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Two critical factors are required for efficient transformation of multiple soybean cultivars: *Agrobacterium* strain and orientation of immature cotyledonary explant

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Abstract An efficient transformation system was developed for multiple soybean [*Glycine max* (L.) Merrill.] cultivars using *Agrobacterium*-mediated gene transfer. A significantly high number of hygromycin-resistant somatic embryos (SEs) was obtained when immature zygotic cotyledons were inoculated with *Agrobacterium tumefaciens* strain KYRT1 and when the abaxial side of explants was oriented upwards (i.e., the adaxial side of explants was in contact with the medium). Most hygromycin-resistant SEs on selective medium were induced along the periphery of the abaxial side of cotyledonary explants. Extended periods of selection (up to 10 weeks post-cocultivation) increased the frequency of somatic embryogenesis, and more than 50% of selected SEs tested positive for β -glucuronidase (GUS). Following maturation and regeneration of selected SEs, ten independent transgenic soybean plants of cv Jack were obtained, and the overall transformation frequency ranged from 1.1 to 1.7%. Six and two transgenic plantlets were obtained from cvs Dwight and Williams, respectively. In addition, transgenic suspension lines were established from cvs Jack, Williams, Dwight, Rend and Ina. Molecular analysis of embryogenic lines and/or transgenic plants, established from different cultivars, confirmed stable integration, expression, and/or inheritance of transgenes in both T₀ and T₁ plants.

Keywords *Agrobacterium tumefaciens* · *Glycine max* · Immature zygotic cotyledons · KYRT1 · Soybean · Genetic transformation

Introduction

Soybean [*Glycine max* (L.) Merrill.] is an important leguminous seed crop as it is an economic source of both oil and protein. Developing an efficient genetic transformation technology for soybean should facilitate physiological and molecular biology studies as well as the improvement of elite cultivars for desirable agronomic traits. Moreover, the rapid ongoing progress in functional genomic studies of the soybean has increased the demand for a routine and efficient transformation system. Although the first production of fertile transgenic soybean plants has been reported about 10 years ago (Hinchee et al. 1988; McCabe et al. 1988; Finer and McMullen 1991), current transformation protocols for soybean remain inefficient and limited to a few cultivars (Trick and Finer 1997).

Various efforts have been made to overcome problems associated with host/tissue specificity of *Agrobacterium* as well as the low transformation efficiency. These include modifying the virulence of *Agrobacterium tumefaciens* strains (Hood et al. 1993; Torisky et al. 1997), sonication of explant tissues to increase the number of infection sites (Santarem et al. 1998; Trick and Finer 1998), and addition of thiol compounds to the cocultivation medium (Ohhoft and Somers 2001; Olhoft et al. 2001). In a comparative study to evaluate virulence of different strains of *A. tumefaciens* on soybean explants, strain KYRT1 was reported to be more virulent than other commonly used strains, including EHA105, GV3850 and LBA4404 (Torisky et al. 1997; Meurer et al. 1998). However, no transgenic plants have been recovered or reported via transformation with strain KYRT1.

Transgenic soybean plants have been developed using either *Agrobacterium*- or particle bombardment-mediated transformation methods in conjunction with either shoot meristems, cotyledonary nodes or cultured embryogenic tissues (Hinchee et al. 1988; McCabe et al. 1988; Finer and McMullen 1991; Di et al. 1996; Trick and Finer 1998). However, these protocols yield low efficiencies of transformation, and are highly genotype-dependent.

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Moreover, observed chimerism and/or sterility in regenerated transformed soybean plants have been problematic.

Immature zygotic cotyledons are not considered ideal target tissues for soybean transformation due to the limited source of explants and low efficiency of embryogenesis (Parrott et al. 1989; Liu et al. 1992). Instead, cultured embryogenic tissues derived from immature cotyledons have been commonly used as target tissues in both *Agrobacterium*-mediated and direct gene-transfer methods (Finer and McMullen 1991; Trick and Finer 1998). However, direct selection and regeneration of transformed somatic embryos on solid-based medium (Parrott et al. 1989) offer several advantages over a regeneration system involving a liquid-based medium (Finer and Nagasawa 1988). A major advantage is the elimination of all lengthy steps for embryogenic culture establishment prior to and following transformation and selection. More importantly, this solid-based medium approach may possibly avoid problems of sterility of regenerated plants often associated with aging embryogenic cultures (Hadi et al. 1996; Simmonds and Donaldson 2000).

Using immature zygotic cotyledons as target tissues for *Agrobacterium*-mediated transformation, and the recovery of transgenic soybean plants have been first reported by Parrott et al. (1989); however, all three primary transformed plants were chimeric. Recently, Yan et al. (2000) obtained fertile transgenic soybean plants using immature zygotic cotyledons. However, the transformation frequency was reported to be low, and recovered plants were likely to be of clonal origin.

In this study, we describe an efficient *Agrobacterium*-mediated transformation system for soybean using immature zygotic cotyledons. The use of an appropriate *A. tumefaciens* strain, combined with the choice of orientation of an explant for transformation, resulted in the development of a highly efficient system for transformation and recovery of fertile transgenic soybean plants.

Materials and methods

Soybean cultivars

Soybean [*G. max* (L.) Merrill.] cvs Jack (MG II), Williams (MG III), Ina (MG IV), Macon (MG III), Dwight (MG III) and Rend (MG IV) were used. All plants were grown under greenhouse conditions with a 14-h photoperiod at 28 °C. Young pods containing immature embryos (5–8 mm in length) were collected, and used for transformation experiments.

