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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation

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Abstract The utility of transformation for soybean improvement requires an efficient system for production of stable transgenic lines. We describe here an improved cotyledonary node method using an alternative explant for *Agrobacterium tumefaciens*-mediated soybean transformation. We use the term "half-seed" to refer to this alternative cotyledonary explant that is derived from mature seed of soybean following an overnight imbibition and to distinguish it from cotyledonary node derived from 5–7-day-old seedlings. Transformation efficiencies using half-seed explants ranged between 1.4 and 8.7% with an overall efficiency of 3.8% based on the number of transformed events that have been confirmed in the T_1 generation by phenotypic assay using the herbicide Liberty® (active ingredient glufosinate) and by Southern analysis. This efficiency is 1.5-fold higher than the cotyledonary node method used in our laboratory. Significantly, the half-seed system is simple and does not require deliberate wounding of explants, which is a critical and technically demanding step in the cotyledonary node method.

Keywords *Agrobacterium tumefaciens* · Cotyledonary node · Glufosinate · Half-seed · Soybean · Transformation

Introduction

The utilization of genetic transformation techniques to introduce useful or novel gene(s) into soybean [*Glycine max* (L.) Merr.] requires an efficient method of transgene integration and regeneration of transformed plants. There are two modes of DNA delivery that are currently utilized by most researchers to transform soybean. One method utilizes particle bombardment of embryogenic tissue with DNA-coated carrier particles of inert materials (Hadi et al. 1996; Santarem and Finer 1999; Droste et al. 2002). This technique often requires a prolonged tissue culture period to prepare target tissue. The other method involves *Agrobacterium*-mediated transformation of plant tissue such as embryonic axis, immature cotyledons or cotyledonary tissue from germinated seedlings (Hinchee et al. 1988; Parrott et al. 1989; Somers et al. 2003; Paz et al. 2004). Parrott et al. (1989) used immature seeds to obtain cotyledonary tissues that were macerated on nylon or steel mesh, infected with *Agrobacterium* and placed on culture medium to generate somatic embryos. Earlier Hinchee et al. (1988) reported *Agrobacterium* infection of the cotyledonary node (CN) area to produce transgenic soybean. Improvements to this CN protocol have been actively pursued to increase efficiency (Clemente et al. 2000; Olhoft et al. 2001; Olhoft et al. 2003; Liu et al. 2004; Paz et al. 2004; Zeng et al. 2004). The CN system involves wounding of explants derived from 5–7-day-old seedlings by making accurate incisions on the adaxial side using a surgical blade. This wounding procedure requires precise cutting of the explant prior to infection. Discrepancies in transformation efficiency via the *Agrobacterium* method have been partially attributed to non-reproducibility of CN wounding procedures among operators (M. Paz, unpublished). Other soybean transformation approaches involving some forms of deliberate wounding on the explant have also been explored resulting in different transformation efficiencies. For example, sonication was used to assist *Agrobacterium*-mediated transformation (SAAT) of both CN explants (Meurer et al. 1998) and immature cotyledons (Santarem et al. 1998; Finer and Finer 2000). Biolistic treatment was used to wound embryogenic tissue derived from cotyledonary explants followed by *Agrobacterium* inoculation of the tissue (Droste et al. 2000). Explant wounding of excised embryonic axes from immature seeds using a multi-needle wounding prong (Ko et al. 2003) and cotyledons using forceps (Yan et al. 2000) have also been reported. Although some of these wounding treatments

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resulted in enhanced transient expression of marker genes, they did not imply the improvement of stable transgenic plant recovery, which has been corroborated using other plant systems (Wroblewski et al. 2005).

In this study we describe an alternative cotyledonary explant derived from mature soybean seed for *Agrobacterium*-mediated transformation. We use the term "half-seed" to refer to cotyledonary tissue obtained from mature seed after overnight imbibition to distinguish it from CN explants derived from 5–7-day-old seedlings. In contrast to the CN technique that requires making precise cuts on the explant for effective transformation and regeneration, the half-seed approach does not involve deliberate manual wounding. The objective of this study was to develop an efficient soybean transformation method using the half-seed explant as a target tissue and demonstrate its efficacy in producing transgenic plants.

