of CP4 EPSPS coding sequence immediately adjacent to the 3' end of the NOS transcription terminator of the CP4 EPSPS gene cassette was identified.

This information was evaluated in the context of expected and observed gene expression products for RR soybean event 40-3-2. First, all newly described DNA sequences are associated with CP4 EPSPS (i.e. a subset of CP4 EPSPS protein coding sequence). Second, the molecular arrangements of the DNA (lack of promoter, lack of complete coding sequences with translation start and stop codons, lack of transcription terminator) indicate that no additional gene expression products should be expected in RR soybean event 40-3-2.

3.0 Molecular Characterization and Gene Expression

A gene is defined as the entire nucleic acid sequence (usually a DNA sequence) that is necessary for the synthesis of a functional polypeptide (protein) or RNA sequence (9). A gene encoding a protein must contain at least three critical elements to be functional: a 5' proximal promoter to initiate transcription of mRNA; a complete open reading frame

[1] Numbering (bp) is according to the complete vector sequence, and not based on position relative to the translation start codon (see Figure 1).

(ORF) that includes both a translation start and translation stop codon to define a protein coding sequence; and a 3' proximal transcription termination element (which includes an mRNA polyadenylation signal). For gene expression (transcription of the DNA sequence into mRNA and subsequent translation of that mRNA into a protein) these components must be present, contiguous and arranged in the correct order (10). This discussion reviews the available information describing: 1) the 250 bp segment of the CP4 EPSPS DNA proximal to the NOS transcriptional termination element at the 3' end of the primary insert and 2) the 72 bp segment of CP4 EPSPS DNA which comprises the second insert. Since neither of these CP4 EPSPS DNA segments represent a gene as defined above, it was concluded that these segments represent non-functional DNA sequences.

3.1 Evaluation of the 250 bp Segment of CP4 EPSPS DNA Proximal to the NOS Transcriptional Termination Element at the 3' End of the Primary Insert

Gene expression data was used to show that only the full length CP4 EPSPS gene on the primary insert was functional. Specifically, northern blotting showed accumulation of only the expected CP4 EPSPS mRNA transcript (8) and western blotting showed accumulation of only the expected CP4 EPSPS protein (2, 6, 11). In the unlikely event that the NOS 3' transcriptional terminator of the CP4 EPSPS transcriptional unit (see Figures 1 and 2) failed to terminate RNA synthesis, then a larger than expected "read-through" mRNA transcript could be produced. A theoretical mRNA such as this would include multiple open reading frames: 1) the full length CP4 EPSPS protein, 2) any putative ORFs in the NOS 3' terminator, and 3) the putative ORF comprising the 250 bp segment of CP4 EPSPS DNA located at the 3' end. Production of read-through transcripts is highly unlikely and without precedent in plants. Moreover, the NOS 3' terminator contains three known polyadenylation signals which signal the end of mRNA transcription by RNA polymerase (12). Furthermore, such a read-through transcript would be significantly larger than the predicted CP4 EPSPS mRNA of ~1.5 Kb. No unexpected transcript of any size other than the full length was detected (8). Finally, the RNA pattern observed in northern blotting was the same (i.e., one ~1.5 Kb hybridizing RNA) for RR soybean event 40-3-2 and a second non-commercial transgenic soybean event (61-67-1) which would not be expected to share the same additional CP4 EPSPS segments described above (8).

Western blot analyses using polyclonal antibodies raised against the CP4 EPSPS protein were conducted with crude protein preparations from RR soybean event 40-3-2 tissue. Since polyclonal antibodies react with numerous antigenic sites (amino acid sequences), if a protein were synthesized that contains sufficient amino acid sequences derived from the CP4 EPSPS gene coding sequence, this partial CP4 EPSPS protein would be expected to be detected by western blotting. No protein was detected in the initial studies other than the full length CP4 EPSPS protein that was encoded by the full length CP4 EPSPS gene contained within the functional insert (2, 6). Subsequent analyses using western blotting methods that were at least ten fold more sensitive than the initial studies confirm that only the expected 46 kDa CP4 EPSPS is produced, and that no larger or smaller protein fragments were detected (11).

