

Development, Identification, and Characterization of a Glyphosate-Tolerant Soybean Line

S. R. Padgette,* K. H. Kolacz, X. Delannay, D. B. Re, B. J. LaVallee, C. N. Tinius, W. K. Rhodes, Y. I. Otero, G. F. Barry, D. A. Eichholtz, V. M. Peschke, D. L. Nida, N. B. Taylor, and G. M. Kishore

ABSTRACT

Glyphosate (*N*-phosphonomethyl-glycine) is the active ingredient in the nonselective herbicide Roundup. The sensitivity of crop plants to glyphosate has limited its in-season use as a postemergence herbicide. The extension of the use of Roundup herbicide to allow in-season application in major crops such as soybeans [*Glycine max* (L.) Merr.] would provide new weed control options for farmers. A glyphosate-tolerant soybean line, 40-3-2, was obtained through expression of the bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS) enzyme from *Agrobacterium* sp. strain CP4. Line 40-3-2 is highly tolerant to glyphosate, showing no visual injury after application of up to 1.68 kg acid equivalent (a.e.) ha⁻¹ of glyphosate under field conditions. Molecular characterization studies determined that the single genetic insert in line 40-3-2 contains only a portion of the cauliflower mosaic virus 35S promoter (P-E35S), the *Petunia hybrida* EPSPS chloroplast transit peptide (CTP), the CP4 EPSPS gene, and a portion of the 3' nontranslated region of the nopaline synthase gene (NOS 3') terminator. Inheritance studies have shown that the transgene behaves as a single dominant gene and is stable over several generations.

WEEED MANAGEMENT is a critical step in maximizing soybean yields and retaining a high quality harvest, free of weed seeds. For effective weed control, the farmer typically selects a herbicide based on several factors: weed spectrum, lack of crop injury, cost, and environmental characteristics. Desirable environmental characteristics include minimal toxicity, low or no groundwater movement, and limited persistence. Few herbicides available today deliver optimal performance in all of these areas. Several classes of broad-spectrum herbicides exist, but most are nonselective and kill or significantly injure crops at the application rates required for effective weed control.

One such nonselective herbicide is glyphosate (*N*-phosphonomethyl-glycine), the active ingredient in Roundup¹ herbicide (Malik et al., 1989). Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Recent advances in plant biotechnology have made it possible to insert a gene into soybeans to provide crop tolerance specifically to glyphosate, and bring the benefits of its use to weed management in soybeans (Barry et al., 1992; Padgette et al., 1995a). Glyphosate-tolerant soybeans (GTS, also referred to as *Roundup Ready* soybeans) can positively impact current agronomic practices in soybean by (i)

offering the farmer a new wide-spectrum weed control option; (ii) allowing the use of an environmentally sound herbicide; (iii) providing a new herbicidal mode of action for in-season soybean weed control with a product for which no weed resistance has developed in almost 20 yr of use (Holt et al., 1993); (iv) offering compatibility with minimum or no-till conservation systems, which result in increased soil moisture, while reducing soil erosion and fuel use; and (v) providing cost-effective weed control.

The development of glyphosate-tolerant crops has been pursued since the early 1980s (Gasser, 1989; Hinchee et al., 1993; Padgette et al., 1989, 1995; Mazur, 1989; Kishore and Shah, 1988; Kishore et al., 1988). The *target-site modification* herbicide tolerance mechanism was utilized for soybeans, whereby a herbicide-insensitive target protein was identified and introduced into the crop by genetic engineering techniques. Glyphosate specifically binds to and blocks the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS) (E.C. 2.5.1.19) (Steinrucken and Amrhein, 1980), an enzyme of the aromatic amino acid biosynthetic pathway (Haslam, 1993). EPSPS catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and phosphate. Glyphosate inhibition of EPSPS thus prevents the plant from making the aromatic amino acids essential for the synthesis of proteins and some secondary metabolites. EPSPS is the only physiological target of glyphosate in plants, and no other PEP-utilizing enzymes are inhibited by glyphosate (Steinrucken and Amrhein, 1984). EPSPS is present in all plants, bacteria, and fungi, but not in animals; animals do not make their own aromatic amino acids, but receive them from plant, microbial, or other animal foods. In plants, EPSPS is localized in the chloroplasts or plastids (della-Cioppa et al., 1986). Upon glyphosate treatment, the GTS plant remains unaffected because the continued action of the introduced glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic amino acids. This is in contrast to the death or severe growth reduction observed upon glyphosate treatment of conventional susceptible soybeans and other plants.

Extensive research indicated that the achievement of high levels of glyphosate tolerance in planta depended on the expression of a glyphosate-tolerant EPSPS with a high catalytic efficiency in the presence of glyphosate (Barry et al., 1992). Extensive work was carried out on

¹ Roundup herbicide and Roundup Ready are registered trademarks of Monsanto Company.

S.R. Padgette, K.H. Kolacz, X. Delannay, B.J. LaVallee, G.F. Barry, D.A. Eichholtz, V.M. Peschke, D.L. Nida, N.B. Taylor, and G.M. Kishore, Monsanto Co., 700 Chesterfield Parkway N., St. Louis, MO 63198; C.N. Tinius, W.K. Rhodes, and Y.I. Otero, Asgrow Seed Co., Kalamazoo, MI 49002. Received 16 Jan. 1995. *Corresponding author (srpadg@ccmail.monsanto.com).

Abbreviations: a.e., acid equivalent; CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4; ELISA, enzyme-linked immunosorbant assay; EPSP, 5-enolpyruvylshikimate-3-phosphate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GTS, glyphosate-tolerant soybeans; GUS, β -glucuronidase; kb, kilobase; kD, kilodalton; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; S3P, shikimate-3-phosphate.

the G101A (glycine to alanine substitution at position 101) petunia EPSPS (Padgett et al., 1991) and other variant EPSPSs (Ruff et al., 1991), but none were identified that were both highly glyphosate tolerant and that bound the PEP substrate comparably to wild-type EPSPS. The naturally occurring EPSPS enzyme from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) was identified from a screen of microorganism cell extracts as having very favorable glyphosate tolerance kinetic parameters, namely high glyphosate tolerance ($\text{appK}_i[\text{glyphosate}] = 2.7 \text{ mM}$) and tight binding of PEP ($\text{appK}_m[\text{PEP}] = 12 \text{ }\mu\text{M}$) (Barry et al., 1992; Padgett et al., 1995a). The $\text{appK}_i[\text{glyphosate}]$ represents the enzyme's capability to bind to and be inhibited by glyphosate. The higher the appK_i value, the more the enzyme is able to resist glyphosate inhibition. The $\text{K}_m[\text{PEP}]$ is a measure of the enzyme's ability to bind PEP—the lower the $\text{appK}_m[\text{PEP}]$, the better the enzyme is able to interact with PEP and catalyze the reaction. Based on these kinetic parameters, and thus the suitability for use in conferring glyphosate tolerance to crops, the gene for CP4 EPSPS was cloned from *Agrobacterium* sp. strain CP4, and expressed in both *Escherichia coli* (Padgett et al., 1989–1990, unpublished results) and several crop plants for further characterization.

The objectives of the research described herein were (i) to produce transgenic soybean lines expressing *Agrobacterium* sp. strain CP4 EPSPS; (ii) to determine whether CP4 EPSPS could confer high levels of glyphosate tolerance to soybean, as suggested by its kinetic parameters; (iii) to determine the expression levels of the CP4 EPSPS protein in the leaves and seeds from the soybean line demonstrating the highest level of glyphosate tolerance; and (iv) to characterize in detail the inserted DNA in the same GTS line. We now report the successful utilization of the CP4 EPSPS gene in generating a GTS line with commercial utility, designated line 40-3-2, along with detailed information on the production and molecular characterization of the line. Results from multi-year yield tests are described in a companion paper (Delannay et al., 1995, this issue). The development of GTS has been recently reviewed (Padgett et al., 1995a).

MATERIALS AND METHODS

Plasmid PV-GMGT04

The CP4 EPSPS gene was engineered for plant expression by fusing the 5'-end of the gene to a chloroplast transit peptide sequence derived from petunia EPSPS (Shah et al., 1986; Gasser et al., 1988; della-Cioppa et al., 1986). This chloroplast transit peptide has been shown previously to deliver bacterial EPSPSs to the chloroplasts of higher plants, which is the site of the aromatic amino acid biosynthetic pathway and the organelle to which the plant EPSPS is targeted (della-Cioppa et al., 1987). Previous studies had shown that it was critical to target glyphosate-tolerant EPSPSs to the chloroplast to obtain the highest levels of in planta glyphosate tolerance. In vitro chloroplast uptake assays verified that the petunia EPSPS chloroplast transit peptide delivered CP4 EPSPS to the chloroplasts (unpublished results, 1989–1990). After the *pre-CP4 EPSPS* protein (containing the chloroplast transit peptide amino-terminal extension) reaches the chloroplast or plastid stroma,

the chloroplast transit peptide is cleaved and degraded, as are the chloroplast transit peptides from other naturally occurring nuclear-encoded chloroplast-targeted proteins (Bartlett et al., 1982). This leaves the *mature* CP4 EPSPS, with no chloroplast transit peptide sequences retained, as the CP4 EPSPS species present in planta (Harrison et al., 1995).

