93-258-01p

Monsanto

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September 14, 1993

Mr. Michael A. Lidsky
Deputy Director, BBEP, APHIS, USDA
6505 Belcrest Road
Federal Building
Hyattsville, MD 20782

Subject: Petition for Determination of Nonregulated Status:
Soybeans with a Roundup Ready™ Gene
Monsanto# 93-089U

Dear Mr. Lidsky:

The Agricultural Group of Monsanto Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding soybeans with a Roundup Ready™ gene. This petition requests a determination from APHIS that the glyphosate-tolerant soybean (GTS) line 40-3-2 and any progenies derived from crosses between line 40-3-2 and traditional soybean varieties no longer be considered a regulated article under regulations in 7 CFR part 340. GTS line 40-3-2 has been field tested at multiple locations since 1991 (USDA# 91-018-01, 91-151-01, 92-007-01, 92-007-02, 92-015-01, 92-037-02, 92-037-06, 92-041-01, and 92-055-01). Copies of the final reports for these field release permits are included in the petition. We have also included copies of the references cited within the petition for your convenience.

Please feel free to contact either Dr. W. M. Strauss (202-783-2460) or myself (314-537-6385) if you need any additional information.

Sincerely,

Diane B. Re

Diane B. Re
Regulatory Affairs Manager

cc: Dr. W. M. Strauss

Petition for Determination of Nonregulated Status:

Soybeans with a Roundup Ready™ Gene

The undersigned submits this petition of 7 CFR 340.6 to request that the Director, BBEP, make a determination that the article should not be regulated under 7 CFR part 340.

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Contains No Confidential Business Information

Summary

The Agricultural Group of Monsanto Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding soybeans with a Roundup Ready™ gene. This petition requests a determination from APHIS that the glyphosate-tolerant soybean (GTS) line 40-3-2 and any progenies derived from crosses between line 40-3-2 and traditional soybean varieties no longer be considered a regulated article under regulations in 7 CFR part 340.

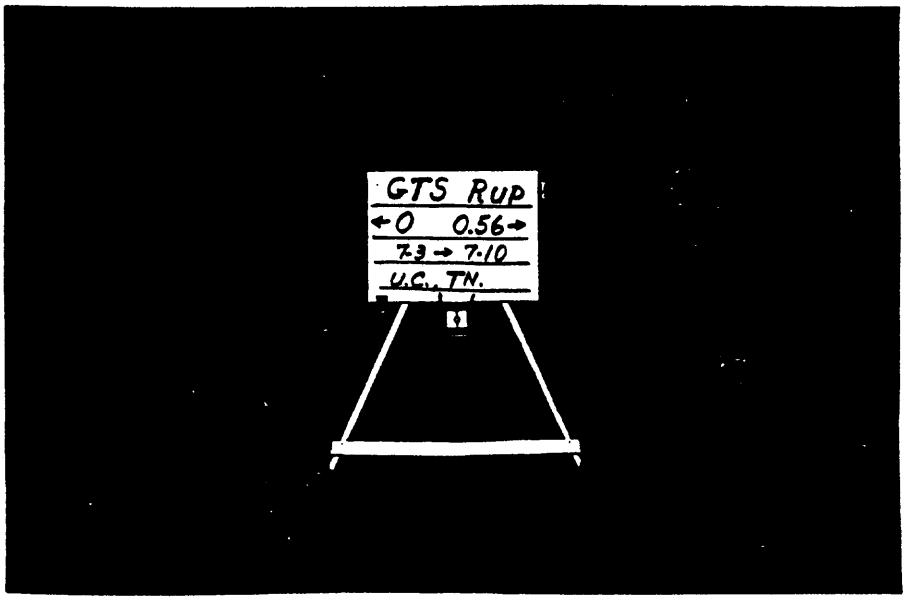
Glyphosate, the active ingredient in Roundup® herbicide, controls weeds due to inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS). EPSPS is an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants (including soybeans) and microorganisms, and is thus ordinarily present in food derived from plant sources. The aromatic amino acid pathway is not present in mammalian, avian, or aquatic lifeforms. Hence glyphosate is only toxic to plants but not other living species, including mammals.

The soybean line for which this determination is requested, GTS line 40-3-2, contains a gene which encodes a glyphosate-tolerant EPSP synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). EPSPSs from a number of bacteria exhibit tolerance to glyphosate. Soybean plants tolerant to glyphosate were produced by stably inserting CP4 EPSPS into the genome of soybean cultivar A5403. Based on polymerase chain reaction (PCR) and Southern blot analysis, it was found that GTS line 40-3-2 contains a single insert of DNA, and that this insert contains the E35S promoter, the petunia EPSPS chloroplast transit peptide, the CP4 EPSPS gene, and the NOS 3' terminator. Upon glyphosate treatment, the soybean plants expressing the CP4 EPSPS are unaffected since the continued action of the glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic compounds.

Soybean plants containing the glyphosate tolerance gene for soybean production would enable the farmer to utilize Roundup herbicide for control of weed pests and take advantage of this herbicide's well-known, very favorable environmental and safety characteristics. GTS can positively impact current agronomic practices in soybean by 1) offering the farmer a new wide-spectrum weed control option; 2) allowing the use of an environmentally sound herbicide; 3) providing a new herbicidal mode of action for in-season soybean weed control; 4) increasing flexibility to treat weeds on an "as needed" basis; 5) offering less dependence on herbicides used before planting; 6) providing an excellent fit with no-till systems, which results in increased soil moisture, while reducing soil erosion and fuel use; and 7) providing cost-effective weed control, not only because Roundup herbicide may be less expensive than most current options, but because total herbicide use may be reduced compared to the

farmer's current weed management program.

GTS line 40-3-2 has been field tested since 1991 at approximately 55 locations under field release permits granted by APHIS (USDA# 91-018-01, 91-151-01, 92-007-01; 92-007-02, 92-015-01, 92-037-02, 92-037-06, 92-041-01, and 92-055-01) and is currently being tested at approximately 90 locations across the U.S. (USDA# 92-335-01, 92-350-01, 92-359-01, 93-011-03, 93-011-04, 93-012-05, 93-012-06, 93-012-07, 93-026-01, and 93-078-01). Data collected from these trials, literature references, and expert opinion letters presented in the following petition demonstrate that the GTS line 40-3-2: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than the non-modified parental varieties; 3) is unlikely to increase the weediness potential for any other cultivated plant or native wild species; 4) does not cause damage of processed agricultural commodities; and 5) is unlikely to harm other organisms that are beneficial to agriculture. Therefore, the Agricultural Group of Monsanto Company requests a determination from APHIS that the GTS line 40-3-2 and any progenies derived from crosses between line 40-3-2 and traditional soybean varieties no longer be considered a regulated article under regulations in 7 CFR part 340.



Pictured are soybeans with a Roundup Ready™ gene. On the right, Roundup controlled a variety of problem weeds, while the unsprayed row on the left shows the kind of weed pressure soybeans face without effective control. The improved soybeans thrived despite the presence of Roundup.

Certification

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

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ABBREVIATIONS AND SCIENTIFIC TERMS

CaMV - cauliflower mosaic virus
CP4 EPSPS - EPSPS from *Agrobacterium* sp. strain CP4
CTP - chloroplast transit peptide
ELISA - enzyme-linked immunosorbant assay
EPSP synthase (EPSPS) - 5-enolpyruvyl-shikimate-3-phosphate synthase
GTS - glyphosate-tolerant soybeans
GUS - ß-glucuronidase
KAN - kanamycin
LAC - ß-d-galactosidase
MAS - mannopine synthase
NOS - nopaline synthase
nptII - neomycin phosphotransferase gene
OD - optical density
oz/A - ounces/acre
PEPSPS - petunia EPSPS
PCR - polymerase chain reaction
SCN - soybean cyst nematode

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I. Rationale for Development of Glyphosate-tolerant Soybean

A. Rationale

Soybean, *Glycine max*, is one of the world's largest plant sources of protein and oil. The soybean plant is a bushy, green legume which farmers plant in late spring, with pods developing in late summer. In 1829, U.S. farmers grew soybeans for soy sauce and until the mid-1940's soybean was used mainly as a forage crop. The establishment of soybean as a major crop in the United States started in 1940 with incentives due to World War II (Norman, 1978). Today, more soybeans are grown in the United States than anywhere else in the world. Farmers in over 29 states grow soybeans, making soybeans the third largest U.S. cash crop (American Soybean Association, 1991). The technology and yield of soybean production in the United States has steadily advanced. According to the American Soybean Association, 57.7 million acres of soybeans were planted in 1990 in the United States, yielding an average of 34 bushels/acre. Yields have increased due to the development of new cultivars, the availability of better field equipment, and the use of selective herbicides that have greatly reduced weed competition.

Several herbicides are currently available to the farmer for weed management in soybeans. Weed management is a critical step to maximize soybean yields and retain a high-quality, weed-free harvest. For effective weed control, the farmer typically selects a herbicide based on several factors: weed spectrum, lack of crop injury, cost, and environmental characteristics. Few herbicides available today deliver optimal performance in all of these areas. Several classes of herbicides are effective for broad-spectrum weed control, but most are either non-selective and kill crop plants or they significantly injure some crops at the application rates required for sufficient weed control.

One such broad spectrum, non-selective herbicide is glyphosate, the active ingredient in Roundup® herbicide (Baird et *al.*, 1971). Glyphosate is the world's largest-selling herbicide, primarily due to its excellent weed control capabilities and its well-known, very favorable environmental and safety characteristics. Recent advances in plant biotechnology have made it possible to insert a gene into soybeans in order to provide crop safety specifically to glyphosate (Gasser *et al.*, 1989; Hinchee, *et al.*, 1992; Padgette *et al.*, 1989; Mazur *et al.*, 1989; Kishore *et al.*, 1988a, b).

The use of glyphosate-tolerant soybeans (GTS) provides an attractive alternative to farmers who wish to increase their options for effective weed control. Roundup herbicide is a foliar-applied, broad spectrum, non-selective, post-emergent herbicide (Baird *et al.*, 1971; Malik *et al.*, 1989). It is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate has favorable environmental features, such as rapid soil binding (resistant to leaching) and biodegradation (which decreases persistence), and extremely low toxicity to mammals, birds and fish (Malik *et*

al., 1989). Recently, glyphosate has been classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739). The use of soybean plants containing a glyphosate tolerance gene for soybean production would enable the farmer to utilize Roundup herbicide for control of weed pests and take advantage of this herbicide's well-known, very favorable environmental and safety characteristics. GTS can positively impact current agronomic practices in soybean by 1) offering the farmer a new wide-spectrum weed control option; 2) allowing the use of an environmentally sound herbicide; 3) providing a new herbicidal mode of action for in-season soybean weed control; 4) increasing flexibility to treat weeds on an "as needed" basis; 5) offering less dependence on herbicides used before planting; 6) providing an excellent fit with no-till systems, which results in increased soil moisture, while reducing soil erosion and fuel use; and 7) providing cost-effective weed control, not only because Roundup herbicide may be less expensive than most current options, but because total herbicide use may be reduced compared to the farmer's current weed management program.

GTS provides an excellent broad-spectrum weed control alternative to farmers. Currently, farmers are using up to 4 to 5 different herbicide families to manage soybean weed problems. One or possibly two treatments of Roundup herbicide at 24 to 32 oz/A will control both annual and perennial weeds which would reduce the time, cost (herbicide and application), and number of herbicide treatments per acre. GTS will also encourage farmers to plant narrow-row (<10 inch) soybeans. University yield data from the north and south indicates a yield increase from planting narrow-row soybeans. However, good weed control is generally difficult under narrow-row cultural practices. GTS will help farmers plant narrow-row soybeans more efficiently and take advantage of increased yields and decreased costs.

More than 150 glyphosate-tolerant soybean lines have been produced and sprayed with Roundup herbicide in order to evaluate tolerance. These lines were originally screened in the greenhouse and rated on the basis of vegetative tolerance. Progeny rows of the best lines were evaluated in the field (USDA# 91-018-01) in 1991 at 24, 48, and 64 oz/A of Roundup herbicide. The commercial use rate of Roundup herbicide for GTS is anticipated to be 24 to 32 oz/A. Several lines were identified which showed no visual damage from the herbicide at any of the selected rates and were yield tested at 18 locations across the United States (USDA# 92-041-01) in 1992. Commercial levels of tolerance were demonstrated in the 1992 yield evaluations. The commercial GTS product will be the result of traditional backcrossing of the glyphosate-tolerance locus to other varieties and maturity groups of soybeans.

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II. The Soybean Family

Description of the Soybean and its Production in the U.S.
Reid G. Palmer, USDA-ARS-FCR

A. History of the Soybean

The soybean is a native to China. Early Chinese history refers to soybeans in books written over 4500 years ago (Hymowitz and Singh, 1987). Soybean was introduced into the USA in 1765 (Hymowitz and Harlan, 1983). Soybean was primarily a forage crop and grown for hay and silage. Successful use of soybean as an oilseed in Europe from 1900 to 1910 promoted interests in its use in the USA. Even though interest in soybean production was on the increase during the 1920's and 1930's, most soybean acres were used for forage. The first cultivars selected from planned cross-pollinations were released in the 1940's. Cultivars selected from the first populations formed by hybridization were used as parents to form populations for additional cycles of selection. The process of utilizing superior progeny from one cycle of selection as parents to form populations for the next cycle continues up to the present time (Burton, 1987).

The USA continues to produce the largest percentage of soybeans in the world but Brazil and Argentina have increased production in recent years and are major exporters (Smith and Huyser, 1987). Soybean production regions in the USA are concentrated in the Midwest and in the Mississippi Valley (Hazera and Fryar, 1981). Production in the USA is forecast at 2.08 billion bushels as of September 1, 1992 (Anonymous, 1992).

B. Taxonomy of the Genus Glycine

The genus Glycine Willd. is a member of the family Leguminosae, subfamily Papilionoideae, and the tribe Phaseoleae. The genus Glycine is of Asian and Australian origin (Lackey, 1981). Glycine is divided into two subgenera, Glycine and Soja (Moench) F. J. Herm. The subgenus Glycine consists of 12 wild perennial species (Hymowitz *et al.*, 1992). These species have wide distribution patterns: Australia, South Pacific Islands, West Central Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan (Hermann, 1962; Newell and Hymowitz, 1978; Hymowitz and Newell, 1981; Grant *et al.*, 1984a, b; Tindale, 1984, 1986 a, b). The subgenus Soja includes the cultivated soybean, G. max (L.) Merrill, and its nearest wild relative, G. soja Sieb. and Zucc., that has been found in China, Taiwan, Japan, Korea, and the former USSR. Both of these species are annuals.

C. Genetics of the Soybean

The genetic structure, and crossability of Glycine species are important considerations in the flow of genes from cultivated to wild annual and to wild perennial species.

Glycine is the only genus in the Phaseoleae where species have diploid chromosome numbers of 40 and 80 but not 20. The unique chromosome number of Glycine is probably derived from diploid ancestors with base number 11, which have undergone aneuploid loss to base number 10 (Lackey, 1988). In the legumes, only 10 of 71 genera are considered completely polyploid and Glycine was considered polyploid (Senn, 1938). The soybean should be regarded as a stable tetraploid with diploidized genomes (Gurley *et al.*, 1979; Lee and Verma, 1984; Skorupska *et al.*, 1989).

During the past decade, extensive cytogenetic studies have been conducted to establish the phylogenetic relationships among the wild perennial species of subgenus Glycine. Intraspecific hybrids within the diploid perennial Glycine species show normal meiosis and are fertile. Hybrids between diploid species having different genome designations produce inviable seed, lethal seedlings, stunted and slow-growing plants that die within a few months or completely sterile plants (Singh and Hymowitz, 1985). Observations on geographical distribution, cytotypes, crossability, and meiotic chromosome behavior in intra- and interspecific F_1 hybrids revealed that various genome complexes evolved through allopolyploidization (Hymowitz *et al.*, 1992).

Species relationships in the subgenus Soja indicated that F_1 hybrids of G. max and G. soja carry similar genomes and are completely fertile or differ by a single reciprocal translocation (Palmer *et al.*, 1987). A form known as G. gracilis, a semi-cultivated or weedy form, known only from Northeast China is somewhat intermediate in morphology between G. max and G. soja (Skvortzov, 1927).

From a taxonomic standpoint, both the wild annual species of subgenus Soja and the wild perennial species of subgenus Glycine are candidates for gene exchange with the cultivated soybean, and therefore potentially useful for broadening the germplasm base of the cultivated soybean. Interspecific hybrids between G. max and G. soja can easily be made. Intersubgeneric hybrids between G. max and the wild perennial Glycine species have been obtained (see Hymowitz *et al.*, 1992 and Hymowitz and Singh, 1992 for reviews). All intersubgeneric hybrids were obtained through in vitro seed culture. The F_1 hybrids have however generally been sterile, and further progeny has been obtained only in a few cases and with great difficulty.

D. Life Cycle of the Soybean

Following vegetative development, the plant enters the reproductive stage during which axillary buds develop into flower clusters. The perfect, polypetalous, zygomorphic flower is approximately 6 mm in diameter when fully opened. The corolla consists of five distinct petals. The largest is posterior (standard), the two next in size (wings) are lateral, and the two keel

petals are obliquely anterior (Carlson and Lersten, 1987).

The androecium consists of 10 diadelphous stamens, all of which are separate at first, but later the filaments of nine of them are elevated as a single structure by the pushing up of a basal region of meristematic tissue, leaving the posterior stamen separate. The style, which is about half the length of the ovary, curves backward toward the posterior stamen and is surmounted by a knob-like stigma which is receptive for pollen at the time the flower opens (Carlson and Lersten, 1987).

The ovule of soybean has two integuments, and both ovule and embryo sac are bent back on themselves. As many as four ovules may be formed. The two polar nuclei fuse before fertilization to form a single large diploid secondary nucleus within the large central cell and in close proximity to the egg apparatus (Carlson and Lersten, 1987).

By the time of pollination, the diadelphous stamens have been elevated so that the anthers form a ring around the stigma. The pollen is shed directly on the stigma, resulting in a very high percentage of self-fertilization (Guard, 1931).

E. Hybridization

Flowers of the female parent are at the proper stage for artificial hybridization one day before anthesis (Fehr, 1987). Emasculation of the female parent is not required since the pollen matures as much as one day after the female is receptive (Walker *et al., 1979).*

Various studies have shown from 0.03 to 3.62% cross-pollination under field conditions (Beard and Knowles, 1971; Caviness, 1966; Garber and Odland, 1926; Weber and Hanson, 1961; Woodworth, 1922). Cross-pollination frequencies vary due to growing season, genotypes, and location of male parent in relation to female parent but are typically less than 1%. Boerma and Moradshahi (1975) reported that most outcrossing occurred with surrounding plants. Nelson and Bernard (1984) reported that a buffer area of approximately 10 meters of soybeans should eliminate almost all pollen contamination into a male-sterile intermating block in southern Illinois.

F. Potential for Outcrossing

1. Outcrossing with wild species

The only wild species that could cross with the cultivated soybean are members of the genus Glycine. No other genus is closely enough related to soybean to allow for the possibility of outcrossing (Hymowitz *et al.* 1992). Therefore, the discussion will concentrate on species of genus Glycine.

a. Hybridization with wild perennial species of subgenus Glycine

The only opportunities for hybridization would occur in Australia, South Pacific

Islands, West Central Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan where the wild perennial species are endemic. Soybean production in these areas is mostly in China and Australia. There are no known reports of successful natural hybridization between the cultivated soybean and the wild perennial species. Thus the possibility of gene transfer is non-existent because hybridization does not occur without in vitro seed culture. Even in those cases, the F_1 plants obtained are generally sterile.

b. Hybridization with the wild annual species of subgenus Soja
The wild annual species, G. soja is found in China, Taiwan, Japan, Korea, and the former USSR. Natural hybridizations between G. soja and the cultivated soybean occurs (Kwon, 1972). In fact, the semi-wild form, intermediate in many phenotypic traits between G. max and G. soja, has been recognized as G. gracilis (Skvortzov, 1927). G. soja is not native to North America and occurs only in research plots. There are no reports of its escape or dispersal from research plots. G. soja has never been found as a weed or naturalized in the USA. Thus the possibility of gene transfer is very low within the United States.

2. Outcrossing to the cultivated soybean
Hybridization among cultivated soybeans is generally less than 1%. Insect activity does increase the outcrossing rate but soybeans are not a preferred plant (Erickson, 1975, 1984). Male-sterile, female-fertile mutants are used in breeding studies but it is very unlikely that chance pollination with transformed soybeans would occur (Burton, 1987). In any case, soybean seeds generally do not survive the winter, and soybean does not establish itself as a volunteer weed in other crops (Appendix III. Expert Opinion Letters). Even if survival occurred, the plants could easily be controlled by a variety of agronomic techniques commonly available.

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III. Description of the Transformation System and Plasmid Utilized

The soybean line for which this determination is requested, GTS line 40-3-2, contains a gene which encodes a glyphosate-tolerant EPSP synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). The plasmid PV-GMGT04, used to transform the parental line A5403 to generate line 40-3-2, contains three genes driven by plant promoters: two CP4 EPSPS genes and a gene encoding ß-glucuronidase (GUS) from *E. coli*. This plasmid was transformed into soybean line A5403 using the particle acceleration method (particle gun).

A. Particle Acceleration Transformation System

Introduction of DNA into the plant tissue by the particle acceleration method has been described previously (McCabe *et al,* 1988; Christou *et al.*, 1988). DNA is precipitated onto microscopic gold particles using a calcium phosphate solution, and dried down under a stream of nitrogen. The coated particles are resuspended in ethanol and spread onto a mylar carrier sheet. The mylar sheet is accelerated by the force of vaporization as 10-15 kilovolts are discharged across a water drop. The mylar hits a stainless steel retaining screen which stops the flight of the sheet but allows the continued flight of the DNA coated particles. The particles penetrate the target plant cells where the DNA is deposited and incorporated into the cell chromosome. The cells are incubated on a plant tissue culture medium containing cytokinin and auxin to induce multiple shoot formation. The shoots which develop from the transformed cells express the phenotype encoded by the genes on the delivered DNA. The DNA contains the chimeric plant expression genes and the ß-glucuronidase (GUS) marker gene (Jefferson *et al.*, 1986). The expression of the GUS gene is used as evidence of transformation. It is detected by a staining method in which the GUS enzyme converts a substrate (5-bromo-4-chloro-3-indolyl ß-D-glucuronide) into a blue precipitate. Plant tissue which produces the blue color after the histochemical reaction is expressing the GUS gene. The majority of the shoots which are regenerated from the shoot tip cells do not contain the gene, therefore GUS screening is necessary to identify the genetically modified tissue. The positive shoots are then grown to maturity, and the resulting plants are screened for glyphosate tolerance phenotype and gene expression.