Western blot analysis showed that the 46 kDa CP4 EPSPS protein[2] and no additional CP4 EPSPS immunoreactive proteins are detected in RR soybean event 40-3-2 (2, 6, 11). If an unexpected read-through mRNA were produced in RR soybean event 40-3-2, no additional proteins would be expected to be made since eukaryotes such as plants typically do not translate multiple open reading frames (ORFs) from single mRNAs. In the exceptionally rare instance where second ORFs contained on a single mRNA[3] have been detected in plants (13, 14), a single polyprotein has been observed, rather than the synthesis of two independent proteins. The presence of multiple translation stop codons between the full length CP4 EPSPS coding sequence and the 250 bp segment of CP4 EPSPS DNA found 3' to the NOS terminator would be expected to prevent the synthesis of a CP4 EPSPS/NOS/CP4 EPSPS combined protein. Western blotting confirmed that only the expected 46 kDa CP4 EPSPS protein is produced by event 40-3-2. No unexpected protein, including any large molecular weight protein potentially produced by the read-through mRNA, was detected by western blotting when using more sensitive western blot methods (11).

3.2 Evaluation of the 72 bp Segment of CP4 EPSPS DNA Comprising the Second Insert

The 72 bp segment of CP4 EPSPS DNA which comprises the second insert in commercial Roundup Ready soybeans does not represent a functional transcriptional unit because it lacks a 5' proximal promoter to initiate transcription of mRNA and a 3’ proximal transcription termination element. Northern blot analysis, using methods at least as sensitive as those used to detect the 72 bp segment of CP4 EPSPS DNA present on the 937 bp *Hin*d III genomic DNA restriction fragment, confirmed that no unexpected CP4 EPSPS containing mRNA accumulates in RR soybean event 40-3-2 tissues (8). Furthermore, the 72 bp segment of CP4 EPSPS DNA does not represent a protein coding sequence because it lacks translation start and stop codons. Likewise, as stated above, no immunoreactive proteins other than the expected 46 kDa CP4 EPSPS protein were observed in RR soybean event 40-3-2 (6, 11).

3.3 Conclusion from the Newly Described DNA Segments

Two corroborating analytical methods were used to exclude gene expression: northern blotting for mRNA detection and western blotting for protein detection were chosen as appropriate methods which corroborate the results of each respective method. Based on both northern blot and western blot analyses, combined with the detailed evaluation of the sequences of the DNA, it was concluded that no new proteins were expected or observed to be produced from either of the newly described CP4 EPSPS DNA segments. Taken

[2] CP4 EPSPS protein has an observed relative molecular mass of approximately 46 kDa in SDS-PAGE or western blot analyses. The specific molecular mass of CP4 EPSPS protein is 47.6 kDa.
[3] Transcripts which contain two or more independently translated ORFs are referred to as polycistronic mRNAs and result in the synthesis of two or more independent proteins.

together, the northern and western blotting data described above confirm the previous conclusion that RR soybean event 40-3-2 contains one functional CP4 EPSPS gene which produces the full-length CP4 EPSPS protein which confers the glyphosate tolerance trait.

4.0 Environmental Assessment

All of the agronomic and environmental studies which support the conclusion that Roundup Ready soybeans do not present a plant pest risk or otherwise pose a risk to the environment were completed with RR soybeans which contained the 250 bp segment of CP4 EPSPS DNA proximal to the NOS transcriptional termination element at the 3' end of the primary insert and the 72 bp segment of CP4 EPSPS DNA located on the 937 bp *Hin*d III genomic DNA restriction fragment. Comparisons of RR soybean event 40-3-2 were made to conventional soybeans (Table 1) with regard to disease and pest characteristics, yield, morphology, weediness, impact on non-target organisms and other characteristics (3, 5). Based on these extensive studies which were conducted and submitted to regulatory authorities for review prior to approval, it was concluded that tolerance to glyphosate is stably inherited and that there are no unexpected plant pest risks or environmental risks associated with RR soybeans. These conclusions are not altered by the additional molecular information discussed above.