The plasmid PV-GMGT04 used to generate the soybean line 40-3-2 contained three bacterial genes driven by plant promoters: two CP4 EPSPS genes and a gene encoding β -glucuronidase (GUS) from *E. coli* (Jefferson et al., 1986) (Fig. 1). PV-GMGT04 is a pUC-Kan vector that was delivered to the donor organism by a particle acceleration transformation system. This vector is a derivative of the high copy *E. coli* plasmid pUC119 (Vieira and Messing, 1987) and was constructed by fusing the 1.3-Kb *FspI-DraI* pUC119 fragment containing the origin of replication to the 1.3-Kb *SmaI-HindIII* Klenow-filled fragment from pKC7 (Rao and Rogers, 1979), which contains the KAN gene (the neomycin phosphotransferase type II gene, *nptII*). The *nptII* gene confers bacterial kanamycin resistance and replaces the ampicillin resistance gene of pUC119. Table 1 lists the genetic elements used to assemble plasmid PV-GMGT04.

DNA Delivery

Introduction of DNA into soybeans by the particle acceleration method was performed by Agracetus Company (Madison, WI), and the methodology has been described previously (Christou et al., 1988; McCabe et al., 1988). Soybean cultivar A5403, a commercial variety developed by Asgrow Seed Company, was used for transformation. The expression of the GUS gene was used as the initial evidence of transformation. GUS expression was detected by a staining method in which the GUS enzyme converts a substrate (5-bromo-4-chloro-3-indolyl β -D-glucuronide) into a blue precipitate (Jefferson et al., 1986).

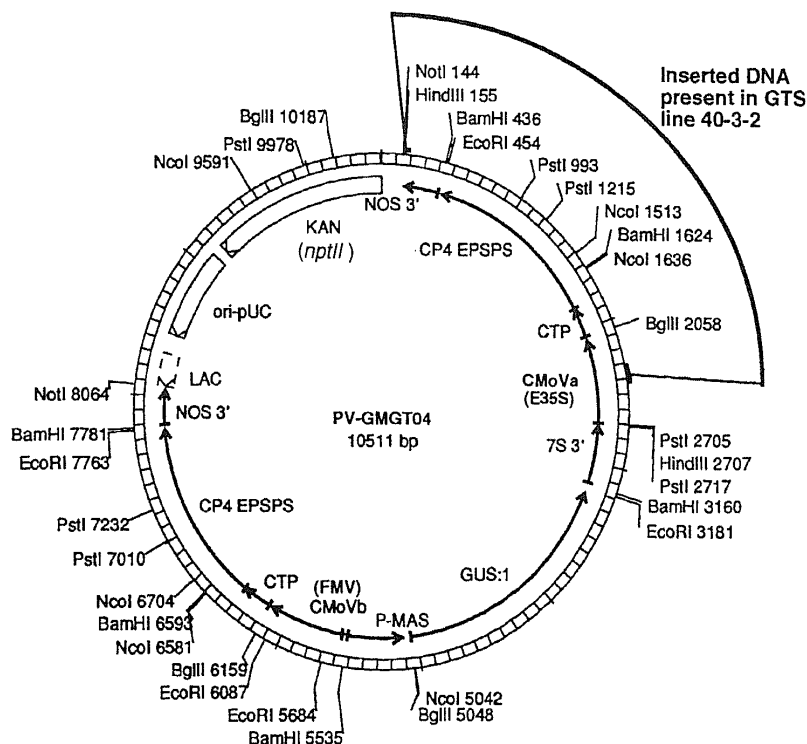
Glyphosate Tolerance of Transformants

R₁ seed from regenerants expressing the GUS gene were planted in a greenhouse at the Monsanto Life Sciences research center in Chesterfield, MO, and treated 2 wk later with a rate of 3.36 kg a.e. ha⁻¹ of glyphosate (equivalent to 1 gallon/acre of the commercial Roundup formulation). A few plants from each R₁ family were left untreated as controls. Individual R₁ families were scored for tolerance to glyphosate 7, 14, and 28 d after spray with a scale of 0 (completely dead) to 10 (no difference from nonsprayed control within the same progeny). Since the R₁ families were segregating for the inserted gene, the scoring was performed only on the tolerant plants (the susceptible plants exhibited extensive chlorosis by the initial 7-d evaluation and subsequently died or remained severely stunted). The segregation ratio was recorded for a preliminary assessment of the mode of inheritance of the glyphosate tolerance trait in each progeny. Individual plants of the highest scoring R₁ families were harvested for further evaluation. See Fig. 2 for a schematic diagram of the lineage of the GTS lines described herein.

Field Evaluation

Progeny from selected single R₁ plants were evaluated in the field in the summer of 1991 at one, two, or three locations, depending on the seed supply available. The locations were the Monsanto experimental farm in Jerseyville, IL, and the Asgrow breeding stations in Marion, AR, and Queenstown, MD. The plots consisted of single rows per R₂ family, 1.5 m long and 0.76 m apart, planted in four separate blocks. Individ-

A. Plasmid Map of PV-GMGT04



B. Schematic diagram of inserted DNA present in GTS line 40-3-2

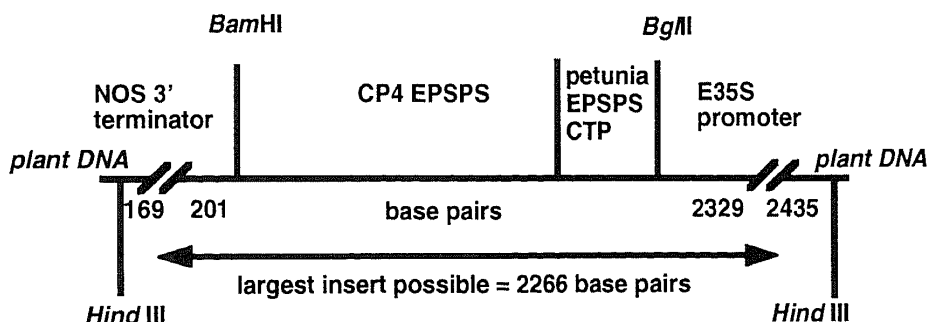


Fig. 1. Plasmid PV-GMGT04 and the inserted DNA in GTS line 40-3-2.

ual R_2 families tracing to the same R_0 plant were planted side by side within each block (approximately 15 seed/m). One block served as nonsprayed control, and the others were sprayed at the V3 stage (Fehr et al., 1971) with 0.63, 1.25, and 1.68 kg a.e. ha^{-1} of glyphosate respectively (equivalent

to 24, 48, and 64 oz./acre of the commercial Roundup herbicide formulation). Damage on nontransgenic control plots ranged from severe stunting at the 0.63 kg ha^{-1} rate to complete death at the higher rates. Again, a rating scale of 0 to 10 was used to evaluate the tolerance of the R_2 families 7, 14, and 28 d

Table 1. Summary of component parts of glyphosate tolerance vector PV-GMGT04.

Genetic element	Size	Function
	Kb	
P-E35S	0.61	The cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985) with the duplicated enhancer region (Kay et al., 1987).
CTP	0.22	The N-terminal chloroplast transit peptide sequence from the <i>Petunia hybrida</i> EPSPS gene (Shah et al., 1986).
CP4 EPSPS	1.36	The C-terminal 5-enolpyruvylshikimate-3-phosphate synthase gene (CP4 EPSPS) from an <i>Agrobacterium</i> species (Barry et al., 1992).
NOS 3'	0.26	The 3' nontranslated region of the nopaline synthase gene (Fraley et al., 1983).
KAN	1.32	The Tn5 neomycin phosphotransferase type II gene (<i>nptII</i>) from plasmid pKC7 (Rao and Rogers, 1979). The <i>nptII</i> confers kanamycin resistance on the bacterial cloning host.
ori-pUC	0.65	The origin of replication from the high copy <i>E. coli</i> plasmid pUC119 (Vieira and Messing, 1987).
LAC	0.24	A partial <i>E. coli lacI</i> coding sequence, the promoter Plac, and a partial coding sequence for β -D-galactosidase or <i>lacZ</i> protein from pUC119 (Yanisch-Perron et al., 1985).
P-MAS	0.42	The TR 2' mannopine synthase promoter region (Velten et al., 1984).
GUS	1.81	The coding region of the <i>E. coli</i> β -glucuronidase gene (Jefferson et al., 1986). The expression of the gene in plants is used as a scoreable marker for transformation.
7S 3'	0.43	The 3' nontranslated region of the soybean 7S seed storage protein alpha subunit (Schuler et al., 1982).
P-FMV	0.57	The figwort mosaic virus 35S promoter (Gowda et al., 1989).

after spray. Ratings of 0 to 2 were characteristic of susceptible plants. Ratings of 3 to 7 indicated a low to intermediate level of tolerance which would be far from acceptable commercially. Ratings of 8 to 10 were characteristic of the best levels of tolerance. A rating of 9 usually indicated very minor differences from the controls.

