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Bialaphos-resistant Transgenic Soybeans Produced by the *Agrobacterium*-mediated Cotyledonary-node Method

S. C. Liu[1, 2], G. C. Zhang[2], L. F. Yang[2], M. Mii[3], J. Y. Gai[1], and Y. L. Zhu[1, 2*]

ABSTRACT

A stable *Agrobacterium*-mediated transformation system was established using bialaphos as the selective agent in soybeans [*Glycine max* (L.) Merr.]. The cotyledonary node explants of the soybean cultivar 'NY-1001' were inoculated with the *Agrobacterium tumefaciens* strain EHA105, harboring the vector pCAMBIA3301 containing the *gus* gene as the reporter gene and the *bar* gene conferring bialaphos resistance. The highest frequency of GUS transient expression (92%) was obtained after inoculation and 4-day co-cultivation with *A. tumefaciens* strain EHA105. Efficient GUS expression was observed in regenerated shoots from explants after 4-day co-cultivation combined with culturing on shoot induction medium (SIM) without bialaphos for 7 days followed by 4 mg.L^{-1} bialaphos for 2 weeks. Bialaphos (4 mg.L^{-1} in SIM; 2 mg.L^{-1} in shoot elongation medium (SEM)) effectively selected the transformants. The putative transformants and escapes could be exactly distinguished by using a half-leaf GUS assay method to detect GUS expression in the elongated resistant shoots, which resulted in the shortening of culture period for the early detection of transformed shoots. The transformation efficiency of this system was 1.06%. The transgenic plants were verified by polymerase chain reaction (PCR), Southern blotting, and herbicide-resistant responses. All four T_0 transgenic plants were fertile and transmitted the phenotypes of both *gus* and *bar* in a 3:1 ratio to their progeny. These results indicate that the established system is suitable for further breeding of herbicide-resistant transgenic cultivars, as well as for functional genomics studies of soybeans.

Keywords: Bar gene, *Glycine max*, Genetic transformation, Herbicide-resistance, Transgene inheritance

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is not only an important source of plant oil and protein (Rafiee *et al.*, 2009) but also used as a model plant in functional genomic studies. Genetic transformation techniques for improving the soybean's agronomic traits require an efficient method of plant regeneration and transgene integration. Regeneration ability and transformation competency are determined by genotype and explant source (Yamada *et al.*, 2012). Two major methods of soybean transformation are commonly used in transgenic studies. One is particle bombardment of embryogenic tissues (Aragão *et al.*, 2000; Finer and McMullen, 1991; Trick and Finer, 1998) and the other involves the *Agrobacterium*-mediated transformation of cotyledonary nodes (Clemente *et al.*, 2000; Hinchee *et al.*, 1988; Paz *et al.*, 2004; Zeng *et al.*, 2004; Zhang *et al.*, 1999). *Agrobacterium*-mediated transformation offers several advantages, such as simple transgene insertions and a low copy number,

[1] National Center for Soybean Improvement, Nanjing Agricultural University, Nanjing 210095, People's Republic of China.
[2] College of Horticulture, Nanjing Agricultural University, Nanjing 210095, People's Republic of China.
[*] Corresponding author; e-mail: ylzhu@njau.edu.cn
[3] Graduate School of Horticulture, Chiba University, 648 Matsudo, Matsudo-Shi, Chiba, 271-8510, Japan.

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stable integration and inheritance, and consistent gene expression over the generations. Embryogenic tissues transformed by particle bombardment require a prolonged tissue culture period compared with *Agrobacterium*-mediated transformations. Additionally, the quality, quantity, and regeneration competency of somatic embryos are largely genotype-dependent (Meurer *et al.*, 2001; Donaldson and Simmonds, 2000; Tomlin *et al.*, 2002). Furthermore, the complex insertion patterns of target genes that occur by particle bombardment may generate sterile lines (Liu *et al.*, 1996; Reddy *et al.*, 2003; Singh *et al.*, 1998). Consequently, *Agrobacterium*-mediated transformation remains an effective method for genetically transforming plants.