B. Properties of the Non-transformed Cultivar, A5403

Glycine max L. cv. A5403 is the cultivar that was genetically modified to be tolerant to Roundup herbicide, and is a commercial variety of Asgrow Seed Company. A5403 is a maturity group V cultivar which combines a consistently high yield potential with resistance to races 3 and 4 of the soybean cyst nematode (SCN). It also combines good standability, excellent emergence, and tolerance to many leaf and stem diseases. A5403 was one of the first group V cultivars with SCN resistance provided to farmers and has received protection under the Plant Variety Protection Act (GTS line 40-3-2 maintains SCN resistance - see section V.D). It should be pointed out that the

commercialization strategy for GTS will be to use traditional backcrossing and breeding to transfer the glyphosate tolerance locus from this cultivar to a wide range of varieties and maturity groups of soybeans.

C. Construction of the Plasmid Utilized for Transformation, PV-GMGT04

PV-GMGT04 is a pUC-Kan vector delivered to the donor organism using the particle gun. This vector is a derivative of the high copy *E. coli* plasmid pUC119 (Vieira and Messing, 1987). The 1.3 Kb FspI-DraI pUC119 fragment containing the origin of replication was fused to the 1.3 Kb SmaI-HindIII Klenow-filled fragment from pKC7 (Rao and Rogers, 1979) which contains the neomycin phosphotransferase type II gene (*nptII*). The *nptII* confers bacterial kanamycin resistance and replaces the ampicillin resistance gene of pUC119. This *nptII* gene is driven by a bacterial promoter which contains bacterial signals which are different from those found in plants, making expression in plant cells unlikely.

The vector PV-GMGT04 is shown in Figure III.1. The description of the DNA elements in PV-GMGT04 is shown in Table III.1.

D. Descriptions of the Open Reading Frames Contained in PV-GMGT04, but not Transferred to Line 40-3-2

As will be described in detail in Section V.B, only a portion of the DNA sequence of PV-GMGT04 was inserted into line 40-3-2. This is because plasmid fragmentation occurs when using the particle acceleration transformation method. Following are the elements which are present on plasmid PV-GMGT04, but not present in the genome of line 40-3-2 (see Table III.1 for additional information on the individual elements):

1. ß-glucuronidase (GUS)

The GUS gene (*uidA*), which has been fully sequenced, was isolated from *E. coli*, a common organism present in the intestinal flora (Jefferson, 1985, 1987). GUS has been used as a scoreable marker in the transformation and regeneration of GTS, and is a 68 KD (monomer molecular mass) acid hydrolase that catalyzes the cleavage of several ß-glucuronides. GUS is an enzyme that has desirable properties for the construction and analysis of gene fusions, or in this case as a scoreable marker. There is little endogenous GUS activity in plants, and there are several easy detection assays available. This makes it a very versatile scoreable genetic marker.

2. Neomycin (kanamycin) phosphotransferase (*nptII*) (KAN)

The *nptII* gene was isolated from the Tn5 transposon, and is found throughout nature, often in soil microorganisms (Leff *et al.*, 1993; Van Elsas *et al.*, 1986).

3. PEPSPS-transit-CP4 EPSPS (driven by the CMoVb promoter)
As will be described in section V.B, the CP4 EPSPS gene copy in PV-GMGT04 driven by the CMoVb promoter was not transferred to the genome of GTS line 40-3-2. CP4 EPSPS will be described in section IV.A.

Table III.1 Summary of Sequences for PV-GMGT04

Genetic Element	Size, Kb	Function
P-E35S	0.61	The cauliflower mosaic virus (CaMV) promoter (Odell, *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1987).
CTP4	0.23	The N-terminal 0.23 Kb chloroplast transit peptide sequence from the *Petunia hybrida* EPSPS gene (Shah *et al.*, 1986).
CP4 EPSPS	1.36	The C-terminal 1.36 Kb 5-enolpyruvylshikimate-3-phosphate synthase gene (CP4 EPSPS) from an *Agrobacterium* species (Barry *et al.*, 1992).
NOS 3'	0.26	The 0.26 Kb 3' nontranslated region of the nopaline synthase gene (Fraley *et al.*, 1983).
KAN	1.32	The neomycin phosphotransferase type II gene (*nptII*) from pKC7 (Rao and Rogers, 1979). The *nptII* confers bacterial kanamycin resistance.
ori-pUC	0.65	The origin of replication from the high copy *E. coli* plasmid pUC119 (Vieira and Messing, 1987).
LAC	0.24	A partial lacI coding sequence, the promoter Plac, and a partial coding sequence for ß-d-galactosidase or lacZ protein (Yanisch-Perron *et al.*, 1985).
P-MAS	0.42	The 0.42 Kb TR 2' mannopine synthase promoter region (Velten *et al.*,1984).
GUS	1.81	The 1.81 Kb coding region of the ß-glucuronidase gene (Jefferson *et al.*, 1986). The expression of the gene in plants is used as a marker for transformation.
7S 3'	0.43	The 0.43 Kb 3' nontranslated region of the soybean 7S seed storage protein alpha' subunit (Schuler *et al.*, 1982).
CMoVb	0.57	The 0.57 Kb 35S figwort mosaic virus promoter (Gowda *et al.*, 1989).
CTP4	0.22	The N-terminal 0.22 Kb chloroplast transit peptide sequence from the *Petunia hybrida* EPSPS gene (Shah *et al.*, 1986).
CP4 EPSPS	1.36	The C-terminal 1.36 Kb 5-enolpyruvylshikimate-3-phosphate synthase gene (CP4 EPSPS) from an *Agrobacterium* species (Barry *et al.*, 1992).
NOS 3'	0.26	The 0.26 Kb 3' nontranslated region of the nopaline synthase gene (Fraley *et al.*, 1983).

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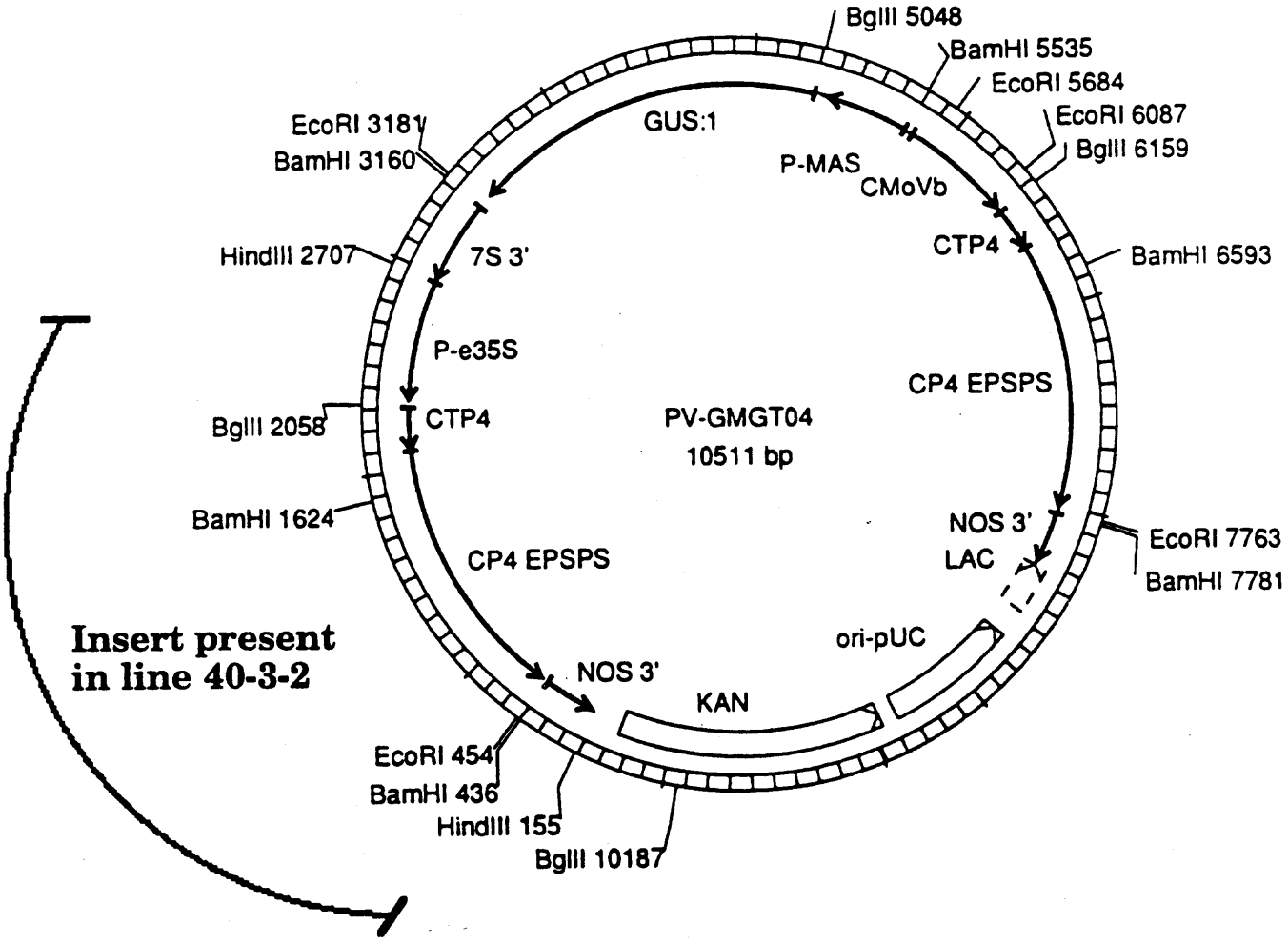

Figure III.1. Plasmid Map of PV-GMGT04

IV. The Donor Gene to be Considered for Non-regulated Status

A. CP4 EPSPS

As will be discussed in section V.B., for line 40-3-2, the only gene transferred from PV-GMGT04 to the A5403 parent soybean line encodes the enzyme EPSP synthase (EPSPS). EPSPS is an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants (including soybeans) and microorganisms (Levin and Sprinson, 1964), and is thus ordinarily present in food derived from plant sources. Genes for numerous EPSPS's have been cloned (Padgette *et al.*, 1989, 1991, and references therein), and active site domains are conserved among the known EPSPSs (Padgette *et al.*, 1988, 1991). Bacterial EPSPSs have been well-characterized with respect to the 3-dimensional X-ray crystal structure (Stallings *et al.*, 1991) and the detailed kinetic and chemical reaction mechanism (Anderson *et al.*, 1990). EPSPSs from a number of bacteria exhibit tolerance to glyphosate (Schulz *et al.*, 1985). CP4 EPSPS thus represents one of many different EPSPSs found in nature.

The herbicide glyphosate kills plants due to inhibition of the enzyme EPSPS (Steinrucken *et al.*,1980). The aromatic amino acid pathway is not present in mammalian metabolic pathways (Cole, 1985). This contributes to the selective action of glyphosate toward plants but not mammals. Glyphosate tolerance can be conferred to plants and microbes by either overproduction of EPSPS or the use of glyphosate-tolerant EPSPSs. Soybean plants tolerant to glyphosate were produced by stably inserting the gene encoding the EPSPS from the bacterium *Agrobacterium* sp. strain CP4, into the chromosome of soybean. The EPSPS from *Agrobacterium* sp. strain CP4 is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most glyphosate-tolerant EPSPSs (Barry *et al.*, 1992; Padgette *et. al.*, 1991). Upon glyphosate treatment, the soybean plants expressing the CP4 EPSPS are unaffected since the continued action of the glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic compounds. The development of glyphosate- and other herbicide-tolerant crops has been documented extensively (Gasser *et al.*, 1989; Padgette *et al.*, 1989; Hinchee *et al.*, 1992; Mazur *et al.*, 1989; Kishore *et al.*, 1988a, b; Duke *et al.*, 1991).

CP4 EPSPS is a 47.6 KD protein consisting of a single polypeptide of 455 amino acids. The gene encoding CP4 EPSPS has been completely sequenced. The enzyme has been expressed in *E. coli* and highly purified. CP4 EPSPS interacts with the EPSPS substrates shikimate-3-phosphate and phosphoenolpyruvate similarly to the plant enzymes, based on steady-state kinetic analyses. In addition, recent results indicate that the 3-dimensional X-ray crystal structure of CP4 EPSPS exhibits the same overall folding pattern as the *E. coli* EPSPS enzyme (Niedhart, D., personal communication).

The isolate CP4 was identified by the ATCC (American Type Culture Collection) as an *Agrobacterium* species, hence the designation *Agrobacterium*

sp. strain CP4. Agrobacteria occur almost worldwide in soils and in the rhizosphere of plants. *Agrobacterium* strains have also been reported in a number of human clinical specimens, but it is believed that these clinical *Agrobacterium* isolates occur either as incidental inhabitants in the patient or as contaminants introduced during sample manipulation (Kersters and De Ley, 1984).

The chloroplast transit peptide (CTP) coding sequence from petunia EPSPS (Shah *et al.* 1986; Gasser *et al.*, 1988) has been fused to the 5'-end of the CP4 EPSPS gene to deliver the CP4 EPSPS to the chloroplasts, the site of EPSPS activity and glyphosate action. Plant expression of the gene fusion produces a pre-protein which is rapidly imported into chloroplasts where the CTP is cleaved and degraded, releasing the mature CP4 EPSPS protein (della-Cioppa *et al.*, 1986).

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V. Genetic Analysis, Agronomic Performance, and Compositional Analysis of Line 40-3-2

A. Description, History, and Mendelian Inheritance of GTS Line 40-3-2

Glyphosate-tolerant soybean line 40-3-2 is an R_1 selection from an original R_0 transformant, 40-3, which was obtained by particle gun bombardment of the Asgrow variety A5403 with vector PV-GMGT04. This vector contains two CP4 EPSPS genes and the GUS marker gene, as described in section III. R_1 progenies from 40-3 R_1 seed were grown in the greenhouse during the winter of 1990-91 and evaluated for glyphosate tolerance by spray test. R_2 seeds from individual R_1 plants were planted in the field during the summer of 1991 (USDA# 91-018-01). Characterization of the R_2 progenies indicated that the original 40-3 R_0 plant had received two inserts located at different positions in the genome. The first insert was responsible for the expression of the GUS marker gene. The second insert had a strong expression of the glyphosate tolerance trait, but did not express the GUS gene. The 40-3-2 R_2 progeny that were selected exhibited strong glyphosate tolerance but no GUS enzyme activity, and did not contain the other active insert, which had been lost through normal genetic segregation. This was indicated by the fact that none of the progenies from line 40-3-2 ever expressed the GUS gene, based on leaf GUS enzyme assays. F_2 progenies of crosses between non-transgenic lines and GTS line 40-3-2 consistently segregate 3 tolerant to 1 sensitive, indicating that this insert behaves as a single dominant gene inherited in a Mendelian fashion (See Table V.1). Subsequent Southern blot analyses have shown that the DNA insert present in line 40-3-2 contains only the DNA of one of the two CP4 EPSPS glyphosate tolerance genes in vector PV-GMGT04, and does not contain an intact GUS gene (see section V.B below). Extensive analyses of line 40-3-2 seed and leaves using a GUS ELISA confirms that no GUS protein is expressed in line 40-3-2.

Progenies of GTS line 40-3-2 were planted for seed increase in Puerto Rico (USDA# 91-151-01) in two successive generations during the winter of 1991-92 (R_3 and R_4 generations), in Argentina in the 1991-92 winter season, and in eighteen yield experiment locations in the United States (USDA# 92-041-01) during the summer of 1992 (R_4 or R_5 generations, depending on the location). Approximately 30 other locations were also planted in 1992 (USDA# 92-007-01, 92-007-02, 92-015-01, 92-037-02, 92-037-06, and 92-055-01). Progenies were also planted in Costa Rica for a seed increase during the winter of 1991-92 (R_3 generation) (USDA# 92-037-02M). GTS line 40-3-2 is currently being tested at approximately 90 sites across the U.S. (USDA# 92-335-01, 92-350-01, 92-359-01, 93-011-03, 93-011-04, 93-012-05, 93-012-06, 93-012-07, 93-026-01, and 93-078-01). When sprayed with Roundup herbicide, all plants have exhibited a high level of glyphosate tolerance, indicating that the gene is stable and was not lost through the successive generations. A backcrossing program is currently in progress to transfer the gene into a wide range of soybean varieties for commercialization. All F_1 seeds resulting from this program were tolerant to Roundup herbicide, as expected, since the donor parent was homozygous for the CP4 EPSPS gene. All data available indicates

that the glyphosate tolerance gene is stably inserted and is transmitted to the progeny as a normal dominant gene.

Table V.1. Segregation Data for F_2 Progeny of GTS Line 40-3-2

Family	Tolerant	Sensitive	X^2 *
1	17	4	0.4
2	10	2	0.44
3	12	4	0
4	16	4	0.27
5	16	5	0.02
6	14	3	0.49
7	18	5	0.13
8	10	4	0.1
9	17	7	0.22
10	6	3	0.33
11	15	4	0.16
12	17	1	3.63
13	10	1	1.48
14	16	5	0.02
15	3	1	0
16	18	3	1.29
17	19	5	0.22
Total	234	61	2.94

* = Uncorrected chi-square goodness-of-fit test for hypothesis of 3:1 segregation. None of the chi-square values are significant at the 95% confidence level ($X^2_{0.05,\ 1\ d.f.} = 3.84$).

B. DNA Analysis of Glyphosate-tolerant Soybean Line 40-3-2

As described in the previous section, line 40-3-2 was derived from particle gun transformation of A5403 soybean with the plasmid PV-GMGT04 (Figure III.1). DNA analyses were performed to address the two key points regarding the DNA insertion event(s) in line 40-3-2: 1) the number of insertion sites where DNA derived from PV-GMGT04 has stably integrated into the plant genomic DNA; and 2) the identity of the DNA elements that are present in the inserted DNA.

1. Number of insertion sites in line 40-3-2

In order to determine the number of insertions in line 40-3-2, Southern blot analysis (Southern, 1975; Church *et al.*, 1984) on isolated genomic DNA (Dellaporta *et al.*, 1983) of line 40-3-2 and the control line A5403 was performed using SpeI, a restriction enzyme that does not cut inside the plasmid PV-GMGT04. The resulting blot was probed with ^{32}P-labelled PV-GMGT04. Since no internal fragmentation of PV-GMGT04-derived DNA can occur with SpeI, the number of bands present in this Southern blot corresponds to the minimum number of inserts. As shown in Figure V.1, a single unique band of high molecular weight DNA was present in the 40-3-2 digest but not in the A5403 control DNA. These results suggest that DNA

derived from PV-GMGT04 was inserted at a single site in the genomic DNA of line 40-3-2. Three additional bands of lighter intensity were present in both the 40-3-2 and A5403 lanes. These represent cross-hybridizing sequences found naturally in A5403 soybean.

The number of insertion sites and the approximate size of the insert were also investigated by Southern blot using three restriction enzymes that cut within the plasmid PV-GMGT04. Southern blot analysis was performed using 40-3-2 and A5403 (control) DNA digested with BamHI, HindIII, and EcoRI (Figure V.2), and the blot was probed with ^{32}P-labelled PV-GMGT04. The number of bands detected in this analysis reveals the number of insertion sites, since no more than two border fragment bands are expected for each insertion. A number of fragments will be common to the plasmid and insert and will vary with the restriction enzyme used. The hybridizing bands observed for 40-3-2 (Figure V.2) are listed in Table V.2, along with the predicted size of the fragments of PV-GMGT04 when cut with these enzymes. In the BamHI digestion of 40-3-2, the 1.2 Kb fragment corresponds to a 1.2 Kb fragment of PV-GMGT04. The two additional hybridizing bands, which do not match in size to any band in the BamHI PV-GMGT04 digest, are border fragments which contain part of the plasmid DNA attached to plant genomic DNA. HindIII cuts twice within PV-GMGT04 but only one hybridizing band is detected in 40-3-2, indicating that at least one or both HindIII sites are absent in the insert. As shown in Figure III.1, an EcoRI site is present in the 1.2 Kb BamHI fragment of PV-GMGT04, in the CP4 EPSPS gene. Since, as shown in Figure V.2 and Table V.2, two hybridizing bands are detected in the EcoRI digestion, it can therefore be concluded that EcoRI cuts once within the CP4 EPSPS gene of the insert to generate two border fragments. The presence of no more than two border fragments detected in any of these three analyses confirms the presence of a single insertion site, since multiple insertions would result in the detection of more than two unique border fragments. The total size of the hybridizing bands is less than 6 Kb in the three digestions, indicating that a fragment of less than 6 Kb of PV-GMGT04 was integrated into the plant genome.

Table V.2. Restriction Analysis of Line 40-3-2 and Plasmid PV-GMGT04

Restriction Fragment Size, base pairs[a]					
BamHI		HindIII		EcoRI	
Plasmid	40-3-2	Plasmid	40-3-2	Plasmid	40-3-2
3166		7959		3202	
	2900		5800		2900
2375		2552		2727	
1536				2503	
1188	1200				1900
1058				1646	
	350			403	

[a] The values for the plasmid PV-GMGT04 are based on calculated sizes (see Figure III.1); the values for line 40-3-2 are estimated from gel migration relative to molecular weight markers (see Figure V.2). Bands present in both the experimental and control lanes are not listed.

