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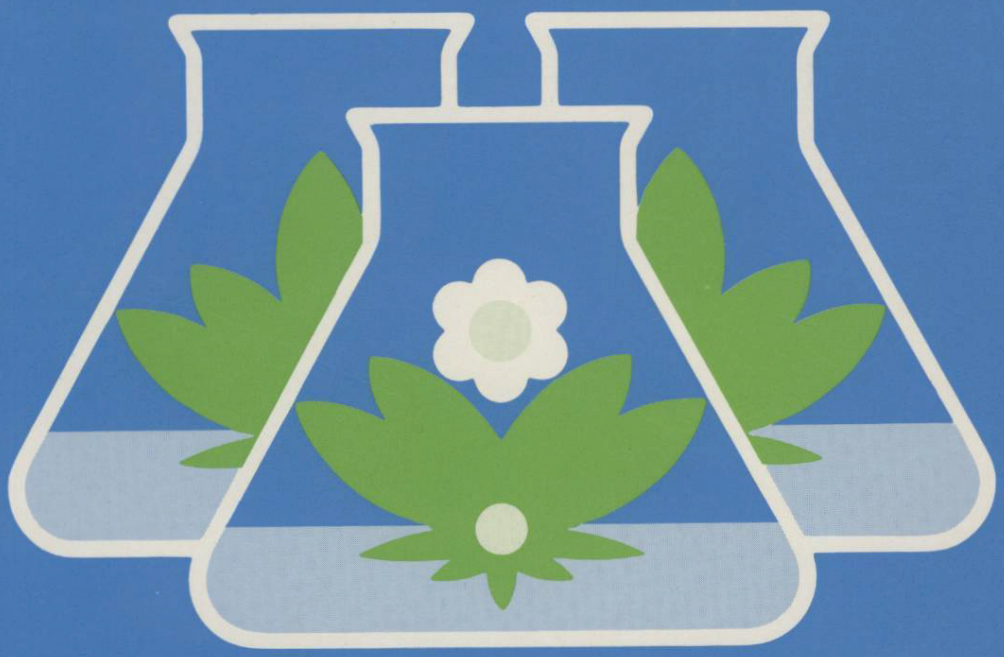

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Aims and Scope

Plant Cell, Tissue and Organ Culture is an international monthly journal that publishes original results of fundamental studies on the behavior of plant cells, tissues and organs in vitro. Topics include growth and developmental, anatomical, histological and ultrastructural aspects, physiology and biochemistry, genetics and breeding, phytopathological aspects and biotechnology. Methodological and technical aspects of the culture of plant cells, tissues and organs will be considered for publication on the basis of novelty and potential contributions to studies of the in vitro biology of these systems.

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The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean

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Key words: Agrobacterium tumefaciens, bar, Glycine max, phosphinothricin

Abstract

The soybean transformation procedure using the *Agrobacterium*-cotyledonary node transformation system and the *bar* gene as the selectable marker coupled with glufosinate as a selective agent is described. Soybean cotyledonary explants were derived from 5 day old seedlings and co-cultivated with *Agrobacterium tumefaciens* for 3 days. Explants were cultured on Gamborg's B5 medium supplemented with 1.67 mg l^{-1} BAP and glufosinate at levels of 3.3 mg l^{-1} or 5.0 mg l^{-1} for 4 weeks. After 4 weeks explants were subcultured to medium containing MS major and minor salts and B_5 vitamins (MS/B5) supplemented with 1.0 mg l^{-1} zeatin-riboside, 0.5 mg l^{-1} GA_3 and 0.1 mg l^{-1} IAA amended with 1.7 mg l^{-1} or 2.0 mg l^{-1} glufosinate. Elongated shoots were rooted on a MS/B5 rooting medium supplemented with 0.5 mg l^{-1} NAA without further glufosinate selection. Plantlets were transplanted to soil and grown to maturity and set seed in the greenhouse. Primary transformants and their progeny were characterized by Southern blot analysis and a leaf paint assay.

Abbreviations: AS – acetosyringone; BAP – 6-benzyl-aminopurine; IAA – indole-3-acetic acid; NAA – naphthalene acetic acid

Introduction

Introduction of agronomically important traits into soybean (*Glycine max* [L.] Merr.) has been accomplished via plant transformation (Trick et al., 1997). Those traits include herbicide tolerance (Padgette et al., 1995), amino acid modification (Falco et al., 1995), virus resistance (Di et al., 1996) and insect resistance (Stewart et al., 1996). Development of more efficient procedures for *Agrobacterium*-mediated soybean transformation could make it possible for routine production of transgenic soybean lines with simple inserts that are inherited as a single functional locus to facilitate breeding.

Agrobacterium-mediated transformation of soybean has been very difficult. Only a few studies on this system have been reported to date. Plant regeneration of soybean via the coyledonary node explant was first reported by Cheng et al. (1980). An *Agrobacterium*-mediated transformation system exploiting this explant was first reduced to practice by Hinchee et al. (1988). Di et al. (1996) used this system to introduced the coat protein of bean pod mottle virus into soybean. Kanamycin is the only selective agent that has been published for use in the system.

The herbicide resistance gene, *bar* (Thompson et al., 1987), has been widely used as a selectable marker in plant transformation systems (D'Halluin et al., 1992). The *bar* gene encodes for phosphinothricin acetyltransferase (PAT) which detoxifies glufosinate, the active ingredient in the herbicide Liberty® (AgrEvo USA). To our knowledge, no work has been reported to date evaluating this selectable marker in a soybean transformation system. Due to the rapid translocation mechanism of glufosinate in soybean tissue through both the xylem and phloem (Shelp et al.,

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1992), we thought that glufosinate selection would provide efficient recovery of transformed tissue with the *Agrobacterium*-cotyledonary system. We report here the use of the *bar* gene as a selectable marker for *Agrobacterium*-mediated transformation of soybean.