An efficient *Agrobacterium*-mediated soybean transformation requires considering several factors, including the plant genotypes, sources of explants, *Agrobacterium* strains, selection agents, and culture conditions (Santarem *et al.*, 1998). In soybeans, transforming the cotyledonary node explants from various genotypes by adding thiol compounds to the co-cultivation medium (CCM) has significantly increased the transformation efficiencies (Liu *et al.*, 2008; Olhoft *et al.*, 2001; Olhoft and Somers, 2001; Paz *et al.*, 2006; Zeng *et al.*, 2004).

Selecting the transformed cells is especially important when using cotyledonary node explants (Olhoft *et al.*, 2007). The *bar* gene from *Streptomyces hygroscopicus* encodes the enzyme phosphinothricin acetyl transferase (PAT) that detoxifies phosphinothricin (PPT) (Botterman and Leemans, 1988), and has been used as a selectable marker for plant transformation (D'Halluin *et al.*, 1992). Both glufosinate and bialaphos were used as selection agents for the *bar* gene because they all contain PPT as the active ingredient and have been used to select herbicide-resistant plants (De Block *et al.*, 1989; Dennehey *et al.*, 1994; Paz *et al.*, 2004; Zhang *et al.*, 1999). In maize, bialaphos is a more potent selection agent than glufosinate (Dennehey *et al.*, 1994; Karaman *et al.*, 2012). Transformed soybeans have been successfully produced using glufosinate as the selective agent of the *bar* gene (Olhoft *et al.*, 2007; Paz *et al.*, 2004; Xing *et al.*, 2000; Yamada *et al.*, 2010; Zeng *et al.*, 2004; Zhang *et al.*, 1999). Paz *et al.*, (2004) assessed the efficacy of two selective agents in soybean transformation and found that glufosinate was more effective. To date, most studies on transgenic herbicide-resistant soybeans focused on glufosinate, but we faced difficulty in using glufosinate as a selective agent in our preliminary study.

The objective of this study was to establish a stable *Agrobacterium*-mediated transformation system using bialaphos as the alternative selective agent in the cotyledonary-node method of soybean transformation.

MATERIALS AND METHODS

Plant Material and Seed Germination

The soybean cultivar 'NY-1001' was used as the experimental host, which was based on the GUS transient expression frequencies of sixteen soybean cultivars (Supplemental Table 1). Dry seeds were surface sterilized with chlorine gas (Di *et al.*, 1996) for 6 h before germinating them on Gamborg's B5 medium (Gamborg *et al.*, 1968) at 25 ℃ under cool white fluorescent lamps (90 $\mu mol.m^{-2}.s^{-1}$) in a 18/6 hour (light/dark) photoperiod for 5 days.

Agrobacterium Preparation

The *Agrobacterium tumefaciens* strain EHA105 carrying the binary vector pCAMBIA3301 was used for soybean transformation. The plasmid pCAMBIA3301 contained the intron-*gus* gene as a reporter gene and the *bar* gene, which confers bialaphos resistance (Ganesan

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Supplemental Table 1. Comparison of GUS transient expression frequencies of cotyledonary node explants after co-cultivation with *A. tumefacines* among different soybean cultivars.

Cultivar	GUS transient expression frequency (%)
NY-1001	70.89 a [a]
Liaoxian	26.65 b
Taiwan75	23.44 bc
Taiwan 292	23.12 bc
Baifenghuang	22.35 bc
Xiafeng 2008	20.27 cd
Lüling No. 7	20.06 cd
Lixiang 95-1	17.43 cd
Ribenqing	17.26 cd
Lüling 303A	13.33 e
Lülingtezao	13.00 e
Lüling No. 7	11.86 e
Zhenong 2	11.52e
Lixiang M-5	11.04 e
Lüling No. 3	7.90 f
Chunfenzao	5.86 f

