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Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant

Margie M. Paz[1], Huixia Shou[1,2], Zibiao Guo[1,3], Zhanyuan Zhang[1,4], Anjan K. Banerjee[1,5] & Kan Wang[1,*]

[1]*Plant Transformation Facility, Department of Agronomy, Iowa State University, Ames, IA 50011–1010, U.S.A.;* [2]*College of Life Science, Zhejiang University, Hangzhou, 310029, China;* [3]*Plant Biology Division, The Samuel Roberts Noble Foundation, Inc., 2510 Sam Noble Parkway, Ardmore, OK 73401, U.S.A.;* [4]*Plant Transformation Core Facility, University of Missouri-Columbia, MO 65211, U.S.A.;* [5]*Department of Horticulture, Iowa State University, Ames, IA 50011-1100, U.S.A.; (*author for correspondence; E-mail: kanwang@iastate.edu)*

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Summary

Conditions affecting *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merr.], including seed vigor of explant source, selection system, and cocultivation conditions, were investigated. A negative correlation between seed sterilization duration and seed vigor, and a positive correlation between seed vigor and regenerability of explants were observed in the study, suggesting that use of high vigor seed and minimum seed sterilization duration can further improve transformation efficiency. Selection schemes using glufosinate or bialaphos as selective agents *in vitro* were assessed. Glufosinate selection enhanced soybean transformation as compared to bialaphos. The use of 6 mg L^{-1} glufosinate during shoot induction and shoot elongation stages yielded higher final transformation efficiency ranging from 2.0% to 6.3% while bialaphos at 4 to 8 mg L^{-1} gave 0% to 2.1% efficiency. Including cysteine and DTT during cocultivation increased the transformation efficiency from 0.2–0.9% to 0.6–2.9%. This treatment also improved T-DNA transfer as indicated by enhanced transient GUS expression. Shoot regeneration and *Agrobacterium* infection were attained in twelve soybean cultivars belonging to maturity groups I-VI. These cultivars may be amenable to genetic transformation and may provide a valuable tool in soybean improvement programs.

Introduction

Plant genetic transformation permits the direct introduction of agronomically useful gene(s) into important crops and offers a significant tool in breeding programs by producing novel and genetically diverse plant materials. It helps alleviate any restriction in traditional crop breeding due to limited availability of germplasm to generate genetic diversity. The natural mechanism of *Agrobacterium*-mediated gene transfer has been widely utilized to genetically modify monocots and dicots (Hansen and Wright, 1999; Fukuoka et al., 2000). *Agrobacterium tumefaciens* is a soil-borne pathogen that has the capacity to infect plants and transfer a segment of DNA (T-DNA) from its tumor-inducing (Ti) plasmid to the plant cell. The T-DNA then integrates into the plant nuclear genome where the genes on the T-DNA are expressed. *A. tumefaciens* has a wide host range although some economically important crops remain recalcitrant (De Cleene & De Ley, 1976; Komari & Kubo, 1999). Advances in transformation techniques have resulted in high transformation frequencies in maize and rice ranging from 5% to 30% (Hiei et al., 1994; Ishida et al., 1996; Zhao et al., 2001; Frame et al., 2002). Advancement in soybean transformation appears to be slow compared to some of the recent improvement in cereal transformation. Reported transformation efficiencies are generally low, and like other recalcitrant crops, the

protocols are often genotype specific (Widholm, 1995; Somers et al., 2003). Increased soybean transformation efficiency, may be achieved by further optimizing the selection system, enhancing explant-pathogen interaction and improving culture conditions to promote regeneration and recovery of transformed plants.

Two major methods have been successful in soybean transformation. The first method utilizes particle bombardment of embryogenic tissue (Trick & Finer, 1998; Maughan et al., 1999; Santarem & Finer, 1999, Droste et al., 2002) and the second method involves *Agrobacterium*-mediated transformation of cotyledonary node tissues (Zhang et al., 1999; Clemente et al., 2000; Olhoft & Somers, 2001; Olhoft et al., 2001). The two methods are distinct, and each has certain advantages and disadvantages. Transformation of embryogenic tissue by particle bombardment requires a prolonged tissue culture period, often yields complex insertion patterns of transgenes into the plant genome, and may result in the regeneration of sterile plants (Liu et al., 1996; Singh et al., 1998; Reddy et al., 2003). On the other hand, *Agrobacterium*-mediated transformation of cotyledonary node tissue utilizes explants derived from 5-day-old seedlings thereby eliminating extensive tissue culture procedures before transformation. However, the infection and regeneration of cotyledonary node tissue of soybean is cultivar dependent (Meurer et al., 1998). This method also requires a properly trained worker who is experienced specifically in making accurate incisions on the explant. The non-reproducibility of the procedures in *Agrobacterium*-mediated transformation of cotyledonary node has been partially attributed to the variation of different operators during the explant preparation (T. Clemente, personal communication).

The development of an effective *Agrobacterium* transformation method for soybean depends on several factors including plant genotype, explant vigor, *Agrobacterium* strain, vector, selection system, and culture conditions (Santarem et al., 1998; Zhang et al., 2000). It has been reported that soybean genotype contributed to variation in susceptibility to *Agrobacterium* and regenerability in tissue culture (Meurer et al., 1998; Donaldson & Simmonds, 2000). In addition, surface sterilization of plant tissue material for *in vitro* tissue culture and transformation is one of the critical steps in carrying out transformation experiments. While a short time of sterilization cannot completely decontaminate explants, prolonged sterilization may cause damage to explants and consequently affect their regenerability (Maruyama et al., 1989).