Table 1 Characteristics examined in the environmental risk assessment for RR soybean event 40-3-2*

Morphology
Seed production (yield)
Disease susceptibility
Insect susceptibility
Weediness (germination, volunteer persistence)
Time to flowering/ pod set
Impact on non-target organisms (insects, birds, fish)
Outcrossing (gene flow)

* Data obtained from over 150 field trials conducted over 3 year period prior to submission to regulatory authorities. (3)

Data published subsequent to regulatory submission includes yield and glyphosate tolerance (15), fungal resistance (16) and weed control (17). These studies confirm the conclusion that tolerance to glyphosate is stably inherited and that there are no unexpected plant pest risks or other risks posed to the environment. Furthermore, RR soybean event 40-3-2 has been crossed with conventional soybean varieties to produce over one thousand Roundup Ready soybean varieties. These RR soybean varieties have been in commercial production on over 100 million acres (40 million hectares) globally since 1996. No unusual plant pest characteristics or unintended environmental effects have been observed that are attributed to the inserted event, as confirmed by the extensive studies developed prior to, and subsequent to, approval and market introduction. Agronomic performance has been as expected and tolerance to glyphosate has been uniform and consistent in these varieties (15-17).

5.0 Food and Feed Safety

5.1 Assessment of CP4 EPSPS DNA Segments

Roundup Ready soybean event 40-3-2 has been approved for planting and/or consumption in a variety of countries worldwide. An assessment of the food and feed safety data developed for soybeans containing the 40-3-2 event established that these soybeans are as safe and nutritious as other commercial soybean varieties. Neither the presence of the 250 bp segment of CP4 EPSPS DNA proximal to the NOS transcriptional termination element at the 3' end of the primary insert nor the presence of the 72 bp segment of CP4 EPSPS DNA which comprises the second insert change the conclusion of this assessment for the following reasons: a) consumption of DNA and RNA *per se* is generally recognized as safe by food safety experts, including the FDA (18) and the FAO/WHO (19); b) the studies were completed on soybeans that contained both segments of CP4 EPSPS DNA (8); c) results of compositional and nutritional analyses established that these soybeans are comparable to other soybean varieties and are not affected by the insertion of DNA from plasmid PV-GMGT04 (4, 5, 20, 21); d) animal feeding studies showed that RR soybeans are as safe and as nutritious as traditional soybeans (22); e) neither segment of CP4 EPSPS DNA represents a functional gene; and f) the safety of CP4 EPSPS protein was established by assessing enzymatic function, history of safe use, digestibility, toxicity and allergenicity (3-6).

5.2 Potential for Pleiotropic Effects

Extensive studies have been conducted with RR soybean event 40-3-2 which demonstrate that there are no unexpected or pleiotropic effects associated with the DNA insertions in this product. Comparisons of the agronomic characteristics of event 40-3-2 with conventional soybeans show no differences in phenotype, yield or other measured parameters (Table 1). No differences in composition were shown in numerous comparisons of Roundup Ready soybeans to conventional soybeans (4, 5, 20). The endogenous allergens of soybeans were not altered by the insertion events (21). No nutritional or functional differences were detected in the animal feeding studies described below (22).

Each of the studies conducted to assess the potential for pleiotropic effects (Table 1 and Table 2), including more recent compositional and crop performance comparisons, supports the conclusion that there are no unexpected effects from the insertion of either the primary or the secondary inserts; and that these soybeans are agronomically, compositionally and nutritionally comparable to conventional soybeans, except for the Roundup Ready trait. In addition, peer reviewed compositional analyses published recently (23-25) have confirmed equivalence of RR soybeans to traditional varieties, thereby confirming the lack pleiotropic effects in RR soybean event 40-3-2.

5.3 Animal Feeding Studies

A series of animal feeding studies were completed previously using diets incorporating seed or processed fractions from RR soybean event 40-3-2. These studies address the nutritional equivalence event 40-3-2 when used as animal feed, the safety of any expressed protein or peptide (or any other newly produced constituent), the potential of any pleiotropic effect caused by the insertion process or site of insertion, and any other constituent that would result from the plasmid PV-GMGT04.

