Glyphosate-Tolerant Cotton: Genetic Characterization and Protein Expression

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Genetically modified cotton lines have been developed that are tolerant to glyphosate, the active ingredient in the herbicide Roundup. The new lines were generated by *Agrobacterium tumfaciens*-mediated transfer of a gene encoding 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium* sp. CP4 (CP4 EPSPS). Lines were screened via greenhouse spray tests and field evaluations to identify agronomically acceptable lines with a commercial level of tolerance to glyphosate. Two lines were characterized. Lines 1445 and 1698 were transformed with different vectors that encode for the CP4 EPSPS and the neomycin phosphotransferase II (NPTII) marker protein. Both lines contain a single DNA insertion that segregates in a typical Mendelian fashion. Line 1445 contains a single copy of the CP4 EPSPS gene, whereas the line 1698 contains two copies of the CP4 EPSPS gene at a single insertion site. The stability of each DNA insertion was demonstrated by Southern analysis across the R_3 and R_5 generations. The expression levels of the CP4 EPSPS and NPTII were quantitated by ELISA in leaf and seed samples collected in 1993 and 1994 field trials. The use of glyphosate-tolerant cotton will enable the grower to take advantage of additional weed management alternatives.

Keywords: *Cotton; genetically modified; herbicide tolerant; Roundup*

INTRODUCTION

Cotton is the leading plant fiber crop worldwide, typically grown in arid regions of the tropical or subtropical areas (Niles and Feaster, 1984). Control of weeds in the cotton crop is essential, as they compete with the crop for sunlight, water, and nutrients. Failure to control weeds within the crop results in decreased yield and reduced crop quality. Weeds also reduce the efficiency of the mechanical harvest and may impact the quality of the lint. Present weed management systems include cultural and mechanical practices with herbicides to overcome the competitive effect. The qualities of the herbicide, such as the spectrum of weeds controlled, lack of crop injury, and environmental impacts, are important considerations. Few herbicides possess optimal characteristics in all of these areas. Several classes of herbicides are efficaceous for broad-spectrum weed control, but they tend to be nonselective and may kill or significantly injure crop plants at the application rates required for sufficient weed control.

One such herbicide is glyphosate, the active ingredient in Roundup herbicide. Roundup herbicide is a broad-spectrum, nonselective, postemergent herbicide (Baird, 1971; Malik, 1989). It is highly effective against the majority of annual and perennial grasses and broadleaved weeds. Glyphosate has favorable environmental features, such as rapid soil binding which enables it to resist leaching and biodegradation which decrease the persistence in nature. It also demonstrated extremely low toxicity to mammals, birds, and fish (Malik, 1989). However, due to the sensitivity of the crop plant to glyphosate, it cannot currently be used over the top of the crop for weed control. The tools of biotechnology have made it possible to develop crop plants that are tolerant to glyphosate (Barry et al., 1992; Padgette et al., 1996a,b). Use of glyphosate-tolerant cotton would enable the farmer to spray Roundup herbicide over the top of the cotton crop and take advantage of this herbicide's desirable features. Glyphosate-tolerant cotton provides an excellent broad-spectrum weed control alternative for the grower.

Cotton was modified to express a gene that encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium sp*. CP4 (CP4 EPSPS). Glyphosate binds to the plant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and blocks aromatic amino acid synthesis (Steinrucken and Amrhein, 1980; Haslam, 1993). EPSPS is present in plants, bacteria, and fungi but not in animals (Levin and Sprinson, 1964). The plant EPSPS is localized in the chloroplasts or plastids (della-Cioppa et al., 1986). Expression of the glyphosate-tolerant EPSPS targeted to the chloroplast confers glyphosate tolerance to the crop plant.

Two lead lines of glyphosate-tolerant cotton (GTCot, also referred to as Roundup Ready cotton, a trademark of Monsanto Co.), lines 1445 and 1698, have been identified and characterized. Both lines contain genes encoding CP4 EPSPS and the neomycin phosphotransferase II (*nptII*) selectable marker gene (Fraley et al., 1983).

In this paper, characterization of both GTCot lines with respect to the genetic insert, segregation of the glyphosate tolerance trait, and expression levels of the additional proteins is reported. The nutritional composition of the seed and oil have been evaluated and are reported in an accompanying paper (Nida et al., 1996). The characterization and composition data are part of the safety assessment to gain regulatory acceptance of the glyphosate-tolerant lines.