2. Identity of the DNA elements present in the insertion of 40-3-2

For particle gun transformations, it is not possible to predict, *a priori*, plasmid cleavage sites which give rise to plant genomic DNA insertions, because there is no defined point of recombination. Therefore, a combination of PCR and Southern blot analyses was used to characterize the single insert present in line 40-3-2.

a. PCR analysis for the pUC origin of replication

To analyze for the presence of the pUC origin of replication (ori-pUC), we employed the polymerase chain reaction (PCR) (Mullis and Faloona, 1987; McPherson *et al.*, 1991). A 5′ oligonucleotide and a 3′ oligonucleotide, identical in sequence to segments of the 5′ and 3′ end sequences of the ori-pUC, were employed in the reactions, using as templates genomic DNA from GTS lines 40-3-2, 61-137 (a GTS line positive for pUC), and the control line, A5403. As shown in Figure V.3, genomic DNA from line 61-137 produced a band of the expected size (671 bp), as did DNA from the plasmid PV-GMGT04. However, there was no ori-pUC PCR band for either 40-3-2 or the control line. These results establish that an intact ori-pUC is not present in line 40-3-2.

b. PCR analysis for the nptII gene

PCR analysis was also used to test for the presence of the *nptII* gene (Kan^r) in line 40-3-2. For this experiment, four oligonucleotide primers were used: a 5′ and a 3′ oligonucleotide for the extreme ends of the *npt II* gene, and 5′ and 3′ oligonucleotides for internal sequences of the gene. Genomic DNA from GTS lines 40-3-2 and 61-137, and the control line, A5403, were used as templates. The four oligonucleotides were used in combination with each other for a total of four experiments, pairing the 5' and 3' ends, the 5' end and 3' internal, the 3' end and 5' internal, and both internal primers, respectively. As shown in Figure V.4, the gel lanes corresponding to the parent plasmid PV-GMGT04, as well as

line 61-137, produced the correct size PCR products in all cases. However, the experimental line 40-3-2 as well as the control line, A5403, showed none of the predicted *nptII* PCR products in any of the reactions. These results establish that an intact *nptII* gene is not present in line 40-3-2.

c. *Southern blot analysis for CP4 EPSPS, E35S, NOS 3', CMoVb, and GUS*
In order to confirm which PV-GMGT04 vector elements (CP4 EPSPS, E35S, NOS 3', CMoVb and GUS) are present in line 40-3-2, a series of Southern blots were performed on genomic DNA from line 40-3-2 and control line A5403, using element-specific probes.

i. CP4 EPSPS:
A Southern blot was performed using A5403 and 40-3-2 DNA cut with HindIII, or BglII and EcoRI (double digest). The blot was probed with ^{32}P-labelled CP4 EPSPS coding region. As shown in Figure V.5, Panel A, a single band of 5.8 Kb in the HindIII digest of 40-3-2 hybridized with the CP4 EPSPS gene (lane 5), indicating that the CP4 EPSPS gene (or gene fragment), as expected (based on the glyphosate-tolerant phenotype), is present in line 40-3-2. This 5.8 Kb band in the HindIII digest was also evident as the only band cross-hybridizing with the entire PV-GMGT04 plasmid for the line 40-3-2 HindIII digestion, in Figure V.2. Since the CP4 EPSPS probe is predicted to hybridize with a 2552 bp HindIII band in PV-GMGT04 (Figure III.1), and no fragment of this size was detected in 40-3-2, it is clear that at least one of the PV-GMGT04 HindIII sites was not transferred to line 40-3-2. In the BglII, EcoR1 double digest lane of 40-3-2 (lane 3 of Figure V.5, Panel A), a band of 1.6 Kb hybridized with the CP4 EPSPS probe, indicating that indeed an intact CP4 EPSPS gene is present in 40-3-2 (based on the band size calculated from Figure III.1).

ii. E35S promoter:
A Southern blot was performed using A5403 and 40-3-2 DNA cut with BamHI, and probed with ^{32}P-labelled E35S promoter DNA. The E35S element, or a portion of it, is present in line 40-3-2 (lane 3 of Figure V.5, Panel B,) since a single band of 2.9 Kb was detected in the 40-3-2 lane, which corresponds to the border fragment detected in Figure V.2 and discussed above. Since E35S is located on a 1536 bp BamHI fragment of PV-GMGT04 (Figure III.1), and no fragment of this size was detected in 40-3-2, it is clear that the BamHI site at map number 3160 of Figure III.1 is not present in line 40-3-2.

iii. NOS 3':
A Southern blot was performed using A5403 and 40-3-2 DNA cut with HindIII, and probed with ^{32}P-labelled NOS 3' terminator. At least a portion of the NOS 3' element is present in 40-3-2 (Figure V.6, lane 10) since a single band of 5.8 Kb was detected in 40-3-2, which corresponds to the border fragment detected in Figure V.2 and discussed above. Two sets of double digestions with EcoRI and BglII, and EcoRI and HindIII were done with A5403 and 40-3-2 DNA to determine the approximate site of insertion. The data (Figure V.6) indicated that a 0.8 Kb fragment hybridized to the NOS 3' probe in the HindIII, EcoRI digest where the map predicted size is 0.3 Kb (lane 5), and in addition a 1.2 Kb

fragment hybridized to the NOS 3' probe in the EcoRI, BglII digest where the predicted size is 0.8 Kb (lane 3). These results demonstrate that the HindIII site at map number 155 and that the BglII site at map number 10187 are not present in the insert of 40-3-2.

iv. CMoVb promoter:
A Southern blot was performed using A5403 and 40-3-2 DNA cut with HindIII, and probed with ^{32}P-labelled CMoVb promoter. As shown in Figure V.7, Panel A, no band was detected in line 40-3-2, indicating that the CMoVb promoter DNA was not transferred to the genome of line 40-3-2. GTS line 61-67-1, which contains the CMoVb promoter, gave a positive signal in this analysis.

v. GUS:
A Southern blot was performed using A5403 and 40-3-2 DNA cut with HindIII, and probed with ^{32}P-labelled GUS coding region. As shown in Figure V.7, Panel B, no band was detected in 40-3-2, indicating that GUS is not present in line 40-3-2. GTS line 61-67-1, which contains the GUS gene, gave a positive signal in this analysis.

3. Summary of the DNA analysis of line 40-3-2
Based on the PCR and Southern blot data presented above, we conclude that GTS line 40-3-2 contains a single insert of DNA derived from PV-GMGT04, and that this insert contains the E35S promoter (or a portion), the petunia EPSPS chloroplast transit peptide, the CP4 EPSPS gene, and the NOS 3' terminator (or a portion). This conclusion is based on the following data: 1) the positive detection of E35S, NOS 3' and the CP4 EPSPS gene by Southern analysis; 2) the lack of ori-PUC and *nptII* signals by PCR analysis; and 3) the lack of CMoVb and GUS signals by Southern analysis. The precise ends of the insert of 40-3-2 have not been determined. However, from the data presented above, it can be concluded that one end of PV-GMGT04 DNA incorporated into the line 40-3-2 soybean genome falls between the HindIII site at map number 155 and the BamHI site at map number 436 (Figure III.1). The other end of the PV-GMGT04 DNA incorporated into the line 40-3-2 soybean genome falls between the BglII site at map number 2058 and the HindIII site at map number 2707 (Figure III.1). This boundary is based on the fact that since the single HindIII band in line 40-3-2 is 5.8 Kb, and the EcoRI, HindIII fragment from Figure V.6 was 0.8 Kb, then the remaining size of the HindIII fragment is 5.0 Kb, not 2253 bp, as would be predicted if the HindIII site at map number 2707 were present. Thus, the maximal size of the PV-GMGT04 DNA contained in line 40-3-2 is 2.5 Kb. This conclusion is shown schematically in Figure V.8. The DNA sequence (and the corresponding deduced protein sequence) of the HindIII fragment representing the largest possible insert from PV-GMGT04 in the line 40-3-2 genome is detailed in Appendix 1.

C. GTS Line 40-3-2 Field Tests for Analytical Evaluation

In order to generate plant material for expression and quality analysis, a field test was planted on March 9, 1992 at the Asgrow Breeding Station, Isabela, Puerto Rico (USDA permit #91-151-01) (R_4 plants to yield R_5 seed).

Literature data has established that soybeans grown in Puerto Rico are comparable in composition to soybeans grown in the continental United States (Bravo *et. al.*, 1980; de Cianzio *et. al.*, 1985; Hawkins *et. al.*, 1983; LeRoy *et. al.* 1991). The soybean lines tested consisted of four genotypes: control line A5403 and GTS line 40-3-2, as well as two additional GTS lines. Each of the four genotypes was planted in three separate plots arranged according to a randomized complete block design, for a total of 12 plots in three blocks. Fertilization and pest and disease control measures were used as needed to maximize yield and conform to constraints of the test protocol (all plots were treated identically). No adverse effects from environmental or other conditions occurred. Soybean leaf and seed samples were collected from the plots. Leaflets were sampled at approximately one month and two months post-planting, using middle leaflets of young fully-expanded trifoliates of six plants randomly selected throughout each plot. Samples were pooled and analyzed for CP4 EPSPS expression. Seed samples were collected at harvest and analyzed for CP4 EPSPS expression and quality characteristics.

Field tests to supply additional soybean tissue for analyses were performed at nine sites across the U.S. in 1992 (USDA# 92-037-06 and 92-007-02). Leaf samples were collected at eight of the nine sites. Line 40-3-2 and the control line A5403 were sampled (one plot / site), as well as one additional GTS line. Shown in Table V.3 are the GTS field sites sampled in 1992 for analysis. Plant samples were collected similarly to that described for the Puerto Rico plots, except that additional monthly leaf samples (after the initial sampling) were taken at two of the eight sites.

Table V.3 1992 GTS Field Sites Sampled for Analysis

Site	Generation Seed	
	Planted	Produced
Isabela, Puerto Rico	R4	R5
Newport, Arkansas	R5	R6
Proctor, Arkansas	R5	R6
Winterville, Georgia	R5	R6
Martinsville, Indiana	R4	R5
Washington, Louisiana	R5	R6
Greenville, Mississippi	R5	R6
Macon, Missouri	R4	R5
Seven Springs, North Carolina	R5	R6
Marion, Arkansas	R5	R6

D. Disease and Pest Characteristics

Line 40-3-2 has been field tested in the U.S. in 1991 (USDA# 91-018-01), in Puerto Rico (USDA# 91-151-01) and Argentina in the 1991-92 winter season, and in the U.S. in 1992 (USDA# 92-007-01, 92-007-02, 92-015-01, 92-037-02, 92-037-06, 92-041-01, and 92-055-01). Detailed monitoring for the disease and insect susceptibility of line 40-3-2, versus the control A5403 line, was performed at the sites listed in Table V.4. No differences in disease or insect infestation or severity were detected between the glyphosate-tolerant lines (including line 40-3-2) and the control line, A5403 (Table V.4). See Appendix II for USDA final reports, and Appendix III for example monitoring forms. Private breeders and/or agronomists were responsible for collecting this data and reporting their findings. Plots were evaluated in the same fashion as a typical soybean breeder would examine his/her plots to decide on the acceptability of a new line for commercial release. Soybean breeders normally walk through a representative number of plots of the variety to be released to visually check for the appearance of possible disease symptoms such as spotted leaves, leaf necrosis, yellowing, or wilting of the plants. They also make notes of any other undesirable characteristics that may be noticeable. In addition, in specific markets with important diseases such as cyst nematodes, they conduct greenhouse tests for susceptibility to those diseases. In tests conducted by Asgrow Seed Company in Marion, Arkansas, no differences could be detected in the sensitivity of line 40-3-2 to cyst nematodes as compared to A5403. As stated above, no differences in disease or insect infestation or severity have been detected between line 40-3-2 and the control line, A5403.

E. Yield Characteristics of Line 40-3-2

The yields of the field plots from the Puerto Rico seed increase described in section V.C above were determined. There was no significant difference in the yields (95% confidence level) between line 40-3-2 and the A5403 control line.

In the summer of 1992, the first wide-scale GTS yield trials were performed. A seven-site yield trial was performed to evaluate line 40-3-2 (untreated with glyphosate) versus the parental line, A5403. At three of the seven sites, there was a statistically significant yield reduction for line 40-3-2, with an average yield reduction of 11.5% over those three sites (95% confidence level). At the remaining four sites, there was no statistically significant difference in yield between lines A5403 and 40-3-2. Further yield tests will be conducted to determine whether this initial yield observation is valid. In 1993, additional GTS yield tests will be performed to obtain more data regarding the yield of line 40-3-2 using the 40-3-2 active insert backcrossed into other varieties. This result is further discussed in Section VII, "Statement of Grounds Unfavorable."

Table V.4 1992 GTS Field Sites Reporting Line 40-3-2 Status

Site	Difference in Susceptibility versus A5403 Control	
	Disease	Insect
Isabela, Puerto Rico	No	No
Newport, Arkansas	No	No
Proctor, Arkansas	No	No
Winterville, Georgia	No	No
Elk City, Kansas	No	No
Tamms, Illinois	No	No
Martinsville, Indiana	No	No
Sheridan, Indiana	No	No
Danville, Iowa	No	No
Webster City, Iowa	No	No
Washington, Louisiana	No	No
LaCenter, Kentucky	No	No
Conklin, Michigan	No	No
Greenville, Mississippi	No	No
Macon, Missouri	No	No
Steele, Missouri	No	No
York, Nebraska	No	No
New Holland, Ohio	No	No
Seven Springs, North Carolina	No	No
Elko, South Carolina	No	No
Arlington, Tennessee	No	No
Skippers, Virginia	No	No
Tallassee, Alabama	No	No
Loxley, Alabama	No	No
Marion, Arkansas	No	No
Stuttgart, Arkansas	No	No
Plains, Georgia	No	No
Jerseyville, Illinois	No	No
Newburgh, Indiana	No	No
Sidney, Iowa	No	No
Madisonville, Kentucky	No	No
Baton Rouge, Louisiana	No	No
St. Joseph, Louisiana	No	No
Snow Hill, Maryland	No	No
Queenstown, Maryland	No	No
Stoneville, Mississippi	No	No
Tunica, Mississippi	No	No
Matthews, Missouri	No	No
Florence, South Carolina	No	No
Union City, Tennessee	No	No

An additional 18-site yield test was performed in 1992 on line 40-3-2, using seven Roundup herbicide application regimes applied post-emergent. Analysis of the data revealed only three minor reductions in yield (95% confidence level), relative to line 40-3-2 untreated, out of 126 comparisons. These minor variations are to be expected for analysis at the 95% confidence level, and we

do not consider them meaningful. These yield results support the high level of glyphosate tolerance exhibited by line 40-3-2.

F. Expression Levels of the CP4 EPSPS Protein

The levels of CP4 EPSPS in the GTS lines and control line A5403 were determined in the seed and leaf samples by ELISA. Given in Appendix IV are the descriptive features of the ELISA developed to detect and quantitate CP4 EPSPS. As can be seen in Appendix IV., the extraction variability for leaf tissue was 26% (data generated over six month time period with ten time points). Although this value appears high, it represents the "worst-case" of assay variability, and takes into account variability resulting from individual extractions and day-to-day assay variability. Assay of the quality control sample on different days yielded a lower variability, 21%. In any case, the site-to-site variability due to environmental conditions is greater, as expected, than the variabilities associated with either the leaf or seed ELISAs for CP4 EPSPS. As described above, DNA analysis of line 40-3-2 indicates that there are no GUS or *nptII* genes present in line 40-3-2; therefore CP4 EPSPS is the only additional protein expressed in the line. Although analyses were performed for the GUS protein, no GUS was detected and this data will not be discussed further. CP4 EPSPS expression data is presented below.

1. Seed expression

Seed expression results of CP4 EPSPS are shown in Table V.5. The mean CP4 EPSPS expression level for line 40-3-2 was 0.239 µg/mg tissue (fresh weight) at the single Puerto Rico field site, and 0.288 µg/mg tissue (fresh weight) across the nine U.S. field sites. These expression levels correspond to 0.024-0.029% of the fresh weight of the soybean seed as CP4 EPSPS.

Table V.5 CP4 EPSPS Expression in Soybean Seeds

Line	Field test	# Sites	Expression, µg/mg fresh weight		
			Mean	Std. dev.	Range[c]
40-3-2	U.S.[a]	9	0.288	0.066	0.186-0.395
	Puerto Rico[b]	1	0.239	0.018	0.179-0.303
A5403	U.S.[a]	9	ND[d]		
	Puerto Rico[b]	1	ND		

[a] Single extracts of seed samples from each of the nine sites were analyzed by ELISA, using two different loads of total extracted protein on the ELISA plate (total of 18 values per line). However, means and standard deviations reported are of the 9 sample means.

[b] Duplicate extracts of seed samples from each of the three plots were analyzed by ELISA, using two different loads of total extracted protein onto the ELISA plate (total of 12 values per line). The means and standard deviations shown are for the means of the 3 plots.

[c] "Range" denotes the lowest and highest individual assay for each line.

[d] ND = Below the threshold of detection of the assay, which is OD(405) = 0.09 in the ELISA.

2. Leaf expression

The CP4 EPSPS leaf expression results from both collection dates from the Puerto Rico field test are shown in Table V.6. For the Month 1 collection, the mean CP4 EPSPS concentration in the leaf tissue of line 40-3-2 was 0.495 µg/mg tissue (fresh weight). At Month 2, the average CP4 EPSPS concentration in line 40-3-2 leaves was 0.657 µg/mg tissue. Between the first and second month timepoints, there is a small difference in expression. Note that only two leaf samplings were possible for the Puerto Rico field test, due to the short growing season (planted 3/9/92; harvested 6/22/92).

As also shown in Table V.6, the U.S. field trial samples yielded a similar value for the initial mean CP4 EPSPS leaf sample expression, 0.443 µg/mg fresh weight (8 sites). However, the second leaf sampling indicated a reduced mean expression level, 0.264 µg/mg. Subsequent monthly leaf samplings at two sites gave a mean CP4 EPSPS expression level of 0.333 µg/mg (0.217-0.446 µg/mg range) and 0.468 µg/mg (0.085-0.851 µg/mg range), for Months 3 and 4, respectively. Based on both the U.S. and Puerto Rico data, the maximum CP4 EPSPS leaf expression level observed (based on individual assay values) was 0.851 µg/mg leaf fresh weight (0.085 % fresh weight of the soybean leaf as CP4 EPSPS).

Table V.6 CP4 EPSPS Expression in Soybean Leaves

Line	Field test	Month	# Sites	Expression, µg/mg fresh weight Mean	Std. dev.	Range[d]
40-3-2	U.S.[a]	1	8	0.443	0.098	0.251-0.789
	Puerto Rico[b]	1	1	0.495	0.027	0.474-0.526
40-3-2	U.S.[c]	2	2	0.264	0.293	0.046-0.480
	Puerto Rico[b]	2	1	0.657	0.138	0.523-0.798
A5403	U.S.[a]	1	8	ND[e]		
	Puerto Rico[b]	1	1	ND		
A5403	U.S.[c]	2	2	ND		
	Puerto Rico[b]	2	1	ND		

[a] Single extracts of leaf samples from each of the eight sites were analyzed by ELISA, using two loads of total extracted protein on the ELISA plate, with the exception that one site had three loads analyzed, one had one loading amount analyzed, and one had four loads total of a duplicate extract analyzed. The mean and standard deviation shown are for the means of the eight sites.

[b] Single extracts of leaf samples from each of the three plots were analyzed by ELISA, using one load of total extracted protein on the ELISA plate (total of 3 values per line).

[c] Single extracts of leaf samples from each of the two sites were analyzed at two loadings each, for a total of four values.

[d] "Range" denotes the lowest and highest individual assay for each line.

[e] ND = Below the threshold of detection of the assay, which is OD(405) = 0.09 in the ELISA.

G. Compositional Analyses of the Transformed Soybeans

During the last several decades, the soybean has been developed as a major source of protein. Soybeans are capable of producing the greatest amount of protein per unit of land of any major plant or animal source used as food by people today (Considine and Considine, 1982). In terms of domestic usage of the U.S. soybean crop, almost 98% is used for animal feed, mainly in the form of soybean meal. Food products containing soybean protein include baked goods, confections, meat products, textured foods, and nutritional supplements (American Soybean Assn., 1992; Waggle and Kolar, 1979). The amino acid profile of soy protein is unusually well-rounded for a plant protein (Erdman, 1988). Especially important is the soybeans' high content of essential amino acids, particularly lysine, leucine, and isoleucine (Coppock, 1974). Extensive literature information exists on the nutritional value of soy protein (Liener, 1972; Wilcke *et al.*, 1979; Wolf, 1983). Soybean oil is also extensively used in the food industry, in products such as cooking oil and salad dressings. In fact, soybean oil is currently the major edible oil used in the U.S. (Mounts, 1988).

Compositional (proximate) analyses were performed on the soybean seeds from line 40-3-2 and the A5403 control line. Compounds measured were protein, fat, moisture, fiber, and ash. Carbohydrates and calories were calculated from these values. There is a relatively wide range of proximate analysis values for soybeans in the literature, as indicated below:

Component	Literature Range
Protein[a], %	36.9-46.4
Fat[a,b], %	13-26
Crude fiber[a,c], %	4.7-6.48
Ash,%	3.3-6.4
Carbohydrates,%	31.1-43.9

[a] Smith and Circle, 1972
[b] Wilcox, J. R., 1985
[c] Mounts *et al.*, 1987

Proximate analysis results from both the 1992 Puerto Rico field test and the 1992 multi-location U.S. field tests are shown in Table V.7. For the Puerto Rico field experiment, seed results from each of the three plots of each line were statistically analyzed. For the U.S. experiment, seed results from each of the nine sites of each line were statistically analyzed. As shown in Table V.7, there was one statistically significant difference seen between line 40-3-2 and the control line, A5403, in the Puerto Rico field test. This was in the level of protein (43.1% vs. 43.5% for 40-3-2 and A5403, respectively). In the nine-site U.S. field tests, there were three statistically significant differences seen between line 40-3-2 and the control line, A5403: the level of fat was 16.3% in 40-3-2 vs. 15.5% in the control, the ash level was 5.24% in 40-3-2 vs. 5.04% in the control,

and the carbohydrate level was 37.1% in 40-3-2 vs. 38.1% in the control. All three of these minor differences fall within the expected ranges of soybean compositional variability, and do not represent meaningful differences between the glyphosate-tolerant soybean line and the control line.

Additional soybean quality data collected on line 40-3-2 and the control A5403 line include, in addition to the proximates shown in Table V.7, amino acids, fatty acids, stachyose, raffinose, trypsin inhibitor, phytate, phytoestrogens, and lectins. This data will be provided to the FDA in support of the food/feed safety of glyphosate-tolerant soybeans. Based on these results, which includes a statistical analysis on the seed measurements, the quality data obtained demonstrates that the glyphosate-tolerant soybeans are essentially equivalent to the parental control line, A5403.