The *bar* gene encoding a phosphinothricin acetyltransferase enzyme has been utilized considerably as a selectable marker in plant transformation. Two selective agents that have been used for *bar* selection include glufosinate (ammonium salt of phosphinothricin [PPT]) which is the main active component of the herbicide BASTA (BAYER, Germany) and bialaphos (tripeptide-L-phosphinothricyl-L-alanyl-L-alanine) (Tachibana et al., 1986; Thompson et al., 1987). Although they both contain PPT as the base molecule, their chemical structures are different. This difference may affect their efficacy in selecting transformants. It was observed that the effectiveness of these two compounds in selecting maize transformants varied (Dennehey et al., 1994; Wang et al., 2003). In soybean, Zhang et al. (1999) used glufosinate for selection of transformed cultivar Asgrow 3237. The authors reported 0% to 3.0% efficiency based on GUS activity of primary transformants derived from cotyledonary nodes.

Antioxidant reagents such as cysteine, dithiothreitol, ascorbic acid and polyvinyl pyrrolidone have been used in plant transformation optimization to enhance either tissue culture response or transformation efficiency (Perl et al., 1996; Enriquez-Obregon et al., 1999; Frame et al., 2002). Recently, high transformation efficiency was also reported in soybean by adding cysteine and thiol compounds to the cocultivation media (Olhoft & Somers, 2001; Olhoft et al., 2001; Olhoft et al., 2003).

This study assessed the conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. Here we designed experiments to (1) determine the effect of sterilization time on seed vigor and on regenerability of explants, (2) investigate the use of bialaphos or glufosinate as selection agent and to determine an efficient selection scheme for *Agrobacterium*-mediated transformation of soybean cotyledonary nodes, and (3) assess the effect of cysteine and dithiothreitol (DTT) on *Agrobacterium*-mediated transformation in twelve soybean cultivars using different constructs derived from the base vector pTF101.1.

Materials and methods

Plant material

Twelve soybean genotypes were utilized in *Agrobacterium*-mediated transformation experiments. They are

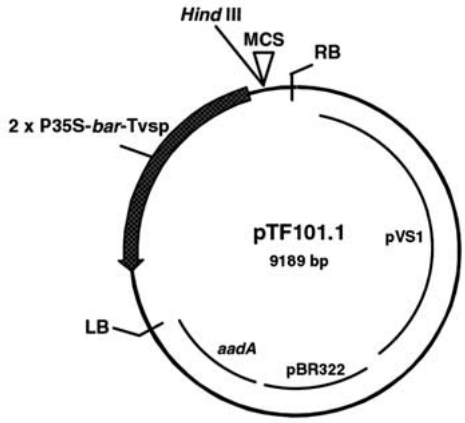

Figure 1. Map of base vector pTF101.1. 2X P35S, double CaMV 35S promoter; *bar,* encoding phosphinothricin acetyltransferase gene; Tvsp, soybean vegetative storage protein terminator; *aadA* gene, encoding aminoglycoside 3' adenyltransferase for resistance to antibiotics spectinomycin and streptomycin; pVS1, origin of replication for *Agrobacterium*; pBR322, origin of replication for *E. coli*; LB – left border; RB – right border; MCS – multiple cloning site. *Hind* III is one of the unique restriction enzyme sites in the MCS of the plasmid.

from six different maturity groups (MG): Bert (MG I), Harosoy, Jack, and Peking (MG II), Thorne, Williams, Williams79, and Williams82 (MG III), Clark (MG IV), Delsoy 5710 and Essex (MG V), and Ogden (MG VI).

The two batches of soybean seeds cv Jack used for the seed vigor and regenerability study were produced in the 1997 field [Plant Transformation Facility (PTF), Iowa] or obtained from Illinois Foundation Seed in 1998 (Illinois).

Transformation vector and Agrobacterium *strain*

Constructs (pTF102, ST14, ST15, ST17, ST18, ST19, ST20 or ST22), all derivatives of base vector pTF101.1, were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986). The base vector pTF101.1 (Figure 1) is a derivative of the pPZP binary vector (Hajdukiewicz et al., 1994) that includes the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens,* a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection. The plant selectable marker gene cassette consists of (1) a double 35S promoter (2x P35S) of the cauliflower mosaic virus (CaMV) (Odell et al., 1985), (2) the tobacco etch virus translational enhancer (Carrington & Freed, 1990), (3) the phosphinothricin acetyl transferase (*bar)* gene from *Streptomyces hygroscopicus* that confers resistance to herbicide phosphinothricin and its derivatives (White et al., 1990) and (4) the soybean vegetative storage protein terminator (Mason et al., 1993). pTF101.1 also contains a multiple cloning site (MCS) to facilitate subcloning of a gene of interest in between the right border region and the plant selectable marker cassette. The MCS includes single restriction sites such as *Bam*H I, *Eco*R I, *Hin*d III, *Sac* I, *Sma* I, and *Xba* I.

The vector pTF102 (Frame et al., 2002) was derived from pTF101.1 by inserting the P35S GUS intron cassette (Vancanneyt et al., 1990) into the *Hin*d III site of pTF101.1. The presence of an intron in the coding region of the *gus* gene ensures no transgene expression in *Agrobacterium,* thereby avoiding mis-interpretation of GUS assay results. All the other transformation constructs (ST14 through ST22) were derived by inserting a gene of interest in the multiple cloning site of pTF101.1.