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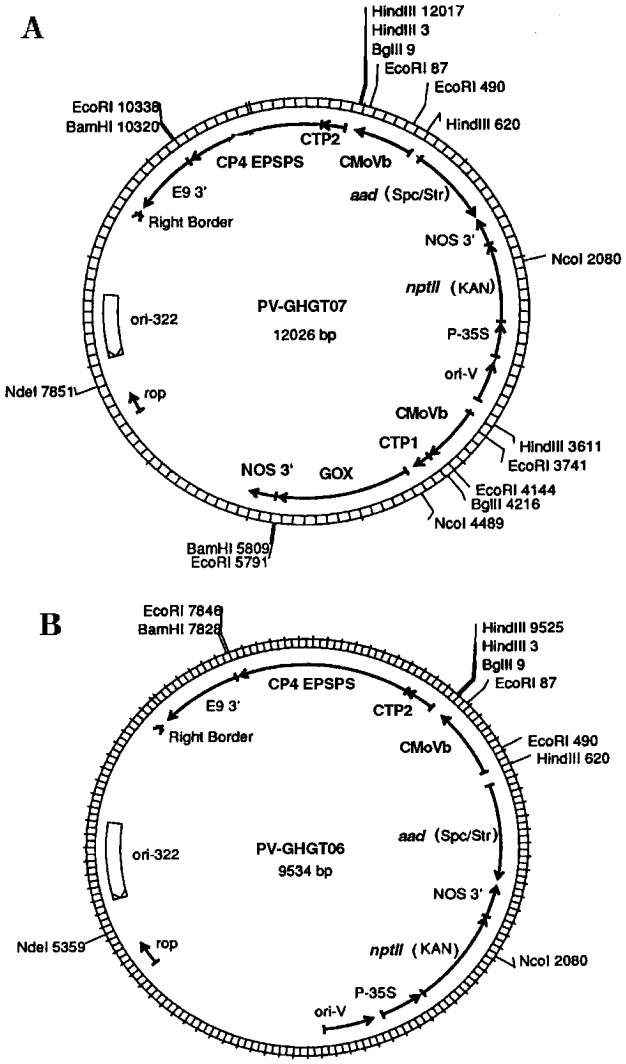

Figure 1. Vector used to transform GTCot line 1445 (A) and GTCot line 1698 (B).

MATERIALS AND METHODS

Production of Glyphosate-Tolerant Cotton Lines. Glyphosate-tolerant cotton (*Gossypium hirsutum L.*) lines 1445 (GTCot 1445) and 1698 (GTCot 1698) were produced by transformation of the Coker 312 variety using *Agrobacterium tumefaciens*-mediated transformation (Klee and Rogers, 1989). Lines 1445 and 1698 were produced with single-border binary plasmid vectors (Bevan, 1984; Wang et al., 1984) PV-GHGT07 (Figure 1A) and PV-GHGT06 (Figure 1B), respectively. Both vectors contain 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) from *Agrobacterium* strain CP4 (Barry et al., 1992) and the neomycin phosphotransferase II selectable marker gene *nptII* (Fraley et al., 1983) which confers resistance to the aminoglycoside antiobiotic kanamycin (KAN).

CP4 EPSPS is driven by a caulimovirus 35S promoter, CMoVb, isolated from figwort mosaic virus (Gowda et al., 1989; Richins et al., 1987). The chloroplast transit peptide from *Arabidopsis thaliana* EPSPS (Klee et al., 1987) is fused to the coding region of CP4 EPSPS to target the protein to the chloroplast, the site of aromatic amino acid biosynthesis. In both vectors, the CP4 EPSPS is followed by a nontranslated region of the pea *rbc-E9* gene (Coruzzi et al., 1984) which directs polyadenylation of the mRNA. Both vectors contain the same cassettes for the *nptII* gene: The cauliflower mosaic virus 35S promoter (P-35S) region (Kay et al., 1985; Odell et al., 1985) drives the expression of the *nptII* gene which is followed by the nopaline synthase (NOS) 3′ region that directs polyadenylation of the mRNA (Fraley et al., 1983; Depicker et al., 1982). Both vectors contain origins of replication required for plasmid replication in *A. tumefaciens* and *Escherichia coli* hosts and a bacterial selectable marker gene driven by a bacterial promoter, aminoglycoside adenylyltransferase (*aad*), which confers spectinomycin/streptomycin (Spc/Str) resistance.