Binary vectors and *Agrobacterium* strains

The plasmid pCAMBIA1305.1 (CAMBIA, Canberra, Australia), a compact binary vector (11.8-kbp), is used in this study. This plasmid contains the *uidA* gene, designated GUS^{Plus}™ with an intron from the castor bean catalase gene to confirm detection of plant-specific β -glucuronidase (GUS) expression, and a hygromycin resistance gene (*hpt*) for selection (Fig. 1). In addition, a chitinase gene expression cassette, under the control of the cauliflower mosaic virus 35S promoter and the NOS-3' terminator, was constructed using pBI221 (Clontech) and a maize chitinase cDNA clone (Wu et al. 1994). This was then subcloned into the *EcoRI*/*HindIII* site of pCAMBIA1305.1, and renamed pCMCHI (Fig. 1).

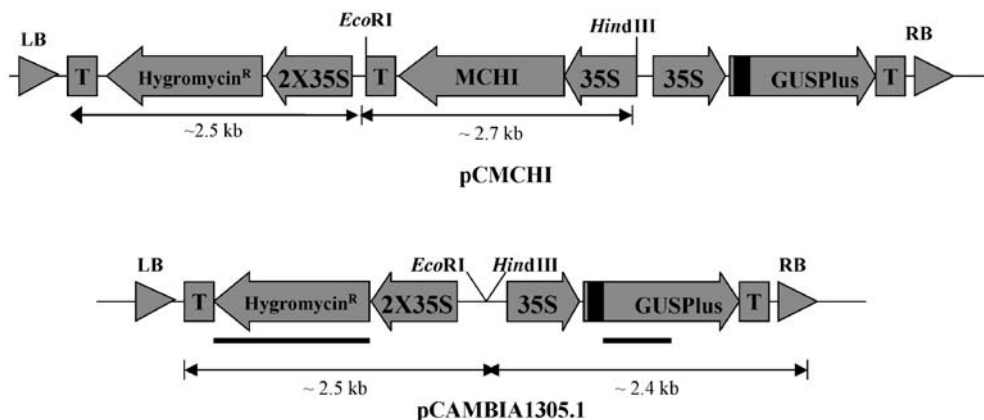
pCAMBIA1305.1 was immobilized into each of three *A. tumefaciens* strains, including KYRT1 (Torisky et al. 1997), EHA105 (Hood et al. 1993) and GV3101 (Koncz and Schell 1986), via electroporation (Duke-Ras and Hooykaas 1995) for initial transformation experiments. The binary vector pCMCHI was introduced into *A. tumefaciens* strain KYRT1. All *A. tumefaciens* strains containing binary vectors were maintained on solid LB media supplemented with 50 mg/l of rifampicin, and 150 mg/l of kanamycin.

A single colony from each bacterial strain was transferred to 5 ml of liquid LB medium with appropriate antibiotics, and grown overnight at 28 °C. Then, 500 μ l of *Agrobacterium* cells were re-cultured in 50 ml of liquid LB medium until OD₆₀₀ of 1.3–1.5. The bacterial culture was centrifuged at 3,000 rpm for 10 min, and the resulting pellet was washed once with liquid MSD40 medium (Finer and Nagasawa 1988). Following a second centrifugation, the pellet was resuspended in liquid MSD40 medium supplemented with 100 μ M of acetosyrigone and OD₆₀₀ was adjusted to 0.5.

Preparation of explants and cocultivation

Young pods were soaked in 70% 2-isopropanol with vigorous shaking for 30 s, and surface-sterilized in 25% Clorox (0.105% sodium hypochlorite) commercial bleach for 20 min, and rinsed three times with sterilized distilled water. Immature seeds were

Fig. 1 Diagrammatic representation of the T-DNA regions of pCAMBIA1305.1 and pCMCHI. The relative location of GUS (*GUSPlus*), hygromycin resistance (*HPT*) and maize chitinase (*MCHI*) genes are shown. 35S, 35S promoter; 2 \times 35S, doubled 35S promoter; RB, right border; LB, left border; T, poly-adenylation sequence



aseptically removed from pods. After cutting-off and removing the side-end containing the embryonic axis, seeds were wounded using a home-made Bio-prong tool that produces an identical number and location of wounding sites on each of the cotyledons. This Bio-prong tool consists of a scalpel blade with a permanently attached sharpened multi-needle wounding prong. Immature cotyledons were then forced out of the seed coat, and incubated in an *Agrobacterium* suspension for 30 min. Then, cotyledons were briefly blotted onto filter paper, placed on 0.2% gelite-solidified MSD40 medium (pH 7.0) containing 100 μ M of acetosyringone, and cocultivated in the dark at 25 °C for 3 to 4 days.

Selection of transgenic embryos and regeneration of plants

Following cocultivation, explants were washed three times in sterilized-distilled water, and then rinsed with a liquid MSD40 medium containing 500 mg/l of cefotaxime. Explants were blotted dry onto a sterile paper towel, and placed on a solid MSD40 medium containing 10 mg/l of hygromycin and 500 mg/l of cefotaxime (D40H10) for 2 weeks. Then, these were transferred to an MSD40 medium containing 25 mg/l of hygromycin (D40H25), and cultured for an additional 2 weeks. Explants, both responding and non-responding to 2,4-D treatment, were continuously cultured onto D20 medium (Bailey et al. 1993) containing 20 mg/l of 2,4-D, 25 mg/l of hygromycin and 500 mg/l of cefotaxime (D20H25). Explants were subcultured on this medium for three times at 2-week intervals. The number of responding explants and somatic embryos produced from each subculture interval were recorded.