The pTF101.1 derived vector systems were maintained on YEP medium (An et al., 1988) containing 100 mg L^{-1} spectinomycin (for pTF101.1 or its derivatives) and 50 mg L^{-1} kanamycin (for pEHA101, a disarmed Ti plasmid, Hood et al., 1986). In every experiment, bacteria cultures used for infection of explants were initiated from plates grown at 28 °C for 2 days. Culture plates of the bacteria were stored for up to one month at 4 °C before being refreshed from long-term, – 80 °C glycerol stocks. Overnight cultures of *Agrobacterium* EHA101 were grown to OD_{650} = 1.0–1.2 at 28 °C, 250 rpm using a shaker incubator (VWR Scientific Products, New Jersey, USA). A bacterial pellet was obtained by spinning the overnight culture at 1500 *g* for 10 min. The pellet was resuspended in 1/10 Gamborg's B5 medium (Gamborg et al., 1968) with 1.67 mg L^{-1} BAP, 0.25 mg $L^{-1}GA_3$, 3% sucrose and 200 μm acetosyringone, pH 5.4. In all experiments, bacteria cell densities were adjusted to OD_{650} = 0.6 to 1.0 using a spectrophotometer prior to infection of cotyledonary nodes.

Seed sterilization and germination

Soybean seeds were surface sterilized for 16 hr or durations specified using chlorine gas produced by mixing 3.5 ml of 12 N HCl with 100 ml commercial bleach (5.25% sodium hypochlorite, Di et al., 1996). Sterilized seeds were germinated on B5 medium with Ferrous-NaEDTA and 2% sucrose, pH 5.8. Fifteen soybean seeds were germinated in a 100×25 mm petri

dish with the hilum proximal to the media. Five dishes were stacked in a plastic bag with four 1-inch slits for aeration. The plastic bag was tied tightly to prevent the lids from opening as the seedlings grow thus, avoiding contamination. Germination plates were incubated in a Percival Biological Incubator under 18-hr photoperiod at 140 μmoles sec^{-1} m^{-2} light intensity (24 °C).

Seed vigor test

Soybean seed vigor was monitored using the standard accelerated aging (AA) test. This test subjects unimbibed seeds to conditions of high temperature and high relative humidity for short periods. The seeds are then removed from the stress conditions and placed under optimum germination conditions.

Forty-two grams of soybean seeds from each sample were weighed and placed on a screening tray which was inserted into an inner aging chamber (Hoffman, Albany, OR, USA) containing 50 ml of water. The inner chamber was then placed into an incubator at 42 °C for three days. AA treated soybean seeds were used for a standard germination test as described in the International Rules for Seed Testing (ISTA, 1999). Briefly, 100 soybean seeds from each treatment were planted in a sand bed on a plate. Plates were put in a germination cart and incubated at 30 °C in the dark. Seven days later, the number of germinating seedlings was scored. Germination rate was calculated as the number of germinating seeds divided by total number of testing seeds multiplied by 100. The experiment was repeated four times.

Infection of explant and culture conditions

Cotyledonary node explants were prepared from 5–6 day old seedlings and infected with *Agrobacterium* as described by Zhang et al. (1999) with modifications. Briefly, cotyledons were excised at the junction of the cotyledons and the hypocotyl (i.e., approximately 5 mm below the cotyledonary node) and a longitudinal cut was made to separate the two cotyledonary explants. Wounding of the explant was achieved by excising the primary shoot and by making 8–10 slices on the cotelydonary node. Explants were inoculated with *Agrobacterium* for 30 min and then co-cultured on resuspension medium, as described above, solidified with 0.5% agar with or without 3.3 mM cysteine and 1 mM dithiothreitol (DTT). Five explants were cultured per 100×15 mm petri dish and the explants were positioned with the adaxial side (upper surface of the cotyledon) on a filter paper laid over the cocultivation media. The cocultivation plates were incubated in the light or dark for 3 or 5 days, respectively, at 24 °C. After cocultivation the explants were washed in liquid shoot induction medium containing B5 salts, Ferrous-NaEDTA, 3% sucrose, 3 mM MES, pH 5.7 and filter-sterilized. B5 vitamins, 1.67 mg L^{-1} BAP, 50 mg L^{-1} timentin (SmithKline Beecham Pharmaceuticals, Pennsylvania, USA), 150–200 mg L^{-1} cefotaxime (Aventis Pharmaceuticals, New Jersey, USA) and 50 mg L^{-1} vancomycin (Abbott Lab., Illinois, USA) were added after autoclaving. The explants were cultured on shoot induction medium (same as above but with 0.8% agar) for 4 weeks with the hypocotyl embedded in the medium and the cotyledonary node region facing upwards. Explants were subcultured to fresh medium after 2 weeks. Big shoots that may have developed from primary shoot were cut and discarded. Following 4 weeks of culture on shoot induction medium, explants were transferred to shoot elongation medium [MS basal salts (Murashige & Skoog, 1962), Ferrous-NaEDTA, 3% sucrose, 3 mM MES, B5 vitamins, 50 mg L^{-1} asparagine, 50 mg L^{-1} glutamine, 0.1 mg L^{-1} IAA, 0.5 mg L^{-1} GA_3, 1 mg L^{-1} zeatin riboside, 50 mg L^{-1} timentin, 150–200 mg L^{-1} cefotaxime and 50 mg L^{-1} vancomycin, pH 5.7] solidified with 0.8% agar. Culture conditions during shoot induction and elongation stages included an 18-hr photoperiod at 140 μmoles sec^{-1} m^{-2} light intensity (24 °C). For herbicide selection, glufosinate-ammonium Pestanal® (Sigma-Aldrich, Missouri, USA) at 3.0 to 6.0 mg L^{-1}, or bialaphos (Shinyo Sangyo Co., Ltd., Japan) at concentrations from 4.0 to 8.0 mg L^{-1} were used. Subculture to fresh medium was done every two weeks. Elongated shoots that were at least 2 cm high were transferred to rooting medium comprised of MS salts, Ferrous-NaEDTA, B5 vitamins, 3 mM MES, 2% sucrose and 0.8% agar, without phytohormone. The rooted plants (R_0 plants) were transferred to soil.