Materials and methods

Plant material

Soybean seeds, genotype A3237 (Asgrow Seed Company; Des Moines, Iowa), were surface sterilized by an overnight exposure to chlorine gas (Di et al., 1996). Sterilized seeds were germinated, in 100 × 20 mm Petri dishes, on Gamborg's B5 basal medium (B5) (Gamborg et al., 1968) supplemented with 2% sucrose, pH 5.8. The plates were stacked 5 high and placed in plastic bags in which 4, approx. 3 inch, slits were made with scissors. Seeds were germinated for 5 days in a growth room, at 24 °C, 18/6 light regime, under a light intensity of approximately 150 μmol s^{-1} m^{-2}. The light source was a mixture of cool white (Sylvania model # F72T12/CW/VHO) and Gro-lux® bulbs (Sylvania model # F72T12/GRO/VHO).

Plant transformation vectors

The binary vector, pPTN101 (Figure 1A), is a derivative of pGPTV-bar (Becker et al., 1992), carrying the GUS cassette from pE7131-GUS (Mitsuhara et al., 1996). The binary vector pPTN125 (Figure 1B) is a derivative of pGPTV-bar (Becker et al., 1992) carrying a GUS cassette under the control of the soybean vegetative storage protein B (*vspB*) promoter (Rhee and Staswick, 1992). An intermediate vector carrying the *bar* gene under the control of the 35S promoter was generated by isolating the *bar* open reading frame (ORF) from pDM302 (Cao et al., 1992) as a *Sma* I fragment and subcloning it into the plant expression cassette pRTL2 (Carrington, 1990). The resultant vector pPTN117 (not shown) contained the *bar* ORF in a translation fusion with the N-terminus of the Tobacco Etch Virus (TEV) polyprotein coupled with the TEV translation enhancer element. A third binary was assembled that harbored a 35S *bar* cassette, a derivative of the *bar* cassette within pPTN117, lacking the N-terminus of the TEV polyprotein, hence, the bar ORF is directly ligated to the TEV translational enhancer element (Carrington, 1990). This latter *bar* cassette was generated by introducing a *Nco* I site at the start of translation of the *bar* ORF, by PCR. Primers used were referred to as BAR-5: 5' CCCGGGATCTACCATGGCTAGCCCAGCCCAGA-ACGAC-3' and BAR-3: 5'-TCATCAGATCTCGGT-GACGG-3'. This manipulation introduced an Ala residue between the Met and Ser residues of the native *bar* protein. The PCR product was subcloned into pPTN117 as a *Nco* I/ *Kpn* I fragment replacing the 5' region of the *bar* ORF within the vector with the engineered fragment. The resultant vector is referred to as pPTN129 (not shown). The 35S *bar* cassette within pPTN129 was sequenced at the University of Nebraska-Lincoln DNA Sequencing Core Research Facility to confirm the integrity of the PCR reaction. The 35S bar cassette from pPTN129 was subcloned as a *Hind* III fragment into the binary vector pPZP202 (Hajdukiewicz et al., 1994) that harbored a 35S GUS cassette, the resultant vector is referred to as pPTN140 (Figure 1C).

Agrobacterium tumefaciens strains

The binary vectors were mobilized into *Agrobacterium tumefaciens* strains EHA105 (Hood et al., 1993) or EHA101 (Hood et al., 1986) by triparental mating (Ditta et al., 1980). Binary vectors pPTN101 and pPTN125 were introduced into EHA105, and transconjugants were selected on 25 mg l^{-1} chloramphenicol [EHA105 chromosomal drug marker] and 50 mg l^{-1} kanamycin [Binary vector drug marker]. Binary vector pPTN140 was introduced into EHA101, and transconjugants were selected on 25 mg l^{-1} chloramphenicol [EHA101 chromosomal drug marker], 50 mg l^{-1} kanamycin [Ti plasmid pEHA101 drug marker], 100 mg l^{-1} spectinomycin [binary vector drug marker] and 100 mg l^{-1} streptomycin [binary vector drug marker]. Integrity of the binary vectors in the respective *Agrobacterium* strains was confirmed by plasmid rescue.

Agrobacterium culturing conditions

Agrobacterium cultures were grown in YEP medium (10 g l^{-1} peptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl, pH 7.0) amended with the appropriate antibiotics to an OD_{650} = 0.6 to 0.8 at 27 °C. The bacterial cultures were centrifuged at 3,500 rpm for 10 min., and the pellets resuspended to a final OD_{650} = 0.6 to 0.8 in 1/10 Gamborg's B5 medium (Gamborg et al., 1968) amended with 1.67 mg l^{-1}BA, 0.25 mg l^{-1} GA_3, 200 μM acetosyringone (AS) and 3% sucrose. The medium was buffered with 20 mM MES, pH 5.4.