Individual R_2 families that appeared homozygous for tolerance to the highest levels of glyphosate utilized in the summer 1991 field tests were harvested in bulk and sent to the Asgrow winter nursery in Isabela, Puerto Rico, for seed increase and further testing. They were planted in single rows, 3 m long and 76 cm apart (25 seeds/m). Each R_1 -derived line was planted in three different blocks, and each block received a different treatment (0, 1.68, and 3.36 kg a.e. ha^{-1}) of glyphosate at V3 stage of growth. The same scoring system was used as for the 1991 summer field trials.

General Laboratory Procedures

The DNA extraction technique utilized was a modification of a previously published procedure (Dellaporta et al., 1983), using one or two leaflets from the first trifoliate leaf of greenhouse-grown plants. An RNase incubation step followed by a phenol/chloroform extraction was added before the final precipitation. Southern blots (Southern, 1975; Church and Gilbert, 1984) were performed as previously described (Sambrook et al., 1989). Polymerase chain reaction (PCR) reactions were performed according to manufacturer's instructions (Perkin-Elmer kit #N801-0055, Perkin-Elmer Corp., Norwalk, CT). PCR reactions were done in 100 μ L total volume, containing 100 pmol of each indicated oligo, 1 μ g of template, deoxynucleotide triphosphates (dNTPs) at 200 μ M, 10 units *Taq* DNA Polymerase (Perkin-Elmer Corp.). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min., 65°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 3.0% agarose gel and visualized by ethidium bromide staining. Protein levels in extracts used for enzyme-linked immunosorbent assay (ELISA) were determined using the method of Bradford (Bradford, 1976) (Bio-Rad protein assay kit, Bio-Rad Laboratories, Richmond, CA) in a 96-well plate format.

ELISA Assays

Extracts of GTS line 40-3-2 and control A5403 seed were prepared for ELISA expression analysis by grinding five to six seeds into a fine powder in liquid nitrogen with a mortar and pestle. The seed powder was then weighed into a 5-mL polypropylene tube and extraction buffer was added (100 mM Tris-HCl, pH 7.8, 100 mM sodium borate, 5 mM magnesium chloride, 0.05% v/v Tween 20, and 0.2% sodium ascorbate). The suspension was homogenized (Brinkmann PT3000 Polytron, setting of 18, Brinkmann Instruments Inc., Westbury, NY) on ice for approximately 30 s. The suspension was then centrifuged for 10 to 15 min and the supernatant (*extract*) was removed and aliquotted into Eppendorf tubes (Eppendorf North America, Inc., Madison, WI). The extracts were either analyzed immediately or stored at -80°C until assayed. The tissue to buffer ratio was determined to be optimal for assay at 1:100 tissue to buffer ratio (30 mg tissue/3 mL buffer) for CP4 EPSPS and 1:20 tissue to buffer ratio (150 mg tissue/3 mL buffer) for GUS. Due to the different tissue to buffer ratios, separate extracts were prepared for each protein ELISA.

ELISAs were developed and validated for the detection of CP4 EPSPS and GUS. Quantitation was accomplished by extrapolation from the logistic curve-fits of the purified mature CP4 EPSPS or GUS standard curves (both standards purified

from *E. coli* overexpression strains). For the CP4 EPSPS ELISA, the double antibody sandwich (primary antibody from goat and secondary antibody from rabbit) was detected with donkey anti-rabbit alkaline phosphatase conjugate followed by development with *p*-nitrophenyl phosphate (pNPP). The assay recognized the non-denatured CP4 EPSPS. The GUS direct double antibody sandwich ELISA utilized a commercially available rabbit anti-GUS antibody (CLONTECH Laboratories, Inc., Corning, NY) and its alkaline phosphatase-conjugate, with pNPP development.

CP4 EPSPS and GUS Enzymatic Assays

The procedure utilized to determine the amount of functionally active EPSPS entailed the use of an HPLC with radioactivity detector, which has been previously described (Padgett et al., 1988; Padgett et al., 1987). The final reagent concentrations in the assay were 50 mM HEPES, 0.1 mM ammonium molybdate, 5 mM potassium fluoride, 1 mM ^{14}C -PEP, and 2 mM S3P, pH 7.0. Reactions were run at 25°C. For EPSPS, 1 unit (U) is defined to be 1 μ mol EPSPS produced/minute at 25°C, under the assay conditions specified. Samples for both CP4 EPSPS and GUS enzymatic assays were extracted with 100 mM Tris Cl, 100 mM sodium borate, 5 mM magnesium chloride, 0.2% sodium ascorbate, pH 7.8, then desalted over spin-desalting columns (Penefsky, 1979).

The enzyme assay described for GUS was a modification of the assay method of Jefferson (Jefferson et al., 1986; Naleway, 1992), based on the GUS-catalyzed formation of *p*-nitrophenol from *p*-nitrophenyl- β -D-glucuronide. The final reagent concentrations in the assay were 8 mM *p*-nitrophenyl- β -D-glucuronide, 49 mM sodium phosphate, 10 mM β -mercaptoethanol, 10 mM EDTA, 0.1% sarkosyl, and 0.1% Triton X-100, pH 7.4. The reactions were run for 1 to 5 min. at 37°C and quenched with 2.5 M 2-amino-2-methyl-1,3-propanediol, and the absorbance measured at 406 nm versus a standard curve of *p*-nitrophenol product. For GUS, 1 unit (U) of activity is defined to be 1 μ mol *p*-nitrophenol produced/min at 37°C, under the assay conditions specified.

RESULTS AND DISCUSSION

Glyphosate Tolerance of the Transformed Soybean Lines

A total of 316 transgenic soybean lines from transformations performed with various glyphosate-tolerance plasmid vectors were obtained between June of 1990 and August of 1991 (Fig. 2). Fourteen lines, each derived from GUS-expressing R_0 plants produced by transformation with construct PV-GMGT04, were evaluated in greenhouse tests during the winter of 1990-1991. One such line, derived from R_0 plant 40-3, showed no damage 14 and 28 d after spray with 3.36 kg a.e. ha^{-1} of glyphosate (score of 10). Its progeny was selected for further evaluation in the field in the summer of 1991, along with the progeny of 25 other lines transformed with other constructs. Each of those R_0 -derived lines was represented by several R_2 families (progeny of individual R_1 plants). Due to limited seed set of the original R_0 transformant, line 40-3 was represented in the field by only four R_2 families. R_2 families 40-3-1 and 40-3-2 came from non-glyphosate-treated R_1 plants used as controls in the greenhouse evaluation, while 40-3-3 and 40-3-4 came from R_1 plants treated with 3.36 kg ha^{-1}