Screening of R_0 plants and progeny testing

One to two weeks after transfer to soil, R_0 plants with 2 trifoliates were screened for putative transformants that expressed the *bar* gene. The upper surface of a leaf was painted with 150 mg L^{-1} glufosinate along the midrib using a cotton bud (Q-tip). To make 150 mg L^{-1} glufosinate, 37.5 μl of Liberty® (concentration of active ingredient glufosinate was 200 mg/ml; AgrEvo, USA) was added to 50 ml of H_2O plus 50 μl Tween 20. Plants were scored based

on the tolerance of the leaf tissue at 3 to 5 days after painting. Glufosinate-resistant R_0 plants were grown in the greenhouse until maturity and seeds were harvested. Subsequently, seeds from R_0 plants were sown and progeny test was done to confirm the presence of the transgene by spraying 2-week old seedlings with 200 mg L^{-1} glufosinate. Plants were scored 1 week after spraying. Southern blot analysis of R_0 and R_1 lines was done based on the method of Sandhu et al. (2001). Ten micrograms of genomic DNA was digested with the appropriate restriction enzyme (e.g. *Hind* III and *Stu* I for construct pTF102 and ST14, respectively) separated on a 0.8% agarose gel and hybridized with the ^{32}P-labeled *bar* fragment or gene of interest (GOI) fragment.

Transient GUS assay of cotyledonary nodes

A histochemical β-glucuronidase assay (GUS assay) was done on cotyledonary nodes after 5 days of co-cultivation to verify T-DNA transfer (Jefferson et al., 1987; Meuer et al., 1998) by incubating them with a 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37 °C. After staining, the explants were destained with 70% ethanol to improve contrast. The level of GUS activity was visually assessed based on the intensity of blue-staining areas.

Results and discussion

Effect of seed vigor on the regeneration rate of cotyledonary nodes

Both dry (chlorine gas) and wet (20% commercial bleach with 1.05% active ingredient, sodium hypochlorite) methods have been used for soybean seed surface sterilization (Lu et al., 1994; Di et al., 1996). Advantages of dry sterilization include low labor input and the convenience in storage of sterilized seeds. While the dry sterilization method is effective for most mature seeds produced from greenhouse and field, there are times when this method requires extra sterilization time to achieve complete decontamination. However, prolonged exposure of soybean seed to chlorine gas may cause adverse effects on seed quality that result in poor germination and regeneration. Here, we conducted experiments to measure the effect of sterilization duration on soybean seed vigor and its correlation with plant regeneration.

Jack seeds produced in the PTF field (1997) and Illinois Foundation Seed (1998) were measured for

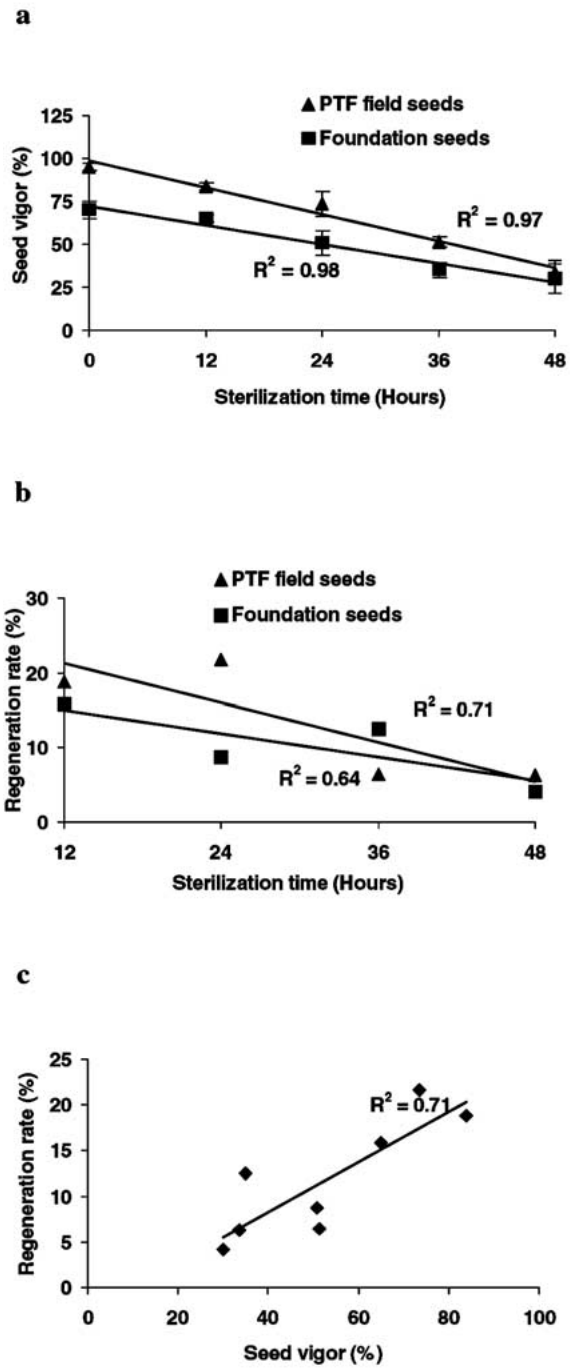

Figure 2. Effect of seed sterilization time on seed vigor and regenerability of soybean cotyledonary nodes. (a) Effect of seed sterilization time on seed vigor. (b) Effect of seed sterilization time on regenerability of explants. (c) Correlation between seed vigor and regeneration rate of cotyledonary nodes after 4 weeks on shoot induction medium. Seed vigor was measured using the accelerated aging test.

seed vigor. While a regular germination test conducted in favorable conditions determines the germinability of seeds, a seed vigor test carried out under stress condition provides information about how strong these seeds are (Delouche & Baskin, 1973). In this experiment, the accelerated aging (AA) test was used to measure the vigor of soybean seeds after sterilization for 0, 12, 24, 36, and 48 hours with chlorine gas. The higher the seed vigor, the less damage will be caused by the AA treatment. Results showed that the PTF field seeds were more vigorous than the Illinois Foundation seeds tested. As can be seen from Figure 2a, the seed vigor of PTF seed was near 95%, while the Foundation seed vigor was about 70% before sterilization. However, both batches of seeds had a 97% germination rate (data not shown). Seed sterilization using chlorine gas significantly reduced seed vigor of both batches and the longer the sterilization time, the lower the seed vigor. After 48 hr of sterilization, seed vigor in both batches of seed was reduced to less than 35%. An inverse correlation between seed vigor and sterilization time was detected in both seed samples (R^2 = 0.98 and 0.97).