PV-GHGT07 also contains a second gene involved in conferring glyphosate-tolerance. The *gox* gene encodes the glyphosate-metabolizing enzyme glyphosate oxidoreductase (GOX), cloned from *Achromobacter sp.* strain LBAA (Barry et al., 1992). The *gox* gene, fused to the CTP from *Arabidopsis thaliana* SSU1A gene (Timko et al., 1988), was driven by the CMoVb 35S promoter and followed by the NOS 3′ region.

The plasmid vectors PV-GHGT06 and PV-GHGT07 were assembled in *E. coli* K-12 cells, mated into a disarmed ABI *Agrobacterium* strain, and used to transform Coker 312-derived hypocotyl sections with modifications as described by Umbeck et al. (1987). Plants were regenerated with modifications described by Trolinder and Goodin (1987). During the transformation process, vector DNA (referred to as T-DNA) was stably transferred to the cotton genome. For single-border vectors, all of the DNA is expected to transfer into the cotton genome since T-DNA typically initiates at the right border of the vector and continues to include the whole plasmid for this type of vector (Klee et al., 1987). It is accepted that T-DNA transferred into plant cells by *Agrobacterium* is irreversible (Huttner et al., 1992).

Greenhouse Screen of New Lines. Glyphosate-tolerant cotton lines were initially selected by greenhouse spray test. Seed from R_0 plants (designated R_1 seed) was collected from the transformed plants, planted in the greenhouse, and treated with up to 128 oz/acre of glyphosate (field equivalent rate) to determine which plants possessed the greatest degree of tolerance. Vegetative injury was scored on a 1−10 visual scale with 1 being the least tolerant and 10 being no different than an unsprayed control. The lines with the most vegetative tolerance were analyzed for effects on pollen fertility by counting the number of nodes to the first boll. The most tolerant line identified up to that point was utilized as a positive control for tolerance comparisons.

Segregation Analyses. Glyphosate applications to progeny of the transformants were used to distinguish tolerant from susceptible plants. A glyphosate solution (1.5−3.0 lb/acre of glyphosate, acid equivalent) was applied with a track sprayer to greenhouse-grown cotton plants in the 3−4 leaf stage. Segregation ratios were determined at least 7 days after application by counting surviving plants versus dead plants. Goodness of fit for single-gene and double-gene insertions was determined using chi square analyses.

Control Cotton Line. The control line, Coker 312, was the parental cotton variety from which the transformed glyphosate-tolerant lines were developed. Seed of Coker 312 was obtained from SeedCo., Inc. (Lubbock, TX).

Glyphosate-Tolerant Cotton Lines 1445 and 1698. For molecular analyses, GTCot 1445 and GTCot 1698 plants were grown from R_3 and R_5 seed, selected from the original R_0 transformants.

Molecular Analyses. *Plant Material.* Plants were grown under normal greenhouse conditions. Young leaf tissue was harvested and immediately frozen on dry ice.

DNA Isolation and Restriction Enzyme Digestion. Leaf DNA was extracted in a similar manner as described by Wendel (1989). Digestions of genomic DNAs by restriction enzymes were performed according to standard laboratory methods (Sambrook, et al., 1989). Restriction enzymes, which cut within both plasmids, used for characterization of the lines included *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Nco*I, and *Nde*I. *Spe*I, which does not cut within either plasmid, was used to determine the number of loci in which the T-DNA integrated in both lines.

Hybridization Probes. The whole plasmid, PV-GHGT06, and the following isolated genetic elements were used as hybridization probes: the *gox* gene, CP4 EPSPS gene, the *nptII* gene, the *aad* gene, the CMoVb promoter, the *ori-V*, and the *ori-322*. With the exception of the whole plasmid, the genetic elements were obtained by digesting plasmids containing these elements, subjecting the digestions to agarose gel electrophoresis, cutting out the appropriate band, and treating it with

the GENECLEAN II KIT (Bio101, Vista, CA). The probes were labeled with [α-^{32}P]d-CTP using the Prime-it II Kit (Stratagene, La Jolla, CA).

Southern Blot Hybrization. The Southern blots were performed as described by Southern (1975) and Bio-Rad Zeta-Probe hybrization conditions. The digested DNA was subjected to electrophoresis on 0.8% agarose gels, transferred to Zeta-Probe GT (BIO-RAD, Hercules, CA) nylon membrane, probed with the appropriate ^{32}P-labeled DNA, and then subjected to autoradiography. A plasmid control, cut with the appropriate restriction enzyme, and molecular weight standards were included on each Southern blot.