Table V.7. Average Proximate Analysis Results for GTS Line 40-3-2 and A5403 Control Seeds in 1992 Field Tests

Component	Puerto Rico[a] (1 site, 3 reps/site)		U.S.[a] (9 sites, 1 rep/site)	
	40-3-2	A5403	40-3-2	A5403
Protein[b,c], %	43.1*	43.5	41.4	41.6
Fat[b,d], %	21.7	20.9	16.3*	15.5
Fiber[b,e], %	4.90	4.85	6.87	7.13
Ash[b,f],%	5.60	5.50	5.24*	5.04
Carbohydrates[b,g], %	29.7	30.1	37.1*	38.1
Calories[b,h], kcal/100g	451	448	431	429
Moisture[i], %	15.17	14.00	8.12	8.12

[a] Values marked with an asterisk (*) are significantly different at the 95% confidence level from the A5403 control line, based on Fisher's Protected Least Significant Difference (LSD) Procedure (Steel and Torrie, 1980). Data from one or more additional GTS line (s) was included in the statistical analysis.
[b] Dry weight basis
[c] Total Kjeldahl Nitrogen-Protein; Official Methods of Analysis of the AOAC, 15th Ed., 988.05.
[d] Fat, Ether Extraction; Official Methods of Analysis of the AOAC, 15th Ed., 920.39C; Foster and Gonzales, 1992; Bhatly, 1985.
[e] Crude fiber; Official Methods of Analysis of the AOAC, 15th Ed., 962.09.
[f] Ash, 550°C; Official Methods of Analysis of the AOAC, 15th Ed., 923.03.
[g] By calculation, on the fresh weight values: % carbohydrate = 100 % - (% protein + % moisture + % fat + % ash)
[h] By calculation, on the fresh weight values, using soybean Atwater factors: calories (kcal/100 g) = (3.47 • % protein) + (8.37 • % fat) + (4.07 • % carbohydrates)
[i] Moisture, F.D., 133°C; Official Methods of Analysis of the AOAC, 15th Ed., 930.15.

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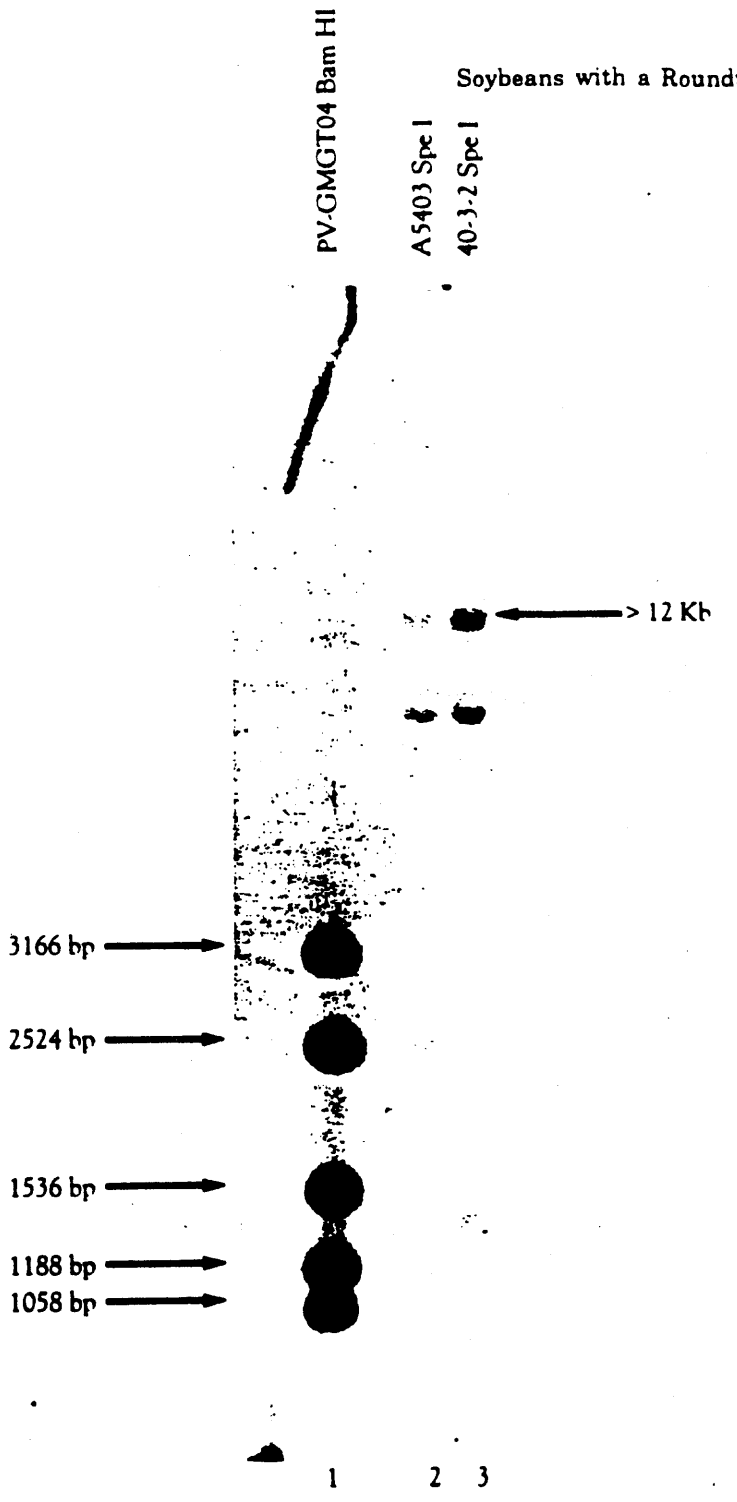


Figure V.1. Southern Blot I of 40-3-2 to Determine the Number of Inserts of PV-GMGT04
Southern blot analysis of PV-GMGT04 plasmid DNA digested with BamHI (lane 1) and soybean genomic control A5403 DNA (lane 2) and GTS 40-3-2 genomic DNA (lane 3) digested with SpeI. Each lane represents 100 pg of plasmid DNA or 5 ug of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P-labelled PV-GMGT04 plasmid DNA and subjected to autoradiography.

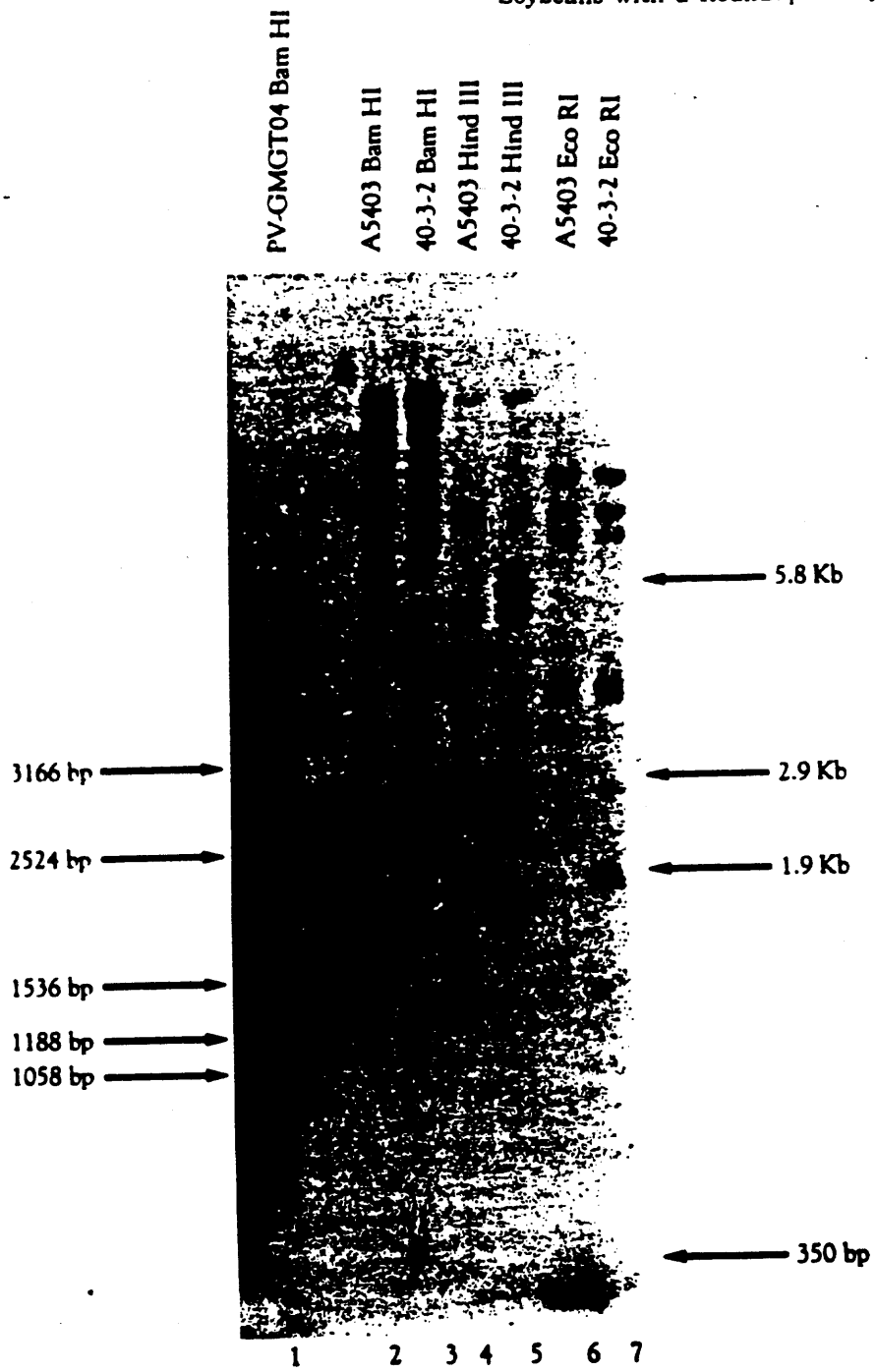


Figure V.2. Southern Blot II of GTS 40-3-2 to Determine the Number of Inserts of PV-GMGT04

Southern blot analysis of PV-GMGT04 digested with BamHI (lane 1), soybean A5403 control DNA digested with BamHI (lane 2) and HindIII (lane 4) and EcoRI (lane 6) and GTS 40-3-2 digested with BamHI (lane 3) and HindIII (lane 5) and EcoRI (lane 7). Each lane represents 100 pg of plasmid DNA or 5μg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P labelled PV-GMGT04 plasmid DNA and subjected to autoradiography.

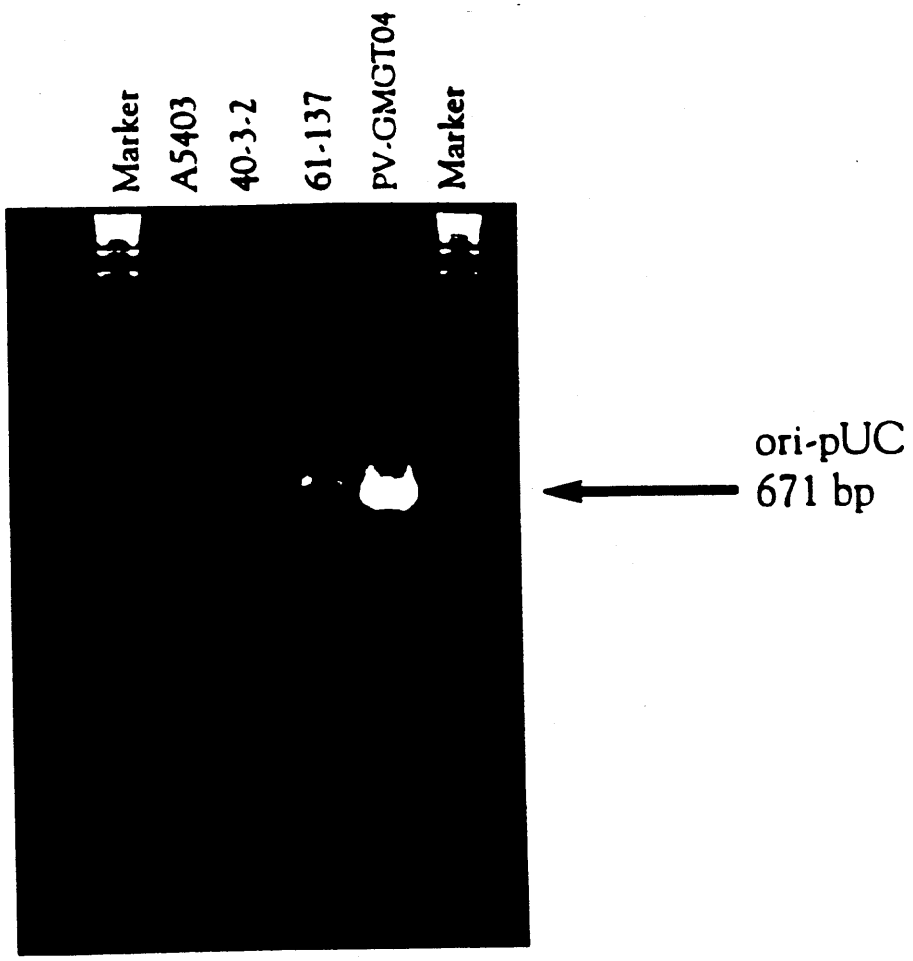


Figure V.3. Ori-pUC PCR Analysis for Line 40-3-2
Genomic soybean DNA from the control line A5403 and the GTS line 40-3-2 were analyzed using PCR to determine the presence or absence of the pUC origin of replication. The positive DNA controls were PV-GMGT04 plasmid DNA and 61-137, a GTS line derived from a vector containing the Ori-pUC. A 5′ and a 3′ oligonucleotide were made identical to the 5′ and 3′ends of the ori-pUC: 1) 5′ ori-pUC (9105 to 9086 base pairs from Figure III.1), CCCCGTAGAAAAGATCAAAGG (21mer); and, 2) 3′ ori-pUC (8478 to 8499 base pairs from Figure III.1), GTTGCTGGCGTTTTTCCATAGC (22mer). Reactions were done in 100µl total volume containing 100 pg of each oligo, 1 µg template, dNTP's at 200 µM, 10 units of Taq® DNA Polymerase (Perkin-Elmer, Norwalk, CT). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 55°C annealing for 1.5 min, and a 72°C extension for 3 min. The cycle was repeated 24 times. Products were separated on a 1.25% agarose gel and visualized by ethidium bromide staining. The lower bands at the bottom of the gel are unused oligos.

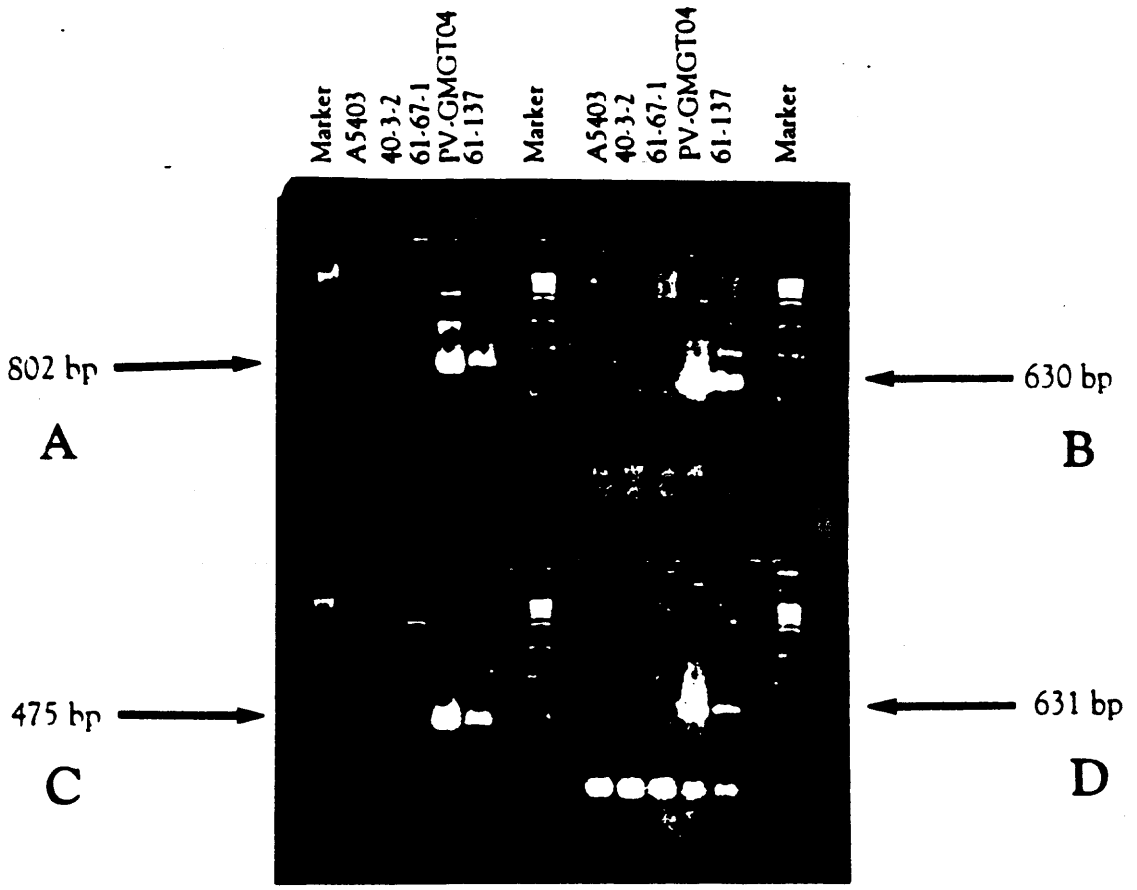
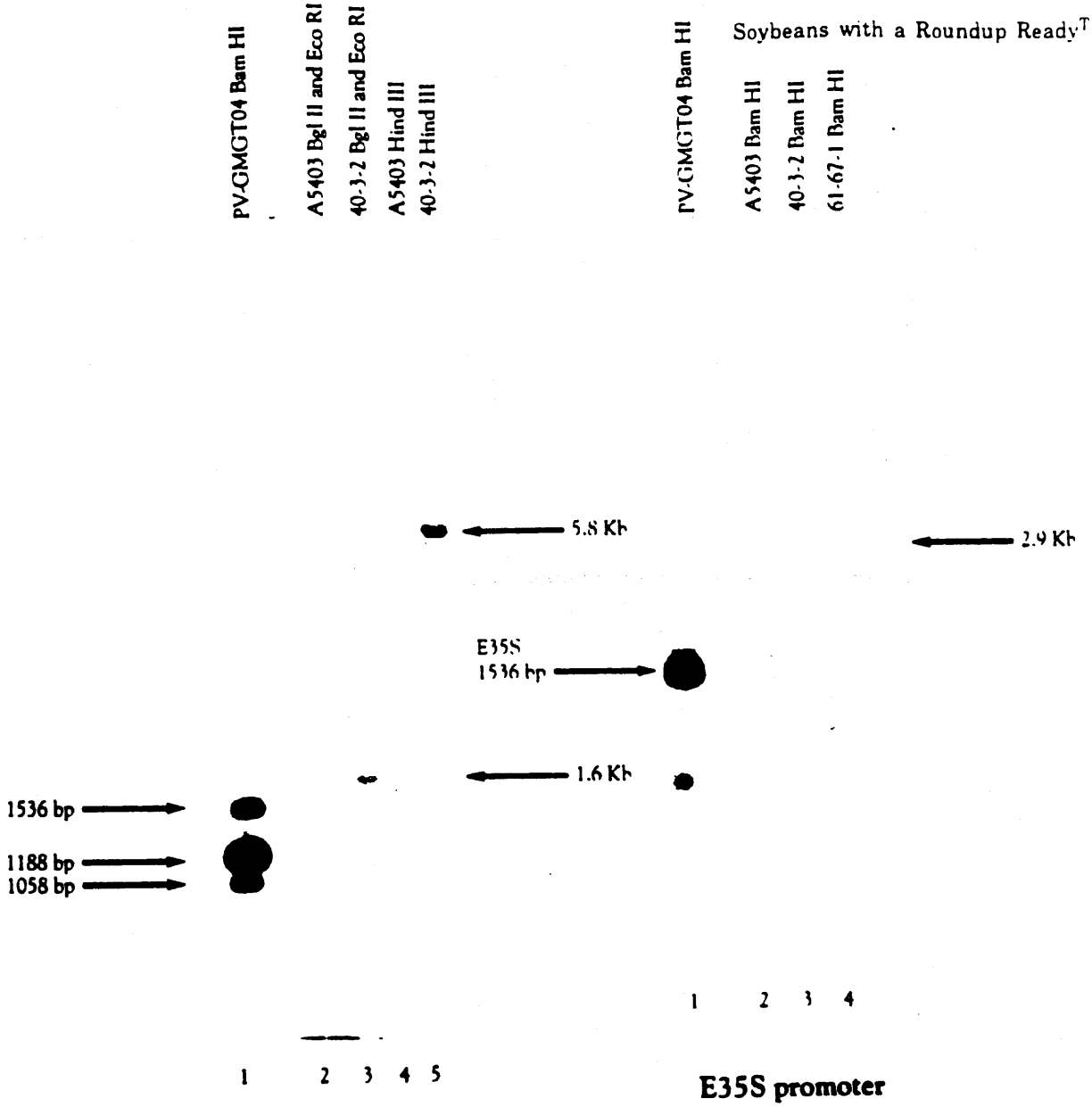


Figure V.4. *Npt II* PCR Analysis for Line 40-3-2
Soybean genomic DNA from the GTS 40-3-2 line was analyzed using PCR to determine the presence or absence of the *nptII* gene. The negative controls were A5403, a control soybean line, and 61-67-1, a GTS line negative for *nptII*. Two positive controls were used: PV-GMGT04 plasmid DNA, and 61-137, a GTS line positive for *nptII*. Four oligonucleotides were used in this analysis: a 5' and a 3' oligo were made identical to the ends of the gene, and a 5' and a 3' oligo were made identical to internal sequences of the gene: 1) *nptII* 5´ end (10159 to 10140 base pairs from Figure III.1), CGCATGATTGAACAAGATGG (20mer); 2) *nptII* 5´ internal (10005 to 9988 base pairs from Figure III.1), CCGACCTGTCCGGTGCCC (18mer); 3)*npt II* 3´ end (9357 to 9370 base pairs from Figure III.1), CCCGCTCAGAAGAACTCG (18mer); and, 4) *nptII* 3´ internal (9511 to 9529 base pairs from Figure III.1), CCGCCACACCCAGCCGGCC (19mer). The predicted product sizes are shown below:

Oligos	Product Size
A = 5' end + 3' end	802bp
B = 5' end + 3' internal	630bp
C = 5' internal + 3' internal	475bp
D = 5' internal + 3' end	631bp

Reactions were done in 100 µl total volume, containing 100 pg of each indicated oligo, 1 µg template, dNTP's at 200 µM, 10 units Taq® DNA Polymerase (Perkin-Elmer, Norwalk, CT) The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 63°C annealing for 1.5 min, and a 72°C extension for 6 min. The cycle was repeated 24 times. Products were separated on a 1.25% agarose gel and visualized by ethidium bromide staining. The lower bands at the bottom of each gel are unused oligos.