Seeds sterilized for 12, 24, 36, and 48 hr were processed for cotyledonary node explant transformation using vector pTF102 and regenerated. Regeneration rates [the number of explants with shoots per 50 infected cotyledonary node explants after 4 weeks of glufosinate selection (5 mg L^{-1})] were recorded. Negative correlations between regeneration rate of explants and seed sterilization duration were detected in both batches of Jack seeds (Figure 2b). Squared correlation coefficients (R^2) were 0.71 and 0.64 for PTF seeds and Illinois Foundation seeds, respectively. Reduced seed vigor caused by chlorine gas sterilization was negatively correlated with the regenerability of explants (R^2 = 0.71, Figure 2c).

It has been reported that explant source contributes to variation in regenerability in tissue culture in other crops such as sunflower (Baker et al., 1999) and maize. In maize, immature embryos from different environments had different tissue culture response for maize transformation (B.R. Frame, personal communication). Dependence on field-derived seed can be problematic due to increased contamination as compared to greenhouse-derived seed. In this study, mature field soybean seeds were sterilized by chlorine gas prior to explant preparation and subsequent *Agrobacterium* infection. While a short duration (< 2 hours) of sterilization cannot completely decontaminate explants, prolonged sterilization (>24 hours) may cause damage to explants and consequently affect their regenerability (Maruyama et al., 1989). Our results demonstrate that regeneration from soybean cotyledonary node explants was correlated with the seed vigor. Minimizing the duration of seed sterilization and using seeds with high vigor can increase the regenerability of the explants and consequently increase the frequency of transformation. Our recommended sterilization duration is between 12-16 hour depending on the cleanliness of seeds. Seed stock that consistently shows contamination in the tissue culture process after 16-hour sterilization should be avoided to ensure a more efficient transformation system.

Effect of selection agents on transformation

The base vector pTF101.1 (Figure 1) containing the *bar* gene has been used as the base vector for most soybean transformation projects in our laboratory. The *bar* gene encodes an enzyme, phosphinothricin acetyltransferase (PAT), that acetylates the free amino group of PPT rendering it inactive and incapable of binding to glutamine synthetase (Thompson et al., 1987; Wehrmann et al., 1996). Glutamine synthetase is required for the production of glutamine and prevents ammonia accumulation in the cell. The *bar* gene has been extensively used as a plant selectable marker in transformation studies. Both glufosinate (ammonium salt of PPT) and bialaphos (tripeptide L-phosphinothricyl-L-alanyl-L-alanine) (Tachibana et al., 1986) were used as selective agents in plant transformation. However, it was reported in other crops that the efficacy for selection of these two compounds varied considerably (Dennehey et al., 1994; Wang et al., 2003). To evaluate the stringency of these two compounds, we performed soybean transformation using ST19 and ST22 constructs. *Agrobacterium*-infected cotyledonary node explants of cv Williams and Williams79 were cultured on regeneration media containing different concentrations of glufosinate or bialaphos. The efficacy of the selective agents was measured by the efficiency of recovery of glufosinate-resistant events at two stages: R_0 plantlets and R_1 progeny. At Stage #1, rooted shoots (R_0) that survived glufosinate or bialaphos selection were transferred to soil and painted with 150 mg L^{-1} glufosinate. Seeds from glufosinate-resistant events were then harvested and planted in the greenhouse for subsequent progeny analysis. At Stage #2, R_1 seedlings were sprayed with 200 mg L^{-1} glufosinate and the segregation ratio was scored one week after the treatment. The number of

Table 1. Efficiency of transformation in soybean cotyledonary nodes subjected to glufosinate or bialaphos selection

Experiment date	Construct	Cultivar	Selection level[a] SI – SE	No. of explants infected	No. of regenerated R_0 plants	No. of Liberty®-resistant events		Transformation efficiency (%)	
						R_0	R_1	Initial[b]	Final[c]
17-8-2001	ST22	Williams79	G6 – G3	50	3	0	0	0.0	0.0
			G6 – G6	50	4	2	1	4.0	2.0
30-8-2001	ST22	Williams79	B4 – B4	48	11	1	1	2.1	2.1
			B6 – B6	48	26	4	1	8.3	2.1
			G6 – G6	48	6	4	3	8.3	6.3
6-9-2001	ST19	Williams79	B4 – B4	50	15	0	0	0.0	0.0
			B5 – B5	50	11	0	0	0.0	0.0
			B6 – B6	50	17	1	1	2.0	2.0
			G6 – G6	50	12	2	2	4.0	4.0
1-8-2002	ST22	Williams	B6 – B6	53	14	0	0	0.0	0.0
			B8 – B8	50	13	1	0	2.0	0.0
			G6 – G3	50	5	2	1	4.0	2.0
			G6 – G6	50	5	2	2	4.0	4.0

[a] Selection level – G6 is glufosinate at 6 mg L^{-1}; B6 is bialaphos at 6 mg L^{-1}. SI – shoot induction stage, SE – shoot elongation stage.