Gene Expression Analysis. Samples were prepared by crushing leaf or seed samples in liquid nitrogen to a fine powder with a mortar and pestle. The tissue powders were homogenized at a 1:20 (w/v) ratio in Tris-borate extraction buffer (TBA) composed of 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 sample was centrifuged and the supernatant removed. The supernatant was evaluated by enzyme-linked immunosorbent asssay (ELISA) immediately or stored frozen at −80 °C.

For the CP4 EPSPS ELISA, a double-antibody sandwich indirect ELISA was developed using the primary capture antibody from goat and secondary detecting antibody from rabbit (Padgette et al., 1996a). For NPTII quantitation, a double-antibody sandwich direct ELISA was developed using a primary antibody produced in rabbits and an antibody conjugated to horseradish peroxidase (HRP) for detection of the NPTII protein (Rogan et al., 1992).

RESULTS AND DISCUSSION

Line Screening. Vegetative effects of glyphosate injury on cotton plants include leaf chlorosis, leaf necrosis, and growth reduction. Vegetative response among transformation events varies from no obvious vegetative effects to death of all vegetative tissue. GTCot line 1445 was initially evaluated in a greenhouse screen with several other lines. Lines 665 and 44 had been identified as vegetatively tolerant in previous screens and were used as positive controls in the greenhouse screens. Both lines received a visual score of 10 for vegetative effects which indicated that no obvious vegetative effects were present. GTCot lines 1445 and 1513 also received a score of 10. No other lines received a vegetative score greater than 8; a vegetative score of 8 or less was deemed not commercially acceptable, and these lines were not evaluated in future tests.

In addition to vegetative effects, glyphosate injury can also be manifested as a lack of pollen shed. In cotton, lack of pollen shed results in boll abortion. Thus, the node of fertility (i.e., the first node with a boll set on the primary fruiting position) was recorded for those lines with an acceptable vegetative response. For negative control line Coker 312, the first fruiting branch is typically found at the fifth or sixth node. For those lines with relatively poor fertility, the node of fertility would be higher than the fifth or sixth node. The node of fertility for GTCot line 1445 was noted as the sixth node, indicating that the fertility effects with this line were minimal for this application timing at this rate (Table 1). Thus, in this particular greenhouse assay, GTCot line 1445 possessed the best combination of vegetative and fertility tolerance to glyphosate.

GTCot line 1698 was screened similarly. In this assay, several lines (1663, 1698, and 1767) and the positive controls (665 and 44) received vegetative scores of 10, indicating no observable vegetative effects (Table 1). On the basis of segregation analysis, however, 1767 contained multiple DNA insertions thereby limiting its utility in a breeding program. The node of fertility for GTCot line 1698 was the fifth node. Thus GTCot line 1698 appeared to possess excellent reproductive tolerance to glyphosate. Line 1663 also possessed promising reproductive tolerance.

Table 1. Vegetative and Fertility Results for the Initial Greenhouse Tests with GTCot Lines 1445 and 1698

line no.	vegetative score[a]
Screen 1	
44[b]	8
665[b]	10
886	7 (5)[c]
1434	5
1435	4
1445	10 (6)
1455	4
1460	7
1464	7
1513	10 (8)
1523	8 (6)
1525	6
Screen 2	
44[b]	9
665[b]	10
1547	8
1663	10 (6)
1698	10 (5)
1744	3
1768	3
1767	10

[a] Visual score on scale of 1−10, 10 as most tolerant, and 1 as least tolerant. [b] Positive control for vegetative tolerance. [c] Score for reproductive tolerance.

The most tolerant lines from the greenhouse assays described above, as well as additional lines from other similar assays, were advanced to a field screen for further evaluation. Through line evaluation trials and yield trials, GTCot lines 1445 and 1698 were selected for commercial development.

Segregation Analyses. Initial segregation data were collected with progeny of the R_0 plants (R_1 plants). The observed R_1 segregation ratios for GTCot line 1445 support the conclusion that the insertional event for this line segregates as a single Mendelian gene locus (Table 2). For example, the R_1 segregation of 11 tolerant plants and 5 susceptible plants fits a single-locus model but does not fit a two-locus model.