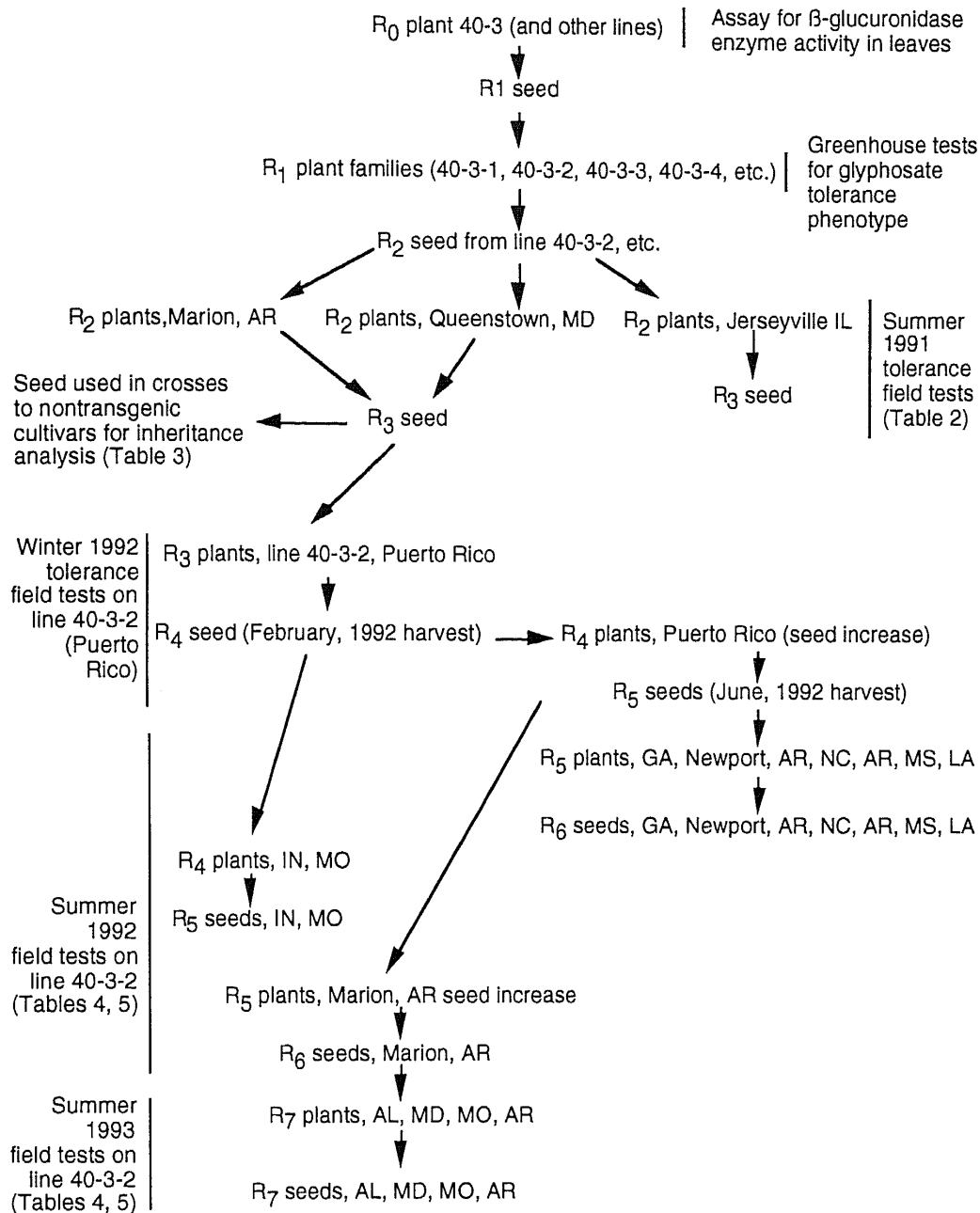


Fig. 2. Lineage of GTS line 40-3-2 seed utilized for glyphosate tolerance and CP4 EPSPS and GUS protein expression experiments.

a.e. of glyphosate. Seed from each of the families were planted at one or two locations, depending on the supply of seed available.

Table 2 summarizes the tolerance scores obtained with each of those four R₂ families. Since some of the R₂ families were segregating for the glyphosate tolerance trait, the scores were based only on the tolerant plants in the plots. When the plots also segregated for differing levels of glyphosate tolerance (as was noticed for lines 40-3-3 and 40-3-4), a single score was given to represent the plants with the highest level of tolerance. Scores for the nontransgenic parent A5403 were generally 1 or 2 for the 0.63 kg ha⁻¹ treatment, and 0 (dead) for the other treatments. Based on the results of numerous experiments on the weed control spectrum of glyphosate, as well as knowledge of the common weeds infecting soybeans, it was expected that the rate of glyphosate necessary to control most weeds under field conditions would generally be between 0.63 and 0.84 kg ha⁻¹

(24–32 oz./acre of commercial Roundup formulation) (unpublished data, 1989–1990). Therefore, tolerance to the highest rate tested in the field in 1991 provided a two- to three-fold safety factor for the soybean crop. Line 40-3-1 showed only a low level of tolerance to glyphosate in all its plants (Table 2). Line 40-3-2 showed little or no damage after application of 1.68 kg ha⁻¹ of glyphosate. No segregation for tolerance was observed in the 40-3-2 line. Lines 40-3-3 and 40-3-4 had a mixture of plants with various levels of tolerance to glyphosate, along with a few dead plants. This mixture of tolerance levels is the main reason for the inconsistencies in the ratings for those lines across treatments and locations. A preliminary conclusion from those observations was that the original R₀ plant 40-3 received two different plasmid DNA insertions, one coding for a high level of glyphosate tolerance, and the other for a low level of glyphosate tolerance. Line 40-3-2 was selected for further evaluation in Puerto Rico during the winter of 1991–1992.

Table 2. Glyphosate tolerance scores of R₁-derived lines from the R₀-derived GTS line 40-3 in 1991 summer tests.

Line location	Treatment		Scores (days after treatment) ^{†,‡}			
	kg ha ⁻¹	7 d	14 d	21 d	28 d	42 d
40-3-1 Jerseyville, IL	0.63	5	7	8	9	8
	1.25	6	6	6	7	8
	1.68	4	4	5	5	6
40-3-2 Marion, AR	0.63	10	10	9	10	
	1.25	10	10	10	10	
	1.68	10	10	10	10	
Queenstown, MD	0.63	9	9	9	9	9
	1.25	9	8	9	10	10
	1.68	8	9	9	8	10
40-3-3 Jerseyville, IL	0.63	6	7	8	9	8
	1.68	7	8	9	9	10
	0.63	10	9	10	10	
Marion, AR	1.25	9	9	8	9	
	1.68	8	9	9	9	
	0.63	6	7	9	10	
40-3-4 Marion, AR	1.25	9	8	8	9	
	1.68	8	8	9	10	

[†] 0 = dead plants; 10 = no difference from unsprayed controls of the same line.

[‡] Damage on nontransgenic control plots (A5403) ranged from severe stunting at the 0.63 kg ha⁻¹ rate to complete death at the higher rates (ratings of 0 to 2).

In the Puerto Rico test, line 40-3-2 had an overall score of 10 after spray with 1.68 kg ha⁻¹ of glyphosate, and 9 after the 3.36 kg ha⁻¹ treatment.

Several plants of lines 40-3-1 and 40-3-2 were examined in the greenhouse for GUS activity to determine if GUS could be used as a marker for backcrossing of the glyphosate tolerance gene. Out of 10 40-3-2 plants sampled, none had GUS activity when assayed by histochemical staining. In contrast, all seven plants tested from line 40-3-1 had GUS activity. This result combined with the field segregation data suggested that line 40-3-2 was homozygous for an insert conferring a high level of glyphosate tolerance but did not contain an active GUS gene. Subsequent DNA and enzymatic analyses (described below) confirmed this to be the case. Furthermore, 40-3-1 appeared to contain inserted DNA responsible for both GUS expression and a weak level of glyphosate tolerance.

Table 3 summarizes the segregation patterns of progeny of crosses between 40-3-2 and 17 nontransgenic cultivars. A consistent 3 tolerant to 1 sensitive ratio

Table 3. Segregation of glyphosate tolerance in F₂ progeny of crosses between GTS line 40-3-2 and 17 nontransgenic cultivars.

Family	Tolerant	Sensitive	χ ² [†]
1	17	4	0.40
2	10	2	0.44
3	12	4	0.00
4	16	4	0.27
5	16	5	0.02
6	14	3	0.49
7	18	5	0.13
8	10	4	0.10
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	0.00
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 (χ_{0.05, 1 d.f.} = 3.84).

was observed among all F₂ progeny, indicating that the glyphosate tolerance in 40-3-2 is conditioned by a single dominant gene. The gene has proven to be stably inherited over multiple selfed and outcrossed generations over the past 3 yr (USDA, 1994, and references therein), and the level of tolerance has remained stable also in those progeny (Delannay et al., 1995).

Characterization of GTS Line 40-3-2

The only proteins which could be expressed from plant promoters present in plasmid PV-GMGT04 are the ones encoded by the CP4 EPSPS and GUS genes (Fig. 1a, Table 1). Expression tests for CP4 EPSPS and GUS proteins were performed by ELISA analysis. Plant samples (leaf and seed) were obtained from field tests in 1992 (8-9 sites) and 1993 (3-4 sites, CP4 EPSPS analysis only) (Fig. 2 and Table 4). As shown in Table 4, CP4 EPSPS is detected in both the seed and the leaf of GTS line 40-3-2. However, GUS was not detected by ELISA in either seed or leaf tissue. These results were supported by enzymatic activity assays performed on seed pools of line 40-3-2 collected from the 1992 field tests: glyphosate-tolerant EPSPS activity was present at 0.025 U/mg but no GUS enzymatic activity was detected. Neither EPSPS nor GUS enzymatic activity was detectable in control A5403 seed extracts. These results indicate, as expected from the glyphosate tolerance phenotype, that at least one of the CP4 EPSPS genes shown in the Fig. 1a plasmid map was incorporated into the line 40-3-2 genome. The lack of GUS activity could indicate either (i) no GUS gene was incorporated into the line 40-3-2 genome; or, (ii) a GUS gene was incorporated which does not produce detectable levels of GUS protein or GUS enzyme activity. To answer this question and to characterize fully the inserted plasmid DNA, Southern blots and PCR experiments were performed on DNA extracted from leaves of line 40-3-2.

Table 4. ELISA analysis of CP4 EPSPS and GUS in GTS line 40-3-2.

Sample†	No. of sites	µg protein/mg tissue fresh weight	
		Mean	Range‡
CP4 EPSPS§			
Leaf¶ 1992	8	0.443	0.251-0.789
Leaf¶ 1993	3	0.415	0.299-0.601
Seed 1992	9	0.288	0.186-0.395
Seed 1993	4	0.201	0.127-0.277
GUS§			
Leaf¶ 1992	8	ND#	-
Seed 1992	9	ND#	-

† All samples were frozen immediately and shipped and stored frozen. Means reported are of the site means. Soybean plant samples for ELISA analysis were generated from nine locations in 1992 (Macon, MO; Washington, LA; Martinsville, IN; Greenville, MS; Newport, AR; Proctor, AR; Winterville, GA; Seven Springs, NC; and Marion, AR) and four locations in 1993 (Gordon, AL; Salisbury, MD; Steele, MO; and Marion, AR). No leaf samples were taken from the Marion, AR, site either year. Seed generations utilized are given in Fig. 2.

