Plant Cell Reports (1998) 18: 180–186

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Factors affecting soybean cotyledonary node transformation

Received: 9 March 1998 / Revision received: 9 July 1998 / Accepted: 28 July 1998

Abstract Cotyledonary node transformation efficiency was evaluated using a sonication assisted *Agrobacterium*-mediated transformation (SAAT) protocol, three dissimilar *A. tumefaciens* strains, and explants derived from 28 diverse cultivars and/or genotypes of soybean [*Glycine max* (L.) Merr.]. The explants were evaluated at 10 and 45 days after co-cultivation for transformation with a binary vector containing both a GUS-intron gene and an NPTII selectable marker. The best overall strain of *A. tumefaciens* was determined to be KYRT1 based on stable GUS transformation of soybean cotyledonary node explants measured at the terminal 45 day evaluation point. SAAT did not increase stable transformation at 45 days post co-cultivation. SAAT was determined to significantly decrease shoot proliferation of some genotypes, but it is unclear what effect this may have on the recovery of transformed shoots. Significant differences were also detected between genotypes for transformation and shoot proliferation frequency.

Key words Soybean · SAAT (sonication assisted *Agrobacterium*-mediated transformation) · *Agrobacterium* · Transformation · KYRT1

Abbreviations *BAP* 6-Benzylaminopurine · *IBA* indole-3-butyric acid

Introduction

Soybean transformation resulting in the inheritance of transgenes in the resulting progeny has been far from routine. Since the first reports of soybean transformation (Hinchee et al. 1988; Parrott et al. 1989; McCabe et al. 1993) only a handful of laboratories have had more than sporadic success (Trick et al. 1997). Protocols utilized to consistently genetically engineer soybeans have been confined to *Agrobacterium*-mediated transformation of cotyledonary nodes, particle bombardment of shoot meristems, and particle bombardment of embryogenic suspension cultures (Di et al. 1996; Hinchee et al. 1988; McCabe et al. 1993; Stewart et al. 1995). Protocols for soybean transformation using cotyledonary node explants offer one of the better methods for regeneration of fertile soybean plants due to a short seed-to-seed generation time and no requirement for the maintenance of parental donor plants or long-term cultures. While there is still a major problem of chimerism using organogenic protocols, with proper selection in the second generation, recovery of transgenic soybeans is possible (Christou 1989).

Agrobacterium-mediated transformation of soybeans has not been without problems as soybean appears to be highly variable in their response to *Agrobacterium spp*. Owens and Cress (1985), using only a single strain of *A. tumefaciens*, demonstrated both significant genotypic and seedling age effects on the tumorigenic response of cut stems. Byrne et al. (1987) in their evaluation of multiple *Glycine* and *Agrobacterium* species identified a significant genotype-by-strain interaction. They next evaluated 11 *A. tumefaciens* strains on the most susceptible soybean genotype, 'Peking', and found significant differences in tumor formation induced by the different *A. tumefaciens* strains. Delzer et al. (1990) found that a significant strain-by-genotype interaction existed when they evaluated 10 early-maturity genotypes with 3 strains of *A. tumefaciens* for tumorigenicity of wounded cotyledons, a non-organogenic tissue. They also evaluated 14-day-old cotyledons of the same genotypes for variation in shoot proliferation over a 30-week period and found no significant differences. Other authors (Mauro et al. 1995; Bailey et al. 1994) have identified genotypes divergent for *Agrobacterium*-induced tumor formation and have made crosses between these genotypes in order to study the heritability of this trait. Both

Communicated by G. Phillips

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groups determined this trait to be quantitative in nature with sufficient genetic variance to permit improvement via selection. It is unclear from any of these studies whether the various scoring assays were indicative of transformability of shoot morphogenic cultures or recovery of regenerated shoots.

In the above studies researchers focused primarily on the wild-type galling response as a measure of genotypic variance of soybean susceptibility to *A. tumefaciens* strains. This could be misleading as tumor formation is a complex process that could be controlled by factors such as tissue growth stage, ability of transformed tissue to proliferate in culture (tissue culture effect), ability of tissue to proliferate in the presence of bacteria, or other factors. There is also no proven connection between wild-type tumor formation on any soybean tissue type and the transformation of tissues specifically able to regenerate whole plants.