Panel A **Panel B**

Figure V.5. Southern Blots Probed for CP4 EPSPS and E35S Promoter in GTS Line 40-3-2

PV-GMGT04 plasmid DNA was digested with BamHI (lane 1 in both Panels). Soybean genomic DNA from A5403 control was digested with BglII and EcoRI (Panel A, lane 2), HindIII (Panel A, lane 4) and BamHI (Panel B, lane 2). GTS line 40-3-2 DNA was digested with BglII and EcoRI (Panel A, lane 3), HindIII (Panel A, lane 5), and BamHI (Panel B, lane 3). GTS 61-67-1, a negative plant control for E35S was digested with BamHI (Panel B, lane 4). Each lane represents 100 pg of plasmid DNA or 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P-labelled coding region of CP4 EPSPS (Panel A), or E35S promoter (Panel B), and then subjected to autoradiography. The smaller mark in lane 1 of Panel B is a dot on the blot and not an additional band.

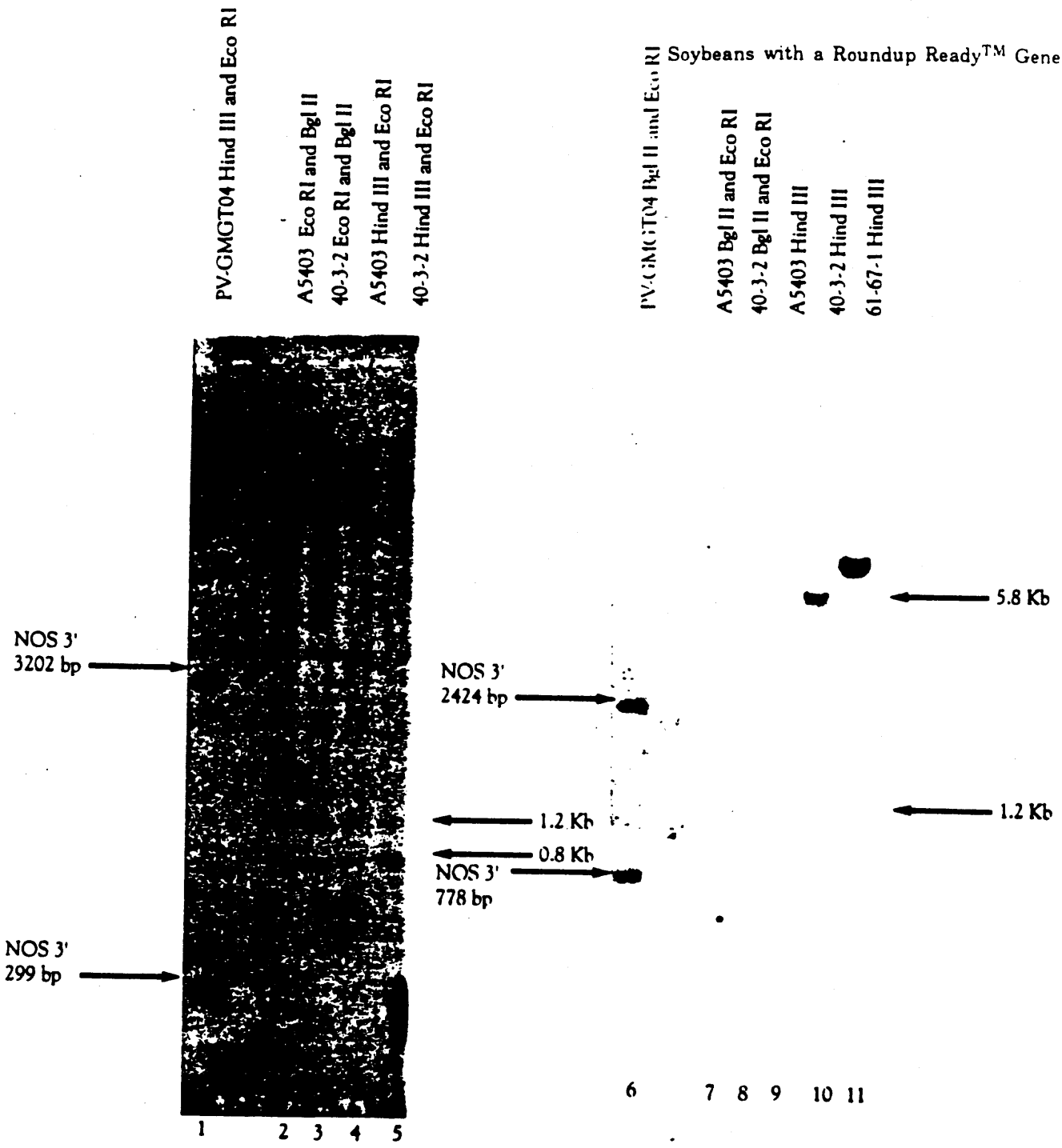


Figure V.6. Southern Blots Probed for NOS 3' Terminator in GTS Line 40-3-2
PV-GMGT04 plasmid DNA was digested with HindIII and EcoRI (lane 1) and BglII and EcoRI (lane 6). Soybean genomic DNA from A5403 control was digested with EcoRI and BglII (lanes 2 and 7), with HindIII and EcoRI (lane 4), and with HindIII (lane 9). GTS line 40-3-2 was digested with BglII and EcoRI (lanes 3 and 8), with HindIII and EcoRI (lane 5) and with HindIII (lane 10). GTS line 61-67-1, a plant positive control for NOS 3' was digested with HindIII (lane 11). Each lane represents 100 pg of plasmid DNA or 5 μg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. Both panels were probed with ^{32}P labelled NOS 3' and then subjected to autoradiography.

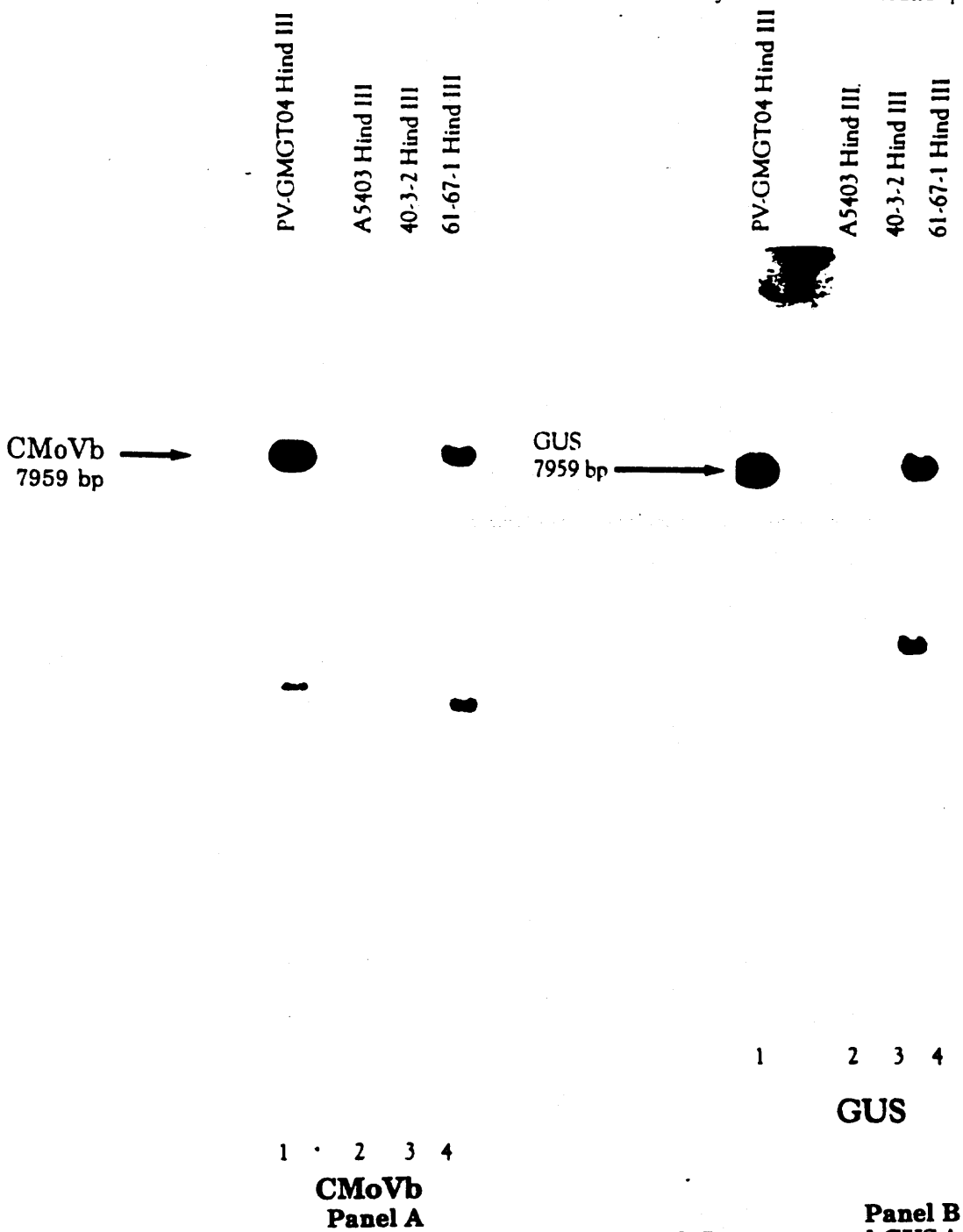


Figure V.7. Southern Blots Probed for CMoVb Promoter and GUS in GTS Line 40-3-2

PV-GMGT04 plasmid DNA was digested with HindIII (Panels A and B, lanes 1). Soybean A5403 control DNA was digested with HindIII (Panels A and B, lanes 2). GTS line 40-3-2 DNA was digested with HindIII (Panels A and B, lanes 3), and GTS line 61-67-1 DNA was digested with HindIII (Panels A and B, lanes 4). Each lane represents 100 pg plasmid DNA or 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P labelled CMoVb promoter (Panel A) or the coding region of GUS (Panel B) and then subjected to autoradiography.

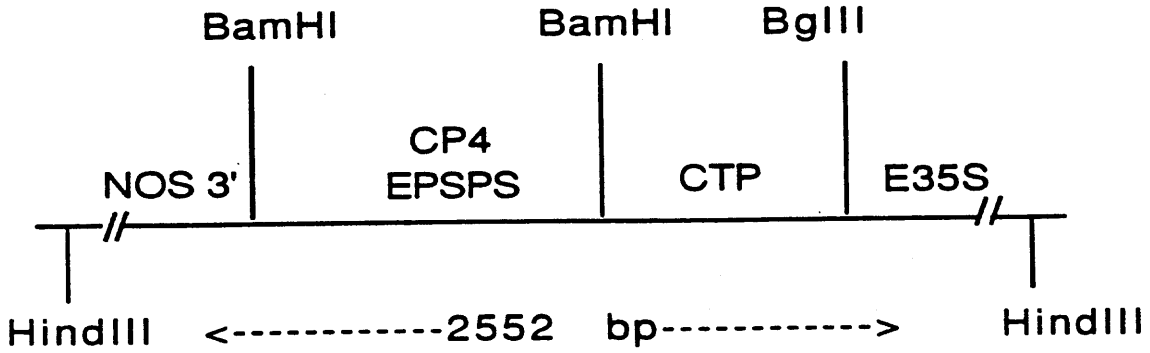


Figure V.8. Schematic of the PV-GMGT04-derived DNA Insert in GTS Line 40-3-2

VI. Environmental Consequences of Introduction of GTS Line 40-3-2

A. The Herbicide Glyphosate

N-(phosphonomethyl) glycine (glyphosate) is an extremely effective broad spectrum, post-emergent herbicide. The primary mode of action of the herbicide is competitive inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway of aromatic amino acid biosynthesis. Glyphosate provides effective control for the majority of the world's worst weeds. It is translocated in the plant via both phloem and xylem. The broad spectrum herbicidal activity is only evident when glyphosate is applied to foliage, as there is little penetration of bark or woody stems (Franz, 1983). Glyphosate is not active when applied to the soil (i.e., glyphosate has no pre-emergence or residual soil activity). Its degradation appears to be mainly microbial (Atkinson, 1985). Glyphosate is essentially nontoxic to mammals and birds (Atkinson, 1985). Environmental impact studies indicate that the herbicide has little direct effect on animal communities (Sullivan and Sullivan, 1979, 1981, 1982). Glyphosate is only slightly toxic to fish and invertebrates, although some of the commercial formulations are more toxic due to the presence of a surfactant (Atkinson, 1985). Effects of the herbicide on soil invertebrates in field situations are minor (Eijsackers, 1985). Although there are numerous reports on the effect of glyphosate on microbial respiration, nitrogen cycling, and cellulolytic activity in soils, no toxicity to any of these microbial processes should be observed at recommended field application rate of the herbicide (Carlisle and Trevors, 1988). There are no reports of problems which have been associated with the use of glyphosate and groundwater contamination (Goldburg et *al.*, 1990). The EPA has classified glyphosate as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739).

B. Current Uses of Glyphosate and other Herbicides on Soybean

Glyphosate is used as a foliar-applied herbicide. Effective for the control of both annual and perennial weeds, it is usually applied before planting to kill winter and early summer weeds. This use fits well in reduced-tillage systems. It is also used as a spot spray at any time during the growing season. Even though highly effective for weed control, the lack of crop selectivity limits widespread use except for spot sprays in the growing crop. Its characteristics of ready translocation in plants and lack of in-crop tolerance resulted in applications via selective equipment (rope wicks), but this technique is practical only on weeds growing taller than the crop. Glyphosate is also used for controlling weeds outside the crop field. We also note that Roundup herbicide is currently labelled for preharvest application on soybeans.

Soybeans are grown primarily in rotation with corn and wheat in both the midwest and southern states. In the northern midwest, it is a straight forward corn/soybean/corn rotation, while in more southern states, it is commonly a fall seeded wheat/double cropped soybean/corn rotation. With considerably

reduced frequency, soybean rotations may include sorghum, tobacco, and cotton. Volunteer soybeans becoming weedy pests in rotational crops is highly unusual for two reasons: 1) the overwintering ability of soybean seed is very poor and they do not survive cool wet soil conditions which occur throughout most of the soybean growing regions of the U.S., and 2) soybeans are generally injured by rotational crop herbicides such as triazines which are commonly used in corn. Most herbicides used in soybeans do not generally result in carryover concerns to rotational crops if label use and geographical restrictions are followed.

Herbicides are used on close to 100% of the soybean acreage in the U.S. (Gianessi and Puffer, 1991). They are applied to soybeans preplant (foliage or soil incorporated applications), at planting (preemergence applications), or after seedlings emerge (postemergence directed or over-the-top).

The number of applications varies in each soybean production region from one to five treatments per growing season. The actual number is dependent upon weed species, population densities, weather, and production economics. In the Corn Belt region, soybean producers generally apply two standard treatments plus one spot spray. No-tillage acreage (increasing in the Corn Belt) commonly receives 3 treatments. In the Delaware, Maryland, and Virginia area, two to three treatments are common. The number increases when Johnsongrass is present. In the southeast, due to the large number of weed genera present, as many as five treatments may be applied. The weed species include a tough annual grass, Texas panicum, tough annual dicots in the Leguminosae family like Florida beggarweed and sicklepod, bermudagrass (a perennial), and the perennial yellow and purple nutsedges. In the midsouth area, severe infestation of the major weed classes (annual grasses and dicots, perennial grasses and dicots) requires three applications per season at minimum to reduce weed interference to acceptable levels. More commonly, four applications plus spot sprays are needed. This is due to the widespread presence of diverse annual species including crabgrass, broadleaf signalgrass, barnyardgrass, cocklebur, several morning glories, prickly sida, spurred annoda, hemp sesbania, and smartweeds. Additionally, Johnsongrass is established throughout the area, itchgrass and wild poinsettia are spreading out of Louisiana, and sicklepod has recently become widely distributed in the area. Each selective herbicide registered for use in soybeans, and other crops, is specific in mode of action and controls only certain weed species. The large number of weed species present, representing diverse botanical families, requires the application of 4 to 5 herbicides in different chemical families for effective weed management. Glyphosate is active at varying levels on all the above, but is currently limited in effectiveness in soybeans due to the lack of crop tolerance.

Several postemergence herbicides are registered for use in soybeans. They are

usually applied when the crop is 4 to 10" or 10 to 15" in height. These herbicides include bentazon, acifluorfen, chlorimuron, fomesafen, fluazifop, imazaquin, imazethapyr, lactofen, sethoxydim, quizalofop, and 2,4-DB. Separate applications of the grass active and dicot active products are made to obtain the best performance from each. Tank mixtures of two or more dicot active products are generally used south of the central Corn Belt in order to obtain the needed control spectrum. Directed sprays of some non-selective products are applied in the southern region in order to safely use these more effective products.

The label for many commonly used herbicides include precautions on seeding depth, application timing, stage of crop growth, etc., to reduce the risk of soybean injury. Restrictions are also placed on planting rotational crops to prevent carryover losses for herbicides which detoxify slowly in the field.

Systems of weed management employed in soybeans vary within and between production regions. No one system is so universal as to be considered dominant; however three different systems, two in the Corn Belt and one in the midsouth, will be described.

Examples of Commonly Used Weed Systems:

1. Traditional Soybean Weed Control System in the Corn Belt:
    a. Fall tillage (chisel plow) of corn stubble.
    b. Disk in the spring.
    c. Apply trifluralin and soil incorporate with field cultivator (twice) before planting.
    d. At planting apply a mixture of metribuzin plus chlorimuron preemergence on a band.
    e. Apply a mixture of bentazon plus acifluorfen postemergence over the top on a band.
    f. Cultivate (one or two times).
    g. Hand hoe and/or spot spray with glyphosate.

2. Lower Management Soybean Weed Control System in the Corn Belt:
    a. Fall tillage (disk) of corn stubble.
    b. Disk in the spring.
    c. Apply pendimethalin and soil incorporate with field cultivator (once) before planting.
    d. Apply imazethapyr postemergence over the top.
    e. Cultivate prior to canopy closure.

3. Traditional Soybean Weed Control Program in the Mid-South:
    a. Fall tillage (disk) of soybean stubble.
    b. Disk in the spring.

c. Apply trifluralin and soil incorporate with disk.
d. Soil incorporate second trip with field cultivator before planting.
e. At planting apply a mixture of metribuzin and chlorimuron preemergence on a band.
f. Apply a mixture of bentazon plus acifluorfen plus 2,4-DB postemergence over the top on a band.
g. Apply a mixture of 2,4-DB plus either linuron or metribuzin postdirected in the band while cultivating.
h. Apply fluazifop postemergence over the top for rhizome Johnsongrass.
i. Cultivate before canopy closure.
j. Apply a second fluazifop treatment postemergence over the top for rhizome Johnsongrass.

C. Glyphosate-tolerant Soybeans

The use of glyphosate-tolerant soybeans (GTS) provides an attractive alternative to farmers who wish to have additional options for effective weed control. Roundup herbicide is a foliar-applied, broad spectrum, non-selective, post-emergent herbicide (Baird *et al.*, 1971; Malik *et al.*, 1989). It is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Use of soybean plants containing a glyphosate tolerance gene for soybean production would enable the farmer to utilize Roundup herbicide for control of weed pests and take advantage of this herbicide's well-known, very favorable environmental and safety characteristics. GTS can positively impact current agronomic practices in soybean by 1) offering the farmer a new wide-spectrum weed control option; 2) allowing the use of an environmentally sound herbicide; 3) providing a new herbicidal mode of action for in-season soybean weed control; 4) increasing flexibility to treat weeds on an "as needed" basis; 5) offering less dependence on herbicides used before planting; 6) providing an excellent fit with no-till systems, which results in increased soil moisture, while reducing soil erosion and fuel use; and 7) providing cost-effective weed control, not only because Roundup herbicide may be less expensive than most current options, but because total herbicide use may be reduced compared to the farmer's current weed management program.

GTS provides an excellent broad-spectrum weed control alternative to farmers. Currently, farmers are using up to four or five different herbicide families to manage soybean weed problems. One or possibly two treatments of Roundup herbicide at 24 to 32 oz/A will control both annuals and perennials which would reduce the time, cost (herbicide and application), and number of herbicide treatments per acre. GTS will also allow farmers to plant narrow-row (<10 inch) soybeans. University yield data in the north and south indicates a yield increase from planting narrow-row beans. However, good weed control is generally difficult under narrow-row cultural practices. GTS will help farmers

plant narrow-row soybeans more efficiently and take advantage of increased yields and decreased costs.

D. The Likelihood of the Appearance of Glyphosate-resistant Weeds

Several decades ago, herbicide resistant weeds were virtually unknown. Today there are some 109 herbicide resistant weed biotypes with over half of them resistant to triazines (Le Baron, 1991). Major factors which can contribute to the development of resistant weeds include: a single target site and a specific mode of action, broad spectrum of activity, long residual activity and the capacity to control weeds year-long, and frequent applications without rotation to other herbicides or cultural control practices. Using these criteria and based on current use data, glyphosate is considered to be a herbicide with low risk for weed resistance (Benbrook, 1991).