To regenerate whole plants, green somatic embryos induced on explants were carefully removed under a dissecting microscope, and directly placed on a solid maturation medium (MSM6) (Bailey et al. 1993). The maturation medium was supplemented with 10 mg/l of hygromycin and 500 mg/l of cefotaxime (MSM6H10). After 8 weeks of maturation, with 2-week subculture intervals, mature somatic embryos were then desiccated in empty Petri dishes (100 mm \times 15 mm) for 5–7 days. A small piece (approximately 10 mm³) of MSM6 medium was also placed in dishes to allow gradual desiccation of embryos. Then, these desiccated embryos were transferred to MS0 regeneration medium (Parrott et al. 1988) containing 500 mg/l of cefotaxime until germination. Regenerated plantlets were transferred to magenta boxes containing a soil mix (1:1:1 of soil, peat and perlite), and covered with a plastic bag for 1–2 weeks. The plastic bag was gradually opened to allow for acclimatization of plantlets. Acclimatized plants were then transferred to the greenhouse for flowering and seed set.

To establish proliferative embryogenic suspension cultures, transformed green somatic embryos collected from explants were individually transferred to 12-well plates (Corning no. 353043) containing 2 ml of FG medium (Finer and Nagasawa 1988) for 4 weeks. The FG medium contained MS basal salts (Sigma), 60 g/l of sucrose and 5 mg/l of 2,4-D, but was supplemented with 15 mM of glutamine instead of asparagine. Individual embryogenic clusters developing in each well were then transferred to 125-ml Erlenmeyer flasks containing 35 ml of liquid FG medium supplemented with 5 mg/l of hygromycin and 500 mg/l of cefotaxime. These were cultured on a gyratory shaker at 125 rpm, and subcultured to fresh medium once every 10 days to establish embryogenic transgenic lines. All cultures described above were maintained at 23 to 26 °C under a 23 h photoperiod.

Assay for β -glucuronidase activity

The histochemical GUS activity (Jefferson et al. 1987) was measured using either whole explants, selected somatic embryos at 4–10 weeks post-cocultivation, suspension culture lines derived from selected somatic embryos, or immature seeds from transgenic plants. Tissues were incubated for 24 h at 37 °C in a buffer consisting of 0.1 M NaPO₄ (pH 7.0), 0.1 M potassium ferrocyanide, 0.1 M ferrocyanide, 0.2 M EDTA (pH 7.0), 0.05% triton X-100 with 500 mg/l of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-

glu) (Gold Biotechnology, St. Louis). Following incubation, tissues were destained with 100% ethanol, if necessary, and then visually observed for blue staining.

Analysis of soybean genomic DNA

Total genomic DNA was isolated from either transformed plants or embryogenic suspension culture lines using a Phytopure DNA extraction kit (Amersham). Ten to twenty micrograms of high-molecular-weight DNA was completely digested with *Hind*III (a single restriction site in the plasmid). The digested DNA fragments were fractionated on a 0.8% agarose gel, and were blotted onto a Zeta-probe membrane using 0.4 N of NaOH. The 820-bp PCR-fragment containing the GUS coding region and the 1,090-bp *Xho*I fragment containing the HPT coding region of pCAMBIA1305.1 were labeled with α [³²P]-dATP using a random primer DNA labeling system (Amersham), and were used as probes for hybridization. Prehybridization and hybridization were carried out in a hybridization solution (6 \times SSC, 5 \times Denhardt's, 10 mM of EDTA, 0.1% SDS, 200/l of denatured salmon sperm DNA) for overnight at 65 °C. The membrane was washed twice with 1 \times SSC/0.1% SDS and 0.1 \times SSC/0.1% SDS, respectively, at 65 °C. Hybridized membranes were exposed to Kodak XAR-5 film at –70 °C for 2–3 days.

Results

Effects of *Agrobacterium* strains and explant orientation

Among the three *Agrobacterium* strains and the two explant orientations used, immature cotyledons transformed with strain KYRT1 harboring the binary vector pCAMBIA1305.1 (Fig. 1) and subsequently incubated with their abaxial sides oriented upwards, were the most responsive on the selection medium, and resulted in the highest frequency (23%) of embryogenesis (Fig. 2). A mean of 1.9 hygromycin-resistant SEs per responding explant was obtained. Based on the GUS assay, 63% of hygromycin-resistant SEs obtained from these explants were GUS-positive. On the other hand, only 6% of explants transformed with strain KYRT1 and placed with the adaxial side oriented upwards were responsive. None of the stomatic embryos (SEs) obtained from these explants tested positive for GUS (Fig. 2).

The embryogenic response of cotyledons, transformed with either strain EHA105 or strain GV3101, was either low or completely inhibited, regardless of the explant orientation (Fig. 2). Only 3% and 2% of explants transformed with strains EHA105 and GV3101, respectively, and placed with the adaxial side oriented upwards were responsive. All SEs obtained from responding explants tested negative for GUS. Interestingly, explants cocultivated with strains EHA105 and GV3101 showed bacterial overgrowth, while those cocultivated with strain KYRT1 showed no bacterial overgrowth during cocultivation. The abaxial side of explants transformed with strain KYRT1 turned gelatinous and brown in color with collapsed cells, while that of explants transformed with strain GV3101 remained relatively hard and pale-green in color without any observed collapsed cells. Explants transformed with strain EHA105 showed intermediate