Materials and methods

Seed materials

Mature soybean seeds of cultivar Thorne, Williams, Williams79 and Williams82, were utilized in *Agrobacterium*-mediated transformation experiments. Soybean seeds were surface sterilized for 16 h using chlorine gas produced by mixing 3.5 ml of 12 N HCl and 100 ml commercial bleach (5.25% sodium hypochlorite, Di et al. 1996) in a tightly sealed desiccator.

Transformation vector and *Agrobacterium* strain

The vectors pTF102 (Frame et al. 2002) and pTF101.1 (Paz et al. 2004) were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986). pTF102 was derived from base vector pTF101.1 by inserting a *Hind* III fragment containing a *gus*-intron cassette driven by a CaMV 35S promoter (Vancanneyt et al. 1990) into pTF101.1. The *gus*-intron cassette prevents background GUS activity derived from contaminating *Agrobacterium* in plant tissue culture. pTF101.1 has the following key elements: (1) double CaMV 35S promoter (Odell et al. 1985); (2) tobacco etch virus translational enhancer (Carrington and Freed 1990) at the 5′ end of the *bar* gene; (3) soybean vegetative storage protein terminator (Mason et al. 1993) cloned to the 3′ end of the *bar* gene; (4) a selectable marker gene (*bar* gene for resistance to herbicide phosphinothricin, White et al. 1990) for selection during plant transformation; and (5) a multiple cloning site (MCS) to facilitate subcloning of a gene of interest (GOI) between the T-DNA right border region and the *bar* gene. The base vector pTF101.1 is a derivative of the pPZP binary vector (Hajdukiewicz et al. 1994) and contains a selectable marker gene (*aad*A gene for resistance to antibiotics spectinomycin and streptomycin) for bacterium, origin of replication for *E. coli*, and right and left border fragments of *A. tumefaciens* T-DNA. Constructs ST42 and ST43 were derived from vector pTF101.1 by inserting a GOI in the MCS.

Agrobacterium culture and infection medium

Construct EHA101(pTF102, pTF101.1, ST42 or ST43) was grown on YEP (An et al. 1988) containing 50 mg l^{-1} kanamycin, 100 mg l^{-1} spectinomycin and 25 mg l^{-1} chloramphenicol at 28°C for 2 days. Single colonies of *Agrobacterium* were obtained from the plate and inoculated into 2-ml liquid YEP containing antibiotics (starter culture) for 8 h at 28°C, 250 rpm. Subsequently, 300 µl of the 2-ml starter culture was transferred to a 250-ml YEP culture, and grown overnight to $OD_{650} = 0.7$ to 1.0 at 28°C, 250 rpm using a shaker incubator. On the day of infection, a bacterial pellet was obtained by spinning the overnight culture at 3500 rpm for 10 min and resuspended in infection medium containing 1/10 Gamborg's B5 medium (Gamborg et al. 1968) with 7.5 µM 6-benzylaminopurine (BAP), 0.7 µM gibberellic acid (GA_3), 20 mM MES [2-(*N*-morpholino)ethanesulfonic acid], 3% sucrose and 200 µM acetosyringone, pH 5.4. Bacteria cell density was adjusted to $OD_{650} = 0.7$ to 0.8 using a spectrophotometer before infection of half-seed explants.

Explant preparation and infection

Disinfected seeds were soaked in sterile distilled water overnight for about 16 h (100 seeds in a 100×25 mm Petri dish). A longitudinal cut along the hilum was made to separate the cotyledons, and the seed coat was removed. The embryonic axis found at the junctions of the hypocotyl and the cotyledon was excised to obtain the half-seed explant (Fig. 1, *panel* a-1 & a-2). About 60–100 half-seed explants were immersed in a 100×20 Petri dish containing *Agrobacterium* suspension culture. Half-seed explants were inoculated with *Agrobacterium* for 30 min, at room temperature (RT). After inoculation, explants were placed adaxial side (flat side) down on cocultivation medium lined with filter paper (six explants per plate). The cocultivation medium contained 1/10 B5 salts, B5 vitamins, 7.5 µM BAP, 0.7 µM GA_3, 20 mM MES, 3% sucrose, 200 µM acetosyringone, 100–400 mg l^{-1} cysteine and 154 mg l^{-1} dithiothreitol, pH 5.4 with 0.425% Noble agar (Fisher Scientific, USA). Cocultivation was continued for 5 days at 24°C under 18 h photoperiod (140 µmoles s^{-1} m^{-2}).