‡ "Range" denotes the lowest and highest individual assay for each plot. § No CP4 EPSPS or GUS proteins were detected in the A5403 parental control line samples (grown at identical locations) in either leaf or seed samples.

¶ The center leaflet from the fully expanded third trifoliolate of six plants randomly selected from different rows in various locations in each treatment plot were collected and pooled by plot.

ND = not detected.

DNA Analysis of GTS Line 40-3-2

In order to determine the number of DNA insertions from plasmid PV-GMGT04 in line 40-3-2, Southern blot analysis was performed on isolated genomic DNA of lines 40-3-2 and its parent A5403 using *SpeI*, a restriction enzyme that does not cut inside the plasmid PV-GMGT04. The presence of a single unique band of high molecular weight DNA was suggested in the 40-3-2 digest, but not in the A5403 control DNA, when probed with ³²P-labeled PV-GMGT04 DNA (data not shown). However, three faint background bands were detected in control A5403 DNA as well as in the 40-3-2 DNA, which complicated the conclusion of a single insert based on the *SpeI* digest alone. Other experiments were therefore required to prove the presence of only a single insert in line 40-3-2.

Using Southern blot analyses, DNA from the following genetic elements was used as ³²P-labelled probes: the CP4 EPSPS coding region, the E35S promoter, the CMoVb (FMV) promoter, the NOS 3' nontranslated region, and the GUS coding region. The Southern blot analyses showed that the CP4 EPSPS gene and a portion of the E35S promoter are present in line 40-3-2 (Fig. 3). The CMoVb promoter and the GUS gene are not present in this line (Fig. 4). DNA from line 61-67-1, a GTS line which contains both the GUS gene and the CMoVb-CP4 EPSPS cassette (but no E35S promoter), was used as a control (Fig. 3 and 4). PCR was used to test for the presence or absence of the *ori*-pUC and the *nptII* gene. The results indicated that neither the *ori*-pUC nor the *nptII* gene are present in line 40-3-2 (data not shown). The plasmid map in Fig. 1a schematically shows the DNA from PV-GMGT04 which inserted into line 40-3-2.

The absence of the GUS protein and gene in the 40-3-2 GTS line seems contradictory to the fact that GUS was

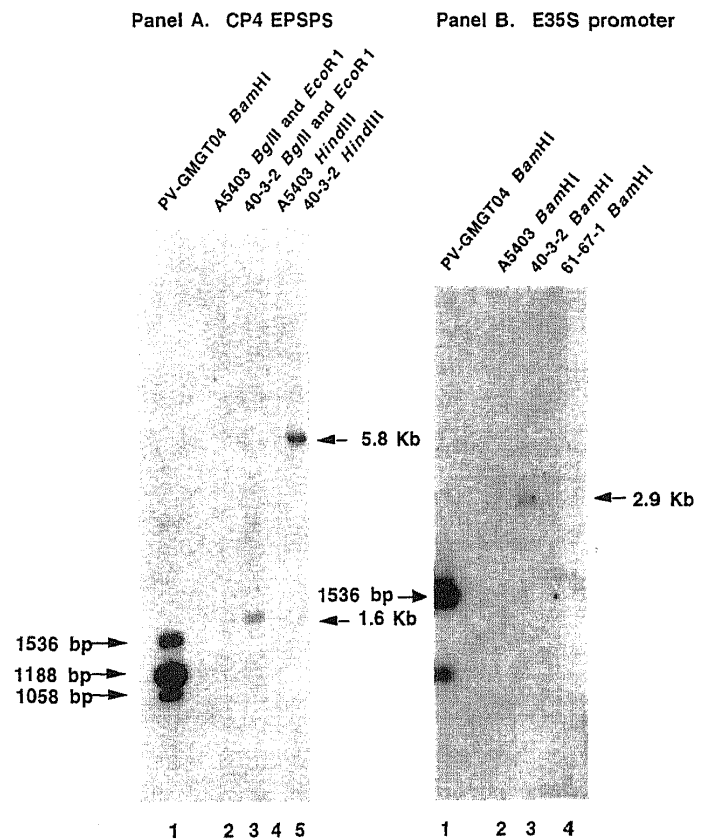


Fig. 3. Southern blots probed for CP4 EPSPS and E35S promoter in GTS line 40-3-2. PV-GMGT04 plasmid DNA was digested with *Bam*HI (Lane 1 in both panels). Soybean genomic DNA from A5403 control was digested with *Bgl*III and *Eco*RI (Panel A, Lane 2), *Hind*III (Panel A, Lane 4) and *Bam*HI (Panel B, Lane 2). GTS line 40-3-2 DNA was digested with *Bgl*III and *Eco*RI (Panel A, Lane 3), *Hind*III (Panel A, Lane 5), and *Bam*HI (Panel B, Lane 3). GTS 61-67-1, a negative plant control for E35S, was digested with *Bam*HI (Panel B, Lane 4). Each lane represents 100 µg 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 ³²P-labeled 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.

utilized as a scoreable marker in the transformation process. However, in light of the standard production and analysis of the R₂ progenies of the parental line 40-3 (described above), it became apparent that the likely explanation was that the original 40-3 R₀ plant had received two DNA inserts located at different positions in the genome. This explanation postulates that Insert 1 was responsible for the expression of the GUS marker protein, and that Insert 2 had a strong expression of the glyphosate tolerance trait, but did not express the GUS protein. Since the 40-3-2 R₂ progeny exhibited strong glyphosate tolerance but no GUS enzyme activity or gene, 40-3-2 contained only Insert 2, while Insert 1 was likely lost through normal genetic segregation.

Fine-structure analysis of the 5' and 3' ends of the PV-GMGT04 DNA integrated into the genomic DNA of line 40-3-2 was accomplished through PCR. If the sequences of the DNA corresponding to selected PCR primer pairs are present in line 40-3-2 genomic DNA, product(s) will be produced similar in size to those produced in the PCR reaction using PV-GMGT04 as the

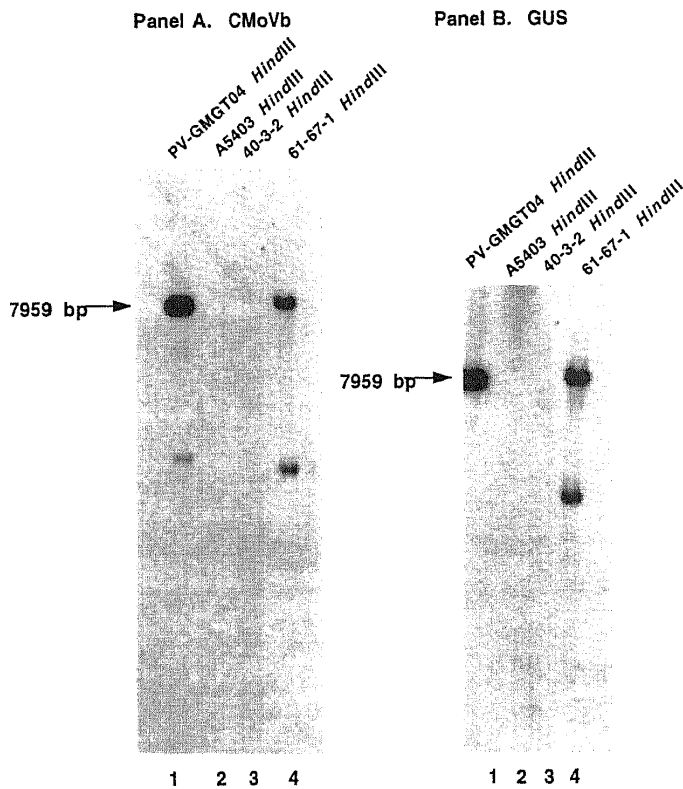


Fig. 4. Southern blots probed for CMoVb promoter and GUS in GTS Line 40-3-2. PV-GMGT04 plasmid DNA was digested with *Hind*III (Panels A and B, Lane 1). Soybean A5403 control DNA was digested with *Hind*III (Panels A and B, Lane 2). GTS line 40-3-2 DNA was digested with *Hind*III (Panel A and B, Lane 3), and GTS line 61-67-1 DNA was digested with *Hind*III (Panels A and B, Lane 4). Each lane represents 100 pg plasmid DNA or 5 μ g 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 labeled CMoVb promoter (Panel A) or the coding region of GUS (Panel B) and then subjected to autoradiography.

control template. If only one (or none) of the primer sequences is present in line 40-3-2 genomic DNA, then no product of the predicted size will be produced.