We designed experiments to evaluate the effect, as measured only on the regenerable nodal region of soybean cotyledonary explants, of a mechanical procedure called "sonication-assisted *Agrobacterium*-mediated transformation" or SAAT (Trick and Finer 1997) and to compare a previously untested disarmed strain of *A. tumefaciens*, KYRT1 (Torisky et al. 1997) to other *A. tumefaciens* strains currently being used in transformation protocols. The SAAT procedure developed by Trick and Finer (1997) has shown promise for increasing the transformation efficiency of many crops recalcitrant to *Agrobacterium*-mediated transformation, especially soybean. This procedure mechanically disrupts and wounds cells via sonic wave effects and is thought to permit a more thorough penetration of *Agrobacterium* into the explant tissues.

Agrobacterium strain comparisons involved KYRT1, a disarmed strain of the Chry5 wild-type strain of *A. tumefaciens* reported by Kovacs and Pueppke (1993) to be "supervirulent" on soybean, and two other commonly used disarmed *A. tumefaciens* strains for transformation of soybean cotyledonary node explants.

Materials and methods

Plasmid construction and transfer

All experiments were conducted using the binary vector pBISN1 (Narasimhulu et al. 1996), obtained from S. B. Gelvin, Purdue University. The pBISN1 plasmid has a β-glucuronidase (GUS) gene interrupted by the ST-LS1 intron that renders the gene non-expressible in prokaryotic cells. The pBISN1 construct also utilized the *NPTII* plant selectable marker within the T-DNA. This binary plasmid was transferred into *A. tumefaciens* strains via electroporation (Dulk-Ras and Hooykaas 1995) and maintained by selection on Kanamycin for the bacterial expressed *NPTII* gene outside of the T-DNA.

Agrobacterium spp. culture

Agrobacterium tumefaciens strains used for transformation were EHA 105 (Hood et al. 1993), KYRT1 (Torisky et al. 1997), and LBA 4404 (Hoekema et al. 1983). Glycerol stocks of each *A. tumefaciens* strain were thawed and then streaked onto solid LB media, pH 7.0, containing 100 mg^{-1} Rifampicin and 100 mg^{-1} Kanamycin and single, isolated colonies were used to start 5-ml liquid LB media cultures. These cultures were grown overnight with shaking at 27°C/200 rpm. The following morning 500-ml cultures were started with 25 µl of the preceding 5-ml overnight culture and again incubated with shaking for approximately 24 h at 27°C/200 rpm until the optical density at 600 nm (OD^{600}) was less than or equal to 0.5, which is equivalent to mid-log phase growth. The resulting culture was centrifuged at 4500 rpm for 10 min to yield a bacterial pellet. The pellet was washed once in liquid co-cultivation media [B5 salts and vitamins (Gamborg et al. 1968), 44 µ*M* BAP, 1 µ*M* IBA, 100 µ*M* acetosyringone, 30 mg^{-1} sucrose, pH 5.5], centrifuged as before, then resuspended in liquid co-cultivation medium to an OD^{600}=0.25, 0.50, or 1.0 before use for the first experiment and standardized to OD^{600}=1.0 for all future experiments. Bacterial suspensions were prepared immediately prior to use.

Soybean cotyledonary node transformation protocol

Soybean seed were pre-washed by brief agitation in sterile water to which one drop of Liqui-nox (Alconox, New York, N. Y.) was added. Following agitation, the water was decanted and the seed briefly air dried and then sterilized in a sealed desiccator with chlorine gas liberated from a reaction between 100 ml of Chlorine bleach (5.25% Na-hypochlorite) and 5 ml 12 *N* HCl. Seed were kept in the desiccator for 12 h and then germinated for 4 days before transfer to a sterile glass petri plate for preparation. The preparation of cotyledonary nodes began with complete removal of the seed coat and removal of the radicle 5 mm below the junction with the seedling cotyledon. Each seed was then bisected to yield 2 identical explants (Fig. 1A). Explants were prepared in groups of 50 that were plated adaxial side down on half-strength B5 medium (Gamborg et al. 1968) until needed for transformation. Explants were bisected no more than 3 h in advance of co-cultivation.