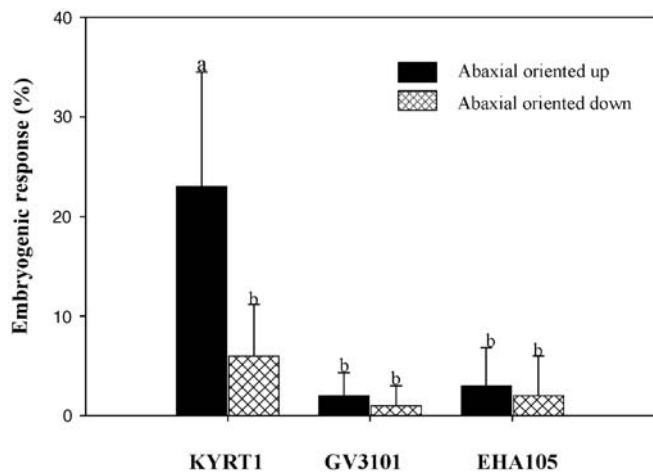


Fig. 2 Effects of *Agrobacterium* strains (KYRT1, GV3101 and EHA105) and explant orientation (abaxial side oriented up or down) on embryogenic response (% explants with SEs) of immature cotyledons (cv Jack) 4 weeks after cocultivation. For each treatment, 25 explants were used with four replications. Means were compared using Fisher's Least Significant Difference Test ($\alpha = 0.05$)

morphological response to those transformed with strains KYRT1 and GV3101 (data not shown).

Globular somatic embryos were first observed on immature cotyledons 4 weeks following cocultivation. These SEs formed mostly singly, but sometimes they formed in clusters (Fig. 3a and b). Hygromycin-resistant globular SEs, greenish-yellow to green in color, were readily distinguishable from hygromycin-susceptible escapes that were yellowish to white in color. Most GUS-positive SEs showed uniform blue staining of varying intensities (Fig. 3c).

Embryogenic regions along immature cotyledons were observed. SEs were mainly initiated along the margin areas of cultured cotyledons when the abaxial side was oriented upwards (Fig. 3a and b). Whereas, SEs were initiated adjacent to wounding sites in the middle region of the cotyledon when the adxial side of the cotyledon was oriented upwards (Fig. 3d). The influence of the explant orientation on induction of somatic embryogen-

esis was also confirmed by the histochemical GUS assay, whereby the location of strong GUS activity along the cotyledon was consistent with the location of somatic embryogenesis and explant orientation. The highest level of GUS expression was always observed along the margin areas of the abaxial side of the explant, while either low or no GUS activity was observed in the mid-region of the cotyledon or around wounding sites (Fig. 3e).

Sustained induction of somatic embryogenesis over extended periods of selection

Transformation of immature cotyledons of soybean cv Jack with strain KYRT1 consistently showed a higher embryogenic response when the abaxial side of the explant was oriented upwards. As SEs and embryo-like structures were observed on cultured cotyledons after 4 weeks following cocultivation, all explants were placed back to MSD20 medium supplemented with 25 mg/l of hygromycin. These explants were subcultured bi-weekly to fresh medium. Following extended periods of selection, from 6 to 10 weeks, additional SEs were induced on previously responding explants. Moreover, SEs were induced on those explants previously deemed non-responsive (Table 1). The embryogenic potential of cotyledonary explants continued to be high up to 8 weeks following cocultivation, and resulted in a combined total frequency of embryogenesis of 31% at 10 weeks following cocultivation. The percentage of GUS-positive SEs at different selection periods ranged between 53% and 66% (Table 1).

To assess the proliferative embryogenic capacity of SEs induced over the 10-week selection phase, ten green somatic embryos from each selection period were randomly picked to establish embryogenic suspension culture lines. Ten proliferative embryogenic culture lines from a total of 40 hygromycin-resistant SEs were established in FG liquid medium. Seven of these lines were confirmed to be GUS-positive. All of transgenic suspension culture lines were stably maintained for more than 6 months. These long-term transgenic embryogenic culture lines showed uniform blue-staining following the

Table 1 Embryogenic response on the abaxial surface of explants (cv Jack) at different selection intervals using *Agrobacterium*, KYRT1:pCAMBIA1305.1

Selection intervals ^a	Responding explants (%) ^b	No. of Hyg ^R SEs	GUS assay		
			No. of SEs tested ^c	No. of GUS + SEs	Transformation frequency (%)
4 weeks	29 (11%)	42	32	21	66%
6 weeks	21 (8.3%)	39	29	18	62%
8 weeks	25 (9.9%)	64	54	29	53%
10 weeks	4 (1.6%)	18	8	5	63%
Total	79/253	163	123	73	59%

^a Selection intervals represent weeks after cocultivation

^b No. of explants with embryogenic response represent transformed explants that gave rise to at least one somatic embryo during the selection period

^c Ten hygromycin-resistant somatic embryos from each selection intervals were taken to establish embryogenic suspension lines