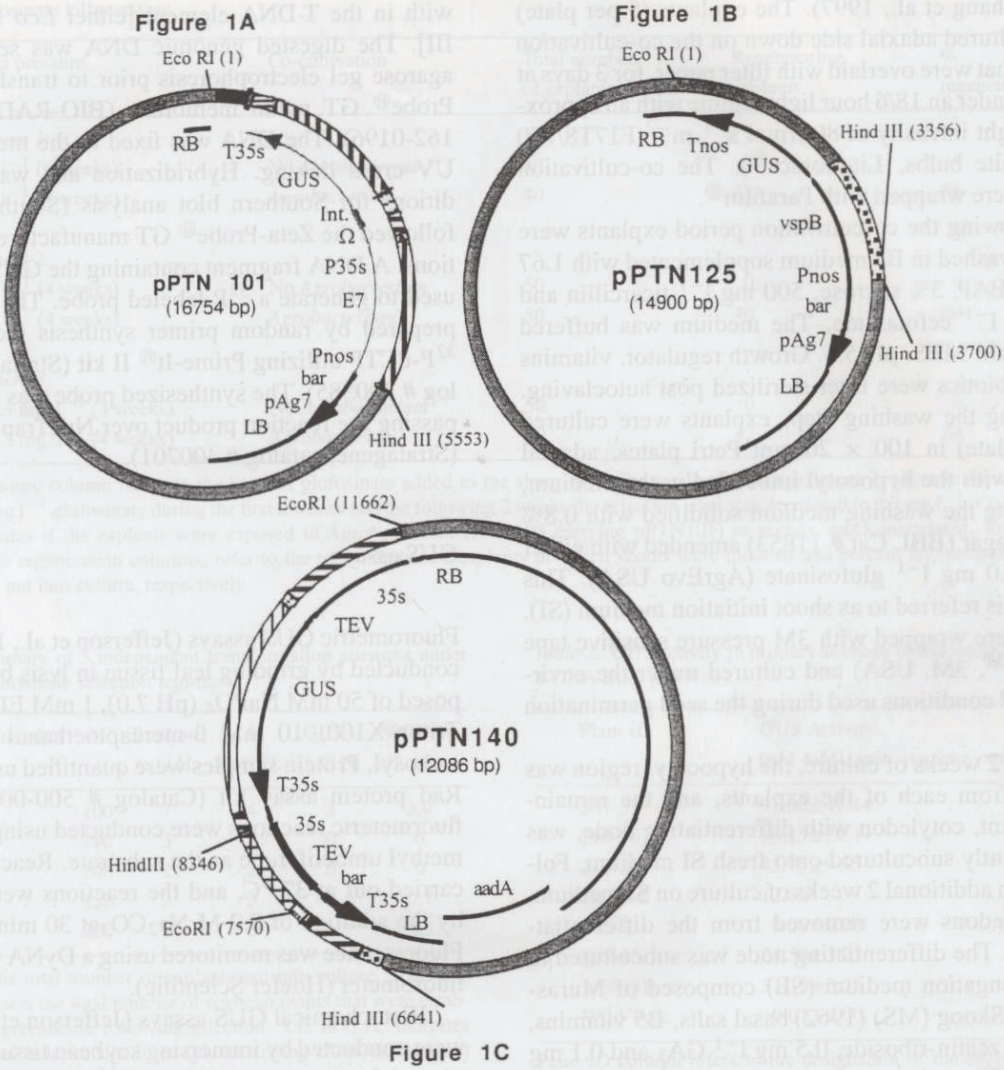

Figure 1. Binary vectors utilized in the experiments. See Materials and methods section for description of the binary vectors. Abbreviations: RB – right border, LB – left border, T35s and pAg7 are polyadenylation signals, Pnos – nopaline synthase promoter, aadA – bacterial drug resistance marker for spectinomycin and streptomycin.

All growth regulators, vitamin components and AS were filter sterilized post autoclaving.

Plant transformation

Cotyledonary explants were prepared from the 5-day-old soybean seedlings by making a horizontal slice through the hypocotyl region, approximately 3–5 mm below the cotyledon. A subsequent vertical slice was made between the cotyledons, and the embryonic axis was removed. This manipulation generated 2 cotyledonary node explants. Approximately 7–12 vertical slices were made on the adaxial surface of the explant about the area encompassing 3 mm above the cotyledon/hypocotyl junction and 1 mm below the cotyledon/hypocotyl junction. Explant manipulations were conducted with a No. 15 scalpel blade.

Explants were immersed in the *Agrobacterium* inoculum for 30 min and then co-cultured on 100 × 15 mm Petri plates containing the *Agrobacterium* resuspension medium solidified with 0.5% purified agar (BBL Cat # 11853). The co-cultivation plates were overlaid with a piece of Whatman #1 filter paper (Mullins et al., 1990; Janssen and Gardner,

1993; Zhang et al., 1997). The explants (5 per plate) were cultured adaxial side down on the co-cultivation plates, that were overlaid with filter paper, for 3 days at 24 °C, under an 18/6 hour light regime with an approximate light intensity of 80 μmol s^{-1} m^{-2}(F17T8/750 cool white bulbs, Litetronics®). The co-cultivation plates were wrapped with Parafilm®.

Following the co-cultivation period explants were briefly washed in B5 medium supplemented with 1.67 mg l^{-1} BAP, 3% sucrose, 500 mg l^{-1} ticarcillin and 100 mg l^{-1} cefotaxime. The medium was buffered with 3 mM MES, pH 5.6. Growth regulator, vitamins and antibiotics were filter sterilized post autoclaving. Following the washing step, explants were cultured (5 per plate) in 100 × 20 mm Petri plates, adaxial side up with the hypocotyl imbedded in the medium, containing the washing medium solidified with 0.8% purified agar (BBL Cat # 11853) amended with either 3.3 or 5.0 mg l^{-1} glufosinate (AgrEvo USA). This medium is referred to as shoot initiation medium (SI). Plates were wrapped with 3M pressure sensitive tape (Scotch™, 3M, USA) and cultured under the environmental conditions used during the seed germination step.