The transformation protocol was based on the protocol of Townsend and Thomas (1993). Explants were exposed adaxial side up and macerated through the meristem and cotyledonary nodes to disrupt primary shoot morphogenesis and provide a wound site for the *Agrobacterium*. Experimental units, composed of 10 explants placed in 16×125-mm borosilicate glass tubes and covered with the previously prepared *Agrobacterium* suspension, were either sonicated or left immersed in inoculum. Sonication was accomplished with a water-bath sonicator (Model PC5, L&R Manufacturing Co, Kearny, N. J.) with both vertical and horizontal wave output switches in the on position. Tubes of explants were sonicated by lowering into the water-bath chamber and holding in place for 6, 60, or 600 s. After sonication or immersion, each tube of explants was removed to a plate of solid co-cultivation media (as above with the addition of 5 mg^{-1} agarose) and incubated at 21°C in the dark for 72 h. All 10 explants from each tube were transferred to a single plate that served as the experimental unit.

After removal from co-culture, explants were transferred to liquid counter-selection medium (B5 salts and vitamins, 5 µ*M* BAP, 1 µ*M* IBA, 30 mg^{-1} sucrose, pH 5.7, augmented with 50 mg^{-1} kanamycin sulfate, 100 mg^{-1} Vancomycin, 100 mg^{-1} Timentin, and 500 mg^{-1} Mefoxin) and cultured at 27°C, 150 rpm for 4 h to remove visible bacteria. After liquid washing, explants were moved to solid counter-selection medium (as above with 6 mg^{-1} agarose) and subcultured every 10 days for a total of three subcultures. After two subcultures, cotyledons were separated from the shoot morphogenic tissue and discarded. The remaining shoot-containing explants were transferred to fresh media without selective agent. No explants were maintained after 45 days.

β-Glucuronidase assay

One half of the total number of soybean cotyledonary nodes on a plate were destructively assayed for GUS expression (Jefferson et al. 1987) at 10 days post co-cultivation, with the remaining half as-

Fig. 1A–D Transformation of soybean cotyledonary nodes. **A** Cotyledonary node explant after bisection. **B** Cotyledonary node at 10 days after co-cultivation with *A. tumefaciens* showing area of scoring (*circle*) and non-scorable GUS foci (*arrow*). **C** Proliferating shoots from cotyledonary node after 6 weeks of culture on Kanamycin-containing (50 mg^{-1}) media. **D** Cotyledonary node of 'Stonewall' cultivar showing GUS-positive transgenic shoots. *Bar* (in all figures): 5 mm

sayed at 45 days post co-cultivation in experiments 2 and 3. During experiment 1 (initial optimization) all explants were assayed at 10 days post co-cultivation. Entire cotyledonary nodes were placed in glass vials and covered with assay buffer (50 m*M* Na-phosphate, 500 µ*M* potassium ferriccyanide, 500 µ*M* potassium ferrocyanide, 10 m*M* EDTA, 0.05% Triton X-100, pH 7.0) containing 100 mg^{-1} X-gluc (Jersey Lab Supply). Tissue was incubated 24 h at 37°C with gentle agitation. Following incubation the assay buffer was decanted, and the tissue was destained with 70% ethanol.

Cotyledonary nodes were scored under a Zeiss SV8 dissecting scope, counting only those GUS foci that fell within 2 mm of the cotyledonary node (Fig. 1B). This prevented the inclusion of transformation events that formed on cotyledons (Fig. 1B) or the cut end of the hypocotyl, tissues known to be non-organogenic. The response variables recorded on a per plate basis were foci per cotyledonary node (Foci/CotN) and percentage response (Resp/CotN). Foci per cotyledonary node was determined as the total number of qualifying foci per plate at either the 10- or 45-day assay point divided by the number of cotyledonary nodes assayed at that point. Percentage response was determined as the number of cotyledonary nodes with at least 1 qualifying GUS focus divided by the number of cotyledonary nodes assayed, again calculated separately for the 10- or 45-day sampling point.