[a] The different small letters indicate significant difference by Tukey's test at $P< 0.05$ after arcsine transformation.

et al., 2012); both genes are under control of the cauliflower mosaic virus (CaMV) 35S promoter. The binary vector pCAMBIA3301 was transformed into the *A. tumefaciens* strain EHA105 using the freeze-thaw method (An *et al.*, 1988). Transformants were selected on YEB medium containing 50 $mg.L^{-1}$ kanamycin (Ti plasmid pCAMBIA3301 drug marker) and 50 $mg.L^{-1}$ rifampicin (EHA105 chromosomal drug marker) (Sokhandan-Bashir *et al.*, 2012). The integrity of the binary vector in the *Agrobacterium* strains was confirmed by bacterial PCR and restriction enzyme digestion.

A. tumefaciens glycerol stocks of EHA105/pCAMBIA3301 stored at -80 ℃ were streaked on solidified YEP medium containing 50 $mg.L^{-1}$ rifampicin and 50 $mg.L^{-1}$ kanamycin and were incubated at 28 ℃ until they formed colonies. A single colony was inoculated in 5 mL liquid YEP medium containing two types of antibiotics (rifampicin and kanamycin), and shaken overnight at 175 rpm at 28 ℃ by a desktop thermostat oscillator (THZ-C, Taicang Huamei Biochemistry Instrument Factory, Suzhou, China). Then, 500 µL cultures was transferred into 50 mL of fresh YEP medium, and shaken at 175 rpm at 28 ℃ until the OD_{650} reached 0.8. The *Agrobacterium* cultures were centrifuged at 3,500 rpm for 10 minutes, and the pellet was re-suspended in a liquid co-cultivation medium (CCM), containing 1/10 B5 medium, 1.67 $mg.L^{-1}$ BAP, 0.25 $mg.L^{-1}$ GA_3, 200 µM acetosyringone, 20 mM MES and 3% sucrose, pH 5.4 (Olhoft *et al.*, 2003). The liquid *Agrobacterium* suspension was adjusted to an OD_{650} of 0.8 before infection.

Bialaphos Response Test

Table 1. Effect of different concentrations of bialaphos on the regeneration of soybean cv. NY-1001.

Bialaphos concentration ($mg.L^{-1}$)	Total no. of treated explants	No. of differentiated shoots	Regeneration efficiency (%) [a]
1	60	31	51.7a
2	60	25	41.7b
3	60	8	13.3c
4	60	3	5.0d
5	60	0	0.0e
6	60	0	0.0e

[a] The regeneration efficiency was calculated [(No. of differentiated shoots/Total no. of treated explants)×100%]. Percentage data were subjected to an arcsine square root transformation (Bartlett, 1947), and followed by Tukey's test at $P< 0.05$.

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A range of selection concentrations was evaluated using sixty explants for each experiment. The experiment was conducted with three replications. The cotyledonary node explants were cultured on shoot regeneration medium (SRM) (B5 medium, 1.67 mg.L^{-1} BAP, 3% sucrose, 0.7% agar, pH 5.6) supplemented with bialaphos (Cat.#B0178-100, Goldbio, USA) at concentrations of 1, 2, 3, 4, 5, or 6 mg.L^{-1}. After 2 weeks, the numbers of adventitious shoots were counted to determine growth inhibition.

Plant Transformation

Explant Inoculation

The cotyledonary node explants were prepared from 5-day-old seedlings (Zhang *et al.*, 1999). Two explants were obtained from a single seedling in the following manner: the seedlings were excised by keeping 2-3 mm of the hypocotyls below the cotyledons. Two cotyledons were separated vertically along the hypocotyl axis, and the preformed axillary shoots were removed completely with a #23 scalpel blade (Shanghai Medical Instruments Ltd., Corp., China). Then, explants were wounded by making 5-7 slight horizontal cuts in the meristematic tissues of the cotyledonary node.