After 2 weeks of culture, the hypocotyl region was excised from each of the explants, and the remaining explant, cotyledon with differentiating node, was subsequently subcultured onto fresh SI medium. Following an additional 2 weeks of culture on SI medium, the cotyledons were removed from the differentiating node. The differentiating node was subcultured to shoot elongation medium (SE) composed of Murashige and Skoog (MS) (1962) basal salts, B5 vitamins, 1 mg l^{-1} zeatin-riboside, 0.5 mg l^{-1} GA_3 and 0.1 mg l^{-1} IAA, 50 mg l^{-1} glutamine, 50 mg l^{-1} asparagine, 3% sucrose and 3 mM MES, pH 5.6. The SE medium was amended with either 1.7 or 2.0 mg l^{-1} glufosinate. The explants were subcultured biweekly to fresh SI medium until shoots reached a length greater than 3 cm. The elongated shoots were rooted on Murashige and Skoog salts with B5 vitamins, 1% sucrose, 0.5 mg l^{-1} NAA without further selection in either Magenta boxes® or Sundae cups (Industrial Soap Company, St. Louis MO).

Southern blot analysis

Total DNA was extracted from soybean leaf tissue as described by Dellaporta et al. (1983). Ten or 20 μg of DNA were digested with a restriction enzyme for which only one recognition sequence is present with in the T-DNA element [either *Eco* RI or *Hind* III]. The digested genomic DNA was separated by agarose gel electrophoresis prior to transfer to Zeta-Probe® GT nylon membrane (BIO-RAD catalog # 162-0196). The DNA was fixed to the membrane by UV cross linking. Hybridization and washing conditions for Southern blot analysis (Southern, 1975) followed the Zeta-Probe® GT manufacturer's instructions. A DNA fragment containing the GUS ORF was used to generate a ^{32}P-labeled probe. The probe was prepared by random primer synthesis incorporating ^{32}P-dCTP utilizing Prime-It® II kit (Stratagene, catalog # 300385). The synthesized probe was purified by passing the reaction product over NucTrap® columns (Stratagene, catalog # 400701).

GUS assays

Fluorometric GUS assays (Jefferson et al., 1987) were conducted by grinding leaf tissue in lysis buffer composed of 50 mM $NaPO_4$ (pH 7.0), 1 mM EDTA, 0.1% Triton-X100, 10 mM ß-mercaptoethanol and 0.1% sarkosyl. Protein samples were quantified using a Bio-Rad protein assay kit (Catalog # 500-0002). GUS fluormeteric reactions were conducted using 1 mM 4-methyl umbeliferone as the substrate. Reactions were carried out at 37 °C, and the reactions were stopped by the addition of 0.2 M Na_2CO_3at 30 min intervals. Fluorescence was monitored using a DyNA Quant 200 fluorometer (Hoefer Scientific).

Histochemical GUS assays (Jefferson et al., 1987) were conducted by immersing soybean tissue in a substrate solution composed of 0.1 M $NaHPO_4$ buffer (pH 7.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6$, 10 mM EDTA and 800 mg l^{-1} X-Gluc. Methanol and Triton-X100 were subsequently added to final concentrations of 20% and 0.06%, respectively. Soybean tissue was allowed to incubate in the X-Gluc substrate for 3 to 8 hours at 37 °C. The tissue was subsequently cleared in 70% ethanol prior to visualization.

Leaf painting. Progeny were screened for tolerance to the herbicide Liberty® by application of a 0.1% solution of the herbicide with a cotton swab to the upper surface of the first unifoliate leaf 3 weeks post germination. A 200 g l^{-1} stock of Liberty® was diluted in water for the leaf paining assay. Leaf tissue was scored for herbicide tolerance 5 days post application.

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Table 1. Glufosinate kill response.

Selection pressure	Co-cultivation	Total number of explants	Regenerating explants	% regeneration
Experiment 1				
3.3 mg l^{-1} (4 weeks)	No *Agrobacterium*	50	2	4
3.3 mg l^{-1} (4 weeks)	*Agrobacterium*	40	34	85
Experiment 2				
3.3 mg l^{-1} (4 weeks)	No *Agrobacterium*	50	0	0
3.3 mg l^{-1} (4 weeks)	*Agrobacterium*	50	40	80
Experiment 3				
5.0 to 3.3 mg l^{-1} (4 weeks)	No *Agrobacterium*	50	0	0
5.0 to 3.3 mg l^{-1} (4 weeks)	*Agrobacterium*	50	42	84

Selection pressure column refers to the level of glufosinate added to the shoot induction medium. In Experiment 3, shoot initiation medium contained 5 mg l^{-1} glufosinate during the first 2 weeks and the following 2 weeks the selection level was decreased to 3.3 mg l^{-1}. Co-cultivation column indicates if the explants were exposed to *Agrobacterium* (EHA105 carrying pPTN101) or merely mock-inoculated. Regenerating explants and% regeneration columns, refer to the total number of explants in which shoots were initiated and percent regeneration based on total explants put into culture, respectively.

Table 2. Summary of 5 independent transformation attempts under the higher glufosinate selection regime.

Experiment number	N	Total no. in G.H.	I.E. in G.H.	GUS+ in G.H.	%GUS+ efficiency
246	100	7	4	3	3.0
247	100	3	3	0	0
248	200	2	2	0	0
249	150	3	3	1	0.7
252	200	9	3	1	0.5

N refers to the total number of explants put into culture. Total No. in G.H. indicates the total number of soybean plants that were established in the greenhouse following selection. 'I.E. in G.H.' indicates the number of independent lines (plants arising from independent explants) represented. 'GUS+ in G.H.' represents the total number of independent soybean transformants co-expressing GUS. %GUS+ Efficiency indicates the efficiency of recovering independent soybean transformants co-expressing GUS.

Table 3. GUS activity in primary soybean transformants carrying T-DNA from pPTN101.