In another experiment, 13 glyphosate-tolerant 1445 plants were grown to maturity, and the progeny were analyzed to determine the number of plants homozygous and heterozygous for the glyphosate-tolerance trait. The susceptible plants were eliminated in the spray test; therefore, the only remaining genotypic classes were heterozygous and homozygous positive. The results in Table 2 showed that five plants were homozygous and eight were heterozygous for glyphosate tolerance. These data also support the single-locus model. The data for all eight heterozygotes were pooled; these data (157 tolerant:58 susceptible) fit the expected ratio for a single Mendelian locus.

Actual segregation in the R_1 generation for line 1698 also fits the expected ratios for a single locus. The R_1 segregation of 38 tolerant:8 susceptible supports the expected ratio for a single locus but does not fit for a double insert (Table 3). Progeny analyses of 34 tolerant plants derived from line 1698 showed that 10 plants were homozygous and 24 plants were heterozygous for the glyphosate tolerance trait. This also supports the presence of a single genetic insertion. Similarly, the pooled segregation for the 24 heterozygous plants of 213 tolerant:68 susceptible fits a 3:1 ratio as expected for a

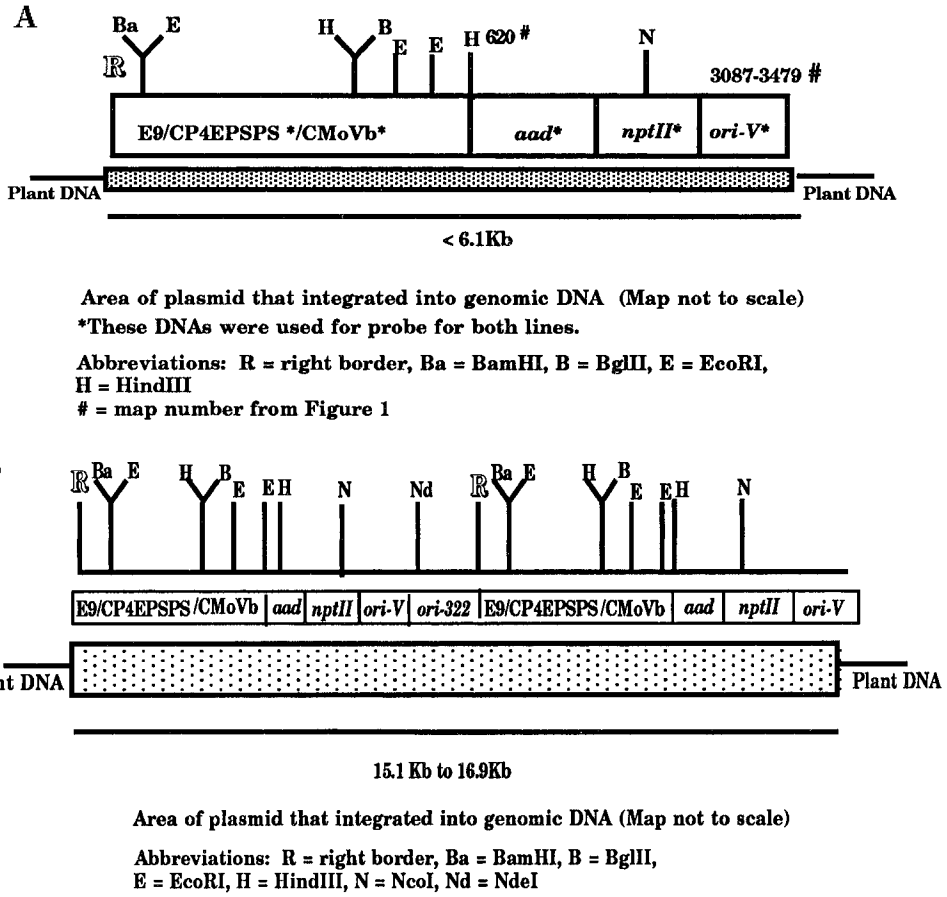

Figure 2. Schematic diagrams of molecular characterization of line 1445 (A) and line 1698 (B).