Soybean cultivars used

Experiment 1 (SAAT optimization) used cv 'Dyna-Gro 3303'. Experiment 2 used cvs 'Dyna-Gro 3303', 'Dyna-Gro 3256', and 'Pioneer 9341'. Experiment 3 used cvs 'Calhoun', 'CF 492', 'Clark', 'Columbus', 'Corsoy', 'Crawford', 'Cutler', 'Dawson', 'Essex', 'Gordon', 'Harosoy', 'Hutcheson', 'Leflore', 'Ozzie', 'Peking', 'Pershing', 'Ripley', 'Sibley', 'Stafford', 'Stonewall', 'Thomas', 'Twiggs', 'Williams', 'York', and 'Young'. 'Dyna-Gro 3256' and 'Dyna-Gro 3303' were obtained from J. Becton, United Agri-Products. 'Dawson', 'Ozzie', and 'Sibley' were obtained from J. H. Orf, University of Minnesota. 'Gordon', 'Twiggs', and 'Thomas' were obtained from H. R. Boerma, University of Georgia. 'Leflore' was obtained from T. C. Kilen, USDA, Stoneville, Mississippi. 'Stonewall' was obtained from D. B. Weaver, Auburn University. 'York' was obtained from G. R. Buss, VPI and State University. 'Young' was obtained from J. W. Burton, North Carolina State Univ. 'Pioneer 9341' was obtained from Duane Frederking, Pioneer Hybrid International, Inc. All other cultivars were provided by T. W.

Table 1 Initial optimization of SAAT (sonication-assisted *Agrobacterium*-mediated transformation) with *A. tumefaciens* EHA 105:pBISN1 and soybean cultivar 'DG 3303'

	Inoculum OD^{600}			Sonication time (s)			Explant orientation	
	0.25	0.5	1.0	6	60	600	Wound up	Wound down
Foci/CotN[a]	6.92	7.96	9.96	5.03	4.21	15.87	8.43	9.26

[a] Foci/CotN is the average number of GUS-positive foci per cotyledonary node explant at 10 days post co-cultivation

Pfeiffer (University of Kentucky). Experiment 4 utilized the cultivars of experiment 3, with the exception of 'Crawford', 'Cutler', 'Dawson', 'Ozzie', and 'Stafford', for which there were insufficient seed for further experimentation.

Experimental design

Experiment 1 was conducted as an unreplicated optimization trial. Simple means were calculated, but no means separation tests were conducted. Data from those treatments yielding no recordable data (SAAT without *A. tumefaciens*, SAAT greater than 600 s, *A. tumefaciens* without a binary plasmid) were omitted.

Experiment 2 was conducted as an incomplete block design with all treatments except cultivar appearing on each day. Two of three cultivars were represented on each day such that each cultivar was represented twice over the three experimental dates. Data were analyzed using the PROC GLM function of SAS (SAS Institute 1988). Treatment means were separated with Fisher's Protected Least Significant Difference.

Experiment 3 was conducted as a 5×5-quadruple lattice design concatenated such that all treatment combinations were represented at least three times over the five experimental dates with no genotype occurring more than twice on any given day. Data were again analyzed using the PROC GLM function of SAS (SAS Institute 1988), and means were separated with Duncans Multiple Range Test.

Experiment 4 was a completely random design prepared after the analysis of experiment 3. The purpose was to evaluate the cultivars of experiment 3 for variations in shoot production in the absence of selection. Data were analyzed using the PROC GLM function of SAS (SAS Institute 1988).

Results and discussion

Previous studies have investigated genotype and genotype-by-strain interactions in *Agrobacterium*-mediated soybean transformation protocols. These studies have utilized many different soybean tissue types and have not always lent themselves to direct correlations between the response variable scored and the ability to recover transgenic plants. Our effort was focused on evaluating multiple strains of *A. tumefaciens* and a mechanical wounding procedure over a wide array of soybean genotypes, using response variables that should be directly correlated with transgenic shoot recovery.