Attached in Appendix V are opinions from several academists located across the soybean growing belt regarding the likelihood of the development of glyphosate-resistant weeds, shifts in weed populations, and overwintering of soybeans. These experts are in agreement that it is highly unlikely that weed resistance to glyphosate will become a problem as a result of the commercialization of glyphosate-tolerant soybeans. Glyphosate has been used for over 20 years in various preplant, directed, spot or post harvest weed management systems with no known reports of weed resistance. This is most likely due to biological and chemical properties demonstrated by glyphosate and the use patterns of the herbicide. Glyphosate essentially has no residual activity in the soil and is quickly broken down by microorganisms in the soil. Also, there is no other herbicide on the market today that has the same mode of action as glyphosate. The experts also tend to agree that eventually one will see a shift in weed populations due to the use of glyphosate in soybeans; however, this would occur with any new herbicide. In fact, any significant change in weed management systems will cause a shift in weed species, but usually these shifts cannot be related to a single variable (combination of tillage, rotation, herbicides, etc.). Finally, soybeans have no innate dormancy, therefore, overwintering is rare. Due to this lack of dormancy (selected for in commercial soybean seed), soybean seeds germinate quickly with adequate temperature and moisture, so all seed that might shatter and/or fall to the ground due to harvest losses eventually will germinate, emerge and be killed by frost during the fall/early winter of the year that they were produced. Even in the unlikely case that some seed did survive, there are several other methods which can be used to remove glyphosate-tolerant soybeans. All field release permits stipulate that the field sites be monitored for one year after harvest for volunteers. Very few, if any, volunteers have been noted for GTS line 40-3-2 (Appendix II. USDA Final Reports; Appendix III. Example Monitoring Forms), and were destroyed by alternate means if observed.

E. Weediness Potential of the Line 40-3-2

The introduction of herbicide tolerance genes to a cultivar should not increase the "weediness" of the plant. A general consensus of the traits common to many weeds was developed by Baker (1974). They include: 1) germination requirement fulfilled in many environments; 2) discontinuous germination and great longevity of seed; 3) rapid growth through vegetative phase to flowering; 4) continuous seed production for as long as growing conditions permit; 5) self-compatibility but not completely autogamous and apomictic; 6) when cross-pollinated, unspecialized visitors or wind pollinated; 7) high seed output in favorable environment and some seed production in a wide range of environments; 8) adaption for short- and long-distance dispersal; 9) if perennial, vegetative production or regeneration from fragments and brittleness (so not easily removed from the ground); and 10) ability to compete interspecifically by special means (rosette formation and presence of allelochemicals). Not all weeds have all of these characteristics. Soybean possesses few of the characteristics of plants that are notably successful weeds. It is an annual crop which is considered to be a highly domesticated, well-characterized crop plant that is not persistent in undisturbed environments without human intervention. G. max cv. A5403, the cultivar which has been genetically modified is not considered to be a weed, and introduction of the glyphosate tolerance trait into this cultivar has not imparted any new "weedy" characteristics. No increase was noted with the transformed cultivar with respect to the number of seeds produced (yield data), and no changes were noted with respect to the germination characteristics of seeds, final stands, or disease or insect susceptibility (Appendix II, USDA Final Reports; Appendix III, Example Monitoring Forms). A preliminary experiment was performed to measure the plant height of line 40-3-2 versus the parental A5403 line. Two out of four sites reported no significant difference, while two sites reported a slight increase in height of line 40-3-2 (16%). This minor observation in height is being investigated further; however, this difference is still within the range of traditional soybean cultivars.

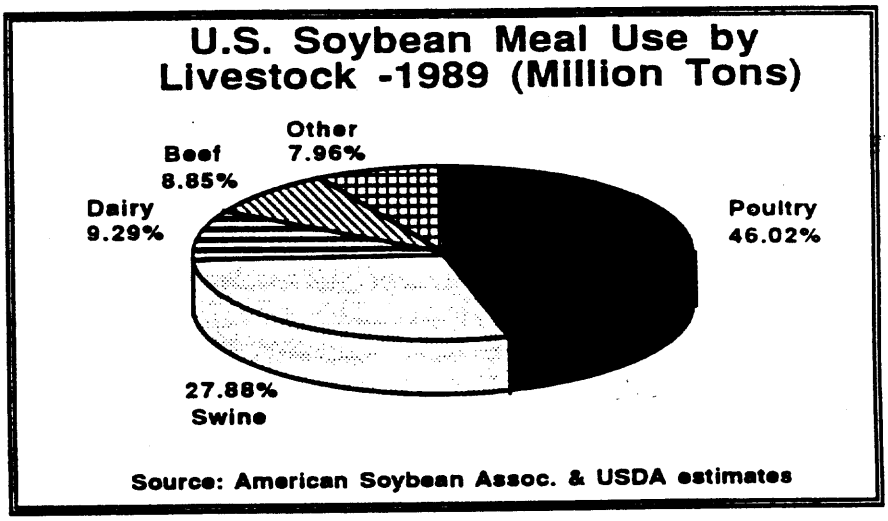
F. Effects of Glyphosate-tolerant Soybean on Nontarget Organisms

GTS line 40-3-2 has been field tested at numerous sites across the U.S. since 1991 and the plants show no toxicity towards insects, birds, or other species that frequent soybean fields (Appendix II, USDA Final Reports; Appendix III, Example Monitoring Forms). We have also performed feeding studies using either raw soybeans or soybean meal on cows, rats, chickens, quail, and catfish with no adverse effects noted (data to be submitted to FDA). As discussed earlier in section IV, the EPSPS enzyme is present in plants (including soybeans) and microorganisms and therefore is ubiquitous in nature and is ordinarily present in food derived from plant sources.

G. Soybean-based Products and Human/Animal Exposure

1. Soybean processing and export - There are three major markets for soybean products: beans, oil and meal. A 60-pound bushel of soybeans yields about 48 pounds of protein-rich meal and 11 pounds of oil (American Soybean Association, 1991). Soybeans are most commonly processed commercially by the following procedure (Lester, 1988): 1) cleaning; 2) drying and tempering; 3) cracking; 4) aspirating to remove the hulls/or fiber; 5) heating (conditioning); 6) flaking (ruptures oil cells); 7) solvent extraction of the oil with hexane; 8) solvent evaporation; 9) refining of the crude soybean oil; 10) desolventizing-toasting of the "spent" soybean flakes (cooking at 210-212°F which denatures most proteins and enzymes, e.g. protease inhibitors); 11) drying / cooling the meal; and 12) screening and grinding of the meal to a uniform grade. The soybean meal contains the protein fraction of the soybeans. The average yield of meal from bulk soybeans is 79%. The United States has become the major exporter of soybeans to world markets. About 35% of the United States soybean crop has been exported as beans to be processed in the importing countries. More than one-half goes to countries in Western Europe, but the largest single importer continues to be Japan. The United States also exports the major products, meal and oil.

2. Animal exposure - The primary use of soybean meal is as a protein supplement for animal and poultry feeds. The use of soybean meal in animal feeds accounts for almost 98% of the processed meal. Shown at right is an estimate of the livestock feed distribution of soybean meal in the U.S. in 1989 (America Soybean Association, 1991).



3. Human exposure - Soybean oil is a major component of the edible oil market and is processed into a variety of products for human consumption. Since proteins are generally water soluble, they are not expected to be a component of refined soybean oil. In fact, studies have concluded that only traces of protein, if any, are found in refined and deodorized soybean oil (Tattrie and Yaguchi, 1973). The average yield of oil from bulk soybeans is 18%. Major

uses are for cooking and salad oils, shortening and oleo margarine. As a source of oil for food products, soybean oil encounters competition from other vegetable oil sources such as sunflower, palm, peanut, cottonseed, rapeseed, coconut and olives. There is a small amount of oil employed in certain inks, paints, varnishes and resin products.

All soybean products going into human food are processed to inactivate natural anti-nutritional components such as trypsin inhibitors, found in all soybeans. Human food usage of soybean protein products accounts for only about 2% of the total meal production. The soybean is the highest natural source of dietary fiber. Soy hulls are processed into fiber bran breads, cereal and snacks. Soy milk is used as an alternative in infant nutrition for the nearly 7% of all infants exhibiting some degree of intolerance of cow's milk. The food industry is increasingly using products derived from soybeans as additives to manufactured food products. They are used to influence the physical structure, stability or texture of products. Soybean flour, either full fat flour or defatted flour, is added to bakery goods. Soy protein concentrates are incorporated in some meat products as an extender, but also in a textured form to simulate meat. Lecithin, a phosphatide removed from crude soybean oil, is used as a natural emulsifier, lubricant, and stabilizing agent (Norman, 1978; American Soybean Association, 1991).

H. Indirect Plant Pest Effects on Other Agricultural Products

Soybeans are not consumed raw by humans, but are subjected to a number of processing steps including cooking and extraction to remove oil (see above). Exposure to elevated temperature is anticipated to denature CP4 EPSPS so that it will not survive processing. Studies conducted at Monsanto on CP4 EPSPS indicate that the enzyme is thermolabile, losing 100% of its activity upon incubation at 65°C (149°F) for 15 minutes. The heat treatments used for processing soybeans exceed these experimental conditions. We have submitted our GTS soybeans to processing (toasting) conditions similar to those used commercially and have detected no residual EPSPS enzymatic activity in the toasted meal.

The only route of exposure to CP4 EPSPS in soybean will be via oral ingestion. If the enzyme survives processing it would then be subject to the hostile environment of the gastrointestinal tract. The gastrointestinal tract is designed to digest ingested dietary proteins by conversion to amino acids and small peptides, which are absorbed by the intestinal tract. This is accomplished through the combined action of acid conditions and pepsin in the stomach and further action of bile acids and enzymes (trypsin, chymotrypsin, carboxypeptidases, etc.) in the intestinal tract. Our own studies have shown that CP4 EPSPS is digested readily by trypsin. We have experimentally confirmed the digestibility of CP4 EPSPS by examining the rate of degradation *in vitro* using simulated gastric and intestinal fluids (*The United States*

Pharmacopeia, 1990). Purified CP4 EPSPS has also been fed to rodents, with no dose-related effects observed.

Therefore, based on 1) the specificity of CP4 EPSPS, 2) the rapid temperature inactivation of EPSPS (by processing), 3) the rapid degradation of ingested proteins, and 4) the normal occurrence of similar plant proteins in plants and animal feed and food, no adverse effects are predicted if this enzyme is ingested as a minor constituent in food.

I. Potential for Outcrossing

1. Outcrossing with wild species

The only wild species that could cross with the cultivated soybean are members of the genus Glycine. No other genus is closely enough related to soybean to allow for the possibility of outcrossing. Therefore, the discussion will concentrate on species of genus Glycine.

a. Hybridization with wild perennial species of subgenus Glycine

The only known perennial species of Glycine occur in Australia, South Pacific Islands, West Central Pacific Islands, China, Papau New Guinea, Philippines, and Taiwan where the wild perennial species are endemic. Soybean production in these areas is mostly in China and Australia. There are no known reports of successful natural hybridization between the cultivated soybean and the wild perennial species. Thus the possibility of gene transfer is non-existent because hybridization does not occur without in vitro seed culture. Even in those cases, the F_1 plants obtained are generally sterile.

b. Hybridization with the wild annual species of subgenus Soja

The wild annual species, G. soja is found in China, Taiwan, Japan, Korea, and the former USSR. Natural hybridizations between G. soja and the cultivated soybean occurs (Kwon, 1972). In fact, the semi-wild form, intermediate in many phenotypic traits between G. max and G. soja, has been recognized as G. gracilis (Skvortzov, 1927). G. soja is not native to North America and occurs only in research plots. There are no records of its escape or dispersal from research plots. G. soja has never been found as a weed or naturalized in the USA. Thus the possibility of gene transfer is very low within the United States.

2. Outcrossing to the cultivated soybean

Hybridization among cultivated soybeans is generally less than 1%. Insect activity does increase the outcrossing rate but soybeans are not a preferred plant (Erickson, 1975, 1984). Male-sterile, female-fertile mutants are used in breeding studies but it is very unlikely that chance pollination with transformed soybeans would occur. In any case, soybean seeds generally do not survive the winter, and soybean does not establish itself as a volunteer

weed in other crops. Even if it did, the plants could easily be controlled by current herbicides which are presently active against soybean.

3. Transfer of genetic information to organisms with which it cannot interbreed

As stated in the USDA's Interpretative Ruling on Calgene, Inc., Petition for Determination of Regulatory Status (FR 57, No.202, pp 47608-47616, October 19, 1992) "There is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from a plant to other organisms. Evidence presented in the Calgene petition and supplementary information and summarized in the FR Notice suggests that, based on limited DNA homologies, transfer from plants to microorganisms may have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of the CP4 EPSPS gene to a microbe would not pose any plant pest risk. EPSPS genes are naturally found in all microorganisms. These microbes are already tolerant to glyphosate under natural growth conditions where the microbes can obtain the aromatic amino acids from the surrounding environment. Some are tolerant because the microbes already carry genes that encode an EPSPS that is tolerant to glyphosate. Indeed, as described earlier in this document, the CP4 EPSPS gene we transferred to soybeans to produce glyphosate-tolerant soybeans was isolated from an *Agrobacterium* sp., a representative of naturally occurring soil microbes. Based on these considerations transfer to microbes is quite unlikely and of no significant consequence from a plant pest point of view.

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Monsanto

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Phone: (314) 694-1000

November 19, 1993

Mr. Michael A. Lidsky
Deputy Director, BBEP, APHIS, USDA
6505 Belcrest Road
Federal Building
Hyattsville, MD 20782

Subject: Petition for Determination of
Nonregulated Status:
Soybeans with a Roundup Ready™ Gene
USDA# P93-258-01
Monsanto# 93-089U

Dear Mr. Lidsky:

The Agricultural Group of Monsanto Company submitted a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding soybeans with a Roundup Ready™ gene. This petition was accepted for review as of September 15, 1993. We would like to provide USDA/APHIS with supplemental information we have obtained from our 1993 field trials which further addresses the yield characteristics of glyphosate-tolerant soybean (GTS) line 40-3-2 which was discussed in sections VE. Yield Characteristics of Line 40-3-2, page 36 and VII. Statement of Grounds Unfavorable, page 64 of the petition.

As stated in the petition, in the summer of 1992, the first wide-scale GTS yield trials were performed. A seven-site yield trial was performed to evaluate line 40-3-2 (untreated with glyphosate) versus the parental line, A5403. At three of the seven sites, there was a slight yield reduction for line 40-3-2. In 1993, additional GTS yield tests were performed to obtain more data regarding the yield of line 40-3-2 using the 40-3-2 active insert backcrossed into other varieties. This is the information we would like to provide the agency which conclusively demonstrates that no yield penalty is associated with the glyphosate tolerance insert present in line 40-3-2. Therefore, we no longer know of any unfavorable grounds associated with the genetic insert of GTS line 40-3-2 and request that the line and any progenies derived from crosses between line 40-3-2 and traditional soybean varieties no longer be regulated under 7 CFR part 340.6.

Detailed below is a summary of the 1993 yield experiments which we based our conclusions from.

In 1992, yield comparisons of unsprayed 40-3-2 and its parent A5403 showed a slightly lower yield for 40-3-2 at three of the seven locations evaluated. This suggested the possibility that a slight yield penalty was associated with the Roundup Ready™ gene insert present in line 40-3-2. Another more likely possibility was that unrelated changes occurred during the transformation and tissue culture process that led to the production of line 40-3-2. As long as those changes are not tightly linked to the Roundup Ready™ gene (which is likely to be the case, since soybean has 20 pairs of chromosomes where somaclonal variation can occur), they can be easily eliminated through the breeding process used to generate new soybean varieties with the Roundup Ready™ gene. Two sets of experiments were set up to test for linkage between the potential yield reduction and the Roundup Ready™ gene:

1) Yield analysis of isopopulations:

   F_2 progenies of crosses between line 40-3-2 and eight different soybean cultivars were used to generate eight pairs of isopopulations with and without the Roundup Ready™ gene. Those progenies were segregating for the Roundup Ready™ gene, and potentially also for any independent somaclonal variation that could have occurred during the transformation and tissue culture process. For each of the progenies, about 75 single F_2 plants were harvested individually and a sample of their F_3 offspring was tested for expression of the Roundup Ready™ gene. As expected, about 50% of the F_3 offsprings segregated for the gene, while 25% were homozygous for the gene (homozygous positives) and the remaining 25% had lost the gene through segregation (homozygous negatives). For each F_2 progeny, all homozygous positive F_3 offsprings were bulked together, and the same was done with the homozygous negatives. Since each pair of bulk F_3 progenies would segregate overall for a similar mixture of genes (including any somaclonal variation unlinked to glyphosate tolerance) except for the Roundup Ready™ gene, they represent isopopulations. The only significant difference between the members of each pair is the presence or absence of the Roundup Ready™ gene. If a somaclonal variation affecting yield is present but unlinked to the gene (and the Roundup Ready™ gene itself does not have any yield effect), there should be no significant difference in yield between the two members of each pair. On the other hand, a significant yield difference in all pairs of isopopulations would indicate that a yield reduction is associated with the glyphosate tolerance gene, either because of the gene itself, or because of the site of insertion of the gene or a tightly linked somaclonal variation.

The Table below summarizes the results obtained with this experiment. Six pairs of isopopulations were tested at Jerseyville, Illinois (USDA# 93-012-05), along with a control pair consisting of 40-3-2 and its parent A5403, three of the six pairs were tested at Monmouth, IL (USDA# 93-012-05) and two additional pairs were tested at Stuttgart, Arkansas (USDA# 93-350-01).

Location	Isopopulation	RR+ bu/acre	RR- bu/acre	
Jerseyville, IL	1	42.6	42.9	ns
	2	43.2	38.8	ns
	3	44.8	46.2	ns
	4	41.7	44.4	ns
	5	42.8	45.7	ns
	6	41.1	43.2	ns
	40-3-2/ A5403	41.8	47.3	*
Monmouth, IL	2	27.8	26.2	ns
	3	26.0	23.7	ns
	5	26.3	28.4	ns
Stuttgart, AR	7	27.6	27.5	ns
	8	27.6	28.7	ns

ns = nonsignificant
* = significantly different at the 0.05 probability level

A significant yield difference was found only within the 40-3-2/A5403 pair, as had been observed at other sites in 1992. In contrast, no significant difference was found in yield between the positive and negative members of each isopopulation. This confirms that the genetic traits affecting yield in line 40-3-2 are similarly distributed in each member of the isopopulation pairs, and therefore are not linked to the Roundup Ready™ gene. Those traits could be readily eliminated through traditional breeding programs aimed at developing new varieties with the Roundup Ready™ gene, using line 40-3-2 as a source for the gene.

2) Yield evaluation of new breeding lines developed from 40-3-2 crosses:

Several seed companies are in the process of incorporating the Roundup Ready™ gene into their breeding programs as part of their effort to develop new soybean varieties tolerant to Roundup® herbicide. As part of the traditional processes involved, they will routinely evaluate the yield of thousands of progenies carrying

the gene and progressively select the highest yielding lines for seed increase and eventual commercialization. Although each line developed through that process is unique and thus cannot be compared directly to any corresponding line that does not contain the Roundup Ready™ gene, one way to determine that no negative yield effect is associated with the gene is to compare the overall yield distribution of the Roundup Ready™ lines to that of the lines developed through their traditional breeding program. Since those types of data are highly proprietary to those seed companies and accidental release to their competitors could be damaging to their competitiveness, they have not shared the actual data with us. However, Asgrow Seed Company has confirmed to us that the yield distribution of more than 3,000 glyphosate-tolerant progenies they tested in 1993 did not differ from what they got through their traditional program, and they were able to select Roundup Ready™ elite lines that yielded as well as or better than their check varieties, as they would have expected to see if no yield penalty was associated with the gene.

Monsanto felt that the additional information demonstrating that no yield penalty is associated with the glyphosate tolerance insert present in line 40-3-2 should be considered as significant supplemental information and should be considered during the review process. Therefore, we no longer know of any unfavorable grounds associated with the genetic insert of GTS line 40-3-2. Please feel free to contact either Dr. W. M. Strauss (202-783-2460) or myself (314-537-6385) if you need any additional information.

Sincerely,

Diane B. Re

Diane B. Re
Regulatory Affairs Manager

cc: Dr. W. M. Strauss

VII. Statement of Grounds Unfavorable

As stated earlier in this petition, in the summer of 1992, the first wide-scale GTS yield trials were performed. A seven-site yield trial was performed to evaluate line 40-3-2 (untreated with glyphosate) versus the parental line, A5403. At three of the seven sites, there was a statistically significant yield reduction for line 40-3-2, with an average yield reduction of 11.5% over those three sites (95% confidence level). At the remaining four sites, there was no statistically significant difference in yield between lines A5403 and 40-3-2. Further yield tests will be conducted to determine whether this initial yield observation is valid. In 1993, additional GTS yield tests will be performed using the 40-3-2 active insert backcrossed into other varieties and maturity groups of soybeans.

Monsanto and our seed company partners plan to commercialize GTS products resulting from the transfer of the glyphosate tolerance locus in line 40-3-2 into new soybean varieties through traditional breeding methods. Standard soybean breeding requires the evaluation of the progenies of the original crosses over several years before selecting the commercial lines. Thus, this standard practice in soybean breeding to eliminate or enhance certain traits by crossing is likely to remove any slight yield reduction (if indeed the initial observation is confirmed) associated with line 40-3-2. The value and benefits of glyphosate-tolerant soybeans (including yield), relative to alternate technologies, will ultimately determine the success of the product. Since there is no evidence that line 40-3-2 has any plant pest characteristics, we request that the line and any progenies derived from crosses between line 40-3-2 and traditional soybean varieties no longer be regulated under 7 CFR part 340.6 in order to provide the necessary flexibility required for continued commercial development.

VIII. Appendices

Appendix I. Maximum Nucleotide Sequence of PV-GMGT04 in GTS Line 40-3-2 and Translation of the Transit Peptide and CP4 EPSPS.

Restriction enzyme sites of HindIII, EcoRI, BglII,and BamHI are included (in the sequence of the 40-3-2 insert, the restriction sites start with the first base pair in each site). The start site of PEPSPS transit peptide is shown at nt 2050 and the start site of CP4 EPSPS is shown at nt 1834. See Figure III.1 for map positions.

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Pages 71-74

Appendix II. Example USDA Final Report for Field Trial

1991 GLYPHOSATE TOLERANT SOYBEAN EXPERIMENTS

JERSEYVILLE, ILLINOIS
MARION, ARKANSAS
QUEENSTOWN, MARYLAND
OXFORD, INDIANA
STONINGTON, ILLINOIS

(USDA PERMIT #91-018-01)

FINAL REPORT

Xavier Delannay
Monsanto Co.

These experiments consisted of an evaluation of transgenic soybeans engineered to be tolerant to glyphosate. The experiment was conducted at four breeding locations of Asgrow Seed Company and at an experimental farm of Monsanto Company.

Experimental layout:

The original protocol allowed for the conduct of up to four separate experiments. The third experiment (Evaluation of selections from the 1990 field tests) did not take place.