Culture conditions and regeneration

After cocultivation half-seed explants were washed in liquid shoot induction (SI) medium (B5 salts, B5 vitamins, MSIII iron stock, 3% sucrose, 3 mM MES, 5.0 µM BAP, 50 mg l^{-1} timentin, 200 mg l^{-1} cefotaxime and 50 mg l^{-1} vancomycin, pH 5.7). The explants were subsequently cultured (flat side up) on SI medium solidified with 0.7% agar. The SI medium contained 0–6 mg l^{-1} glufosinate (glufosinate-ammonium Pestanal®, Sigma-Aldrich). The base of the explant (i.e., the part of the explant from where the embryonic axis was removed) was embedded in the medium. Shoot induction was carried out at 24°C with 18 h

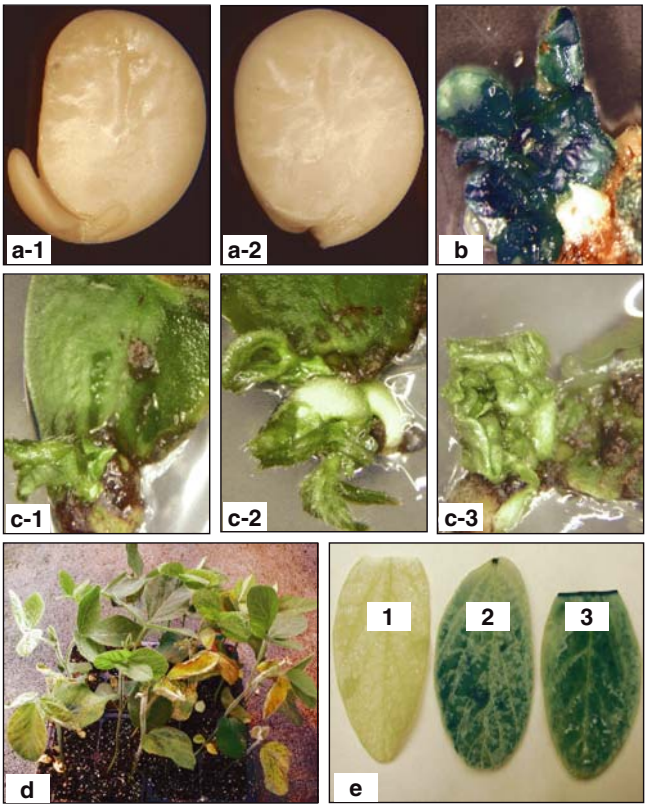

Fig. 1 *Agrobacterium*-mediated transformation of half-seed explants and regeneration. (**a**) Half seed explant, 1–with embryonic axis, 2–after embryonic axis was removed; (**b**) GUS positive shoots during regeneration stage; (**c**) shoot formation, c-1 minimal, c-2 moderate; c-3 good; (**d**) One week after Liberty® spraying T_1 plants were scored: green plants were herbicide resistant; yellow (dead) plants were sensitive; (**e**) GUS assay of T_1 plants prior to herbicide spraying showed 1-herbicide sensitive plant was negative, 2 and 3- resistant plants were GUS positive

photoperiod (140 μmoles s^{-1} m^{-2}) in a Percival Biological Incubator. After 14 days, explants were transferred to fresh SI medium containing 6 mg l^{-1} glufosinate for *bar* selection. After 4 weeks of culture on SI medium, explants were transferred to shoot elongation (SE) medium containing MS basal salts (Murashige and Skoog 1962), MSIII iron stock, 3% sucrose, 3 mM MES, B5 vitamins, 50 mg l^{-1} asparagine, 100 mg l^{-1} L-pyroglutamic acid, 0.1 mg l^{-1} IAA, 0.5 mg l^{-1} GA_3, 1 mg l^{-1} zeatin riboside, 50 mg l^{-1} timentin, 200 mg l^{-1} cefotaxime, 50 mg l^{-1} vancomycin, pH 5.7, and solidified with 0.7% agar. Selection during the shoot elongation stage was 6 mg l^{-1} glufosinate. Starting at 2 weeks after transfer of half-seed explants to SE medium, elongated shoots (>2.5 cm) were obtained. Each individual shoot was dipped in sterile IBA (1 mg l^{-1}) then transferred to rooting medium (MS salts, MSIII iron stock, B5 vitamins, 3 mM MES, 2% sucrose, pH 5.6 and 0.7% agar) without glufosinate. After 2 weeks, rooted plantlets were rinsed with water to wash off the agar medium and then, transplanted to soil (Redi-Earth Peat-Lite Mix, Scotts-Sierra Horticultural Products Company) in jiffy pots. The plants (T_0) were grown at 24°C, 18 h photoperiod for 1–2 weeks then, transferred to the greenhouse.