Cotyledonary nodes were scored so that only the GUS foci within 2 mm of the cotyledonary node (Fig. 1B) were included in order to omit transformation events on tissues known to be non-organogenic. GUS staining was often observed on other parts of the explant and, while incapable of leading to transgenic plants, was considered a form of positive control for *A. tumefaciens* transformation. This GUS staining was observed primarily on the cut end of the hypocotyl or where wounding had been inadvertently done to the cotyledon during explant manipulation. The use of a binary construct carrying an intron-interrupted GUS gene (Narasimhulu et al. 1996) also ensured that observed staining was due only to plant cell expression and to not contaminating *A. tumefaciens*.

The initial transformation optimization experiment performed to determine the best *Agrobacterium* concentration, sonication time, and explant orientation during co-culture (Table 1) was done using only 1 genotype and strain combination ('Dyna-Gro 3303' and EHA 105). Differences in the number of GUS-stained spots were observed by increasing sonication times to 600 s, but few explants survived to 10 days when sonicated at the 600-s level. The SAAT protocol when used with other soybean explants, as well as tomato and tobacco leaf disks (data not shown), showed that the balance between regeneration and tissue sensitivity to sonication must be determined empirically. This concurs with the results of Trick and Finer (1997). For the remaining experimentation the sonication time was standardized at 6 s based on the initial optimization of transient GUS expression and continued tissue proliferation. This standardized sonication time was determined as that level that led to a perceived increase in GUS foci without any perceived decrease in explant viability. There was no direct comparison of sonication to non-sonication in experiment 1. However, when sonication was not used in conjunction with *A. tumefaciens* in our lab (Torisky et al. 1997) the number of GUS-positive foci was approximately 1/10 of that seen with sonication in this experiment unpublished data showed that tissues exposed to sonication alone, a disarmed *A. tumefaciens* strain without a binary vector, or a combination of sonication and disarmed *A. tumefaciens* without binary vector would not assay positive for GUS even when incubated with assay buffer for extended periods of up to 7 days. For this reason, and to maximize the number of meaningful strain, SAAT, and genotype comparisons, negative controls were not included in further experiments.

Significant interactions were not detected in the second and third experiments between the several factors analyzed (SAAT, *A. tumefaciens* strain, soybean genotype) that affect recovery of transgenic soybean plants from cotyledonary node explants, with the exception of genotype × day in experiment 2. The one interaction detected was likely due to an extremely high response, relative to the other 2 cultivars, exhibited by 'Pioneer 9341'. The lack of interactions throughout the rest of the experiments permitted a more direct comparison between treatment factors rather than combinatorial factors.

Table 2 Effects of SAAT and *Agrobacterium* strain on transient and stable transformation of soybean cotyledonary nodes as evaluated with GUS assay (*N. D.* not determined)

Variable	10-Day (transient) expression		45-Day (stable) expression		
	Foci/CotN[a]	Resp/CotN[b]	Foci/CotN[a]	Resp/CotN[b]	Shoots[c]
Strain					
Exp. 2					
KYRT1	0.63[d] a	23.6 a	0.82 a	26.1 a	N. D.
EHA 105	0.24 b	15.6 a	0.16 b	10.7 b	N. D.
LBA4404	0.08 c	4.7 b	0.17 b	2.8 b	N. D.
Exp. 3					
KYRT1	0.61 a	12.2 a	0.68 a	7.0 a	1.14 b
EHA 105	0.43 a	8.2 a	0.29 b	4.6 a	1.50 a
SAAT					
Exp. 2					
Sonication	0.34 a	16.4 a	0.44 a	14.4 a	N. D.
Immersion	0.31 a	12.2 a	0.28 b	13.7 a	N. D.
Exp. 3					
Sonication	0.65 a	12.9 a	0.46 a	7.2 a	0.97 b
Immersion	0.37 b	7.2 b	0.48 a	4.6 a	1.71 a

[a] Foci/CotN is the average number of GUS-positive foci per cotyledonary node explant
[b] Resp/CotN is the percentage of GUS-positive cotyledonary nodes of the total cotyledonary node explants
[c] Shoots is the average number of shoots per cotyledonary node recorded at 45 days
[d] Means not followed by the same letter within each column are significantly different at the 5% level