Table 2. Segregation Data and Analyses of Self-Pollinated Progeny of GTCot Line 1445[a]

single insert			double insert	
obsd	expected	chi square	expected	chi square
		R_1 Plants[b]		
11:5	12:4	0.33[c]	15:1	4.26[d]
		R_1 Progeny		
5:8	4.3:8.7	0.17[c]		
		Pooled Heterozygotes[b]		
157:58	161.3:53.7	50.45[c]		

[a] Data expressed as R_1 homozygotes:R_1 heterozygotes. [b] Data expressed as glyphosate tolerant:glyphosate susceptible. [c] Not significant at $p = 0.05$ (chi square value = 3.84). [d] Significant at $p = 0.05$ (chi square value = 3.84).

Table 3. Segregation Data and Analyses of Self-Pollinated Progeny of GTCot Line 1698[a]

single insert			double insert	
obsd	expected	chi square	expected	chi square
		R_1 Plants[b]		
38:8	34.5:11.5	1.42[c]	43.1:2.9	9.57[d]
		R_1 Progeny		
10:24	8.5:25.5	0.35[c]		
		Pooled Heterozygotes[b]		
213:68	210.8:70.3	0.09[c]		

[a] Data expressed as R_1 homozygotes:R_1 heterozygotes. [b] Data expressed as glyphosate tolerant:glyphosate susceptible. [c] Not significant at $p = 0.05$ (chi square value = 3.84). [d] Significant at $p = 0.05$ (chi square value = 3.84).

single locus. Thus, the segregation data for both GTCot lines 1445 and 1698 support the presence of a genetic insertion which functions as a single Mendelian locus.

Molecular Characterization. Genomic DNAs of Coker 312 and glyphosate-tolerant lines 1445 and 1698 were analyzed by Southern blots. The plasmid PV-GHGT06 and the following isolated genetic elements were used as hybridization probes: the *gox* gene, CP4 EPSPS gene, the *nptII* gene, the *aad* gene, the CMoVb promoter, the *ori-V*, and the *ori-322*. Figure 2 (parts A and B) shows a schematic diagram of the T-DNA in lines 1445 and 1698, respectively.

Number of Loci in Which Plasmid DNA Inserted into the Genome. All of the Southern blots described in this section were probed with ^{32}P-labeled PV-GHGT06. When GTCot line 1445 genomic DNA was cut with *Bam*HI, an enzyme which cuts once within PV-GHGT06, two bands were observed at 13 and 10.8 kb, which were identified as border fragments (e.g., fragments containing cotton genomic DNA) because both bands were larger than the plasmid control cut with this enzyme. GTCot line 1445 revealed a single band of 23 kb when digested with *Spe*I, an enzyme which does not have a site in the plasmid DNA, indicating the T-DNA inserted at a single locus (data not shown).

When GTCot line 1698 DNA was digested with *Bam*HI, two bands were observed on the Southern blot. One band was 9.5 kb, an expected size if the entire plasmid was inserted, and a band of 19 kb was identified as a border fragment which contained additional DNA from the plasmid. Line 1698 genomic DNA digested with *Spe*I showed a single band of 25 kb which demonstrated that the T-DNA integrated at a single locus (data not shown).

Genetic Elements. Line 1445 was generated from Coker 312 transformed with PV-GHGT07, but this line did not express the GOX protein, as evaluated by both ELISA and Western blot (data not shown). A Southern

Figure 3. Southern blot of line 1445 probed with *gox*. Lane 1 represents approximately 100 pg of plasmid DNA. Lanes 2 and 3 represent approximately 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-labeled *gox* and then subjected to autoradiography.

blot was performed to determine if the *gox* gene was present. Figure 3 shows a Southern blot containing PV-GHGT07 DNA, genomic DNA from Coker 312, and GTCot line 1445 cut with *Nco*I and *Eco*RI and then probed with ^{32}P-labeled *gox*-coding region. Since the plasmid DNA has a band at the expected position, and neither Coker 312 nor line 1445 has a similar band, it was concluded that the *gox* gene was not present in line 1445. Southern blots of genomic DNA from line 1445 were probed with the following ^{32}P-labeled DNA probes: CMoVb, CP4 EPSPS, *aad*, *nptII*, *ori-V*, and *ori-322*. The analyses showed that the T-DNA contained CMoVb, CP4 EPSPS, *aad*, *nptII*, and a portion of *ori-V*. The T-DNA stopped before all of the *ori-V* was integrated. The *ori-322* also was not included in the T-DNA (Figure 2A).

The Southern blot analyses of line 1698, probed with the same DNA probes as line 1445, showed that the T-DNA contained the entire plasmid plus additional plasmid DNA through at least a portion of the *ori-V* (Figure 2B).