The animal feeding studies included two independent four week studies in rats (one with unprocessed and one with processed soybeans), a four week dairy cow study, a six week chicken study, a ten week catfish study and a five day quail study. Animals were fed either unprocessed or processed soybeans (dehulled, defatted, toasted). Included in these studies were control groups fed a non-commercial RR soybean event (61-67-1) and the non-modified parental soybean line (A5403) from which both Roundup Ready events were derived. Results from all groups were compared using conventional statistical methods to detect differences between groups in measured parameters.

All three soybean samples tested provided similar growth and feed efficiency for rats, chickens, catfish and quail (22). Milk production, composition and rumen fermentation parameters for dairy cows were also comparable across all groups (22). Results for other parameters measured in each feeding study were also similar across all groups. When compared to the US population as a whole, the levels of soybean consumption in these animal feeding studies were 100 fold or more higher than the average human daily consumption of soybean-derived foods in the US (26), where significant consumption has occurred since the introduction of RR soybeans into the marketplace in 1996.

These studies all confirmed the food and feed safety and nutritional equivalence of diets from RR soybean event 40-3-2 to diets from the control soybean varieties. If the presence of the 250 bp segment of CP4 EPSPS DNA at the 3' end of the primary insert or the presence of the 72 bp segment of CP4 EPSPS DNA comprising the secondary insert could result in gene function or create pleiotropic effects, no measurable effects were observed. The nutritional value or wholesomeness of RR soybean event 40-3-2, even when fed to animals at levels much higher than humans would encounter in the diet, was the same as conventional varieties of soybeans.

5.4 Applicability of Data to Fragments of CP4 EPSPS Protein

If a protein or peptide would be unexpectedly produced from the 250 bp segment of CP4 EPSPS DNA proximal to the NOS transcriptional termination element at the 3' end of the primary insert or from the 72 bp segment of CP4 EPSPS DNA located on the 937 bp *Hin*d III genomic DNA restriction fragment, these proteins or peptides would contain amino acid sequences of the CP4 EPSPS protein. The safety of such products has already been addressed — bioinformatics, digestive fate and acute oral toxicity studies performed

with CP4 EPSPS protein establish that CP4 EPSPS protein and its proteolytic fragments are not homologous to toxins or allergens, are rapidly digested and are non-toxic (3-7). This conclusion was made on the basis that bioinformatics assessments include the entire sequence of a protein, including all segments or fragments; and on the basis that protein digestion *in vitro* and *in vivo* creates a diverse (and transient) population of protein fragments. Thus, during safety and nutritional feeding studies, as the result of protein digestion, animals would have been exposed to both full-length CP4 EPSPS and its proteolytic fragments.

The digestive fate study showed that the CP4 EPSPS protein and its proteolytic fragments generated in simulated gastric and simulated intestinal fluids were rapidly degraded. These data, combined with validation data from these digestibility assays, and data from the amino acid sequence comparisons described above, support the conclusion that CP4 EPSPS sequences do not pose risks relative to the toxicity and allergenicity of potentially produced proteins or protein fragments (6).

The acute oral mouse gavage study performed with CP4 EPSPS protein showed that the CP4 EPSPS protein was not orally toxic. This was expected due to the long history of safe consumption of homologous EPSPS proteins in plants, fungi and microbes. Furthermore, the long history of safe consumption and the acute oral mouse gavage study also establish that proteolytic fragments of the CP4 EPSPS protein are safe for consumption (6).