Sonication treatments had different results between experiments 2 and 3 (Table 2). Sonication had no significant effect on transient (10 days) GUS expression in experiment 2 in which only 3 soybean genotypes were evaluated. At 45-days post co-cultivation the number of stable foci per cotyledonary node became significant when the non-SAAT treatment essentially remained static while the response variable of the SAAT treatment increased. This could possibly be explained by the proliferation of foci that were undetectable at 10 days. This significant increase did not affect percentage response of stable transformation (45 days) in the same experiment. In experiment 3, across a much broader range and number of genotypes, sonication significantly increased both measures (Foci/CotN and Resp/CotN) of transient (10 days) transformation, but this increase was not evident among either of the stable (45 days) transformation measures (Table 2).

Statistical analysis of shoots per cotyledonary node (Shoots) determined that SAAT significantly decreased the number of regenerating shoots. This was an unplanned measurement recorded only in experiment 3 when there appeared to be visible differences. This measurement was not recorded at the 10-day point (as secondary proliferative shoots are just being initiated at that time) but was recorded at the 45-day time point when there were visual differences detected between genotypes. It is not clear what the effect of this was on transformation, but it seems likely that a reduced production of shoots would reduce the likelihood of recovering transgenic shoots.

Comparison of the *A. tumefaciens* strains KYRT1, EHA 105, and LBA 4404 in experiment 2 showed that KYRT1 consistently produced a greater transformation response on explants than LBA 4404 (Table 2). The magnitude of this increased transformation response was approximately five- to tenfold. For this reason LBA 4404 was excluded from further use in experiment 3. KYRT1 often significantly increased stable (45 days) transformation over EHA 105 although this difference was usually not evident at the 10-day measurement. In experiment 3 strain KYRT1 showed a significant decrease relative to EHA 105 in shoot production. This decrease in shoot production may be offset with KYRT1's increased transformation potential.

Due to the apparent low interdependence between transient and stable evaluations, genotype comparisons in experiment 3 were only conducted on the stable (45 days) data. Few differences were found in transformability when the 25 genotypes selected for this study were compared (Table 3), as measured by foci per cotyledonary node (Foci/CotN) and percentage of responding cotyledons (Resp/CotN). This was due to the high variability and low values for response variables. Only those genotypes with more than 0.8 foci per cot node or that had a 5% or higher response could be declared significantly different from non-responsive genotypes. It is of interest to note that 'Peking', selected as the most susceptible cultivar by both Byrne et al. (1987) and Hinchee et al. (1988) and used as the susceptible parent in crosses with "resistant" genotypes by Mauro et al. (1995), was not different from the public cultivars often considered recalcitrant to transformation via *Agrobacterium*. While not conclusive, it appears that the use of hypervirulent strains of *A. tumefaciens*, such as KYRT1 and EHA 105, can overcome the genotypic resistance found in some cultivars of soybean.

A major difference observed between cultivars in experiment 3 was the number of shoots per cotyledon (shoots). A high number of shoots were observed in several cultivars despite placement on a selective media containing Kanamycin for over 6 weeks (Fig. 1C). The number of shoots displayed by cv 'Twiggs' was significantly higher than that of the other 23 cultivars, and yet none of these were scored

Table 3 Evaluation of 25 soybean genotypes for stable expression of the GUS-intron gene and shoot production on selective media