Copy Number of CP4 EPSPS. Analysis of the number of loci in which T-DNA integrated into line 1445 indicated that there was only one locus into which PV-GHGT07 DNA integrated into the genome of line 1445. It was concluded that there is a single copy of CP4 EPSPS present in this line (Figure 2A). The Southern blot analysis showed that the T-DNA integrated into line 1698 at a single locus contained the entire plasmid DNA plus an additional segment of between 5.6 and 7.4 kb more of plasmid DNA. There are two copies of CP4 EPSPS present in line 1698. There is one copy present in the complete plasmid DNA and an additional copy on the extension of the plasmid DNA. The schematic diagram in Figure 2B illustrates this.

Stability of the Glyphosate-Tolerant Gene and the T-DNA. In the Southern blot shown in Figure 4, panel A, the GTCot line 1445 (lanes 3 and 4) and GTCot line 1698 (lanes 5 and 6) show that CP4 EPSPS was stably maintained during the life cycle of the cotton plant from the R_3 generation through the R_5 generation. In panel B, GTCot line 1445 (lanes 3 and 4) and GTCot line 1698 (lanes 5 and 6), the blot showed a similar banding pattern between the R_3 and R_5 generations when probed with the *nptII* gene. Given this evidence, it was concluded that the T-DNA has been stably maintained for at least these 3 generations.

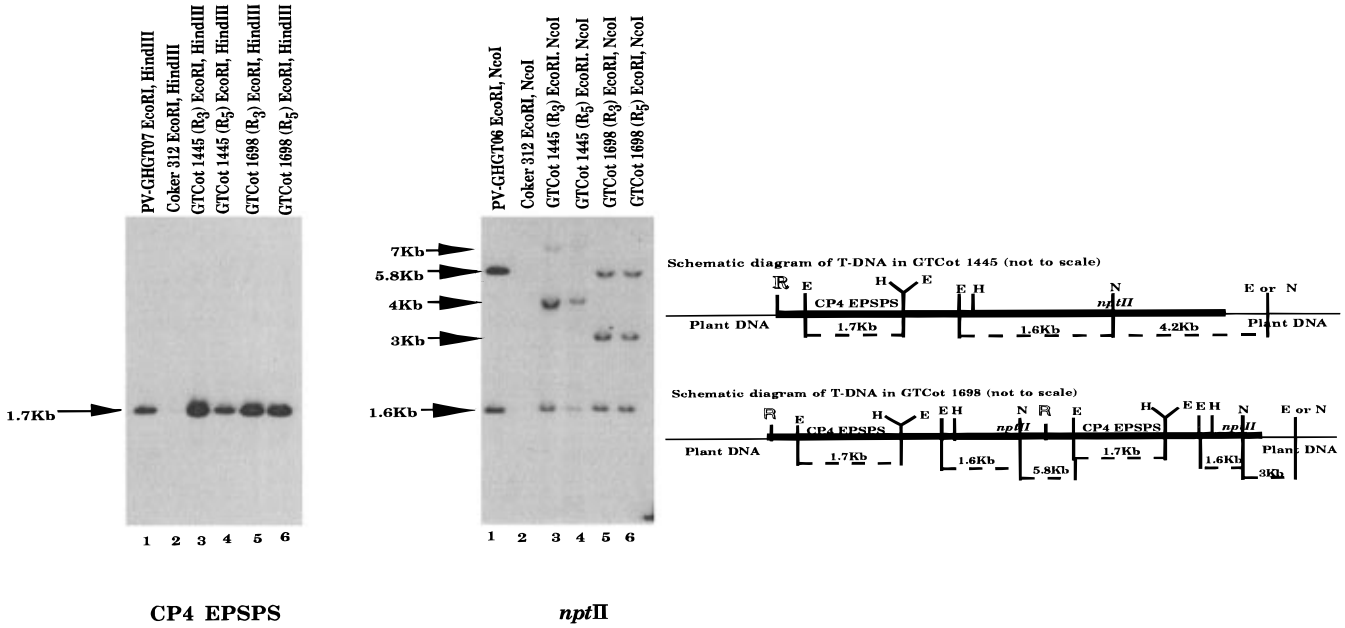

Figure 4. Southern blots of lines 1445 and 1698 probed with CP4 EPSPS- and *nptII*-coding regions. Each lane represents approximately 100 pg of plasmid DNA or approximately 5 μg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with [^{32}P]CP4 EPSPS-coding sequence (A) or *nptII* DNA (B) and then subjected to autoradiography. Abbreviations: R = right border, E = *Eco*RI, H = *Hin*dIII, N = *Nco*I.