To determine the NOS 3' terminator boundary, the PCR primers f through k were utilized (Fig. 5). Figure 5 shows a schematic diagram of the location of these primers on the PV-GMGT04 plasmid and the predicted PCR product sizes utilizing combinations of these primers to determine the NOS 3' boundary. In the PCR reactions, the positive control was PV-GMGT04 plasmid DNA, the negative control was line A5403 genomic DNA, and the test substance was line 40-3-2 genomic DNA. Figure 5 shows that a product is formed with GTS line 40-3-2 DNA using primers f + g, f + h, and f + i, but not with primers f + k. Therefore the 3' end point of the inserted DNA must lie between map numbers 201 and 169 (Fig. 1). We concluded that one end of the inserted DNA sequence is within that 32bp sequence of the NOS 3' terminator. The NOS 3' polyadenylation site is located at map numbers 266 to 278 (Fig. 5, Fig. 1b) (Bevan et al., 1983) and therefore remains intact in GTS line 40-3-2.

To determine the E35S 5' boundary, the PCR primers a through e were utilized, (Fig. 6; locations on the plasmid and the predicted PCR product sizes are shown). Two products are generated for each set of primers with

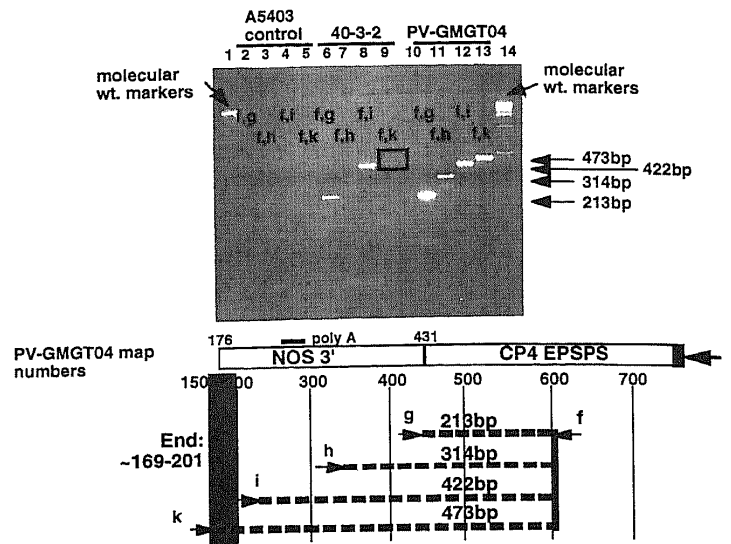


Fig. 5. PCR analysis of 3' end of the PV-GMGT04 plasmid DNA inserted into GTS line 40-3-2. PCR reactions were carried out as described in MATERIALS AND METHODS. Lanes 1 and 14 are molecular weight markers; Lanes 2, 3, 4, and 5 contain A5403 as template plus the combinations of primers as listed; Lanes 6, 7, 8, and 9 contain 40-3-2 as template plus the combination of primers as listed; Lanes 10, 11, 12, and 13 have PV-GMGT04 as template plus the combination of primers as listed. The lower bands at the bottom of each lane are unused oligos. The primers are indicated in the figure with a letter above the arrowhead. Primer f is a constant in all the reactions. The dashed lines indicate the predicted sized product that would be produced using Primer f with Primers g, h, i, and k with plasmid PV-GMGT04 DNA as template in the PCR.

PV-GMGT04 DNA as template. This is because the E35S sequence contains a 252bp direct repeat (*enhancer region*). The 252 base pair direct repeat starts at map site 2691 and ends at map site 2440 (Fig. 6, Fig. 1a). If both sets of sequences are present in line 40-3-2 (i.e., if the entire E35S promoter would be present), then both products will be produced. If only one set of the repeated sequences is present, then only one product will be produced. Figure 6 shows a schematic drawing of the primers used in the PCR and the size of the predicted products. In the PCR reactions, the positive control was PV-GMGT04 plasmid DNA, the negative control was line A5403 genomic DNA, and the test substance was line 40-3-2 genomic DNA. Since a product is formed in GTS line 40-3-2 with primers a + b, a + e, and a + c (Fig. 6), and no product is produced with primer a + d, the end point of the inserted DNA must lie between map numbers 2329 and 2435 (Fig. 1). It is thus concluded that one end of the inserted DNA sequence lies within that 106 bp sequence interval of the E35S promoter. The data demonstrate that the enhancer region of the E35S promoter was not integrated into the genomic DNA of line 40-3-2 since the enhancer region is between map site 2440 and 2691 (Odell et al., 1985; Kay et al., 1987). A schematic diagram of the inserted DNA in GTS line 40-3-2 is shown in Fig. 1b.

As described above, F₂ progenies of crosses between other soybean lines and GTS line 40-3-2 consistently segregate 3 tolerant to 1 sensitive, establishing that the 40-3-2 insert behaves as a single dominant gene inherited in a Mendelian fashion (Table 3). The glyphosate toler-

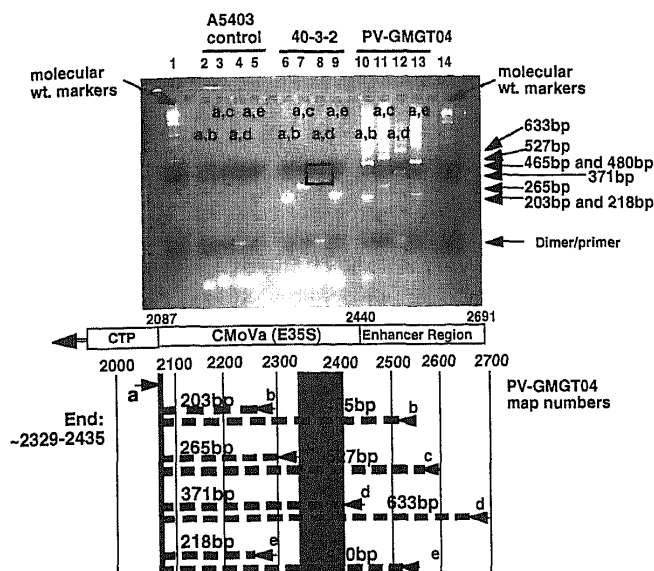


Fig. 6. PCR analysis of 5' end of the PV-GMGT04 plasmid DNA inserted into GTS line 40-3-2. PCR reactions were carried out as described in MATERIALS AND METHODS. Lanes 1 and 14 are molecular weight markers; Lanes 2, 3, 4, and 5 contain A5403 as template plus the primers as listed; Lanes 6, 7, 8, and 9 contain 40-3-2 as template plus the primers as listed; Lanes 10, 11, 12, and 13 have PV-GMGT04 as template plus the primers as listed. The lower bands at the bottom of each lane are unused oligos. The primers are indicated in the figure with a letter above the arrowhead. Primer a is a constant in all the reactions. The dashed lines indicate the predicted size product that would be produced using Primer a with Primers b through e with plasmid PV-GMGT04 DNA as template in the PCR.

ance phenotype and Mendelian transmission have been consistent for more than seven generations of line 40-3-2 soybean tested to date. The genetic stability was further confirmed by DNA analyses. A Southern blot was performed with *Hind*III to cut the genomic DNA of line 40-3-2 and the parental A5403 genomic DNA and then probed with ³²P labelled PV-GMGT04. The blot showed a single 5.8-Kb band in 40-3-2. As discussed above, neither *Hind*III site originally present in plasmid PV-GMGT04 (at positions 155 and 2707) is present in the DNA inserted in line 40-3-2. Therefore, the two *Hind*III sites bordering the 5.8-Kb fragment are located in the plant genome, on either side of the inserted DNA. This fragment was therefore appropriate to monitor as an indicator of the inserted DNA's stability in line 40-3-2, because both inserted and border DNA are present in the band.

Line 40-3-2 DNA from both R₃ and R₆ plants and DNA from line A5403 were cut with *Hind*III and then subjected to Southern blot analysis. The reference plasmid, PV-GMGT04, was cut with *Eco*RI and included on the blot. Figure 7 shows the Southern blot probed with ³²P-labelled PV-GMGT04. All of the expected band sizes of PV-GMGT04 are seen on the blot. The 5.8-Kb *Hind*III band referred to above was evident when either R₃ generation or R₆ generation line 40-3-2 were probed with plasmid PV-GMGT04. The fact that this same size band is present in both generations of 40-3-2 indicates that the plasmid DNA insert and the plant border DNA are stably maintained throughout the plant life cycle over four generations.