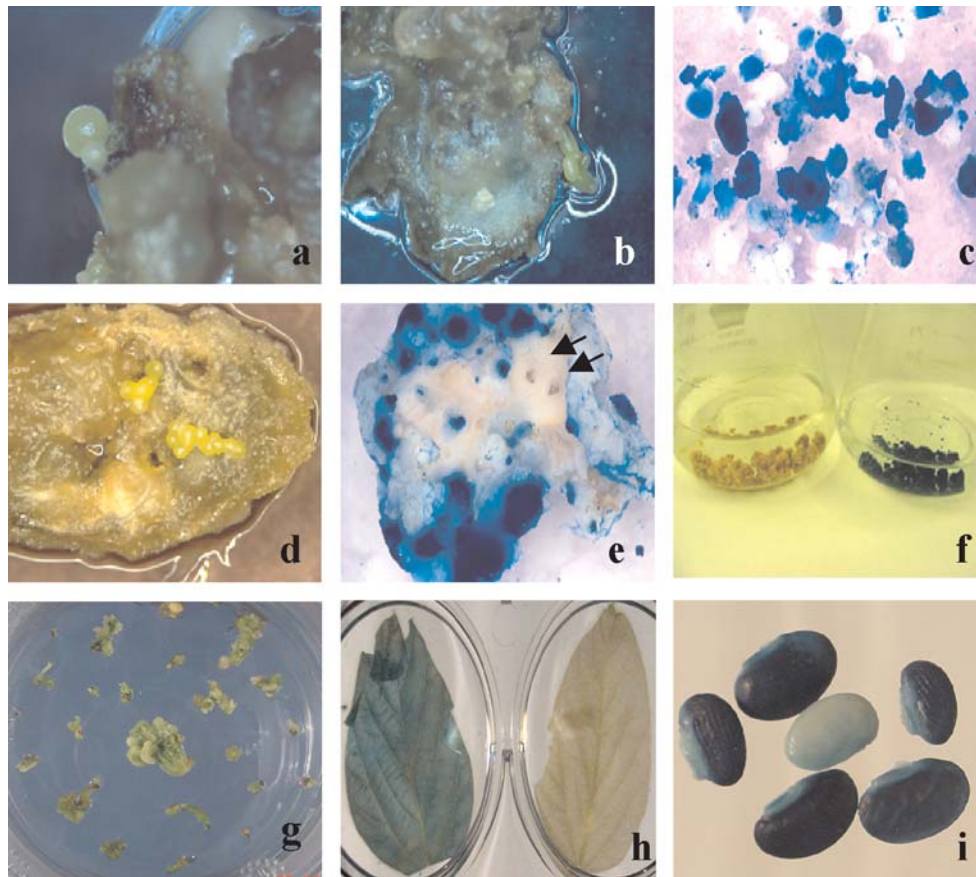


Fig. 3a–i Transformation, somatic embryogenesis under hygromycin selection, and recovery of transgenic plants. **(a)** Green globular and **(b)** mass of globular or oval-shaped somatic embryos emerging along the margins of an immature cotyledon under hygromycin selection 4 weeks after cocultivation. **(c)** Mixture of transformed and untransformed primary somatic embryos collected from responded cotyledons 4 weeks after cocultivation. **(d)** The adaxial side, which was placed up on the medium, produced somatic embryos mainly along the wounding sites. **(e)** Immature zygotic embryo showing dark-blue staining mainly along the margins of a

cotyledon 4 weeks after cocultivation under selection. *Arrows* indicate the wounding sites. **(f)** GUS expression in long-term embryogenic suspension culture (right) exhibiting uniform blue-staining. **(g)** Mature somatic embryos 8 weeks after culture on a selective maturation medium (MSM6H10). **(h)** GUS expression on a leaf from a putative transgenic plant growing in the greenhouse. **(i)** GUS expression in immature seeds of a primary transformant. Mixture of GUS-positive and negative was detected due to segregation

histochemical GUS assay. Chimerism (mixtures of transformed and untransformed embryogenic clumps), previously observed in some of the initial suspension culture lines, was not detected (Fig. 3f).

Recovery of putative transgenic plants of cv Jack

All hygromycin-resistant SEs, resulting from three separate transformation experiments using immature cotyledons of cv Jack transformed with either KYRT1:pCMCHI or KYRT1:pCAMBIA1305.1, were placed onto a maturation medium (MSM6) supplemented with 10 mg/l of hygromycin, and subcultured bi-weekly (Table 2). After 8 weeks of maturation, the frequency of mature SEs surviving on hygromycin selection (10 mg/l) ranged from 23% to 25% in three independent experiments. Mature SEs exhibited diverse morphology ranging from a single

dicotyledonous embryo to abnormal embryogenic clusters (Fig. 3g).

All mature embryos were subjected to a desiccation treatment, followed by germination on an MS0 regeneration medium (Parrott et al. 1988), and conversion into plantlets having both shoots and roots. A total of 15 putative transgenic plants, including each of four and three plants originating from same embryogenic cluster, were recovered from three independent experiments (Table 2). Any plantlets originating from the same cluster of mature SEs were counted as a single transformation event, and this was later confirmed in molecular analysis. Therefore, the overall transformation frequency (number of putative independent transformants per total number of immature cotyledons) ranged from 1.1% to 1.7% (Table 2). Histochemical GUS assays revealed strong enzymatic activities in either leaf tissues or immature seeds of all recovered plants (Fig. 3h and i).