Phenotypic screening of transgenic plants

T_0 plants with at least two trifoliates were screened in the greenhouse using an herbicide paint assay to identify putative transformants that expressed the *bar* gene. The upper surface of a leaf was treated with 150 mg l^{-1} glufosinate (herbicide Liberty®, Bayer CropScience, USA) along the midrib using a cotton bud (Q-tip). The Liberty®-treated leaf tissue was scored for tolerance to glufosinate at 3–5 days after herbicide application. Glufosinate-resistant T_0 plants were grown in the greenhouse until maturity and seeds were harvested. Fourteen seeds from T_0 plants were sown together with seeds from a *bar* gene expressing transgenic line as positive control and non-transgenic soybean as negative control. The progeny tests were done by spraying 2-week-old seedlings with 250 mg l^{-1} Liberty® to confirm the presence of the *bar* transgene in T_1 progeny. When the T_0 plant produced fewer than 14 seeds, all available seeds were sown. T_1 plants were scored for resistance or sensitivity to Liberty® 1 week after spraying.

Molecular analysis

Southern blot analyses of T_0 and T_1 generations of transgenic events were carried out. Total genomic DNA was extracted using the CTAB method based on the method by Doyle and Doyle (1987). Ten micrograms of genomic DNA was digested with *Xho* I or *Hin*d III, the restriction products were separated on a 0.8% agarose gel and transferred to a Magnacharge nylon membrane (Osmonics Inc.). The membranes were hybridized with ^{32}P-labeled *gus* fragment for 18 h at 65°C. After hybridization, the membranes were washed in solutions of SDS (sodium dodecyl sulfate) detergent and SSC (sodium chloride and sodium citrate solution) to remove excess probe from the membranes. The Southern blots were then exposed to X-ray film (Fujifilm Medical Systems) at −80°C for 2–3 days and subsequently developed.

Statistical analysis

Completely random design (CRD) was used to analyze the effect of BAP (cytokinin treatments) on regeneration. Data were scored using nonparametric values based on shoot formation. Each soybean explant (half-seed) was considered as an experimental unit and eight levels of BAP were tested. Regeneration data were analyzed with ANOVA and Duncan's multiple range test (p=0.05) using the Statistical Analysis System package (SAS Institute v. 9.1).

Results and discussion

Transformation efficiency using half-seed explants

Primary regenerants (T_0 plants) produced from *in vitro* culture of half-seed explants infected with *Agrobacterium*

Table 1 Efficiency of *Agrobacterium*-mediated transformation using half-seed explants of cultivar Thorne and vector pTF102

Experiment no	No. of explants infected	No. of glufosinate-resistant events in T_1 generation[a]	Final transformation efficiency (%)[b]
1	65	3	4.6
2	115	10	8.7
3	228	11	4.8
4	250	8	3.2
5	210	3	1.4
6	100	4	4.0
7	151	4	2.6
8	60	2	3.3
		Mean transformation efficiency	4.1
		Standard error	0.8