In conclusion, expression of the CP4 EPSPS gene

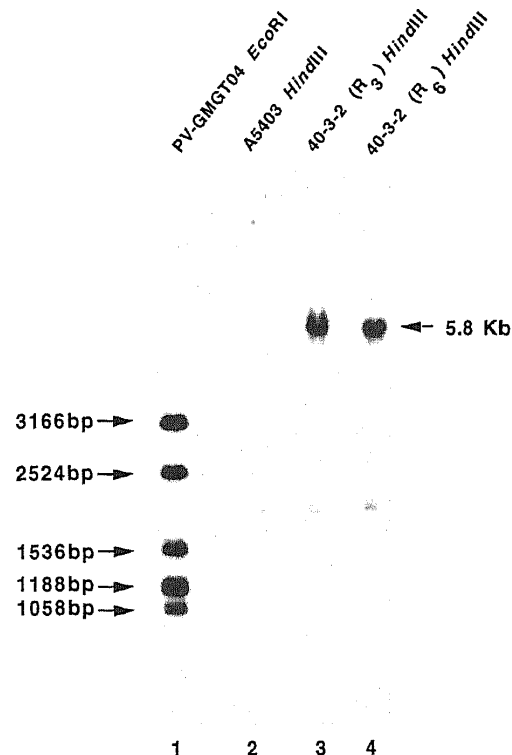


Fig. 7. Southern blot of GTS line 40-3-2 generations R₃ and R₆ probed with PV-GMGT04. PV-GMGT04 plasmid DNA was digested with *Eco*RI (Lane 1). Soybean A5403 control genomic DNA (Lane 2), line 40-3-2 (R₃) genomic DNA (Lane 3), and line 40-3-2 (R₆) genomic DNA (Lane 4) were digested with *Hind*III. Each lane represents 100 µg 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 membrane was probed with ³²P-labelled PV-GMGT04 plasmid DNA and then subjected to autoradiography.

confers a high level of glyphosate tolerance to soybeans, as exemplified by the data presented herein on line 40-3-2. Extensive molecular characterization indicates that line 40-3-2 contains a portion of the E35S promoter, the petunia EPSPS chloroplast transit peptide, the CP4 EPSPS gene, and a portion of the NOS 3' terminator. No other plasmid-derived DNA sequences are present. Yield tests have demonstrated that line 40-3-2 is tolerant to applications of Roundup herbicide in excess of levels required for weed control, with a very wide window of application (Delannay et al., 1995). Extensive efforts are continuing with seed companies to develop a wide range of commercial soybean cultivars containing the glyphosate tolerance locus present in line 40-3-2. In parallel to those breeding efforts, new weed control systems are being developed to include this new use of glyphosate for the soybean grower. Finally, numerous experiments have been conducted to support amending the Roundup herbicide label to include foliar application to soybeans, as well as to establish the environmental, food and feed safety of the new transgenic crop (Padgett et al., 1995b; Harrison et al., 1995; and Hammond et al., 1995).

ACKNOWLEDGMENTS

The authors would like to thank all of our colleagues and collaborators at Monsanto, Asgrow Seed Company, and numerous academic and private research groups for their contributions to this project.

REFERENCES

- Barry, G., G. Kishore, S. Padgett, M. Taylor, K. Kolacz, M. Weldon, D. Re, D. Eichholtz, K. Fincher, and L. Hallas. 1992. Inhibitors of amino acid biosynthesis: Strategies for imparting glyphosate tolerance to crop plants. p. 139-145. *In* B.K. Singh et al. (ed.) *Biosynthesis and molecular regulation of amino acids in plants*. Am. Soc. Plant Physiologists, Rockville, MD.
- Bartlett, S.G., A.R. Grossman, and N.-H. Chua. 1982. In vitro synthesis and uptake of cytoplasmically-synthesized chloroplast proteins. p. 1081-1091. *In* M. Edelman et al. (ed.) *Methods in chloroplast molecular biology*. Elsevier Biomedical Press, Amsterdam.
- Bevan, M., W.M. Barnes, and M.-D. Chilton. 1983. Structure of transcription of the nopaline synthase gene region of T-DNA. *Nucl. Acids Res.* 11:369-385.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Christou, P., D.E. McCabe, and W.F. Swain. 1988. Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiol.* 87:671-674.
- Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81:1991-1995.
- Delannay, X., T.T. Bauman, D.H. Beighley, M.J. Buettner, H.D. Coble, M.S. DeFelice, C.W. Derting, T.J. Diedrick, J.L. Griffin, E.S. Hagood, F.G. Hancock, S.E. Hart, B.J. LaVallee, M.M. Loux, W.E. Lueschen, K.W. Matson, C.K. Moots, E. Murdock, A.D. Nickell, M.D.K. Owen, E.H. Paschal II, L.M. Prochaska, P.J. Raymond, D.B. Reynolds, W.K. Rhodes, F.W. Roeth, P.L. Sprankle, L.J. Tarochione, C.N. Tinius, R.H. Walker, L.M. Wax, H.D. Weigelt, and S.R. Padgett. 1995. Yield evaluation of a glyphosate-tolerant soybean line after treatment with glyphosate. *Crop Sci.* 35:1461-1467 (this issue).
- della-Cioppa, G., S.C. Bauer, B.K. Klein, D.M. Shah, R.T. Fraley, and G. Kishore. 1986. Translocation of the precursor of 5-enolpyruvyl-shikimate-3-phosphate synthase into chloroplasts of higher plants in vitro. *Proc. Natl. Acad. Sci. USA.* 83:6873-6877.
- della-Cioppa, G., S.C. Bauer, M.T. Taylor, D.E. Rochester, B.K. Klein, D.M. Shah, R.T. Fraley, and G.M. Kishore. 1987. Targeting a herbicide-resistant enzyme from *Escherichia coli* to chloroplasts of higher plants. *Bio/Technology* 5:579-584.
- Dellaporta, S.L., J. Wood, and J.B. Hicks. 1983. A plant DNA miniprep: Version II. *Plant Mol. Biol. Reporter* 1:19-21.
- Fehr, W.R., C.E. Caviness, D.T. Burmood, and J.S. Pennington. 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. *Crop Sci.* 11:929-931.
- Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, G.R. Galluppi, S.B. Goldberg, N.L. Hoffmann, and S.C. Woo. 1983. Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA.* 80:4803-4807.
- Gasser, C. 1989. Genetically engineering plants for crop improvement. *Science* 244:1293-1299.
- Gasser, C.S., J.A. Winter, C.M. Hironaka, and D.M. Shah. 1988. Structure, expression, and evolution of the 5-enolpyruvylshikimate-3-phosphate synthase genes of petunia and tomato. *J. Biol. Chem.* 263:4280-4289.
- Gowda, S., F.C. Wu, and R.J. Shepard. 1989. Identification of promoter sequences for the major RNA transcripts of Figwort Mosaic and Peanut Chlorotic Streak Viruses (Caulimovirus group). *J. Cell. Biochem.* 13D (supplement):301.
- Hammond, B.G., V.L. Vicini, G.F. Hartnell, M.W. Naylor, C.D. Knight, E. Robinson, R.L. Fuchs, and S.R. Padgett. 1995. The feeding value of soybeans fed to rats, poultry, catfish, and dairy cattle are not altered by incorporation of glyphosate tolerance. *J. Nutr.* (in press).
- Harrison, L.A., M.R. Bailey, M. Naylor, J. Ream, B. Hammond, D.L. Nida, B. Burnette, T.E. Nickson, T. Mitsky, M.L. Taylor, R.L. Fuchs, and S.R. Padgett. 1995. The expressed protein in glyphosate-tolerant soybeans, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested and is not toxic to mice upon acute gavage administration. *J. Nutr.* (in press).
- Haslam, E. 1993. Shikimic acid: Metabolism and metabolites. John Wiley and Sons, Chichester, England.
- Hinchee, M.A. W., S.R. Padgett, G.M. Kishore, X. Delannay, and R.T. Fraley. 1993. Herbicide tolerant crops. p. 243-263. *In* S. Kung et al. (ed.) *Transgenic plants Vol. 1*. Academic Press, Inc., Orlando.
- Holt, J.S., S.B. Powles, and J.A.M. Holtum. 1993. Mechanisms and agronomic aspects of herbicide resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:203-229.
- Jefferson, R.A., S.M. Burgess, and D. Hirsch. 1986. β -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* 83:8447-8451.
- Kay, R., A. Chan, M. Daly, and J. McPherson. 1987. Duplication of the CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* 236:1299-1302.
- Kishore, G., and D. Shah. 1988. Amino acid biosynthesis inhibitors as herbicides. *Annu. Rev. Biochem.* 57:627-663.
- Kishore, G., D. Shah, S. Padgett, G. della-Cioppa, C. Gasser, D. Re, C. Hironaka, M. Taylor, J. Wibbenmeyer, D. Eichholtz, M. Hayford, N. Hoffmann, X. Delannay, R. Horsch, H. Klee, S. Rogers, D. Rochester, L. Brundage, P. Sanders, and R.T. Fraley. 1988. EPSP synthase: From biochemistry to genetic engineering of glyphosate tolerance. p. 37-48. *In* P.A. Hedin et al. (ed.) *Biotechnology for crop protection*. ACS Symposium Series no. 379, Am. Chem. Soc., Washington, DC.
- Malik, J., G. Barry, and G. Kishore. 1989. The herbicide glyphosate. *BioFactors* 2:17-25.
- Mazur, B. 1989. The development of herbicide resistant crops. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40:441-470.
- McCabe, D.E., Swain, W.F., Martinell, B.J., and Christou, P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* 6:923-926.
- Naleway, J.J. 1992. Histochemical, spectrophotometric, and fluorometric GUS substrates p. 61-76. *In* S.R. Gallagher (ed.) *GUS protocols: Using the GUS gene as a reporter of gene expression*. Academic Press, San Diego.
- Odell, J.T., F. Nagy, and N.-H. Chua. 1985. Identification of DNA sequences required for activity of the Cauliflower Mosaic Virus 35S promoter. *Nature* 313:810-812.
- Padgett, S.R., G. della-Cioppa, D.M. Shah, R.T. Fraley, R.T., and G.M. Kishore. 1989. Selective herbicide tolerance through protein engineering. p. 441-476. *In* J. Schell et al. (ed.) *Cell culture and somatic cell genetics of plants*, vol. 6. Academic Press, New York.
- Padgett, S.R., Q.K. Huynh, S. Aykent, R.D. Sammons, J.A. Sikorski, and G.M. Kishore. 1988. Identification of the reactive cysteines of *Escherichia coli* 5-enolpyruvylshikimate-3-phosphate synthase and their nonessentiality for enzymatic catalysis. *J. Biol. Chem.* 263:1798-1802.
- Padgett, S.R., Q.K. Huynh, J. Borgmeyer, D.M. Shah, L.A. Brand, D. Re, B.F. Bishop, S.G. Rogers, R.T. Fraley, and G.M. Kishore. 1987. Bacterial expression and isolation of *Petunia hybrida* 5-enolpyruvylshikimate-3-phosphate synthase. *Arch. Biochem. Biophys.* 258:564-573.
- Padgett, S.R., D.B. Re, G.F. Barry, D.E. Eichholtz, X. Delannay, R.L. Fuchs, G.M. Kishore, and R.T. Fraley. 1995a. New Weed Control Opportunities: Development of glyphosate-tolerant soybeans. *In* S.O. Duke (ed.) *Herbicide resistant crops*. CRC Press, Boca Raton, FL (in press).
- Padgett, S.R., D.B. Re, C.S. Gasser, D.A. Eichholtz, R.B. Frazier, C.M. Hironaka, E.B. Levine, D.M. Shah, R.T. Fraley, and G.M. Kishore. 1991. Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. *J. Biol. Chem.* 266:22364-22369.
- Padgett, S.R., N.B. Taylor, D.L. Nida, M.R. Bailey, J. MacDonald, L.R. Holden, and R.L. Fuchs. 1995b. The composition of glyphosate-tolerant soybean seeds is equivalent to conventional soybeans. *J. Nutr.* (in press).
- Penefsky, H.S. 1979. A centrifuged-column procedure for the measurement of ligand binding by beef heart F₁. *Meth. Enzymol.* 56: 527-530.
- Rao, R.N. and S.G. Rogers. 1979. Plasmid pKC7: A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. *Gene* 7:79-82.
- Ruff, T., D. Eichholtz, D. Re, S. Padgett, and G. Kishore. 1991.