Fifty explants were incubated in the 25 mL *A. tumefaciens* suspension for 30 minutes. Explants (15 per plate) were plated adaxial side down on sterile #1 Whatman paper (GE Healthcare, UK) placed on solid co-cultivation medium (CCM) (1/10 B5 medium, 1.67 mg.L^{-1} BAP, 0.25 mg.L^{-1} GA_3, 200 µM acetosyringone, 20 mM MES, 3% sucrose, 1 mM dithiothreitol, 1 mM L-cysteine, 1 mM $Na_2S_2O_3$, 0.7% agar, pH 5.4) in Petri dishes (90 diameter×20 mm deep).

Evaluation of Co-cultivation Time

A range of co-cultivation times was evaluated using fifty explants for each experiment. The experiment was conducted with three replications. The inoculated cotyledonary node explants were cultured on CCM for different periods (2, 3, 4, and 5 days) in the dark. After co-cultivation, the explants were first cultured on SIM (B5 medium, 1.67 mg.L^{-1} BAP, 500 mg.L^{-1} carbenicillin, 3% sucrose, 3 mM MES, 0.7% agar, pH 5.6) without bialaphos for 7 days, and then on fresh SIM containing 4 mg.L^{-1} bialaphos for 2 weeks. GUS expression of the adventitious shoots was examined after 3 weeks of shoot induction. The optimum co-cultivation time was evaluated based on GUS expression in the cotyledonary nodes and regeneration of the bialaphos-resistant shoots.

Selection and Regeneration

After co-cultivation, the explants were washed with liquid SIM (without bialaphos) 4-5 times. The explants (five per plate) were cultured on SIM without bialaphos for 7 days with the adaxial side up (Olhoft *et al.*, 2006). Explants that showed initiation of shoot differentiation were transferred to fresh SIM with 4 mg.L^{-1} bialaphos. Explants were then subcultured every 2 weeks on the same selection medium.

After cultivation on selection medium for 4 weeks, explants with differentiated shoots were transferred to shoot elongation medium (SEM) (MS salts, MS iron (100 µM ferrous sulfate, 100 µM NaEDTA), B5 vitamins, 1 mg.L^{-1} zeatin, 0.5 mg.L^{-1} GA_3, 0.1 mg.L^{-1} IAA, 100 mg.L^{-1} pyroglutamic acid, 50 mg.L^{-1} asparagine, 300 mg.L^{-1} carbenicillin, 2 mg.L^{-1} bialaphos, 3% sucrose, 3 mM MES and 0.8% agar, pH 5.6). The explants were subcultured with fresh SEM every 2 weeks for 2 months. Elongated GUS positive shoots (3-4 cm in height) were excised for rooting on rooting medium (RM) (1/2 B5 salts, MS iron, 0.5 mg.L^{-1} IAA, 3% sucrose and 0.8% agar, pH 5.6) for 3 weeks. The regenerated plantlets that grew with healthy roots were transferred to artificial substrate

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(50% vermiculite/50% peat) in pots in the greenhouse to mature.

GUS Expression Analysis

A GUS histochemical assay was used not only for optimizing the transformation procedure by detecting the GUS expression efficiency in the cotyledonary nodes, but also for identifying the transgenic events. The fresh plant materials were placed in the GUS buffer [80 mM Na_2HPO_4 (pH 8.0), 8 mM Na_2EDTA, 0.8% (v/v) Triton X-100, 1.6% (v/v) dimethyl sulfoxide, 20% (v/v) methanol, 0.38 mM $K_4Fe(CN)_6$, 1 mM X-gluc (Goldbio, USA)] for overnight at 37 ℃ (Jefferson *et al.*, 1987) and the chlorophyll was then removed in 95% ethanol. Photographs were taken using a stereomicroscope (SZX10, Olympus Corporation, Japan) equipped with a CCD camera (DP72, Olympus Corporation, Japan).