[a]Resistant to 250 mg l^{-1} Liberty®

[b]Final transformation efficiency = (No. of glufosinate-resistant T_1 events/No. of explants infected) × 100

and selected on medium containing 6 mg l^{-1} glufosinate, were screened by leaf painting with Liberty® herbicide (active ingredient glufosinate). Subsequent evaluation of T_1 progeny by spraying with 250 mg l^{-1} Liberty® was also carried out. Final transformation efficiency based on the number of Liberty®-resistant events in the T_1 generation per number of explants infected ranged between 1.4 and 8.7% (Table 1). Forty-five independent transformation events were confirmed based on their resistance to the herbicide, giving an overall efficiency of 3.8% (45/1179). Chi-square analysis for goodness of fit for a 3:1 segregation ratio of glufosinate resistance was carried out for 30 of the 45 events. Segregation ratios showed the expected Mendelian inheritance in the T_1 progeny consistent for segregation of a single locus conferring glufosinate resistance in 26 of 30 events while four events indicated abnormal segregation ratios (data not shown). Deviation from the Mendelian segregation of 3:1 could be due to multiple loci, or due to chimerism of the T_0 transformant. The generation of chimeric soybean plants by *Agrobacterium*-mediated transformation has been reported previously by Clemente et al. (2000) and Olhoft et al. (2003).

Transgene inheritance was verified in the T_1 generation using a histochemical GUS assay (Fig. 1e) and by molecular analysis. Southern blot analysis of Liberty®-resistant T_1 plants was carried out for 32 of 45 events. One glufosinate-resistant T_1 plant was randomly selected from each event and leaf tissues were collected for DNA extraction. Total genomic DNA was digested with *Xho* I and the Southern blot was hybridized with a *gus* probe. Since there is a single restriction site of *Xho* I in the T-DNA region of pTF102 vector (Fig. 2a), the presence of one hybridization band suggests a single transgene integration site while multiple bands suggest multiple integration loci. All 32 plants analyzed contained DNA fragments that hybridized to *gus,* confirming meiotic transmission of the transgene (Fig. 2b). The majority of these events (20/32) had a single copy of the transgene based on the presence of a single *gus* band. Several plants showed more than one hybridization band which may indicate multiple transgene integrations and could reflect the approximate transgene copy number. Southern blot analysis was also carried out using *Hin*d III digestion of total genomic DNA in additional progeny of events pTF102-56, -62 and -105, and all genotypes exhibited the presence of the *gus* fragment after hybridizing with *gus* probe (Fig. 2c).

Tissue culture condition and cultivar comparison

Alternative parameters tested in the half-seed treatment

Explants were prepared by splitting the seed and removing the embryonic axis, causing a break on the cotyledonary tissue. This break may provide an entry point for *Agrobacterium* during infection. In an effort to enhance *Agrobacterium* infection of half-seed explants in the absence of making incisions on the tissue, we examined the effect of a sucrose plasmolyzing pretreatment on transformation efficiency. Plasmolysis of plant cells has been employed to promote gene transfer via electroporation (Wu and Feng 1999; Koscianska and Wypijewski 2001). In our study, half-seed explants were placed in 1/10 MS liquid medium (Murashige and Skoog 1962) with 1.0 M sucrose (from this point forward 1/10 MS liquid medium with 1.0 M sucrose will be referred to as 1.0 M sucrose) for varying periods of pretreatment (0, 30, or 60 min at RT) prior to infection with *Agrobacterium*.

Our results show that 1.0 M sucrose had negative effect on transformation. Final transformation efficiency based on the number of glufosinate-resistant T_1 plants per number of explants infected, decreased from 7.0% to from 0.6 to 2.4% following sucrose pretreatment (Table 2). Moreover, shoot regeneration declined following sucrose pretreatment. Regeneration frequency, which was based on the number of explants producing shoots per number of explants infected, decreased from 59% in the absence of sucrose pretreatment, to 45 and 31% following 30 and 60 min pretreatments, respectively (Table 2). These results suggest that sucrose pretreatment may have caused permanent injury to plant cells that reduced their susceptibility to *Agrobacterium* or that may have reduced the ability of plant cells to attain their organogenic potential. We also examined the effect of vacuum infiltration on T-DNA delivery and shoot regeneration. Vacuum (24 in of Hg) was applied for 15–45 min during *Agrobacterium* infection of half-seed explants after which explants were placed on cocultivation medium for 5