- Effects of amino acid substitutions on glyphosate tolerance and activity of EPSPS. *Plant Physiol. Suppl.* 96:94.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schuler, M.A., E.S. Schmitt, and R.N. Beachy. 1982. Closely related families of genes code for alpha and alpha prime subunits of the soybean 7S storage protein complex. *Nucl. Acids Res.* 10: 8225-8244.
- Shah, D., R. Horsch, R., H. Klee, G. Kishore, J. Winter, N. Tumer, C. Hironaka, P. Sanders, C. Gasser, S. Aykent, N. Siegel, S. Rogers, and R. Fraley. 1986. Engineering herbicide tolerance in transgenic plants. *Science* 233:478-481.
- Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-517.
- Steinrucken, H.C., and N. Amrhein. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl shikimic acid-3-phosphate synthase. *Biochem. Biophys. Res. Comm.* 94:1207-1212.
- Steinrucken, H.C., and N. Amrhein. 1984. 5-Enolpyruvylshikimate-3-phosphate synthase of *Klebsiella pneumoniae*: Inhibition by glyphosate [N-(phosphonomethyl)glycine]. *Eur. J. Biochem.* 143: 351-357.
- USDA. 1994. APHIS-USDA Petition 93-258-01 for determination of nonregulated status for glyphosate-tolerant soybean line 40-3-2: Environmental assessment and finding of no significant impact. USDA, Washington (announced in Fed. Reg. 59 (99):26781-26782, May 24, 1994).
- Velten, J., L. Velten, R. Hain, and J. Schell. 1984. Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J.* 3:2723-2730.
- Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Meth. Enzymol.* 153:3-11.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-119.

Yield Evaluation of a Glyphosate-Tolerant Soybean Line after Treatment with Glyphosate

X. Delannay,* T. T. Bauman, D. H. Beighley, M. J. Buettner, H. D. Coble, M. S. DeFelice, C. W. Derting, T. J. Diedrick, J. L. Griffin, E. S. Hagood, F. G. Hancock, S. E. Hart, B. J. LaVallee, M. M. Loux, W. E. Lueschen, K. W. Matson, C. K. Moots, E. Murdock, A. D. Nickell, M. D. K. Owen, E. H. Paschal II, L. M. Prochaska, P. J. Raymond, D. B. Reynolds, W. K. Rhodes, F. W. Roeth, P. L. Sprankle, L. J. Tarochione, C. N. Tinius, R. H. Walker, L. M. Wax, H. D. Weigelt, and S. R. Padgett

ABSTRACT

Transformation of soybean [*Glycine max* (L.) Merr.] with a gene encoding a glyphosate-tolerance 5-enolpyruvylshikimate-3-phosphate synthase enzyme from *Agrobacterium* sp. strain CP4 resulted in the development of glyphosate-tolerant line 40-3-2. Glyphosate (N-phosphonomethyl glycine) is the active ingredient of Roundup herbicide. Line 40-3-2 was yield tested at 17 locations in 1992, 23 locations in 1993, and 18 locations in 1994. At those locations, broadcast applications of glyphosate at various rates were made over 40-3-2 or its derivatives from early vegetative growth to pod fill. No significant yield reduction was observed as a result of the glyphosate treatment at any of the locations. Development of glyphosate-tolerant soybean promises to provide the farmer with access to a new weed control system that should result in lower production costs and reliable weed control under a wide range of conditions.

X. Delannay, C.W. Derting, B.J. LaVallee, P.J. Raymond, P.L. Sprankle, L.J. Tarochione, and S.R. Padgett, Monsanto Co., 700 Chesterfield Parkway N., St. Louis, MO 63198; T.T. Bauman, Purdue Univ., West Lafayette, IN 47906; D.H. Beighley, Jacob Hartz Seed Co., Stuttgart, AR 72160; M.J. Buettner, T.J. Diedrick, K.W. Matson, C.K. Moots, A.D. Nickell, E.H. Paschal II, W.K. Rhodes, C.N. Tinius, and H.D. Weigelt, Asgrow Seed Co., Kalamazoo, MI 49002; H.D. Coble, North Carolina State Univ., Raleigh, NC 27695; M.S. DeFelice, Univ. of Missouri, Columbia, MO 65211; J.L. Griffin, L.M. Prochaska, and D.B. Reynolds, Louisiana State Univ. Agric. Center, Baton Rouge, LA 70803; E.S. Hagood, Virginia Polytechnic Institute and State Univ., Blacksburg, VA 24061; F.G. Hancock, Mississippi State Univ., Stoneville, MS 38776; S.E. Hart, and L.M. Wax, Univ. of Illinois, Urbana, IL 61801; M.M. Loux, Ohio State Univ., Columbus, OH 43210; W.E. Lueschen, Univ. of Minnesota, Lamberton, MN 56152; E. Murdock, Clemson Univ., Clemson, SC 29634; M.D.K. Owen, Iowa State Univ., Ames, IA 50011; F.W. Roeth, Univ. of Nebraska, Clay Center, NE 68933; and R.H. Walker, Auburn Univ., Auburn, AL 36849. Received 18 Jan. 1995. *Corresponding author (xxdela@ccmail.monsanto.com).

GENETIC ENGINEERING promises to provide plant breeders and agronomists with tools not previously available because of the limitations of the gene pools accessible through conventional plant breeding. Since geneticists are no longer restrained by interspecific barriers, genes now can be accessed from virtually any living organism, modified for optimum expression in specific tissues or plant parts, and inserted into the genome of most crop species (Gasser and Fraley, 1989). In soybean, one of the first practical applications of genetic engineering has been the development of tolerance to glyphosate, the active ingredient in the herbicide Roundup¹ (Padgett et al., 1995, this issue).

Roundup herbicide is a broad-spectrum compound widely used in a variety of weed control programs throughout most of the world (Baird et al., 1971; Malik et al., 1989). The active ingredient, glyphosate (N-phosphonomethyl glycine), inhibits a specific enzyme in the metabolism of aromatic amino acids, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), resulting in plant death. This enzyme is present in plants, bacteria, and fungi, but not in animals. Due to its mode of action, glyphosate is highly effective against the majority of annual and perennial grasses and broadleaved weeds. Other advantages of glyphosate are its relative low cost and its favorable environmental features: It binds to the

¹Roundup is a registered trademark of Monsanto Company.

Abbreviations: a.e., acid equivalent; CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4.