[b] Initial transformation efficiency = No. of Liberty®-resistant R0 events / No. of explants infected.

[c] Final transformation efficiency = No. of Liberty®-resistant R_1 events / No. of explants infected.

glufosinate-resistant R_0 or R_1 events per total number of cotyledonary node explants infected was referred to as the Initial Transformation Efficiency (ITE) and the Final Transformation Efficiency (FTE), respectively. Transformants obtained in these experiments were further confirmed by Southern or northern blot analyses in all glufosinate-resistant R_1 events. The Liberty® screening results in the R_1 generation had been well correlated with these molecular analyses for the presence of the transgene in our system (data not shown).

Table 1 summarizes the results obtained from four transformation experiments using two pTF101.1 derived constructs (ST19 and ST22) with two different cultivars (Williams79 and Williams). Overall ITE and FTE ranged from 0–8.3% and 0–6.3%, respectively. However, the ITE for glufosinate as the selective agent across experiments was 4.0% (12/298) while the ITE for bialaphos was 2.0% (7/349). We could clearly see a trend showing that glufosinate performed better as a selective agent compared with bialaphos in transformation efficiency. Notably, the use of glufosinate selection in the regeneration media decreased the number of escapes, i.e. non-transgenic shoots that survived herbicide selection *in vitro*. A reduction in the incidence of escape plants was important for efficient use of laboratory resources.

The reason why glufosinate worked better than bialaphos for soybean is not clear. The two compounds have phosphinothricin as the base molecule but with different chemical modifications on their overall structures. Glufosinate is the ammonium salt of PPT (which is the active ingredient of the herbicide BASTA®, BAYER, Germany) while bialaphos is the tripeptide L-phosphinothricyl-L-alanyl-L-alanine (which is the active ingredient of Herbiace®, Meiji Seika Kaisha Ltd., Japan). These different chemical modifications may cause different rates of uptake or transport of the selective agents by plant tissues and cells during selection and regeneration stages (Dennehey et al., 1994), which may make significant difference in final recovery of transgenic plants. Different plant species also respond differently to these two compounds. In maize, bialaphos is a preferred selective agent than glufosinate (Dennehey et al., 1994; Wang et al., 2003).

The optimum level of herbicide selection *in vitro* was 6.0 mg L^{-1} glufosinate (G6) based on the FTE. When G6 was used the FTE ranged from 2.0% to 6.3%. In comparison, B4 (4.0 mg L^{-1} bialaphos), B5 (5.0 mg L^{-1}), B6 (6.0 mg L^{-1}) or B8 (8.0 mg L^{-1}) gave 0% to 2.1% final efficiency. Earlier Zhang et al. (1999) achieved a range of 0% to 3.0% efficiency for the production of GUS positive R_0 plants

Table 2. Segregation ratio for glufosinate-resistance in R_1 progeny

Event	Resistant[a]	Sensitive[b]	χ^2 (3:1)[c]
ST19-11	12	2	0.8
ST19-53	13	0	4.3
ST19-80	11	3	0.1
ST22-3	11	3	0.1
ST22-34	11	3	0.1
ST22-37	14	2	1.3
ST22-42	14	0	4.7
ST22-44	12	1	2.1
ST22-65	11	3	0.1

[a] Resistant to 200 mg L^{-1} Liberty® spray.
[b] Sensitive to 200 mg L^{-1} Liberty® spray.
[c] $\chi^2 = 3.8$ (0.5, 1 df).

using a private cultivar Asgrow 3237 and glufosinate levels of 5.0 mg L^{-1} during shoot induction and 2.0 mg L^{-1} during shoot elongation. In the present study we used a more stringent glufosinate selection regime (6.0 mg L^{-1}) for both the shoot initiation and elongation stages of the public genotypes tested. This selection regime improved final transformation efficiency in our system.

Segregation of the *bar* gene in the R_1 generation was analyzed by phenotypic assay using Liberty® screening. Among 14 glufosinate-resistant R_0 events tested, the R_1 progeny from 9 events were resistant to Liberty®. Segregation ratios for *bar* gene expression followed Mendelian inheritance in 7 of 9 events tested (Table 2), indicating that the *bar* gene was inherited as a single dominant gene in these transgenic events. The segregation ratios of two events (ST19-53 and ST22-42) did not follow the 3:1 ratio. The observed segregation (resistant: sensitive) ratios were 13:0 and 14:0 for ST19-53 and ST22-42, respectively. It is possible that these two lines have the *bar* gene as multiple gene insertion in separate loci (i.e., two loci will be 15:1). It is also possible that we failed to observe the negative segregants due to the small sample size. Further confirmation by molecular analysis is required. On the other hand, the lack of *bar* gene inheritance in the R_1 generation of some events was likely due to either non-germline transformation or chimerism in the R_0 plant (Parrot et al., 1989; Lowe et al., 1995; Olhoft et al., 2003).

Effect of cysteine and DTT on transformation

Olhoft & Somers (2001) reported that the addition of cysteine at 3.3 mM to 8.3 mM in the cocultivation medium increased T-DNA transfer in cultivar Bert cotyledonary nodes based on transient GUS expression. The authors used a binary plasmid in *Agrobacterium* strain AGL1 and PPT selection to obtain 2.1% final transformation efficiency. We conducted several independent experiments to investigate the effect of cysteine and DTT on *Agrobacterium*-mediated transformation of other cultivars (Peking, Thorne and Williams79) using different constructs derived from the base vector pTF101.1. A summary of experiments is presented in Table 3. Our FTE in six different constructs ranged from 0.6% to 2.9% when 3.3 mM cysteine and 1 mM DTT were present in the cocultivation media. In three independent experiments the absence of cysteine and DTT gave 0.2% to 0.9% efficiency. Our results corroborate observations made by Olhoft and Somers (2001) that cysteine and DTT increased transformation efficiency (0.9% without cysteine and 2.1% with cysteine) in Bert. When these two compounds are present, cocultivation should be carried out in the dark because light incubation resulted in severe browning of the explants when compared to those tissues cocultivated in media without cysteine (data not shown). It is not clear why light caused more browning of the tissues in medium containing cysteine. Cysteine is a known inhibitor of polyphenoloxidase and peroxidase and enzymatic browning through the action of its thiol group (Olhoft et al., 2001). However, cysteine is light sensitive (MSDS sheet, http://www.biacore.com/pdf/products/msds/eng_LCysteine.pdf), exposing it to light may have diminished its effect as browning inhibitor.