45day (stable) expression					
Genotype	Foci/CotN [a]	Genotype	Resp/CotN [b]	Genotype	Shoots [c]
Williams	1.66 [d] a	Peking	18.3 [a]	Twiggs	4.01 [a]
Crawford	1.61 a	Dawson	16.0 a	Stonewall	3.67 a, b
Peking	1.33 a	Cutler	15.0 a	Young	2.55 b, c
Cutler	1.33 a	Stafford	14.6 a	Dawson	2.23 c, d
Stonewall	1.12 a	Williams	13.7 a	Williams	1.94 c, d, e
Dawson	1.05 a	Clark	12.5 a	Thomas	1.87 c, d, e
Clark	0.81 a	Stonewall	10.0 a	Leflore	1.82 c, d, e
Ozzie	0.50 a, b	Leflore	10.0 a	Columbus	1.49 c, d, e, f
Stafford	0.50 a, b	Crawford	7.5 a	Calhoun	1.42 d, e, f
Leflore	0.37 a, b	Thomas	7.5 a	York	1.20 d, e, f
Thomas	0.33 a, b	Hutcheson	5.7 a	Harosoy	1.00 d, e, f
Sibley	0.28 a, b	Ripley	3.6 a, b	Cutler	1.00 d, e, f
Hutcheson	0.21 a, b	Corsoy	3.3 a, b	Crawford	0.81 d, e, f
Corsoy	0.17 a, b	Sibley	2.9 a, b	Stafford	0.78 d, e, f
Ripley	0.14 a, b	Ozzie	2.5 a, b	Ripley	0.63 e, f
Calhoun	0.11 a, b	Calhoun	2.2 a, b	Essex	0.60 e, f
Gordon	0 b	Gordon	0 b	Clark	0.59 e, f
Pershing	0 b	Pershing	0 b	Sibley	0.58 e, f
CF 492	0 b	CF 492	0 b	Corsoy	0.57 e, f
Columbus	0 b	Columbus	0 b	Ozzie	0.54 e, f
Essex	0 b	Essex	0 b	Gordon	0.51 e, f
Twiggs	0 b	Twiggs	0 b	Peking	0.47 e, f
Harosoy	0 b	Harosoy	0 b	Pershing	0.46 e, f
York	0 b	York	0 b	Hutcheson	0.45 e, f
Young	0 b	Young	0 b	CF 492	0.26 f

[a] Foci/CotN is the average number of GUS-positive foci per cotyledonary node explant
[b] Resp/CotN is the percentage of GUS-positive cotyledonary nodes of the total cotyledonary node explants
[c] Shoots is the average number of shoots per cotyledonary node recorded at 45 days
[d] Means not followed by the same letter within columns are significantly different at the 5% level

GUS-positive. In fact, no GUS-positive staining on any part of the explant was observed on 'Twiggs'. This suggests that either 'Twiggs' has some inherent tolerance to kanamycin or supports previous studies that suggest that kanamycin is a poor selectable marker for soybeans. It also suggests that future studies involving genetic variability to *Agrobacterium* susceptibility may wish to include 'Twiggs'. Another cultivar, 'Stonewall', appeared to also have a high regeneration potential on kanamycin-containing media and yielded 3 GUS-positive shoots (Fig. 1D), 1 uniformly transformed and 2 showing chimeric sectoring, as well as many GUS-positive foci on nodal tissue. These two examples clearly show that significant differences in shoot production were not directly related to transformation potential across cultivars. Cotyledonary node explants of 20 of the 25 genotypes evaluated in experiment 3 (omitting 'Crawford', 'Cutler', 'Dawson', 'Ozzie', and 'Stafford' for lack of seed) were re-evaluated under non-selective conditions, and no significant differences in shoot production per explant were noted (data not shown). This concurs with Delzer et al. (1990) who found no differences in shoot production per cotyledonary node among a collection of early-maturity group genotypes. The results from experiments 3 and 4, when taken together, also suggest that while the susceptibility of soybean to *Agrobacterium* may be low, the present obstacles to the recovery of transgenic fertile plants may also involve cultivar regeneration potential on selective media.

Shoots that were either entirely GUS-positive or displayed sectoring with GUS staining appeared at a frequency of 1–2% of the shoots recovered. Thus, while the transformation of soybean continues to remain low compared to other crops, such as the model systems tobacco, petunia and tomato, the present study highlights the feasibility of obtaining transgenic soybean using the cotyledonary node protocol when hypervirulent strains of *A. tumefaciens* are used in conjunction with soybean genotypes of moderate-to-high regeneration and transformation potential.

Acknowledgments We wish to thank H. Trick and J. Finer for sharing results prior to publication, S. Gelvin for plasmids, C. Redmond, K. McAllister, G. Budziszewski, and W. Parrott for helpful discussions, and T. Pfeiffer and P. Cornelius for experimental design assistance. This research was supported with grants from The United Soybean Board and the Kentucky Soybean Promotion Board. This paper (No. 98-06-33) is published with the approval of the Director of the Kentucky Agricultural Experiment Station.

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