Table 4. Levels of CP4 EPSPS and NPTII Expression in Cotton Leaf and Seed Tissues (μg/mg)

	1993 mean[a] (range)[b]			1994 mean[a] (range)[b]		
characteristic	C312	1445	1698	C312	1445	1698
			CP4 EPSPS			
leaf	ND[c]	0.052 (0.027−0.101)	0.311 (0.169−0.522)	NA[c]	NA	NA
seed	ND	0.082 (0.058−0.117)	0.204 (0.122−0.259)	ND NA	0.060 (0.047−0.077)	0.170 (0.140−0.223)
			NPTII			
leaf	ND	0.045 (0.019−0.084)	0.031 (0.011−0.071)	NA	NA	NA
seed	ND	0.007 (0.005−0.010)	0.004 (0.002−0.011)	ND	0.007 (0.002−0.009)	0.004 (0.002−0.007)

[a] Mean of six replicated samples collected at six sites in the southern United States. Values are expressed as μg of CP4 EPSPS or NPTII protein/mg of fresh weight tissue. [b] Range indicates the maximum and minimum individual value detected. [c] NA = not analyzed, ND = not detectable.

Gene Expression. Expression levels of CP4 EPSPS and NPTII were analyzed in leaf and seed collected in field trials conducted under good laboratory practices (GLP). Six locations were used for each of 2 years, 1993 and 1994. The expression levels were consistent across 1993 and 1994 trials. The levels of the CP4 EPSPS and NPTII proteins are presented in Table 4. The range of CP4 EPSPS in seed from GTCot lines 1445 and 1698 was 0.047−0.117 and 0.122−0.259 μg/mg of tissue, respectively. The range of levels of NPTII in seed from lines 1445 and 1698 were 0.002−0.10 and 0.002−0.011 μg/mg of tissue, respectively. The samples were also evaluated for the AAD protein. None was detected by ELISA or Western blot assay, as expected considering the gene is driven by a bacterial promoter.

The safety of the expressed proteins was demonstrated through acute toxicity tests and digestive fate studies (Harrison et al., 1996; Fuchs et al., 1993). In addition, the expression levels of the additional proteins of CP4 EPSPS and NPTII are extremely low; therefore, exposure to the proteins through animal feed of raw seed would be minimal. The major cottonseed products used for human consumption are cottonseed oil and cellulose produced from linters. Because of the methods of extraction and purification of the oil, protein is not detected in the oil fractions (USDA, 1979). Similarly, the linters are highly processed through stringent conditions that protein would not be expected to survive (*Cottonseed and Its Products*, 1989). Therefore there would be no expected human exposure to these proteins through normal consumption of cottonseed products.

CONCLUSION

The expression of the CP4 EPSPS gene in genetically modified cotton lines 1445 and 1698 confers tolerance to glyphosate, the active ingredient in Roundup herbicide. Segregation data and the molecular analysis of both GTCot lines 1445 and 1698 establish that both lines contain a single insert, inherited in a typical Mendelian fashion. Extensive molecular characterization established that GTCot line 1445 contains the CoMVb promoter region, CP4 EPSPS-coding region, *aad*, *nptII*, and a portion of *ori-V*. The remainder of the plasmid, including the *gox* gene, did not integrate into the genome. GTCot line 1698 contains CMoVb, CP4 EPSPS, *aad*, *nptII*, *ori-V*, and *ori-322*. The data establish that two copies of the CP4 EPSPS integrated at a single locus into the cotton genome. Molecular analyses demonstrated that the insertional event in each line was stable across the R_3−R_5 generations. Expression of the CP4 EPSPS and NPTII proteins was low and consistent across field locations and both years of analyses.

Environmental, food, and feed safety studies have been completed that demonstrate that GTCot lines are substantially equivalent to currently available commercial varieties (Nida et al., 1996). Digestive fate and acute gavage studies establish that both the CP4 EPSPS and NPTII proteins pose no risks to consumer or animals (Harrison et al., 1996; Fuchs et al., 1993). Breeding efforts are underway with GTCot lines 1445 and 1698 to develop commercial cotton varieties containing the glyphosate tolerance trait for new weed control systems.

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