Susceptibility of soybean cultivars to Agrobacterium *infection and regeneration from cotyledonary node*

It is desirable that a transformation protocol could be applicable to a variety of genotypes. This study was conducted to determine the response of different soybean cultivars to *Agrobacterium* infection and their regeneration ability. Ten cultivars from 6 maturity groups were chosen and infected with *Agrobacterium* strain EHA101 containing pTF102. The explants were cocultivated with or without 3.3 mM cysteine and 1 mM DTT. For each experiment, two reproductions were executed. For the first experiment, a total of 45 explants were wounded. Of these, 20 explants were infected with *Agrobacterium* strain pTF102 and

Table 3. Transformation efficiency in soybean cotyledonary nodes infected with *Agrobacterium* and cocultivated with or without cysteine and DTT

Construct	Cultivar	Number of explants infected	No. of Liberty® -resistant events		Final transformation efficiency (%)[a]
			R_0	R_1	
Without cysteine+DTT					
ST14	Thorne	2876	9	5	0.2
ST15	Thorne	400	1	1	0.3
pTF102	Peking	337	3	3	0.9
With cysteine+DTT					
ST17	Thorne	490	6	3	0.6
ST18	Thorne	1076	18	10	0.9
ST19	Thorne	330	3	3	0.9
ST20	Williams79	532	15	9	1.7
ST22	Williams79	529	12	6	1.1
pTF102	Thorne	140	12	4	2.9

[a] Final transformation efficiency = No. of Liberty® resistant R_1 events /No. of cotyledonary nodes infected.

Table 4. Transient GUS expression on cotyledonary node explants of soybean cultivars 5 days after cocultivation with EHA101(pTF102) and shoot regeneration *in vitro*

Cultivar	Maturity group	Without cysteine[a]	With cysteine[a]	Mean level of GUS expression[b]	Regeneration rate (%)[c]	Average degree of regeneration[d]
Bert	I	0/20	17/20	2	88	3.8
Clark	IV	0/20	19/20	2	96	3.4
Delsoy 5710	V	3/20	20/20	3	76	4.0
Essex	V	2/20	20/20	3	88	3.0
Harosoy	II	0/20	20/20	2	68	3.2
Jack	II	1/20	20/20	2	96	4.8
Ogden	VI	13/20	20/20	3	96	3.1
Thorne	III	0/20	17/20	3	92	3.5
Williams	III	0/20	17/20	2	100	4.9
Williams82	III	1/20	20/20	3	96	3.7
TOTAL		20/200	190/200			

[a] No. of cotyledonary nodes showing GUS activity/No. of cotyledonary nodes infected; data were based on 2 experiments.
[b] Levels of GUS expression: 1 – light blue, 2 – moderate, 3 – dark blue; data based on 2 experiments.
[c] No. of cotyledonary nodes with shoots/25 cotyledonary nodes cultured × 100.
[d] Degree of regeneration: 0 – none (Figure 3d-1), 1 – minimal (Figure 3d-2), 2 – slight (Figure 3d-3), 3 – moderate (Figure 3d-4), 4 – good (Figure 3d-5), 5 – profuse (Figure 3d-6).

analyzed for transient GUS assay while the other 25 wounded but not infected explants were cultured on regeneration medium without selection for regenerability assessment. In the second experiment 20 explants were infected and subsequently tested for GUS. Histochemical assays were performed 5 days after cocultivation to assess the presence of transformed cells. The addition of cysteine and DTT in cocultivation media dramatically increased transient GUS activity in all ten cultivars tested wherein 95% activity was obtained when cysteine was added while 10% GUS expression was obtained without cysteine (Table 4). Intense blue staining was observed when cysteine was present while GUS expression without cysteine was limited to sporadic small blue spots (Figure 3a). It is possible that the antioxidant properties of cysteine and DTT that reduce browning of wounded soybean tissue resulted in better recovery of infected tissues. It is also possible that less oxidized tissue could have improved the interaction between *Agrobacterium* and plant cells as