Table 2 Summary of three independent experiments with KYRT1:pCAMBIA1305.1 or KYRT1:pCMCHI

Expt no.	Plasmids in KYRT1	Total no. of explants	Responding explants (%)	No. of SEs per responding explants	Total no. of Hyg ^R SEs	No. of mature SEs (%) ^a	No. of transgenic plants (%) ^b
I	pCAMBIA1305.1	225	82 (36%)	1.9	155	36 (23%)	4 (1.7%)
II	PCMCHI	233	100 (43%)	2.0	199	47 (24%)	3 (1.3%)
III	PCMCHI	267	118 (44%)	1.5	182	46 (25%)	3 (1.1%)

^a No. of mature SEs represent the total number of green and elongating SEs and SE clumps survived on MSM6 medium containing 10 mg of hygromycin and were scored 8 weeks after the maturation period

^b The frequency of independent transformation represents the percentage of the total number of transgenic plants per total number of explants transformed. Transgenic plants derived from the same transformation event were scored as a transgenic plant, based on Southern analysis

Table 3 Embryogenic response of immature cotyledons of five soybean cultivars following transformation with *Agrobacterium* strain KYRT1:pCAMBIA1305.1

Cultivars	Total number of explants	Responding explants (%)	Total no. Hyg ^R SEs	No. of SEs per resp. explants	No. of SEs used for maturation ^a	No. of mature SEs	No. of plantlets converted
Dwight	459	110 (24%)	197	1.8	147	42	6
Williams	488	94 (19%)	139	1.5	78	22	2
Ina	420	20 (5%)	25	1.3	NA	NA	NA
Rend	118	34 (29%)	75	2.2	45	15	0
Macon	348	3 (0.9%)	3	1.0	NA	NA	NA

^a The remaining hygromycin-resistant SEs were used for the destructive GUS assay and the establishment of embryogenic suspension cultures

Transformation of multiple soybean cultivars

Immature cotyledonary explants of all five cultivars, transformed with strain KYRT1 and cultured with the abaxial side oriented upwards, produced green globular SEs through 8 weeks of selection. Genotypic differences in the frequency of somatic embryogenesis were observed among the five soybean cultivars. Immature cotyledons of cvs Dwight, Rend and Williams were highly responsive to embryogenic induction, while those of cvs Ina and Macon exhibited relatively low response to embryogenic induction (Table 3). The frequencies of GUS-positive SEs observed among all cultivars, except for cv Macon, were similar, and ranged from 45% to 59%. Only three hygromycin-resistant SEs from a total of 348 explants of cv Macon were obtained, and a single SE was GUS-positive (data not shown). The percentage of mature SEs surviving on MSM6 medium containing 10 mg/l of hygromycin varied between 28% (cv Williams) to 34% (cv Rend) (Table 3). A total of six and two converted plantlets, developing shoots and roots, was obtained from mature SEs of cvs Dwight and Williams, respectively. Efforts to convert additional plantlets from mature SEs are underway.

The proliferative capacity of hygromycin-resistant SEs among the different cultivars was also evaluated by establishing embryogenic suspension culture lines. Eight to ten initial embryogenic lines out of 18 selected SEs from responding explants of each cultivar were recovered 3 to 4 weeks after culture of SEs in FG liquid medium. Although there were no considerable differences in the initial proliferative embryogenic potential among the four cultivars, the establishment of long-term embryogenic

cultures (more than 6 months) was genotype-dependent. Cv Ina exhibited the highest long-term embryogenic potential (seven transgenic lines); whereas, cvs Dwight, Williams and Rend showed lower embryogenic potentials, resulting in two, three and three transgenic lines, respectively (data not shown). Long-term embryogenic suspension cultures of cvs Dwight and Williams showed high turbidity, tissue browning and lack of friability.

Analysis of transgenic plants for stable integration and inheritance of transgenes

Southern-blot analyses of putative transgenic plants (Table 2) and transgenic suspension lines (Tables 1 and 3) were conducted to confirm stable integration of the introduced *uidA* and *hpt* genes into the soybean genome. The unique *Hind*III site in each of the two plasmids pCAMBIA1305.1 and pCMCHI was used to discriminate integrated from non-integrated forms of the plasmids. The distances of the left and right borders to the *Hind*III restriction site in these two plasmids were indicated in Fig. 1. The pattern in Southern hybridizations using *uidA* and *hpt* probes clearly indicated that transgenes linked with T-DNA were randomly integrated into the soybean genome (Figs. 4 and 5). No DNA hybridizations with these probes were observed in both an untransformed plant (Fig. 4, lane C) and an untransformed embryogenic culture line (Fig. 5, lane C). Several transgenic plants derived from the same embryogenic cluster showed identical integration events, confirming that these plants were derived from the same transformation event (Fig. 4, lanes G2–G5 and G6–G8). All transgenic plants (ten

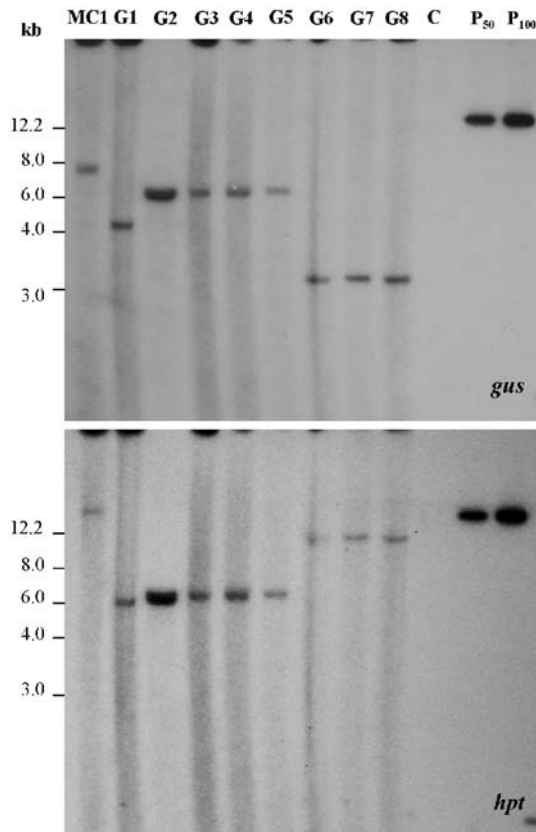


Fig. 4 Southern-blot analysis of genomic DNA from representative T_0 transgenic plants (cv Jack). Total genomic DNA (approximately 10 μ g) was digested with *Hind*III, which recognizes a single site within pCAMBIA1305.1 or pCMCHI, and hybridized with the *uidA* (upper panel) and *hpt* (lower panel) probes. Lanes MC1 and G1–8, with DNAs from transgenic plants transformed with pCMCHI and pCAMBIA1305.1, respectively; C, DNA from wild-type soybean plant; P₅₀ and P₁₀₀ represent *Hind*III-digested DNAs (50 pg and 100 pg) of pCMCHI. The size of the DNA marker is indicated on the left lane