Plant ID	GUS Activity (nM 4-MU/min./μg total protein)
207-5	91.00±41.44
210-16	36.15±29.49
211-2B	43.75±9.55
207-5-2	12.80*
210-11	0.00±0.00
209-15	24.50±3.25
209-15B	31.10±3.25
Wild Type	0.01±0.00

Plant ID column refers to the designation of the soybean putative transformant. Wild type is a A3237 non-transformant. GUS activity is the mean of 2 independent assays ± S.D.
* indicates only 1 tissue sample was assayed.

Results and discussion

Glufosinate response

Kill curve experiments were used to determine the dose response of soybean cotyledonary explants to glufosinate. Our objective was to ascertain glufosinate levels that would allow for differential growth between transformed and non-transformed tissues. Glufosinate levels ranging from 2 mg l^{-1} up to 33 mg l^{-1} were tested. Explants were prepared for these studies as described in M &M, Plant Transformation section. A total of 10 plates were evaluated per glufosinate level per experiment (i.e. 50 explants per treatment). Explants were scored after 2 and 4 weeks on selection. Explants cultured on glufosinate levels greater than 10 mg l^{-1} failed to regenerate (Data not shown). We decided to focus our efforts on selection levels 3.3 or 5.0 mg l^{-1} during shoot initiation. A different response was observed depending on whether the explants were inoculated with *Agrobacterium* and subsequently co-cultivated for 3 days or merely mock inoculated and co-cultivated for 3 days (Table 1). A mock inoculation was conducted by placing the cotyledonary explants in the bacterial resuspension medium [see M & M, *Agrobacterium* culturing con-

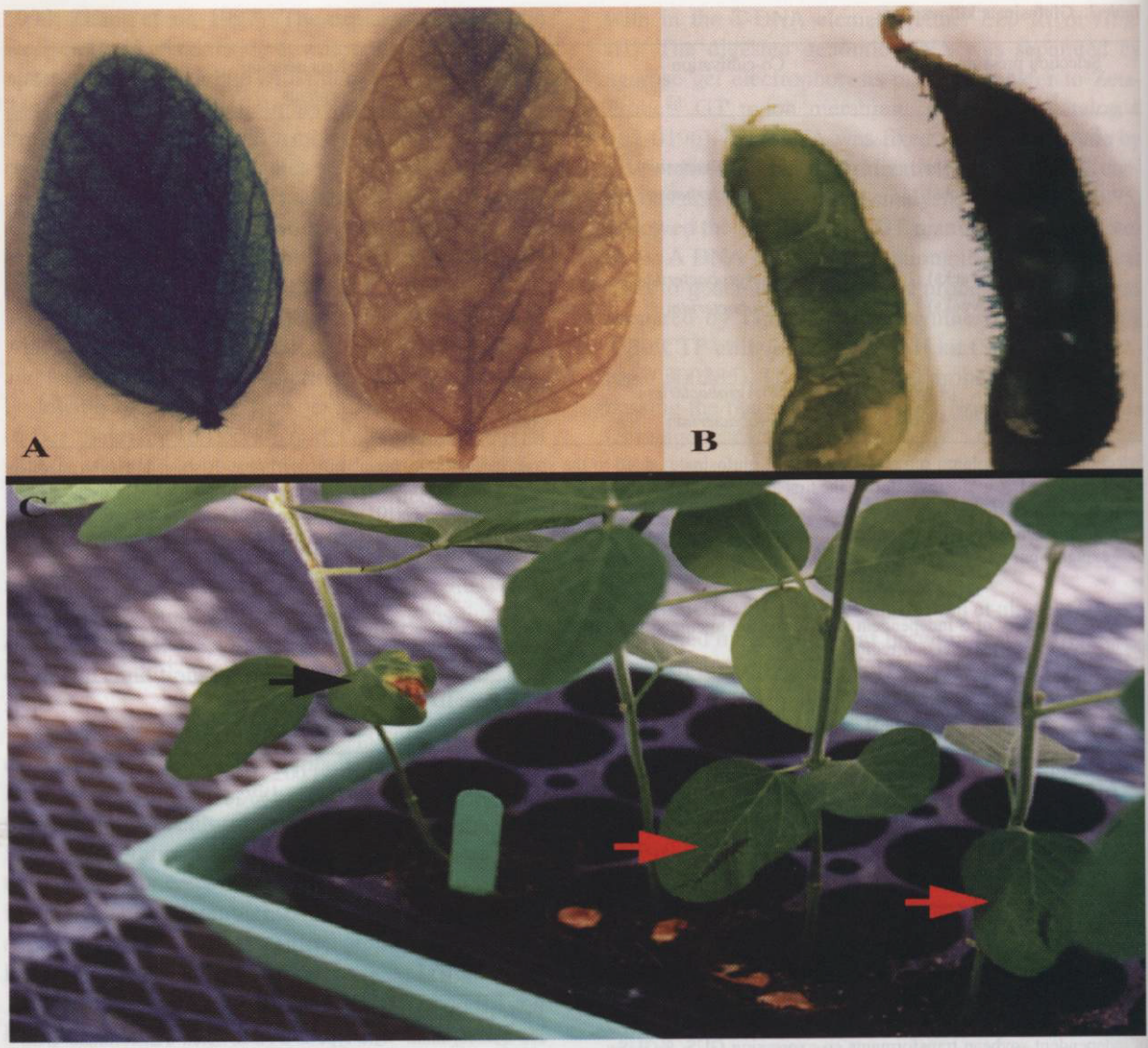

Figure 2. Primary transformants carry the T-DNA of pPTN140. Panel 1A: Histochemical GUS expression in mature leaflet of transformant (left) and control (right). Panel 1B: Histochemical GUS expression in developing pericarp (right) and control pericarp (left). Panel 1C: Progeny screen for herbicide tolerance. Black arrow pointing to herbicide susceptible response in T_1 plants and red arrows pointing to the herbicide tolerant response in T_1 plants following leaf painting with a 0.1% glufosinate solution.