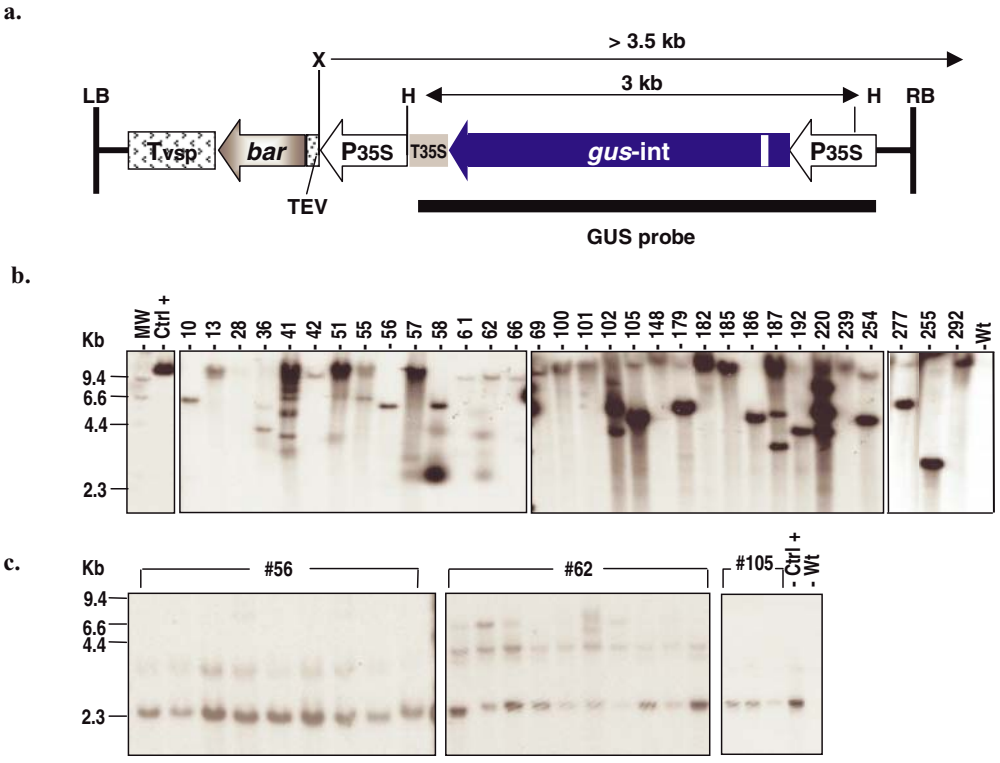

Fig. 2 pTF102 vector and Southern analysis of pTF102 transformants (T_1 progeny). (**a**) T-DNA region of binary vector pTF102. LB, *left border;* RB, *right border;* Tvsp, soybean vegetative storage protein terminator; *bar*, phosphinothricin acetyltransferase gene; TEV, tobacco etch virus translational enhancer; P35S, CaMV 35S promoter; T35S, CaMV 35S terminator; *gus*-int, β-glucuronidase gene containing an intron. H, *Hin*d III; X, *Xho* I. Ten micrograms of genomic DNA were digested with (**b**) *Xho* I enzyme that cuts once in the T-DNA region of the plasmid, or (**c**) *Hin*d III that cuts at two restriction sites in pTF102 to generate a drop out fragment. A DNA fragment from the *gus* gene was used as a probe. Ctrl + is a positive control in which 10 μg of non-transgenic soybean genomic DNA spiked with 63 pg of pTF102 plasmid DNA was loaded. Wt is a non-transformed Thorne plant. T_1 refers to the second generation of transgenic soybean plants

days. The number of explants expressing GUS transient activity (blue spots) declined when vacuum was applied. No regeneration from vacuum-treated explants was obtained. Overall, these results demonstrate that neither sucrose pretreatment nor vacuum infiltration increases transformation frequencies from *Agrobacterium*-mediated transformation of half-seed explants.

Cultivar response

Half-seed explants of Thorne, Williams, Williams79 and Williams82 were infected with *Agrobacterium* and cultured on selective regeneration medium containing 6 mg l^{-1} glufosinate. Transformed T_0 events showing resistance to Liberty® were obtained from all four cultivars (Table 3). Transgenic events of Thorne, Williams, Williams79 and Williams82 have been confirmed in the T_1 generation after Liberty spraying (data not shown). Our results demonstrate the applicability of the half-seed technique to several soybean cultivars.