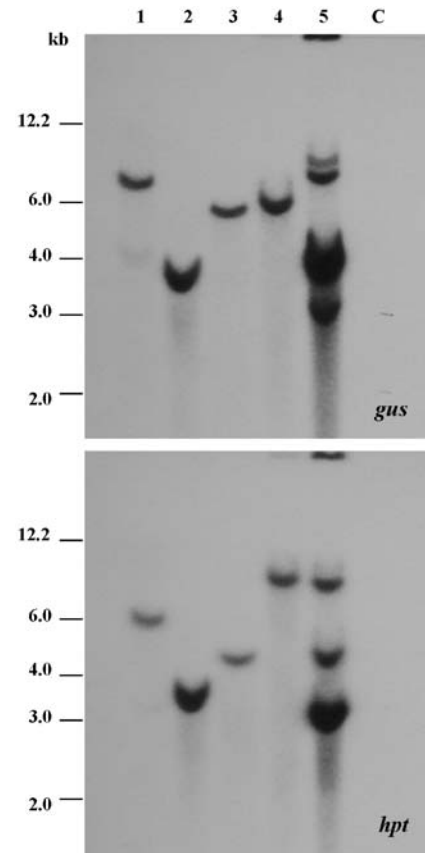


Fig. 5 Southern-blot analysis of genomic DNA from representative transgenic suspension culture lines. *Hind*III-digested DNA from transgenic lines (cvs Ina, Williams and Dwight) transformed with pCAMBIA1305.1 and from a transgenic line (cv Jack) transformed with pCMCHI was hybridized with the *uidA* and *hpt* probes. Lanes 1, 2, 3, 4 and 5; DNA from transgenic suspension lines of cvs Ina, Williams, Dwight and Jack, respectively; C, DNA from an untransformed suspension culture line. The size of the DNA marker is indicated as shown in Fig. 4

independent transformants) showed the presence of a single copy of the introduced transgenes, *uidA* and *hpt*. Most transgenic embryogenic suspension lines, established from somatic embryos of multiple soybean cultivars, including Jack, Williams, Dwight, Ina and Rend, showed simple integration patterns with either one or two transgene copy numbers integrated into the genomic DNA. Only one suspension culture line was identified to possess at least three copies of the transgenes (Fig. 5, lane 5).

The stability of the introduced DNA in primary transformants was evaluated by GUS expression and Southern-blot analysis (Fig. 6). Selfed progeny (T_1) of 3 out of 10 primary independent transformants (T_0) showed the expected 3:1 segregation ratio ($P = 0.8$ – 1.0), suggesting a simple Mendelian inheritance of the *uidA* transgene in both analyses, and indicating that the transgenes are integrated at a single locus (Table 4). Further statistical analysis with larger sample sizes of T_1

Table 4 Segregation for the *uidA* transgene in T_1 soybean seedlings transformed with *Agrobacterium* strain KYRT1 containing pCAMBIA1305.1 and pCMCHI

Transgenic line	Plasmid	GUS expression and Southern analysis		Segregation ratio	Chi-square test ($\alpha = 0.05$)	P (χ^2)
		No. of T_1 plants (+)	No. of T_1 plants (–)			
G1	PCAMBIA1305.1	17	3	3:1	0.053	0.823
G2	PCAMBIA1305.1	12	8	3:1	0.120	0.729
G3	PCAMBIA1305.1	13	7	3:1	0.053	0.823
MC1	pCMCHI	15	5	3:1	0	1.000

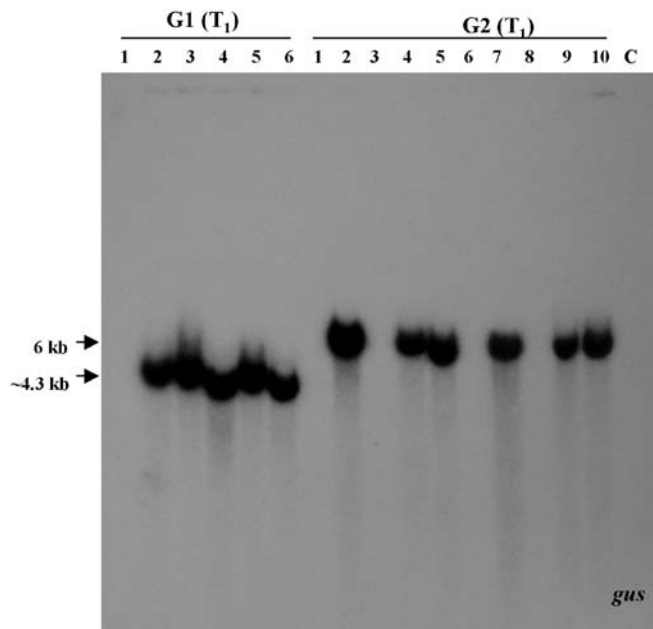


Fig. 6 Southern-blot analysis of soybean genomic DNAs from T₁ plants of two primary transformants (*G1* and *G2* in Fig. 4A) probed with ³²P-labeled *uidA*. Twenty micrograms of genomic DNA from each of T₁ plants was digested with *Hind*III

plants is required to determine the inheritance patterns of transgenes.