6.0 Conclusions

As previously concluded, a single copy of the gene cassette encoding glyphosate tolerance (CP4 EPSPS) is present in RR soybean event 40-3-2. Detailed data confirm that no unexpected gene expression products (mRNA and proteins) or unexpected changes in other components of nutritional or safety importance are produced as a result of the presence of the 72 bp secondary insert nor the presence of the 250 bp segment of CP4 EPSPS DNA at the 3' end of the NOS termination sequence of the primary insert in RR soybean event 40-3-2. In addition, a review of the safety assessment of RR soybean event 40-3-2 confirms that RR soybean event 40-3-2 is as safe and as nutritious as conventional soybean varieties, and does not pose a plant pest risk or otherwise pose a risk to the environment. Factors included in this assessment were: the safety of the genetic elements contained on the transformation vector used to produce event 40-3-2; the history of safe use of the EPSPS family of proteins present in all plants, fungi and bacteria; the functionality and safety assessments of the CP4 EPSPS protein; the assessment of the compositional and nutritional properties of event 40-3-2 (comparing the key nutrients, anti-nutrients and allergens to the parental line and conventional soybeans); by comparing crop agronomic characteristics of event 40-3-2 to the parental and conventional soybeans; and by comparing the toxicological and nutritional characteristics of event 40-3-2 to parental and conventional soybeans in animal feeding studies.

Furthermore, because the newly described sequences were a constituent in the Roundup Ready soybeans used in all the safety assessment studies which addressed the food, feed and environmental safety of event 40-3-2, the recent characterization of these sequences using more sensitive and precise methods than were available for the initial assessment does not alter the conclusion that RR soybean event 40-3-2 is as safe as conventional soybeans. Any risks associated with the production and consumption of RR soybean event 40-3-2 to human health or the environment are no different than those associated with conventional soybean varieties.

Table 2 Analyses conducted for the food and feed safety assessment for RR soybean event 40-3-2 (3)

Safety of the CP4 EPSPS protein

Function and enzyme specificity
Similarity to the family of EPSPS proteins with a long history of safe use and consumption
Lack of amino acid sequence similarity to toxins and allergens
Accumulation levels in various tissues and processed products
Digestibility *in vitro*
Acute oral toxicity in mice

Compositional and Nutritional Analyses

Whole soybeans
- proximate analysis
- amino acid composition
- fatty acid composition
- trypsin inhibitor levels
- lectin levels
- phytoestrogen levels
- urease activity

Toasted Meal
- proximate analysis
- trypsin inhibitor levels
- lectin levels
- phytoestrogen levels
- urease activity
- stachyose & raffinose levels
- phytate levels
- N-solubility

Defatted Flour
- proximate analysis
- trypsin inhibitor levels
- urease activity

Protein Isolate
- proximate analysis

Protein Concentrate
- proximate analysis

Refined, Bleached, Deodorized Soybean Oil
- fatty acid composition

Animal Feeding Studies

Duration	*Test Material*	*Animal*
4 week	Processed soy meal	Sprague-Dawley Rat
4 week	Unprocessed soy meal	Sprague-Dawley Rat
6 week	Processed soy meal	Broiler Chicken
4 week	Cracked whole beans	Dairy Cow
10 week	Processed soy meal	Catfish
5 days	Unprocessed soy meal	Bobwhite Quail