Effect of BAP on regeneration

We examined the effect of varying levels of BAP present in the SI medium on shoot regeneration. Thorne half-seed explants were infected with EHA101(pTF102) and were

Table 2 Transformation efficiency and shoot regeneration on half-seed explants of Thorne transformed with pTF102 at different durations of sucrose pretreatment before infection

Duration of sucrose pretreatment (min)	No. of explants infected	Regeneration frequency (%)[a]	No. of glufosinate® resistant T_1 events	Final transformation efficiency (%)[b]
0	302	59.3	21	7.0
30	293	45.1	7	2.4
60	170	31.2	1	0.6

[a]Regeneration frequency = (No. of explants with one or more shoots/No. of explants infected)×100

[b]Final transformation efficiency = (No. of glufosinate®-resistant T_1 events/No. of explants infected)×100

Data were based on five experiments. T_1 refers to the second generation of transgenic soybean plants

Table 3 Shoot regeneration and transformation efficiency in T_0 generation of different soybean cultivars transformed with *Agrobacterium*

Cultivar	No. of explants infected	Regeneration frequency (%)[a]	No. of glufosinate-resistant T_0 plants	Transformation efficiency (%)[b]
Thorne	413	60	4	1.0
Williams	357	46	13	3.6
Williams79	390	37	7	1.8
Williams82	375	56	17	4.5

[a]Regeneration frequency = (No. of explants with one or more shoots/No. of explants infected)×100
[b]Transformation efficiency = (No. of glufosinate-resistant T_0 events/No. of explants infected)×100
Constructs ST43 and pTF102 were used; data based on six experiments
T_0 refers to the first generation of transgenic soybean plants

cultured on SI medium without BAP or with different BAP concentrations (2.5–100 μM). Use of 5 μM BAP significantly increased the regeneration frequency (number of explants forming shoots per number of explants infected×100) (P=0.05; Table 4). Similar observations were reported by Dan and Reichert (1998) and Saka et al. (1980) where use of 5.0 μM BAP improved regeneration of hypocotyl explants and stem node segments of soybean, respectively. Wright et al. (1986) and Hinchee et al. (1988) also obtained shoot regeneration on cotyledonary explants at 5 μM BAP. Use of 7.5 μM BAP (as routinely used in the CN protocol; Olhoft et al. 2003; Paz et al. 2004) gave the lowest regeneration rate (29%) when applied to the half-seed system. This observation suggests that culture conditions optimized in the CN system were not immediately applicable to the half-seed protocol. By providing an optimum hormonal environment to the explant *in vitro*, the ability of transformed cells to regenerate into plants may be improved.

In our study the capacity of half-seed explants for shoot induction was based on a visual evaluation of the number of shoots formed (i.e., degree of shoot formation: 0 – no shoot; 1 – minimal, Fig. 1c-1; 2 – moderate, Fig. 1c– 2; 3 – good, Fig. 1c-3). The mean degree of shoot formation was calculated using all explants with or without shoots. Because regeneration frequencies were generally low, a lot of the explants were given a score of "0" and this resulted in the mean degree of shoot formation being low for all treatments. When only explants with shoots were scored, the mean degree of shoot formation ranged from 1.8 to 2.8 (data not shown). Shoot formation was enhanced by treatment with BAP (except 7.5 μM treatment) (Table 4). Buising et al. (1994) observed a similar trend on embryonic axes of soybean where the number of shoots per axis increased with higher BAP level. A high concentration of BAP disrupts DNA replication and initially slows down cell division resulting in a break down of apical dominance (Wright et al. 1986) thereby triggering profuse shoot formation.

Half-seed versus cotyledonary node

To compare transformation efficiencies using the half-seed or CN protocols, four independent experiments using different genotypes and constructs were carried out (Table 5). In experiment 1, using pTF102 and cultivar Thorne, a 5.7% transformation rate was obtained using half-seed explants while the CN method did not produce any transformed plants. A similar trend was obtained in experiment 2 using vector pTF102 and cultivar Williams82 wherein the half-seed method generated transgenic events (2.5% transformation efficiency) while the CN protocol produced no transgenic events. In experiment 3, transgenic plants of Williams were generated from both half-seed (5.6%) and CN (5.3%) explants using vector pTF101.1. When

Table 4 Shoot formation on half-seed explants of Thorne transformed with pTF102 at different levels of BAP present in shoot induction medium