1) Evaluation of homozygous lines:

This experiment consisted of a yield evaluation of transgenic lines selected from a field experiment conducted in Isabela, Puerto Rico during the winter 1990-91 (permit # 90-184-01). Two different sets of lines were used, depending on the locations:

- Two independent transformants of Asgrow variety A3322 transformed with pMON10034 and pMON10043 (5-1 and 5-19) were tested in an early maturity experiment at Stonington, IL,

Oxford, IN and Jerseyville, IL. Four sublines of 5-19 were tested at Jerseyville, and only three at Stonington and Oxford. With the two nontransgenic A3322 control entries, there were a total of seven entries at Jerseyville and six at Stonington and Oxford.

- Four independent transformants of Asgrow variety A5403 transformed with pMON10034 (1-1, 1-47, 1-60 and 1-83), two transformed with pMON10047 (3-42 and 3-67), and one transformed with pMON638 (27C) were tested in a late maturity experiment at Marion, AR, Queenstown, MD and Jerseyville, IL. Since there were a few sublines for most transformants, and two nontransgenic A5403 control entries, the total number of entries was 15 at Jerseyville, 14 at Marion and 13 at Queenstown.

Each plot consisted of two rows 16 ft. long, 30 inches apart, with a planting density of about 8 seeds/ft. There were four treatments (0, 24, 48 and 64 oz./acre of Roundup) and three replications, for a total of 12 plots per entry. The experiment was planted more than 20 ft. away from the other nontransgenic soybean experiments conducted at the farm.

The plants were evaluated for glyphosate tolerance and general growth at different times until maturity. At harvest time, each plot was harvested individually using small plot combines. Yield was measured and recorded for each plot.

The lines tested in the early maturity experiment did not have a level of glyphosate tolerance sufficient for further studies. Therefore, no seeds were saved from that experiment and they were all destroyed on the test site in Stonington and Oxford. For the late maturity experiment, only seeds from transformant 1-83 were shipped from Marion and Queenstown to Chesterfield. The other seeds were destroyed on the test sites. The seeds from Jerseyville were all brought to the Monsanto research center in Chesterfield, Missouri (under permit #91-018-03) for weighing and have been kept in cold storage under contained conditions.

2) Evaluation of segregating progenies:

This experiment consisted mostly of a preliminary field evaluation of new selections made in the greenhouse in Chesterfield, Missouri during the winter 1990-91. It was conducted at Jerseyville, IL, Marion, AR and Queenstown, MD. The following lines were involved:

- Two independent lines of Asgrow variety A5403 transformed with pMON638.
- Five independent lines of Asgrow variety A5403 transformed with pMON10034.
- Two independent lines of Asgrow variety A5403 transformed with pMON10047.
- One line of Asgrow variety A5403 transformed with pMON13640.
- Four independent lines of Asgrow variety A5403 transformed with pMON17159.
- Two independent lines of Asgrow variety A5403 transformed with pMON13661.
- Five independent lines of Asgrow variety A5403 transformed with pMON10090.
- Nontransgenic A5403.

Depending on the seed supply, a different number of progenies were planted for each transformant at each location. Each progeny was planted as a single entry. There were a total of 104 entries in Jerseyville, 60 in Marion and 56 in Queenstown. Each entry was divided into four plots, each sprayed with a different rate of glyphosate (0, 24, 48 and 64 oz./acre of Roundup). each plot consisted of a single 5 ft.-long row, and was planted with about 25 seeds. There was no randomization. The experiment

was planted more than 20 ft. away from the other nontransgenic soybean experiments conducted at the farm.

The plants were evaluated for glyphosate tolerance and general growth at different times until maturity. At harvest time, selected plot were harvested individually using a small plot thresher. Seed of the selected lines from Marion and Queenstown were sent to the Monsanto research center in Chesterfield, Missouri and to the Asgrow breeding station in Isabela, Puerto Rico. Those shipments took place under permit # 91-018-03. The seed from Jerseyville were all sent to Chesterfield, Missouri.

3) Crossing block:

This planting consisted of various rows of nontransgenic soybeans for use in crosses with transgenic plants from the previous experiments. Pollen was collected from various plants from plantings 1 and 2, and used for crosses on the nontransgenic lines. The F_1 pods were harvested at maturity and the seeds were shipped either to the Monsanto research center in Chesterfield, Missouri or to the Asgrow breeding stations in Isabela, Puerto Rico and Stonington, Illinois. Only small quantites of seeds were involved in those shipments.

Schedule of major operations:

5/10/91 Seed shipped to Oxford and Stonington

5/14/91 Seed transported and planted in Jerseyville (for $\overline{MG}$ III)

5/21/91 Field planting at Stonington

5/24/91 Field planting at Oxford

5/30/91 Field planting at Jerseyville (first experiment for MG V)

6/03/91 Seed shipped to Marion and Queenstown

6/04/91 Field planting at Jerseyville (second experiment for MG V)

6/05/91 Field planting at Marion

6/06/91 Field planting at Queenstown

9/26/91 Shipment of 81 F_1 seeds from Queenstown to the Asgrow breeding station in Stonington, Illinois

10/10/91 Experiment harvested and destroyed at Marion, Arkansas

10/17/91 Shipment of 40 F_1 seeds from Queenstown to the Asgrow breeding station in Stonington, Illinois

10/18/91 Field harvesting at Oxford, Indiana and Stonington, Illinois. Experimental area destroyed at Stonington, Illinois (the harvested seeds were stored in a cold room at the station until February 1, 1992, when they were spread on the ground in the experimental area and monitored for germination).

10/21/91 Experimental area disked at Oxford, Indiana

10/22/91 Shipment of 55 F_1 seeds from Marion to the Asgrow breeding station in Stonington, Illinois

10/22/91 Shipment of 12 lbs. of transgenic seedsfrom Marion to the Asgrow breeding station in Isabela, Puerto Rico

10/23/91 Shipment of 125 lbs of transgenic seeds from Marion to the Monsanto research center in Chesterfield, Missouri

10/28/91 Shipment of 10 lbs. of transgenic seeds and about 1,500 F1 seeds from Queenstown to the Asgrow breeding station in Isabela, Puerto Rico

10/30/91 Field harvesting at Queenstown, Maryland

11/1/91 Shipment of 9 lbs of transgenic seeds from Marion to the Monsanto research center in Chesterfield, Missouri

11/13/91 Shipment of 172 lbs of transgenic seeds from Queenstown to the Monsanto research center in Chesterfield, Missouri

11/21/91 Experiment plowed under at Queenstown, Maryland

11/26/91 Experiment harvested and disked at Jerseyville, Illinois 590 lbs of transgenic seeds transported from Jerseyville to the Monsanto research center in Chesterfield, Missouri

4/1/92 Germination of soybean seeds observed on the experimental area in Stonington, Illinois. The plants were destroyed and the plot area was worked prior to corn planting. No soybean plants were observed after that.

Plant growth and general observations:

All plots grew normally during the course of the experiment. Except for some minor somaclonal variation in some lines, no obvious differences in growth or yield could be detected between the unsprayed transgenic and nontransgenic plants.

The plots were regularly monitored for *Agrobacterium* infection symptoms. None could be found. No plant damage was observed that could be attributed to birds or rodents, and the plants and remaining seed were destroyed according to the protocol. Nothing unusual was observed during the course of the experiment.

Responses to specific issues:

1) Horizontal movement:

No weed species or other crops that could outcross with soybeans were present in the experimental area, so that transfer of the gene to other species through outcrossing was not possible. As required

in the protocol, the plants were spaced at least 20 ft. away from other soybean plants, so that there was little chance of outcrossing or seed mixture with other soybean experiments.

2) Changes in survival characteristics:

There was no evidence of changes in the survival characteristics of the transgenic soybean plants. The plots were monitored on a regular basis over the winter and the next spring, and no new growth could be observed, except for the Stonington site, where some germination was observed on April 1st, 1992. Those plants were destroyed and no more germination was observed after that.

3) Expression level of the genes:

The plants tested in the field experiment expressed the new EPSP synthase gene from 2- to 10-fold over the normal level of expression of the endogenous EPSP synthase enzyme, depending on the line. In the best lines, this provided for an improvement over the level of glyphosate tolerance observed during the summer 1990 field tests.

4) Stability and inheritance of the new genes:

The transgenic lines included in the field test were either homozygous, or segregated. Most segregating lines showed a normal 3:1 segregation ratio indicative of a single dominant insert, consistent with normal Mendelian genetics. Some lines showed aberrant ratios and are being investigated to gain a better understanding of the genetic mechanism involved. The homozygous lines consistently expressed the gene in their offsprings, showing that the gene is stable in homozygous condition.

5) Published data:

At this point, we are not aware of any published data by Monsanto for this specific test.

Appendix III. Example Monitoring Forms

Field Monitoring for Disease/Insect/Weediness Characteristics

- Make observations at least once every 4 weeks during the growing season.
- Compare control versus transgenic lines for obvious differences using the following criteria:
  - DISEASE (resistance/susceptibility to diseases not specifically engineered to resist).
  - INSECTS (resistance/susceptibility to attack by insects not specifically engineered to resist).
  - WEEDINESS (less susceptibility to herbicides not specifically engineered to resist, unusual proliferation, etc.).
- Record observations below.

Observations about:

Disease NO DIFFERENCES OBSERVED

Insects NO DIFFERENCES OBSERVED

Weediness NO DIFFERENCES OBSERVED

[CBI DELETED]

3-19-93
Date

MARION, ARKANSAS
Study Location

GT SOYBEAN USDA 92-041-01
Study Title

Field Monitoring for Disease/Insect/Weediness Characteristics

- Make observations at least once every 4 weeks during the growing season.
- Compare control versus transgenic lines for obvious differences using the following criteria:
  - DISEASE (resistance/susceptibility to diseases not specifically engineered to resist).
  - INSECTS (resistance/susceptibility to attack by insects not specifically engineered to resist).
  - WEEDINESS (less susceptibility to herbicides not specifically engineered to resist, unusual proliferation, etc.).
- Record observations below.

Observations about:

Disease None

Insects None

Weediness There were weeds but no differences were observed and noted between transgenic and non-transgenic plots. The first two replications were sprayed for cosmetic purposes with 2, 4-DB

[CBI DELETED] _____ 3/19/93 Date

Stuttgart, AR Study Location

GT Soybeans Study Title

Observation Dates: 8/8 - 9/4/92

Field Monitoring for Disease/Insect/Weediness Characteristics

- Make observations at least once every 4 weeks during the growing season.
- Compare control versus transgenic lines for obvious differences using the following criteria:
  - DISEASE (resistance/susceptibility to diseases not specifically engineered to resist).
  - INSECTS (resistance/susceptibility to attack by insects not specifically engineered to resist).
  - WEEDINESS (less susceptibility to herbicides not specifically engineered to resist, unusual proliferation, etc.).
- Record observations below.

Observations about:

Disease No notable differences of transgenic compared to control A5403

Insects No notable differences in insect feeding among lines

Weediness No differences were noted between the transgenic and the control in response to non-glyphosate herbicide. (Pennicle and Classic were sprayed after planting but before emergence.

[CBI DELETED]

8/9/92, 8/11/92
Date

Sidney Ia
Study Location

GT Soybean
Study Title
USDA #92-041-01

Field Monitoring for Disease/Insect/Weediness Characteristics

- Make observations at least once every 4 weeks during the growing season.
- Compare control versus transgenic lines for obvious differences using the following criteria:
  - DISEASE (resistance/susceptibility to diseases not specifically engineered to resist).
  - INSECTS (resistance/susceptibility to attack by insects not specifically engineered to resist).
  - WEEDINESS (less susceptibility to herbicides not specifically engineered to resist, unusual proliferation, etc.).
- Record observations below.

Observations about:

Disease NONE

Insects NONE

Weediness NONE

[CBI DELETED] 11-16-92
Date

Lewiston NC
Study Location

GTS Yield
Study Title

Glyphosate Tolerant Soybeans: U.S. Plant Regulatory Field Tests,
1992 Use Season

Protocol No. 92-01-30-02

[CBI DELETED]

Signature of Investigator [CBI DELETED]

Date 8/8/92

Field Monitoring Observation Log

Visually inspect the genetically modified plots and the non-genetically modified plot every two weeks for disease and insect infestation. Describe any disease or insect infestation that markedly differs in incidence or severity between the genetically modified and non-genetically modified plants in this observation log. Initial and date each observation.

Observation Date	Check If No Differences Noted	Observations
7/3/92	✓	Soybeans emerged TR-1 Best, then TR-3 & Then TR-2
7/6/92	✓	Took stand counts
		TR-1 77,000 plants/A or 5.6 plants/row foot
		TR-2 38,000 or 2.8
		TR-3 64,000 or 4.6
7/13/92		Some damping off disease (Phytophora, Pithium, Rhizoctonia complex) and fine lesions on leaves. Soybeans 1st trifoliate not fully expanded.
7/20/92		Soybeans 3rd Trifoliate fully expanded
7/27/92		Soybeans 6th Trifoliate about ¼ to ½ expanded. <1% foliage damage on leaves & no disease observed
8-3-92		Soybeans beginning 8th trifoliate. Some slight herbicide damage but less than 5%. Good control of broadleaf weeds but grass may recover. Foliage damage due to insects is <1%. Some aerial blight in all plots but early stages only.
8/5/92		Soybeans beginning 10th trifoliate. Could begin to bloom next 1-2 days because flower buds are visible.
8/8/92		Soybeans are blooming. First flower purple. Less than 1% of plants are flowering. Most flowers open per plant was 2.

Glyphosate Tolerant Soybeans: U.S. Plant Regulatory Field Tests,
1992 Use Season

Protocol No. 92-01-30-02

[CBI DELETED]

Signature of Investigator [CBI DELETED]

Date 10/26/92

Field Monitoring Observation Log

Observation Date	Check If No Differences Noted	Observations
8/10/92		No significant problems with insects or diseases
8-17-92		Soybeans just past full bloom which would be late R-2 but prior to R-3. Some foliage damage but less than 1%.
8/30/92	x	Soybeans just beginning ped fill. Wind damage to leaves from Hurricane Andrew. Foliage damage 5%. Also, some plants lodged from high winds estimated to be 5%.
9-1-92		Small looper & beetle population increasing as sprayed the plots.
9-4-92		Checked plots - good insect control. More soybeans are down in CK-2 than CK-1 or 3
9-15-92		Checked plots. Many beetles, worms and stink bugs. Need to spray today.
9/21/92		Checked plots - Control was good. Soybeans in full ped fill and may be approaching leaf drop.
10/1/92		Soybeans maturing, 5-10% leaf drop - 50-60% leaves yellow. No bugs
10/9/92		Soybeans approaching maturity 75-95% leaf drop overall

TIA
DBW
10/26/92

R #

90

Glyphosate Tolerant Soybeans: U.S. Plant Regulatory Field Tests,
1992 Use Season

Protocol No. 92-01-30-02

WDS 8/10/92 ENTRY ERROR
WDS 7/27/92 ENTRY ERROR
32A

[CBI DELETED]

Signature of Investigator [CBI DELETED]

Date 7/27/92

Field Monitoring Observation Log

Visually inspect the genetically modified plots and the non-genetically modified plot every two weeks for disease and insect infestation. Describe any disease or insect infestation that markedly differs in incidence or severity between the genetically modified and non-genetically modified plants in this observation log. Initial and date each observation.

Observation Date	Check If No Differences Noted	Observations
7/27/92	✓	WDS
8/10/92	✓	WDS
8/24/92	✓	WDS
9/7/92	✓	WDS
9/21/92	✓	WDS
10/5/92	✓	WDS
10/19/92	✓	WDS
11/2/92	✓	WDS

WDS 11/11/92

Monitoring for Volunteer Plants

- After harvest and all data is collected, destroy unwanted seed/tubers by the method(s) specified in the permit.
- If available and appropriate for your crop and area, irrigate after harvest to encourage germination.
- One month later, make the initial observation for the number of volunteers (estimates will suffice if the numbers are substantial). During the rest of the offseason, monitor on a monthly basis whenever the weather conditions are favorable for germination. Continue to monitor on a monthly basis for the fallow period specified in the permit or until another transgenic test is planted in the same area.
- Record observations below.
- Remove any volunteer plants by hand weeding, herbicide treatments, or mechanical cultivation.
- Return form to Monsanto after every observation is complete.

Number of volunteers observed 5 plants

Method used to destroy volunteers Hoed plants out by hand.

Comments Field was walked weekly starting the first week of April, 1992. Noted 5 plants the first time, and none after that. Those plants were hoed out, but they would have been destroyed by normal field preparations for corn planting in 1992.

3-18-93
Date

[CBI DELETED]

Stonington, IL 1991-92
Study Location

Glyphosate Tolerant Yield Evaluation
Study Title

Monitoring for Volunteer Plants

- After harvest and all data is collected, destroy unwanted seed/tubers by the method(s) specified in the permit.
- If available and appropriate for your crop and area, irrigate after harvest to encourage germination.
- One month later, make the initial observation for the number of volunteers (estimates will suffice if the numbers are substantial). During the rest of the offseason, monitor on a monthly basis whenever the weather conditions are favorable for germination. Continue to monitor on a monthly basis for the fallow period specified in the permit or until another transgenic test is planted in the same area.
- Record observations below.
- Remove any volunteer plants by hand weeding, herbicide treatments, or mechanical cultivation.
- Return form to Monsanto after every observation is complete.

Number of volunteers observed: None observed

Method used to destroy volunteers: N/A

Comments: Due to late harvest and self cleaning combine we did not note any seed present nor volunteer plants later

[CBI DELETED] _____ 3-20-93 (Date)

Baton Rouge, LA (Study Location) GT3 Yield Study (Study Title)

Unintentional Release of Transgenic Material

If transgenic materials are unintentionally released into the environment (eg. - planting before release permits are obtained; planting or spillage in an area not designated for the release; movement of seed outside of test area by natural causes or vandals), notify Monsanto and the USDA/APHIS Regional Biotechnologist within 24 hours of your knowledge of the release. Record information about the release below.

What was released (seed, leaf tissue, tubers, etc.) N/A

How was it released N/A

Quantities released N/A

Date and time of release (if known) N/A

Steps taken to rectify unintentional release N/A

[CBI DELETED]

3/22/93
Date

Tallassee, AL
Study Location

Field Release GTS Yield Trial
Study Title

USDA # 92-041-01

Monsanto : PS 84

Appendix IV. CP4 EPSPS ELISA Validation Data Summary

I. Precision:

Intraplate Variability:	7.4 % C.V.
Interplate Variability:	7.6 % C.V.
Inter-assay Variability:	≤ 21 % C.V. (determined by QC)
QC Sample Variability:	≤ 21 % C.V. at 0.40 ng/well
Extraction Variability:	29% C.V. from soybean seed 26% C.V. from soybean leaf tissue
Variability in Tissue:	16% C.V. for soybean seed 25% C.V. for soybean leaf tissue

II. Range:

Limit of detection (from spike experiment):	0.010 ng CP4 EPSPS/200 µl/well
Threshold of detection:	OD(405) = 0.090 (reference OD = 655 nm)
Limit of quantitation:	0.13 ng CP4 EPSPS/200 µl/well
Linear range for standard:	0.13 - 0.75 ng CP4 EPSPS/200 µl/well

III. Accuracy:

Extraction Efficiency:	90.2% from soybean seed (1:100 tissue:buffer ratio) 75.1% from soybean leaf (1:20 tissue:buffer ratio)
Spike and Recovery:	122% in soybean seed 73% in soybean leaf

IV. Stability of CP4 EPSPS:

Seed extract:	3 months at -80°C
Seed tissue:	3 months at -80°C, -20°C, and 4°C
Leaf extract:	5 months at -80°C
Leaf tissue:	decrease of 9.5%/30 days

V. Accept/Reject Criteria:

Quality control sample[1]:	± 3 standard deviations from established mean
Value of the blank[2]:	less than 0.306 OD at 405 nm
OD of 0.75 ng/well standard:	> 0.600 OD at 405 nm
Variability in sample replicates:	<8% C.V.

[1]Quality control sample is CP4 EPSPS standard spiked into A5403 extract, aliquotted, and stored at -80°C

[2]Blank value is the absorbance (405 nm) in wells containing only ELISA buffer

Appendix V. Expert Opinion Letters

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY
University Extension

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2104 Agronomy Hall
Ames, Iowa 50011-1010
515 294-1923
FAX 515 294-3163

February 8, 1993

Ms. Diane Re
Monsanto Company
700 Chesterfield Parkway North
Chesterfield, MO 63198

Dear Ms. Re:

Dr. Emilio Oyarzabal requested that I write to you and address several issues concerning the development of glyphosate-resistant soybeans. I have conducted research for one year on these soybeans and feel strongly that this research should continue. It was suggested that I comment on three issues: the development of weed resistance, weed population shifts and overwintering of soybean seed.

Based on the biological and chemical properties demonstrated by glyphosate, it is my opinion that suggested glyphosate use patterns that would develop as the result of glyphosate-resistant soybeans would not result in the development of a resistant weed population. Care should be taken, however, to inform growers that misuse of glyphosate could theoretically result in a resistant weed population. The same could be said for all commercially available herbicides.

Similarly, the use of glyphosate and glyphosate-resistant soybeans will not greatly influence any weed population shifts. Given weed seed dormancy, the soil-seedbank will influence the weed populations more than the judicial use of any herbicide, regardless of the crop genetics. It is important to recognize that soybeans are traditionally grown in rotational schemes that would allow only one year of soybeans consecutively.

Finally, there is no likelihood that soybean seeds will overwinter in the soil. Seed dormancy is a negative trait in crop seed and is bred out of the seed lines. Further, soybeans are self-pollinated. Thus, there is no likelihood that any seed that "volunteered" would ever cross. Importantly, there are no native species with which a soybean could hybridize, even if cross-pollination was possible.

I hope that my assessment of some of the risks commonly associated with the development of herbicide-resistant crops has been helpful. Please contact me if you have any questions.

Sincerely,

Micheal D. K. Owen

Micheal D. K. Owen
Professor and
Weed Science Extension

MDKO:al

University of Illinois
at Urbana-Champaign

Department of Agronomy
W-203 Turner Hall
1102 South Goodwin Avenue
Urbana, IL 61801-4798

217 333-3420
217 333-9817 fax

February 5, 1993

Ms. Diane Re
Monsanto Company
700 Chesterfield Parkway North
Chesterfield, MO 63198

Dear Ms. Re:

Dane Williamson, Monsanto representative who calls on me, mentioned to me some concerns that some people may have concerning the release of glyphosate tolerant soybeans, as related to the possibility of the potential increase of herbicide resistant weeds, weed population shifts, and possible overwintering of soybeans and/or soybean seed in the midwest. He asked if I had any opinions on the matter, and if so, would I convey those opinions to you. My feelings on the matter, based on experience in weed science over the last 25 years, are as follows.