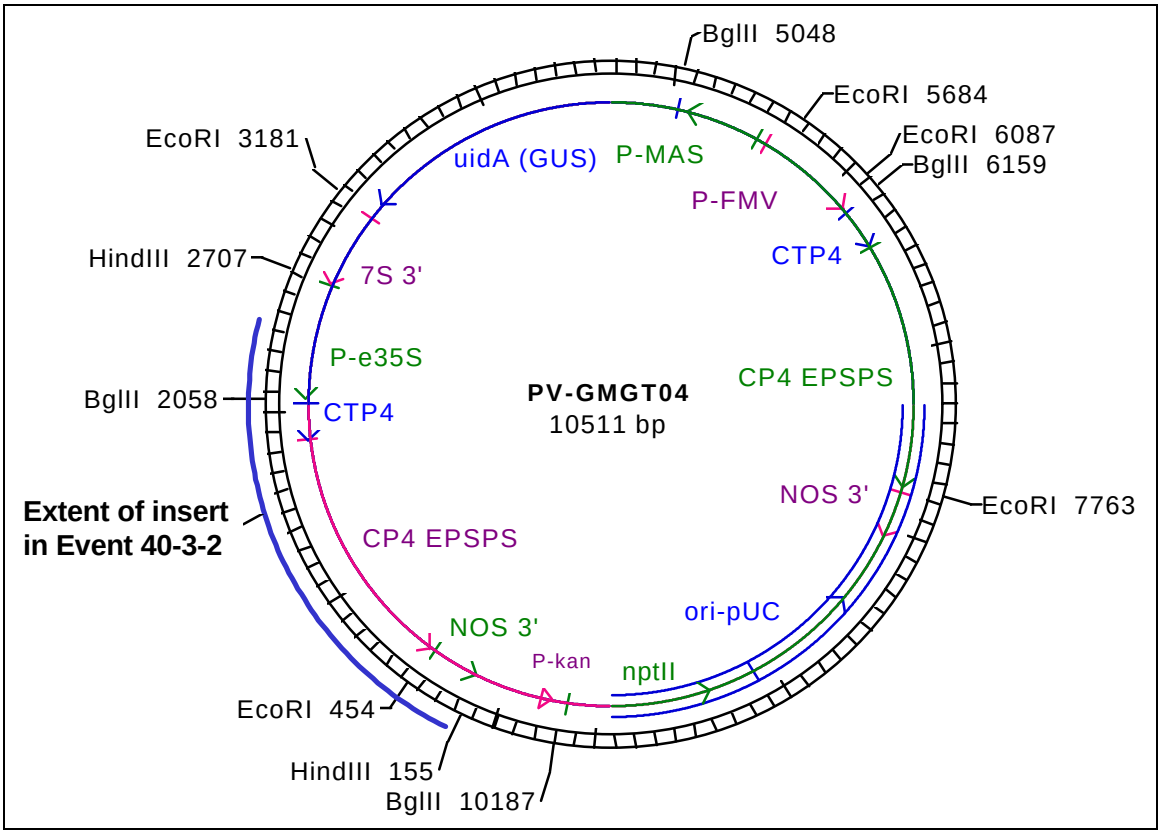

Figure 1 **Plasmid map including genetic elements of vector PV-GMGT04 used in the transformation of RR soybean event 40-3-2.**

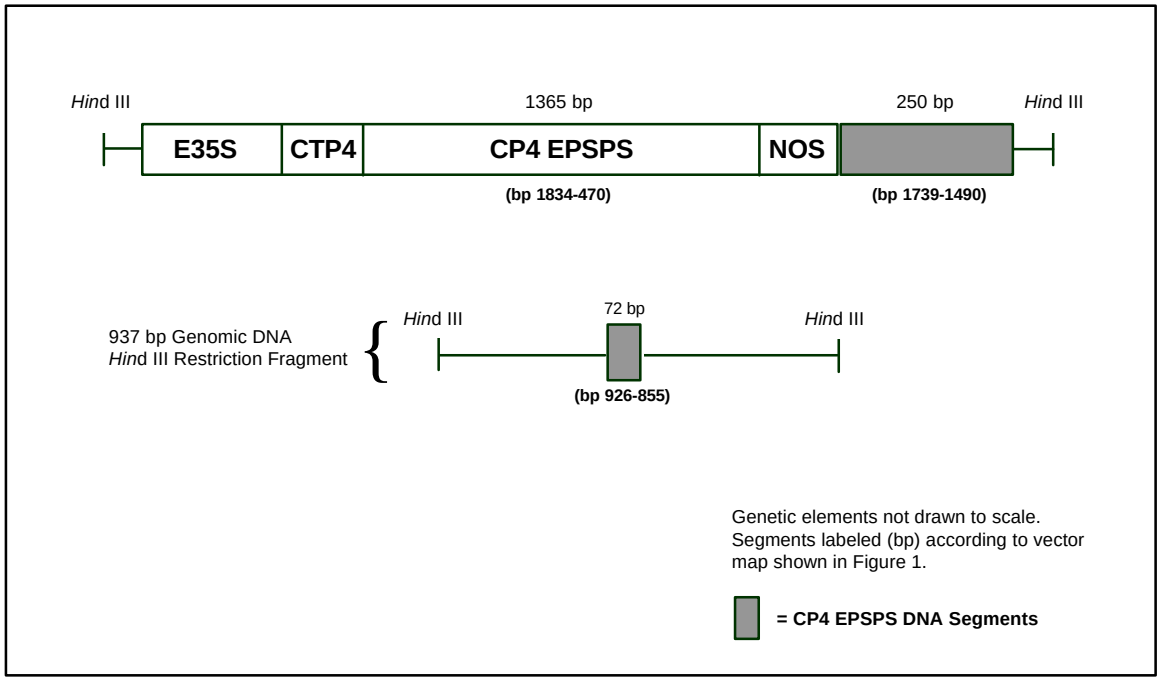

Figure 2 Updated molecular characterization of RR soybean event 40-3-2.