ditions] without the *Agrobacterium*. The explants that were exposed to *Agrobacterium* regenerated on 3.3 or 5.0 mg l^{-1} glufosinate, while those explants that were mock inoculated, were either significantly reduced in regeneration, or regeneration was impeded on the levels of glufosinate tested, across 3 independent experiments (Table 1). This observation suggests that is important to incorporate all the steps of a transformation system when evaluating alternative selection agents and/or conducting experiments to optimize this soybean transformation system.

Selection regimes

Two selection regimes were employed for the recovery of whole plants: (1) 3.3 mg l^{-1} glufosinate during the shoot initiation culture period and stepping down to 1.7 mg l^{-1} during shoot elongation, and: (2) 5.0 mg l^{-1} during the shoot initiation culture period and stepping down to 2.0 mg l^{-1} during shoot elongation. After 4 weeks on SI medium under 3.3 mg l^{-1} glufosinate, 70–80% of the explants survived and were transferred to SE medium. Under the more stringent

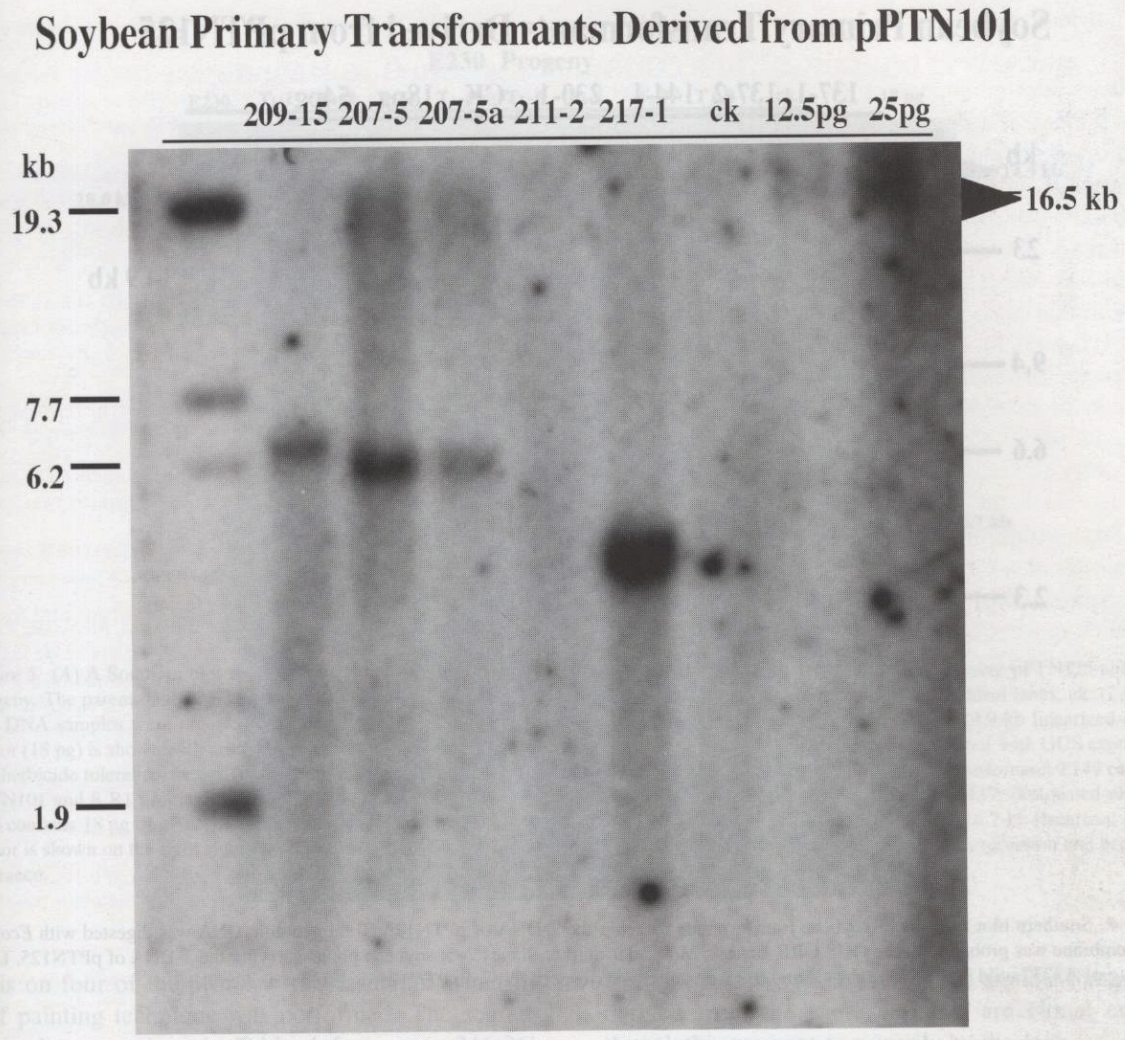

Figure 3. Southern blot analysis of soybean transformants carrying the T-DNA of pPTN101. Total genomic DNA was digested with *Hind* III. The membrane was probed with the GUS ORF. Lane 1: Molecular marker, Lanes 2-6 soybean plants carrying the T-DNA of pPTN101. Lane 7: 10 μg of A3237 wild type DNA. Lanes 8 and 9: are 12.5 pg and 25 pg, respectively, of pPTN101 digested with *Hind* III.

selection regime, 70–80% of the explants regenerated after 2 weeks on SI medium. However, after the second subculture period, only 40–50% of the explants survived and were subcultured to SE medium.