Of all the possible classes of types of herbicides for which weed resistance might develop, glyphosate would seem to be one of the least likely to have resistant weeds develop. To my knowledge, there have been no documented instances of weed resistance to glyphosate to date, and the product has been in use world-wide since the mid-70s. The herbicide has a number of "good" characteristics, in terms of not being likely to have resistant weeds develop. That is, the product has essentially no residual activity in the soil because it is tightly bound to soils and not available to plants, but is broken down by microbial action. Also, as of this time, there is no other herbicide on the market and widely used that has the same mode of action.

Because of some of these same characteristics mentioned above, such as lack of residual control, as well as its being relatively non-selective, I doubt that we can expect to see any significant shift in populations of weeds with repeated use of glyphosate.

As to the likelihood of soybeans overwintering in the midwest, I have never seen that happen. Also, due to the lack of dormancy that is selected for in soybean seed for commercial production, soybean seeds germinate quickly with adequate temperature and moisture, so all seeds that might shatter and/or fall to the ground due to harvest losses, eventually germinate, emerge and are killed by frost during the fall/early winter of the year that they are produced. I have never seen a soybean seedling from a commercial variety emerge and grow in the spring, following a fall seeding or seed shattering at soybean harvest time.

I hope that the these opinions will clarify my thinking on the matter. I see very little if any potential problems of the type mentioned with glyphosate tolerant/resistant soybeans. In fact, the availability of glyphosate tolerant soybeans would appear to be a real plus for us as we develop weed management systems for no-tillage systems, in that we can begin to eliminate the need for some of our more persistent, and less environmentally friendly herbicides from our management systems, with a resulting reduction in pesticide load placed into the environment.

Sincerely,

Loyd M. Wax
Professor of Agronomy
University of Illinois

LMW:sc

Opinions of Dr. Stephen O. Duke (USDA, ARS, Southern Weed Science Laboratory, P. O. Box 350, Stoneville, MS 38776) on several matters regarding glyphosate-resistant soybeans (termed Roundup Tolerant Soybeans by Monsanto Co.)

1. Will weeds become resistant to glyphosate in glyphosate-resistant soybeans?

Given enough time, almost anything can happen. However, I think that resistance to glyphosate is unlikely to evolve to any significant extent by selection pressure exerted through use of this herbicide. By significant, I mean that the odds of weed biotypes evolving that are extremely resistant (such as found for herbicides inhibiting acetolactate synthase or acetyl CoA carboxylase) is very low. I think that for all of the herbicides available, resistance to glyphosate is the least likely to evolve. My opinion is based primarily on biochemical and historical information.

From the research done to produce glyphosate-resistant crops, it is clear that a natural mutation resulting in a glyphosate-resistant EPSP synthase (the primary molecular target site of this herbicide) is not sufficient to impart resistance. A mutation providing amplification of the EPSP synthase gene at some level would also be required. The odds of such a double mutation would be extremely remote. Furthermore, should such an unlikely mutant occur, it would probably be unfit to survive in a natural population and/or only moderately tolerant to glyphosate, eliminating it from a field of glyphosate-resistant soybean. To my knowledge, soybeans do not interbreed with any plant species in North America. So, the possibility of resistance spreading from the crop to weeds is even more remote than resistance evolving through selection pressure.

Furthermore, glyphosate has been used extensively for about 20 years with no reports of glyphosate-resistant weeds. Glyphosate-resistant soybeans will add relatively little to the present and past selection pressure.

2. Will glyphosate-resistant soybeans cause shifts in weed populations?

The answer is yes, eventually this is likely to happen. Any significant change in weed management methods will cause a shift in weed species, from those that are killed to those that are more tolerant to the method of management. This process may be quite slow with glyphosate-resistant soybeans because the weeds that are most tolerant to glyphosate are not common in soybean fields. Such weed population shifts would be extremely slow if the glyphosate-resistant soybeans are rotated with a crop with which other herbicides are used.

Even without changes in herbicides, changes in weed populations appear to occur. These shifts usually cannot be related to a single variable.

3. Will glyphosate-resistant soybeans overwinter to become a weed problem?

This is no more likely than with non-glyphosate-resistant soybeans, unless the soybeans are rotated with a different glyphosate-resistant crop (*e.g.*, glyphosate-resistant corn).

University of Kentucky
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AGRONOMY
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Office: (606) 257-7310
Fax (606) 258-1952

February 26, 1993

Ms. Diane Re
Monsanto Company
700 Chesterfield Parkway North
Chesterfield, MO 63198

Dear Ms. Re:

I support the use of glyphosate tolerant soybeans as an integral part of an overall weed management program. Glyphosate is an environmentally safe herbicide that controls many of the troublesome weeds in soybeans in Kentucky. A major advantage of using glyphosate is the introduction of another herbicide chemistry in the soybean-corn-wheat rotation commonly used in our state.

Sulfonylureas and imidazolinones are commonly used in soybeans and corn for control of common cocklebur, giant ragweed, morningglory species, smooth pigweed and johnsongrass. Johnsongrass is the most troublesome weed in soybean and corn in Kentucky and sulfonylureas will be used for managing this weed in corn. Therefore, it is important to have available other chemistry, such as that offered by glyphosate, to use in the soybean segment of the crop rotation to minimize the potential for developing weed resistance to the herbicides available to Kentucky's farmers.

Johnsongrass resistant to the ACCase inhibitor herbicides has been documented in Mississippi. Glyphosate offers soybean growers another herbicide chemistry to use for managing this troublesome weed in soybean.

The above examples point out the advantage of having glyphosate tolerant soybeans available to the grower to avoid the development of herbicide resistant weeds. I am not aware of any weed having developed resistance to glyphosate and this characteristic, along with its environmentally safe characteristics, make it a desirable herbicide to include for weed management.

I strongly urge that the program for development of glyphosate tolerant soybeans be continued.

Sincerely yours,

William W. Witt

William W. Witt
Professor

OHIO STATE UNIVERSITY

Ohio Cooperative Extension Service

Agronomy
2021 Coffey Road
Columbus, OH 43210-1086
Phone 614-292-2047

February 15, 1993

Diane Re
BB4D Monsanto Company
700 Chesterfield Parkway North
Chesterfield, MO 63198

Dear Ms. Re:

Paul Sprankle asked me to write a letter addressing possible concerns about glyphosate-tolerant soybeans (GTS) and more intensive use of glyphosate in soybean weed control programs. These included: 1) the potential for the seed of GTS soybeans to overwinter and become a weed problem in a subsequent crop, 2) the likelihood of the development of glyphosate-resistant weeds, and 3) possible weed population shifts in response to the use of glyphosate. In my position as an extension weed specialist, I see the development of GTS as beneficial for farmers, due to the low cost and broad-spectrum effectiveness of glyphosate compared to many other soybean herbicides. The activity of glyphosate on perennial weeds, which are becoming more of a problem as no-till acreage increases, will go a long way towards management of these weeds.

Regarding the first of the concerns listed above, I have never seen soybean seed overwinter in the field and germinate the following year to become a weed problem in a subsequent crop. While this does occur for kernels of corn on ears that may be missed by a combine and buried in the soil, it does not occur for soybean seed. I assume the concern here is that volunteer GTS could become a problem which would not be easily controlled, thus resulting in the spread of an organism that has glyphosate tolerance. I see very little potential for this to occur.

To the best of my knowledge, glyphosate-resistant populations of weeds have not developed to date. I am not a specialist in weed physiology and herbicide resistance, but this lack of resistance to date may be due to a combination of several factors. First, glyphosate has not been used continuously (year after year) in row crop production as a sole means of weed control. In row crops, glyphosate is generally used as a burndown treatment to control existing vegetation at planting, and other herbicides are applied with or after glyphosate to control weeds that emerge

after crop planting. Other uses of glyphosate in a row crop rotation generally involve fall applications for perennial weed control. Resistance to herbicides generally develops, primarily in annual weeds, as a result of continuous use of a single herbicide for a number of years, and glyphosate has not been used in this manner. The development of GTS would allow use of glyphosate in soybeans, and it may be possible for weeds to develop resistance to glyphosate in a continuous soybean monoculture where glyphosate is the primary weed control. It would be unlikely that this would occur in any rotation of soybeans with another crop, as glyphosate would not be used as the primary means of control in the other crop. Application of herbicides that have different modes of action across a rotation generally prevents the development of weed resistance. The mode of action of glyphosate may be such that the development of resistance is unlikely, but I believe that the lack of resistant weeds so far is a result primarily of the lack of continuous use of glyphosate.

Weed scientists are concerned about the potential for widespread development of herbicide-resistant weed populations, due to the characteristics of many of the herbicides currently used in corn, soybean, and wheat production. This can be prevented by rotation of crops and herbicides, and we would recommend this for the use of glyphosate as well. When managed properly, glyphosate use in soybeans should result in little risk of glyphosate-resistant weeds developing.

A final concern to be addressed here is the potential for weed population shifts following the use of glyphosate. I would expect that some weed population shifts would occur, but this is to be expected from the use of any herbicide. Every herbicide has a certain spectrum of weed control activity (i.e. it controls some weeds and not others). Those weeds not controlled by a herbicide or combination of herbicides tend to become more prevalent in the treated area, since they do not have to compete with other weeds for growth factors. This is generally overcome by combining herbicides that control as many of the weeds in a field as possible. We have many herbicides available for use in soybeans, and a number of these could be combined with glyphosate to control weeds that are outside glyphosate's spectrum of activity. Alternatively, one could apply a separate application of another herbicide to control weeds that escape treatment with glyphosate. We have to deal with weed population shifts in every field as a result of changes in tillage, rotation, or herbicide use, and the use of glyphosate will not result in anything unusual as far as I can tell.

While the development of GTS is certainly novel, as a weed specialist I consider the possible deleterious effects of glyphosate use in soybeans to be negligiable and manageable. In this respect, the use of glyphosate for weed control in soybeans is no different than that of any other herbicide. Any herbicide must be properly managed to be used effectively over a long

period of time, and glyphosate is no exception.

Sincerely,

Mark M. Loux
Extension Agronomist
Weed Science

University of Wisconsin-Madison

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Improving Agriculture Through Crop Biotechnology, Genetics and Production Research

February 5, 1993

Diane Re
Monsanto Company
700 Chesterfield Parkway North
Chesterfield, MO 63198

Dear Diane:

Knudt Miller provided me with a copy of the three questions posed by the USDA concerning Roundup. Consequently, I have summarized below my answers to those questions.

Question: Likelihood of weed resistance developing to glyphosate

It is highly unlikely that weed resistance to glyphosate will become a serious problem in the future as a result of expected production of glyphosate tolerant (GTS) soybeans. Glyphosate has been widely used in various preplant, directed, spot or post harvest systems for nearly 20 years. Despite that extensive use, I am aware of no plant species which are either naturally tolerant or have developed resistance to glyphosate. By its nature, glyphosate is non-selective. That means that even crops are susceptible to it. When considering other selective herbicide which do not injure specific crops, I know in advance that since certain crops are naturally tolerant, that there is a high likelihood that some naturally tolerant or newly-developed resistant weed will become a problem. This indirect evidence suggests that it is unlikely that weeds will develop resistance to glyphosate.

One plant species which is not always controlled by glyphosate under field conditions is alfalfa. During the 1970's, I tried to exploit that characteristic in order to use glyphosate for quackgrass control in alfalfa fields. Our studies indicated that alfalfa plants were in fact susceptible to glyphosate, but some environmental conditions prevented adequate uptake or translocation of the herbicide to kill alfalfa plants. For several years, I collected surviving alfalfa plants from treated fields and retreated them in the greenhouse. In all but one case, retreatment injured the alfalfa plants. The one exception was a plant which I eventually treated three more times in the greenhouse. It was still not injured. I then took cuttings and rooted them to provide approximately 25 plants for another field study. Since they were derived from cuttings, they were genetically identical. When treated in the

field, all but one of these cuttings was injured by glyphosate. I suspect that the original plant escaped the first application due to inadequate coverage, etc. That first treatment may have stimulated an adaptive physiological response which protected the plant from subsequent treatments. During the process of rooting and transplanting cuttings, the adaptive response was lost. As a result, the second field application injured the plants. Obviously, genetic resistance was not involved.

Later, I had a graduate student grow 'Regen' alfalfa in tissue culture. We tried to use tissue culture selection procedures to create plants resistant to glyphosate. Not only did we fail to create resistant plants, we never even got "resistant" tissue. This again supports the hypothesis that development of weeds resistant to glyphosate is unlikely.

Several other factors also support the theory that weeds are unlikely to develop resistance to glyphosate. Rapid development of herbicide resistance to other herbicides has often been associated with long-term selection pressure resulting from use of chemicals with long periods of soil activity. Characteristics of glyphosate are just the opposite. Glyphosate is bound tightly to soil colloids and thus the chemical has almost no residual soil activity. Once in the soil, glyphosate is quickly degraded by microorganisms. This further reduces the opportunity for residual selection pressure. With the exception of TOUCHDOWN, no other herbicides have the same mode of action as glyphosate. This again reduces the potential for excessive selection pressure creating resistant weeds. TOUCHDOWN is actually just another salt of glyphosate. Thus, it should not be considered as a different herbicide.

Thus, in conclusion, it is unlikely that glyphosate resistant weeds will develop.

Question: Likelihood of weed population shifts as a result of glyphosate-resistant soybeans.

I have no doubt that shifts in weed populations will result from use of glyphosate on GTS soybeans. Such population shifts have resulted from every other weed management practice including handweeding and cultivation. In fact, the nonselective nature of glyphosate action makes it very similar to cultivation; a more effective broadcast chemical cultivation! If applied only once early in a season, late-germinating weed species will increase in population. This shift will be less severe if glyphosate application can be delayed until most weed species have germinated and emerged, or if multiple applications of glyphosate are made in each field. This is not a particular problem. It is simply an example of why no single weed-management strategy will ever be likely to solve all weed problems.

Question: Potential for overwintering of soybean seed in the Midwest.

In answering this question, I am only able to discuss Wisconsin conditions. Because soybean seed has no innate dormancy, overwintering is rare. To survive the winter, soybean seed must be isolated from moisture, oxygen and temperatures exceeding about 40 F. Few situations

will create these conditions in Wisconsin. Occasionally, we find a few volunteer soybean plants in no-till fields which follow soybeans harvested extremely late in a dry fall. Apparently a few seeds may be trapped in dry plant residues and cold weather forces the seed into dormancy before adequate rainfall occurs to cause the seeds to germinate. The number of seeds which might overwinter under such conditions are too small to cause economic problems.

I hope these answers will help satisfy the USDA concerns. The GTS soybeans could be extremely important in Wisconsin. Problems which we have encountered with atrazine in our groundwater demonstrate the potential for problems to arise from other soil-applied chemicals as well. We have also found a few wells contaminated with cyanazine, alachlor, metribuzin and several other chemicals. Controlling weeds with glyphosate applied to GTS soybeans should help protect our groundwater.

Sincerely,

Robert Gordon Harvey
Professor of Weed Science

93-258-01P
Update on Roundup Ready Soybean

MONSANTO
Food • Health • Hope™

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May 15, 2000

John H. Payne, Ph.D.
Assistant Director of Plant Health
U.S. Department of Agriculture / APHIS
4700 River Road
Riverdale, MD 20737

Re: Roundup Ready® Soybean Event 40-3-2, Petition 93-258-01P

Dear Dr. Payne:

The enclosed documents are being provided to the U.S. Department of Agriculture to update the file on Roundup Ready soybean event 40-3-2:

1. "Further Molecular Characterization of Roundup Ready Soybean Event 40-3-2", MSL-16646, May 12, 2000;
2. "Updated Molecular Characterization and Safety Assessment of Roundup Ready Soybean Event 40-3-2", MSL-16712, May 13, 2000; and
3. "Expert Panel Opinion on Roundup Ready Soybean Varieties Derived from 40-3-2", which was prepared by an independent panel of experts on May 2, 2000, following their review of the data.

Monsanto submitted a summary of our safety assessment of Roundup Ready soybean event 40-3-2 to USDA on September 15, 1993 in a petition seeking a determination of non-regulated status. This document informed the USDA of the steps taken by Monsanto to ensure that soybean event 40-3-2 complied with the legal and regulatory requirements that fall within USDA's jurisdiction. Utilizing APHIS regulations at 7 CFR part 340, Monsanto concluded that soybean event 40-3-2 did not pose a plant pest risk and should no longer be regulated.

5/15/2000

On May 19, 1994, the USDA concluded that "glyphosate-tolerant soybean line 40-3-2 does not present a plant pest risk and should therefore no longer be a regulated article under regulations at 7 CFR part 340". On May 24, 1994, the USDA published a notice of the availability of the determination and the environmental assessment and finding of no significant impact (59 FR 26781).

As part of the safety assessment of Roundup Ready soybean event 40-3-2, Monsanto's 1994 submission included a detailed description of the source, identity and function of the genetic material introduced into this event. Based on PCR and Southern blot analysis, it was concluded that Roundup Ready soybean event 40-3-2 contained a single insertion of DNA from the plasmid PV-GMGT04, encoding a portion of the e35S promoter, a chloroplast transit peptide, the CP4 EPSPS coding sequence, and a portion of the NOS 3' transcriptional termination sequence.

Monsanto has subsequently extended the molecular characterization of soybean event 40-3-2 using more sensitive and selective methods as part of our seed quality monitoring and detection method development programs. Based on the results of these additional molecular characterization studies, the 40-3-2 event has been further defined to contain an additional, previously unobserved 250 bp segment of the CP4 EPSPS element on the primary functional insert and a second insert consisting of 72 bp of the CP4 EPSPS sequence. In addition, these recent studies show that the NOS transcriptional termination sequence is intact, and not a partial element as previously reported. A schematic representation of the revised 40-3-2 insert can be found on page 32 of the enclosed molecular characterization report.

Using Southern blotting, genomic cloning, PCR and nucleotide sequencing techniques we have determined that both CP4 EPSPS segments were constituents of Roundup Ready soybean event 40-3-2 throughout the comprehensive safety studies performed on this product, that only the full-length CP4 EPSPS contains elements required for gene expression, that only the full-length CP4 EPSPS protein is detected, and that both CP4 EPSPS segments were present in the common progenitor of all commercial soybean varieties.

Based on these factors, Monsanto has reviewed its safety and environmental assessment of Roundup Ready soybean event 40-3-2 and concluded that the recent characterization using more sensitive and precise methods does not alter the initial conclusion that Roundup Ready soybean event 40-3-2 is as safe as conventional soybeans for use in food and animal feed and does not pose a risk to the environment.

These conclusions have been confirmed by a panel comprised of recognized experts, following a thorough review and evaluation of all of the data; their opinion document is enclosed.

Monsanto requests that the enclosed information be inserted as an addendum to file for Roundup Ready soybeans. If you have any questions regarding this information please contact me or Dr. Russ Schneider.

Sincerely,

Sheila A. Schuette

Sheila A. Schuette, Ph.D.
Director, Regulatory Affairs

Enclosures (3)

[

CBI DELETED

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CBI DELETED

]

Pages 130-180

Roundup Ready Soybeans: Peer-reviewed publications

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A comparative study of the allergenic potency of gene modified (GMO) and wild type soybeans. P.Stahl Skov, E. Andersson, S. B. Andersen, AM. Torp, A.Olesen, U. Bindslev-Jensen, L. K. Poulsen and C. Bindslev-Jensen. Abstract to the European Academy of Allergy and Clinical Immunology Annual Meeting, July 2000, Lisbon, Portugal

Purpose. A large proportion of soybeans grown in USA is now GMO varieties and concern has been raised about the safety of these products to consumers. In order to study the impact on allergenic potency in comparable beans (i.e, grown under similar conditions) genetically comparable except for the newly introduced gene,a study was performed using RAST-inhibition and histamine release (HR) from IgE sensitized patients.
Methods. The allergenicity of 19 different (10 GMO and 9 wild type) soybean extracts were examined blindly by the following two methods:
A) Sera from patients with specific IgE against soybean were used for determining the 50 % RAST inhibition of the different soybean extracts.
B) Histamine release (HR-Test, RefLab, Copenhagen) induced by the extracts were examined on blood from IgE sensitized patients and the treshold dose inducing > 15 ng histamine/ml were recorded.
Results. Both RAST inhibition and histamine release showed variations in the allergenic potency between the individual extracts but these variations were not related to the genetic background of the soybeans. RAST-inhibition: $p > 0.05$; HR: HR < 3.5 fold variation.
Conclusion. Using standard in vitro methods for determination of allergenic potency we were not able to detect any significant difference in the potency between GMO and wild type soybeans.

93-258-01p addendum

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February 5, 2002

James L. White, Ph.D.
Senior Operations Officer, Biotechnology
U.S. Department of Agriculture / APHIS
4700 River Road
Riverdale, MD 20737

Re: Roundup Ready® Soybean Event 40-3-2, USDA-APHIS Petition 93-258-01p

Dear Dr. White:

As followup to the information on Roundup Ready Soybean event 40-3-2 recently communicated to you verbally by Dr. Russell Schneider, the enclosed documents are being provided to USDA-APHIS to update your file on petition 93-258-01p on Roundup Ready Soybean event 40-3-2:

1. "Executive Summary: Characterization and Safety Assessment of the DNA Sequence Flanking the 3' End of the Functional Insert of Roundup Ready Soybean Event 40-3-2"; and
2. Additional Characterization and Safety Assessment of the DNA Sequence Flanking the 3' End of the Functional Insert of Roundup Ready Soybean Event 40-3-2, Dobert *et. al.* (February 1, 2002), MSL-17632.

Monsanto requests that the enclosed information be inserted as an addendum to your file for petition 93-258-01p. If you have any questions regarding this information, please contact me at (636) 737-5532 or Dr. Russ Schneider at (202) 383-2866.

Sincerely

Raymond C. Dobert

Raymond C. Dobert, Ph.D.
Regulatory Affairs Manager

JW
3/2/02

Enclosures (2)
cc: Dr. Russell Schneider
93-089U

Received
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