BAP concentration (μM)	No. of explants infected	Regeneration frequency (%)*	Mean degree of shoot formation#
0	113	47^{bc}	0.58^{bc}
2.5	55	52^{bc}	0.74^{b}
5	111	80^{a}	1.06^{a}
7.5	102	29^{c}	0.37^{c}
15	111	65^{ab}	1.03^{a}
30	108	32^{c}	0.60^{bc}
50	105	32^{c}	0.61^{bc}
100	109	59^{ab}	1.06^{a}

*Regeneration frequency = (No. of explants with one or more shoots/No. of explants infected)×100
#Degree of shoot formation: 0 – No. of shoot, 1 – minimal (Fig. 1c-1), 2 – moderate (Fig. 1c-2), 3 – good (Fig. 1c-3)
Regeneration frequency or mean degree of shoot formation not followed by the same letter differ significantly at the 5% level

Table 5 Transformation efficiency using half-seed or cotyledonary mode explants of soybean that were transformed with *Agrobacterium*

Experiment no	Construct	Cultivar	Type of explant	No. of explants infected	No. of glufosinate-resistant events in T_0 generation[a]	No. of glufosinate-resistant events in T_1 generation[b]	Final transformation efficiency (%)[c]
1	pTF102	Thorne	CN	70	0	0	0
			Half-seed	70	4	4	5.7
2	pTF102	Williams82	CN	80	5	0	0
			Half-seed	119	4	3	2.5
3	pTF101.1	Williams	CN	150	15	8	5.3
			Half-seed	89	5	5	5.6
4	ST42	Williams	CN	150	2	1	0.7
			Half-seed	98	3	1	1

[a]Resistant to 150 mg l^{-1} Liberty®

[b]Resistant to 250 mg l^{-1} Liberty®

[c]Final transformation efficiency = (No. of glufosinate-resistant T_1 events/No. of explants infected) × 100

T_0 and T_1 refere to the first and second generation of transgenic soybean plants, respectivey

construct ST42 and Williams were used in experiment 4 transformation efficiencies were 1.0 and 0.7% utilizing half-seed and CN, respectively. Overall, more consistent transgenic plant recovery was obtained using the half-seed protocol. We also observed fewer escapes among putative transformants derived from half-seed explants in experiments 2 and 3. For example, in experiment 3 all 5 putative T_0 events derived from half-seeds were confirmed in the T_1 generation. Meanwhile, eight transgenics were identified out of 15 putative events generated by the CN protocol. These results suggest that *bar* selection is more effective with the half-seed than the CN protocol. A reduced number of escapes, i.e. non-transgenic shoots that survived glufosinate selection *in vitro* and T_0 Liberty® painting, among half-seed regenerants contributed to an efficient transformation system and efficient use of laboratory resources.

It is not clear why the half-seed and CN methods produced different results in recovering transgenic soybean plants. One possible explanation might be a more effective selection for the *bar* gene in half-seed than CN due to the differences in the age of explants (i.e., cotyledonary tissue from mature half-seed versus CN tissue from 5 to 7-day-old germinating seedlings). Another explanation might be the wounding effect. Precise wounding at the CN area of 5–7-day-old germinating seedlings was believed to facilitate *Agrobacterium* infection by (1) providing entry site for *Agrobacterium* and (2) destroying primary shoots and promoting secondary shoot formation (Townsend and Thomas 1993; Meurer et al. 1998). This wounding on the CN tissues, however, may also trigger plant defense mechanisms such as the production of polyphenol oxidases that results in browning of wounded tissue, which may weaken the *Agrobacterium*-mediated transformation process (Olhoft et al. 2001). Since deliberate wounding was not performed on half-seed explants, it was possible that there were less plant oxidases produced, thereby improving the interaction between *Agrobacterium* and plants cells. Olhoft et al. (2001) described the positive effect of thiol compounds as protective agents against oxidases produced by wounding of CN explants. In our study, we did not observe any significant difference on stable GUS expression in shoots regenerated from half-seeds cocultivated in the presence or absence of cysteine or DTT, i.e. GUS blue shoots were obtained with or without cysteine or DTT (M. Paz, unpublished).

In this study we describe an improved cotyledonary node transformation method using a half-seed explant derived from mature soybean seeds. We demonstrate that it is a robust system when the *bar* gene/bialaphos selection regime is used. Further experiments are needed to investigate the application of this method when using different selectable markers.

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