GUS-positive soybean plants were recovered at frequencies ranging from 0.0% to 0.5% and 0.0% to 3.0%, across independent transformation experiments under the lower and higher stringent selection regimes, respectively. Data from 5 transformation attempts utilizing EHA101 (pPTN140) employing the higher selection regime are given in Table 2. Histochemical GUS expression and herbicide tolerance in transformed progeny carrying pPTN140 are shown in Figure 2. GUS expression of primary transformants derived from the lower selection scheme carrying pPTN101 is quantified in Table 3. The events listed in Table 3 were apparent chimeras (see below), with only one being a germline transformation event, 207-5-2.

Molecular characterization of transgenic plants

Southern blot analyses on primary transformants carrying pPTN101 and pPTN125 are shown in Figures 3 and 4, respectively. The Southern data confirms the integration of the transgenes in the soybean genome.

Ten plants, derived from the lower selection regime, (arising from independent explants) were characterized by Southern blot analysis. From the 10

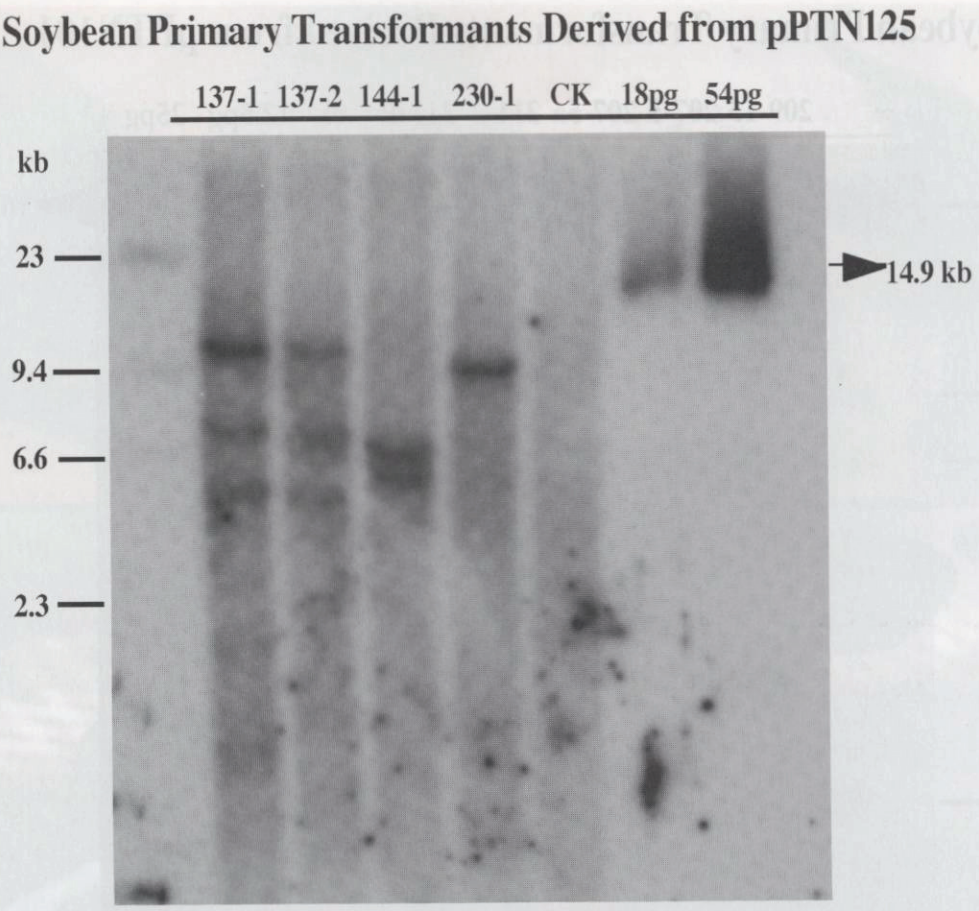

Figure 4. Southern blot analysis of soybean transformants carrying the T-DNA of pPTN125. Total genomic DNA was digested with *Eco*R I. The membrane was probed with the GUS ORF. Lane 1: Molecular marker, Lanes 2–5: soybean plants carrying the T-DNA of pPTN125. Lane 6: 20 μg of A3237 wild type DNA. Lanes 7 and 8: 18 pg and 54 pg, respectively, of pPTN125 digested with *Eco* RI.

Table 4. Segregation analysis on primary transformants derived from glufosinate selection.

Event	T_0 GUS	T_1 GUS^+/HT	T_1 GUS^-/HT	T_1 GUS^-/HS
246-2C	+	4	0	3
252-3A	+	4	0	4
252-3B	+	7	0	1
255-1B	+	0	8	0
230-1	+	5	1	2
Z149	+	3	0	5

Event column refers to the identification number of the soybean transformants. T_0 GUS column indicates the primary transformants as being positive for GUS expression. T_1 GUS^+/HT refers to the number of progeny for which the phenotype was GUS positive and herbicide tolerant. T_1 GUS^-/HT column is the number of progeny for which the phenotype was GUS negative and herbicide tolerant. T_1 GUS^-/HS indicates the number of progeny for which the phenotype was GUS negative and herbicide susceptible.