E35S - 35S transcription promoter
CTP4 - Chloroplast targeting sequence (polypeptide)
CP4 EPSPS - the CP4 EPSPS open reading frame (protein)
NOS - The nopaline synthase 3' transcription terminator

7.0 References

1. Franz, J.E., Mao, M.K., and Sikorski, J.A. (1997) *Glyphosate: A Unique Global Herbicide*. ACS Monograph 189. American Chemical Society, Washington D.C., pp 1-653.

2. Padgette, S.R., Kolacz, K.H., Delannay, X., Re, D.B., LaValee, B.J., Tinius, C.N., Rhodes, W.K., Otero,. Y.I., Barry, G.F., Eichholtz, D.A., Peschke, V.M., Nida, D.L., Taylor, N.B., and Kishore, G.M. (1995). Development, indentification, and characterization of a glyphosate-tolerant soybean line. *Crop Science* **35**: 1451-1461.

3. Re, D.B., Padgette, S.R., Delannay, X., Kolacz, K., Nida, D.L., Peschke, V.M., Derting, C.W. Rogers, S.G., Edwards, J.W., Barry, G.F., and Biest, N.A. (1993) Petition for Determination of Nonregulated Status: Soybeans with a Roundup Ready Gene, submitted to USDA-APHIS.

4. Padgette, S.R., Re, D.B., Hammond, B.G., Fuchs, R.L., Rogers, S.R., Harrison, L.A., Nida, D.L., naylor, M.W., Kolacz, K.H., Taylor, N.B., and Ream, J.E. (1994) *Safety, Compositional and Nutritional Aspects of Glyphosate-tolerant Soybeans: Conclusions Based on Studies and Information Evaluated According to FDA's Consultation Process.* Monsanto Company, St. Louis, Missouri, pp 1-107.

5. USDA. (1994) Petition 93-258-01 for determination of nonregulated status for glyphosate tolerant soybean line 40-3-2: Environmental assessment and finding of no significant impact. *Federal Register* **59**: 26781-26782.

6. Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L., and Padgette, S.R. (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. *Journal of Nutrition* **126**: 728-740.

7. Padgette, S.R., Re, D.B., Barry, G.F., Eicholtz, D.E., Delannay, X., Fuchs, R.L., Kishore, G.M., and Fraley, R.T. (1996) New weed control opportunities: Development of soybeans with a Roundup Ready gene. *In* Duke, S.O. (ed.) *Herbicide-Resistant Crops. Agricultrual, Economic, Regulatory and Technical Aspects*. CRC Lewis Publishers., pp 53-84.

8. Lirette, R.P., Cavato, T.A., Reiser, S.E., Beazley, K.A., Borovkova, I.G., Higgins, M.R., Albee, L.E. and Dixon, D.A. (2000) Further characterization of glyphosate tolerant soybean event 40-3-2. Monsanto Technical Report: MSL-16646. St. Louis, Missouri.

9. Darnell, J., Lodish, H., and Baltimore, D. (1990) *Molecular Cell Biology, 2nd Edition*. Scientific American Books, New York, N.Y, p. 344.

10. Beebee, T. and Burke, J. (1988) Gene structure and transcription. *In:* Rickwood, D., and Male, D. (Series Eds.), *In Focus*. IRL Press, Oxford. pp. 1-77.

11. Rogan, G.J., Dudin, Y.A., Lee, T.C., Magin, K.M., Astwood, J.D., Bhakta, N.S., Leach, J.N., Sanders, P.R., and Fuchs, R.L. (1999) Immunodiagnostic methods for detection of 5-enolpyruvylshikimate-3-phosphate synthase in Roundup Ready soybeans. *Food Control* **10**: 407-414.