Discussion

Previous efforts undertaking *Agrobacterium*-mediated transformation of soybean have been characterized with low efficiency of transformation and high genotype-dependent influences (Parrott et al. 1989; Yan et al. 2000). In this report, a reliable protocol for soybean transformation has been developed. This protocol is characterized by a high efficiency of transformation, and is applicable to multiple elite soybean genotypes. It has been determined that a particular orientation of immature cotyledons (abaxial side placed upward on the medium) along with an appropriate strain of *A. tumefaciens* (KYRT1) are critical for induction of embryogenesis under hygromycin selection.

Santarem et al. (1997) have indicated that cotyledonary explants incubated with the adaxial side oriented upwards on the induction medium have exhibited a higher frequency of embryogenesis than those oriented downwards. Thus, previous protocols for soybean transformation with immature cotyledons have relied on placing explants with the adaxial side oriented upwards on the selection medium (Santarem et al. 1998; Yan et al. 2000). However, this is in contrast to the results obtained in this study whereby embryogenesis of immature cotyledons on the selection medium is higher when the abaxial side is oriented upwards.

Collins et al. (1991) have previously described the effects of explant orientation on the location of embryogenesis along immature cotyledons whereby SEs are reported to initiate along either the margins or central portions of either the abaxial side or the adaxial side of cotyledons (oriented upwards), respectively. Santarem et al. (1997) have also reported that SEs are mainly observed adjacent to wounding sites on the adaxial side of immature cotyledons. These observations are mostly consistent with our results, even though explants in this study have been incubated on the selection medium following transformation. GUS staining patterns along the adaxial side of explants have verified these explant orientation effects; i.e., strong blue staining is observed along the margins, while no blue-staining is observed around wounding sites of the abaxial side of immature cotyledons (Fig. 3d). These findings suggest that cells along the margins of cotyledons are competent to undergo somatic embryogenesis under hygromycin selection up to 10 weeks or longer following cocultivation. It is not clear whether somatic embryogenesis along the margin areas is inhibited when the adaxial side of cotyledons is oriented upwards. This may be due to the poor contact of the cells in those areas with the medium, thus limiting the influence of 2,4-D in inducing cell competence for embryogenesis as explants tend to curl upward and away from the medium.

The hypervirulent strain EHA105 has been commonly used for soybean transformation (Trick and Finer 1997, 1998; Simmonds and Donaldson 2000; Yan et al. 2000), although Meurer et al. (1998) reported that strain EHA105 was as effective as strain KYRT1 in promoting cotyledonary node transformation. However, in this study, comparisons among three *A. tumefaciens* strains showed that strains GV3101 and EHA105 were ineffective in producing transgenic SEs (Fig. 2). The highest number of hygromycin-resistant SEs was observed in explants transformed with strain KYRT1. Moreover, it is important to indicate that although immature cotyledons inoculated with strain KYRT1, exhibited partial browning and necrosis with collapsed cells during selection, explants remained highly competent for both transformation and subsequent embryogenesis. In fact, this observed morphology of explants served as a positive indicator for embryogenesis of cotyledons under selection. Bacterial overgrowth of explants transformed with strains GV3101 and EHA105, observed during cocultivation and selection periods, might have contributed to inhibition of somatic embryogenesis and to the inefficiency of transformation. The use of 500 mg/l of cefotaxime was effective in killing bacterial cells of strain KYRT1, but not those of strains EHA105 and GV3101. This bacterial overgrowth most likely contributed to detrimental effects on explants during cocultivation and throughout selection.

The levels of hygromycin concentration (10 mg/l and 25 mg/l) used in induction media, MSD40 and MSD20, did not inhibit the induction of non-transformed SEs. More than 40% of hygromycin-resistant SEs collected from explants during each selection interval were later

deemed as escapes (Table 1). However, continuous selection of explants on MSM6 maturation medium containing 10 mg/l of hygromycin for 8 weeks was quite effective in eliminating non-transformed SEs. This was likely due to the fact that individual SEs were in direct contact with the maturation medium containing hygromycin. Our results showed that all regenerated plants through this selection regime were confirmed to be transgenic using both GUS assay and Southern-blot analysis. In addition, all ten transgenic plants were identified to have integrated a single copy of the transgene. Simple integration patterns (1–2 copies) have also been observed in all transgenic suspension culture lines, except for one line (Fig. 5).

In this study, a high transformation frequency (1.1–1.7%) of cv Jack was obtained. This frequency is approximately 100-fold higher than that previously reported by Yan et al. (2000), and is also comparable to that reported (0.7–2.0%) by Hinchee et al. (1988). All transgenic plants recovered from this protocol are fertile and appear to be normal. This is probably due to their relatively short in vitro culture duration, about 4–5 months from initiation of somatic embryos to the transfer of regenerants to soil, compared to the liquid-based regeneration protocol that requires a period of more than 9 months (Finer and Nagasawa 1988). As many mature SEs, that germinate but do not convert into plantlets, produce secondary SEs on a regeneration medium, attempts to regenerate plants from these secondary SEs are underway. This may also contribute to increasing the frequency of soybean transformation obtained thus far.

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