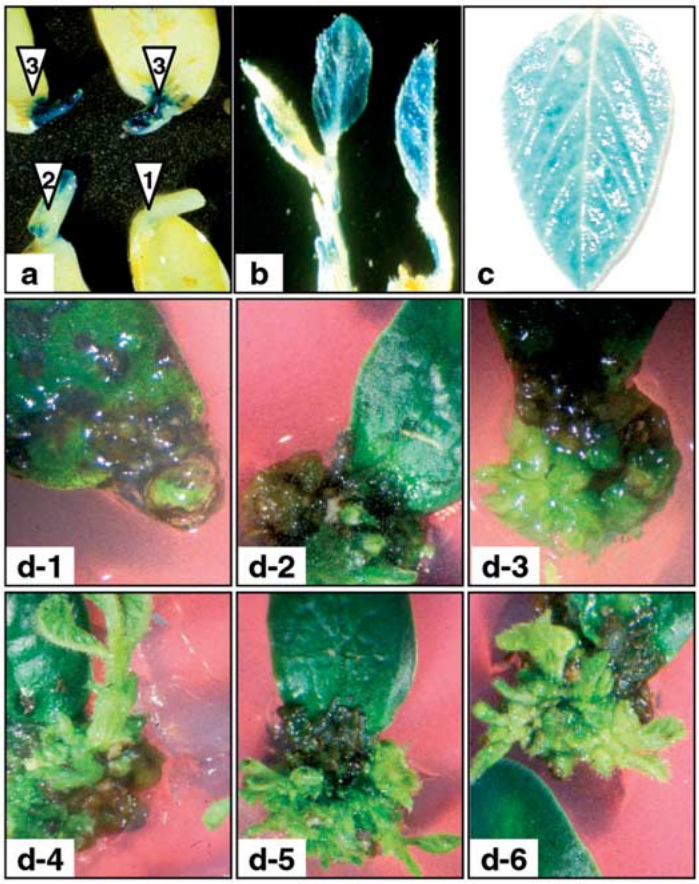

Figure 3. Transient and stable GUS expression, and regeneration of soybean cotyledonary nodes. (a) Level of GUS expression on soybean cotyledonary nodes. 1-light blue, 2-moderate blue, 3-dark blue; (b) and (c) GUS positive leaf tissues of R_0 plants; (d) shoot regeneration, d-1, no shoot; d-2, 1-minimal; d-3, slight; d-4, moderate; d-5, good; d-6, profuse.

suggested by Olhoft et al. (2001). The positive effect of cysteine, DTT and other thiol componds as protective agent against oxidases in plant tissue was described by Peach and Velten (1992) and Olhoft et al. (2001). Likewise, Enriquez-Obregon et al. (1999) described the antinecrotic property of cysteine in rice transformation. Our regeneration test of non-infected explants on shoot induction media without selection showed that the cultivars tested had moderate to profuse shoot regeneration (Table 4). The degree of regeneration on cotyledonary node explants ranged from none to profuse (Figure 3d). Enhanced regenerability was observed in cultivars Williams and Jack. Previously, Jack was also shown to have good tissue culture and transformation response (Zhang et al., 2000). Our observations suggest that twelve soybean genotypes, ten listed in Table 4 and two other cultivars (Peking and Williams79), were amenable to *Agrobacterium* transformation and may provide a valuable tool in soybean improvement programs.

Southern analysis of transformants

Southern blot analysis was done for further confirmation of glufosinate-resistant plants. For construct ST14, restriction enzyme digestion of total genomic DNA was carried out using *Stu* I, which cut once in the T-DNA region (Shou et al., 2004). For construct pTF102 (Frame et al., 2002), a derivative of pTF101.1, single restriction enzyme *Hind* III (in the T-DNA region) was used (Figure 1). ^{32}P-labeled *bar* and GOI were used as probes for the transformants generated from pTF102 and ST14, respectively. Transgenes were detected in all herbicide resistant R_0 plants as exemplified in Figure 4. Because *Stu* I cuts only once in the T-DNA region, the hybridization band could be greater than 1.9 kb, which is the restriction fragment

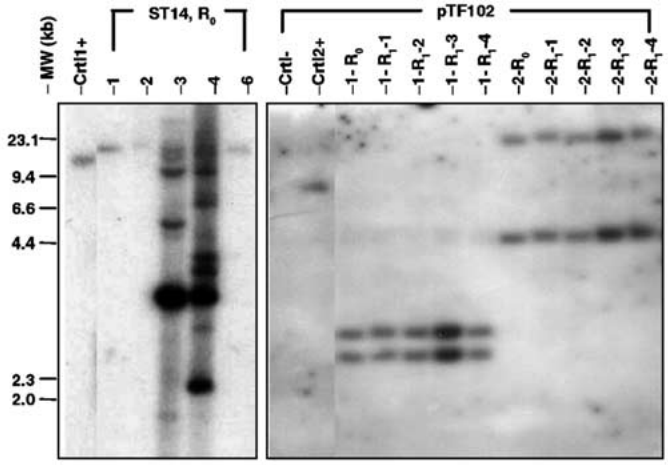

Figure 4. Southern blot analysis of transgenic soybean events. Ten micrograms of genomic DNA were digested with *Hind* III (pTF102 transformants) or *Stu* I (ST14 transformants). Both enzymes cut once in the T-DNA region of the corresponding plasmid. A DNA fragment from the GOI or *bar* gene was used as a probe for ST14 and pTF102 transformants, respectively. Ctr+ is a positive control in which 10μg of non-transgenic soybean genomic DNA and 63 pg of ST14 or pTF102 plasmid DNA was loaded. R_0 and R_1 refer to the first and second generations of transgenic soybean plants, respectively.

size from *Stu* I to the right border (Shou et al., 2004). As shown in Figure 4, *Stu* I restriction digestion of genomic DNA obtained from ST14 transgenic materials generated different band sizes (> 1.9 kb) when hybridized with the GOI probe. The number of bands can reflect the different sites of transgene integration and is an estimate of transgene copy number. While three events had a single copy of the GOI gene, two ST14 events had multiple copies.

To assess transmission of the transgene from the R_0 to R_1 generation, Southern analysis was also conducted in herbicide pre-screened progeny of two pTF102 events. *Hind* III restriction digestion (Figure 1) generated hybridization bands greater than 2.3 kb in pTF102 transformants. Events pTF102-1 and pTF102-2 contained double and single copies in the R_0 transformants, respectively (Figure 4). Their progeny contained the same hybridization pattern as the R_0 plants in both events, indicating that the transgene was transmitted to the next generation. The transgenic nature of these events was also examined by northern blot and reverse transcriptase-PCR analyses in both R_0 and R_1 plants (Shou, unpublished). Herbicide screening results in the R_1 generation were well correlated with molecular analysis for the presence of the transgene in our system.

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