Southern positive plants, 3 transmitted the transgenes on to the progeny, while the others were non-germ-line events. Segregation analysis for two of these events, E230 and Z149, is shown in Table 4. Molecular analysis of the progeny from these events is shown in Figure 5. Herbicide tolerance and GUS expression correlated with the presence of the approximately 10-kb and 8-kb GUS-hybridizing fragments (Figure 5), in progeny derived from events E230 and Z149, respectively. An exception to the correlation between the molecular and phenotypic segregation analysis was observed in event E230. Individual number 4 (T_1-4, Figure 5A) carried the 10-kb hybridizing fragment, yet the observed phenotype was GUS-negative and herbicide tolerant.

Ten plants, derived from the higher selection regime, (arising from independent explants) were also characterized by Southern blot analysis. Progeny ana-

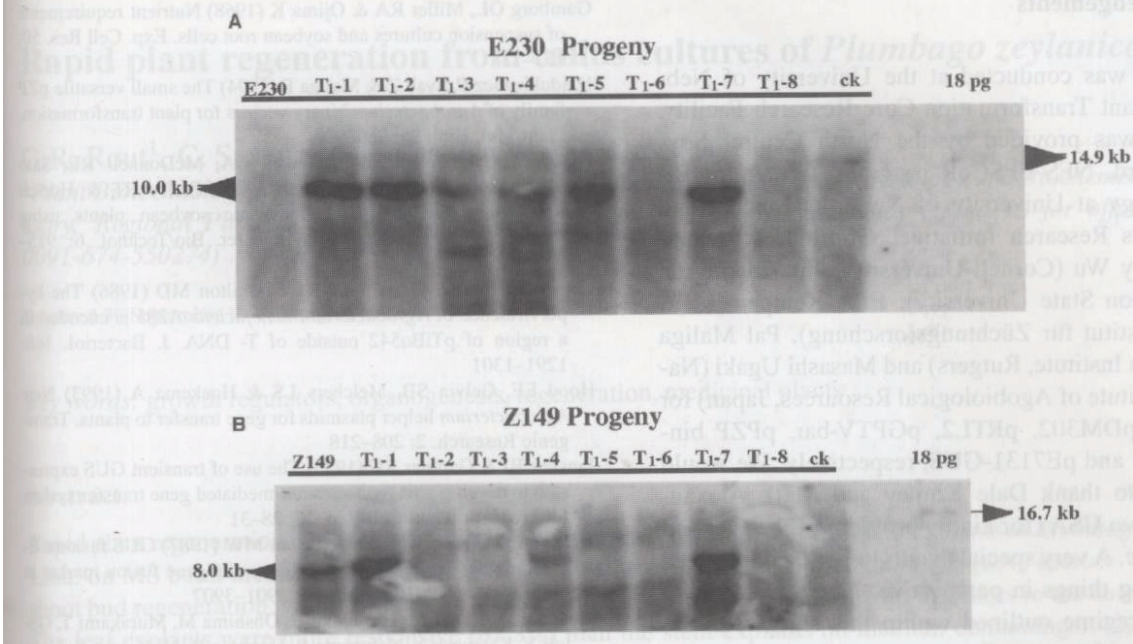

Figure 5. (*A*) A Southern blot analysis on a primary transformant E230 (same event as in Figure 4 and Table 4) carrying pPTN125 and 8 T_1 progeny. The parent, lane 2, E230, had less DNA loaded (~5 μg) as compared to the progeny, designated T_1, and control lanes, ck, (2 0 μg). The DNA samples were digested with *Eco* RI (cuts once within the T-DNA) and probed with the GUS ORF. The 14.9-kb linearized binary vector (18 pg) is shown with arrow on right side. The approximate 10-kb hybridizing fragment (arrow left side) correlated with GUS expression and herbicide tolerance, except for T_1 number 4, which we can not explain. (*B*) Southern blot analysis on a primary transformant Z149 carrying pPTN101 and 8 R1 progeny. Lane 1: parent, Z149. Lanes 2-9: T_1 plants derived from Z149. Lane 10, control, A3237, designated ck. Last lane contains 18 pg of pPTN101. The DNA samples were digested with *Eco* RI and probed with the GUS ORF. The 16.7-kb linearized binary vector is shown on the right side. The approximate 8.0-kb hybridizing fragment (arrow left side) correlated with GUS expression and herbicide tolerance.

lysis on four of the primary transformants using the leaf painting technique was performed. The segregation data are given in Table 4 for events 246-2C, 252-3A, 252-3B, and 255-1B The data demonstrate that these plants transmitted the transgenes to the next generation.

Generally a single plant is recovered from one explant. However, we have taken to soil multiple plants derived from the same explant. In Figure 3, events 207-5 and 207-5a were derived from the same explant and appear to carry the same integration event. Similarly, event 137 had two plants recovered from a single explant (137-1 and 137-2; Figure 4) and both of these appear to be derived from the same integration event. In one case, a GUS-negative plant, 211-2 (Figure 3) recovered under the lower stringency selection regime, appears to be an escape, while another plant derived from the same explant was positive for GUS expression, both histochemically and fluorometrically (211-2B, Table 2). For this reason our transformation frequencies do not include multiple plants derived from the same explant. Hence, we assume that plants derived from the same explants are clonal events, though this may not necessarily be the case.

Conclusion

Agrobacterium-mediated transformation of soybean using the cotyledonary node axillary meristem as the target for gene transfer was first achieved using kanamycin as a selective agent. Here we have demonstrated the utility of the *bar* gene, employing glufosinate as the selective agent, in the system. Germ-line transformation events were recovered at frequncies up to 3% using a selection regime of 5 mg l^{-1} glufosinate during the shoot initiation stage and 2 mg l^{-1} during shoot elongation. The time frame from explant in culture to collecting T_1 seed from a primary transformant ranges from 7 to 10 months under the conditions outlined in this study.

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