12. Depicker, A., Stachel, S., Dhaese, P., Zambryski, P., and Goodman, P.M. (1982) Nopaline synthase: transcript mapping and DNA sequence. *Journal of Molecular and Applied Genetics* **1**: 561-573.

13. Garcia-Rios, M., Fujita, T., LaRosa, P.C., Locy, R.D., Clithero, J.M., Bressan, R.A., and Csonka, L.N. (1997) Cloning of a polycistronic cDNA from tomato encoding gamma-glutamyl kinase and gamma-glutamyl phosphate reductase. *Proceedings of the National Academy of Sciences* **94**: 8249-8254.

14. Futterrer, J. and Hohn, T. (1996) Translation in plants -rules and exceptions. *Plant Molecular Biology* **32**: 159.

15. Delannay, X., Bauman, T.T., Beighley, D.H., Buettner, M.J., Coble, H.D., DeFelice, M.S., Derting, C.W., Diedrick, T.J., Griffin, J.L., Hagood, E.S., Hancock, F.G., Hart, S.E., LaVallee, B.J., Loux, M.M., Lueschen, W.E., matson, K.W., Moots, C.K., Murdock, E., Nickell, A.D., Owne, M.D.K., Pashal, E.H., Prochaska, L.M., Raymond, P.J., Reynolds, D.B., Rhodes, W.K., Roeth, F.W., Sprankle, P.L., Tarochione, L.J., Tinius, C.N., Walker, R.H., Wax, L.M., Weigelt, H.D., and Padgette, S.R. (1995) Yield evaluation of a glyphosate-tolerant soybean line after treatment with glyphosate. *Crop Science* **35**: 1461-1467.

16. Sanogo, S., Yang, X.B., and Scherm, H. (2000) Effects of Herbicides on *Fusarium solani* f. sp. *glycines* and development of sudden death syndrome in glyphosate-tolerant soybean. *Phytopathology* **90**:57-66.

17. Nelson, K.A. and Renner, K A. (1999) Weed management in wide- and narrow-row glyphosate resistant soybean. *Journal of Production Agriculture* **12**:460-465.

18. United States Food and Drug Administration. (1992) Foods derived from new plant varieties. *Federal Register* **57**: 22984-23005.

19. FAO/WHO (1996) *Biotechnology and Food Safety*. The Food and Agriculture Organization and The World Health Organization, Geneva, pp 1-27.

20. Padgette, S.R., Taylor, N.B., Nida, D.L., Bailey, M.R., MacDonald, J., Holden, L.R., and Fuchs, R.L. (1996) The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. *Journal of Nutrition* **126**: 702-716.

21. Burks, A.W. and Fuchs, R.L. (1995) Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. *Journal Allergy Clinical Immunology* **96**: 1008-1010.

22. Hammond, B.G., Vicini, J.L., Hartnell, G.F., Naylor, M., Knight, C.D., Robinson, E.H., Fuchs, R.L., and Padgette, S.R. (1996) The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *Journal of Nutrition* **126**: 717-727.

23. List, G.R., Orthoefer, F., Taylor, N., Nelsen, T., and Abidi, S.L. (1999) Characterization of phospholipids from glyphosate-tolerant soybeans. *Journal of the American Oil Chemsists Society* **76**: 57-60.

24. Taylor, N.B., Fuchs, R.L., MacDonald, J., Shariff, A.R., and Padgette, S.R. (1999) Compositional analysis of glyphosate-tolerant soybeans treated with glyphosate. *Journal of Agricultural and Food Chemistry* **47**: 4469-4473.

25. Lappe, M.A., Bailey, E.B., Childress, C., and Setchell, K.D.R. (1999) Alterations in clinically important phytoestrogens in genetically modified, herbicide-tolerant soybeans. *Journal of Medicinal Food* **1**:241-245

26. USDA (1993) *United States Department of Agriculture Continuing Surveys of Food Intake by Individuals (CSFII)* 1989-1992.