Basta-resistant Analysis

The herbicide resistance of T_0 putative transgenic plants was identified by painting 0.5% Basta® (200 $g.L^{-1}$ glufosinate-ammonium, BAYER, Germany) solution with a cotton swab on the upper surface of the leaves. The leaves were scored for herbicide tolerance 10 days after application.

Plant Genomic DNA Isolation and PCR Analysis

Genomic DNA was isolated from leaves (0.2 g) of transgenic and control plants using the SDS method (Ma and Sorrells, 1995). Primers for *bar* and *gus* genes were designed according to Choi *et al.* (2007). The 413-bp *bar* coding region was amplified using primers (forward 5′-GCACCATCGTCAACCACTAC-3′ and reverse 5′-TGAAGTCCAGCTGCCAGAAAC-3′). The 944-bp *gus* coding region was amplified using primers (forward 5′-TGGTGACGCATGTCGCGCAAGAC-3′ and reverse 5′-GGTGATGATAATCGCCTGATGCAG-3′). PCR reactions were conducted using a thermal cycler (TProfessional, Biometra, Germany). Each PCR reaction mixture contained 5.0 µl of the 10x *Taq* DNA polymerase buffer, 4.0 µl of dNTP mix (2.5 mM), 3.0 µl of $MgCl_2$ (25 mM), 1.0 µl of each primer (20 µM), 0.2 µl of *Taq* DNA polymerase ($5U.\mu l^{-1}$) (Takara, Japan), and 1 µl of template DNA ($50\ ng.\mu l^{-1}$) in a 50 µl final volume. The amplification program for the *bar* primers was as follows: 95 ºC for 5 minutes for initial denaturation, then 30 cycles at 95 ºC for 2 minutes for denaturation, 55 ºC for 1 minute for primer annealing, and extension at 72 ºC for 1 minute, followed by a final extension at 72 ºC for 10 minutes. For the *gus* fragment, the PCR cycle was as follows: 95 ºC for 5 minutes for initial denaturation, then 30 cycles at 95 ºC for 45 seconds for denaturation, 55 ºC for 45 sec for annealing, and extension at 72 ºC for 45 seconds, followed by a final extension at 72 ºC for 10 minutes. The amplified products were separated by electrophoresis in a 1% agarose gel and were stained with $0.5\ \mu g.ml^{-1}$ ethidium bromide and visualized under UV light in gel documentation apparatus (JS-380, Shanghai Peiqing Science and Technology Co., Ltd., China).

Southern Blotting Analysis

Genomic DNA was extracted from soybean leaf tissues using the SDS protocol. Genomic DNA (20 µg) was completely digested overnight with *Eco*R I (Promega, USA). Digested DNA fragments were separated by electrophoresis in a 0.8% agarose gel and transferred to a Hybond-N^+ nylon membrane (GE Healthcare, UK). Probe labeling and Southern hybridization were performed using DIG High Prime DNA Labeling and Detection Starter Kit I

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according to the manufacturer's instructions (Roche Applied Science, Germany). The 413-bp fragments of *bar* were labeled with digoxigenin-dUTP and used as the probe for hybridization.

Progeny Segregation Analysis

The progeny derived from independent transgenic T_0 soybean plants were evaluated for GUS expression and tolerance to the herbicide by testing leaves of self-pollinated plants 3 weeks after sowing. The chi-squared (χ^2) analysis was conducted to determine if the observed segregation ratio was consistent with Mendelian segregation in the T_1 generation.

Statistical Analysis

The data (Tables 1 and 2) were presented as the mean of three replicates and were tested using analysis of variance (ANOVA) with SPSS 20 software (SPSS Inc., Chicago, USA). Levels of significance were indicated by Tukey's test at $P< 0.05$.

RESULTS

Selection of Bialaphos Concentration

Soybean cotyledonary node explants derived from 5-day-old seedlings were transferred to SRM containing various concentrations of bialaphos to determine the appropriate selection concentration (Table 1). The number of explants with regenerated shoots significantly decreased with increasing concentrations of bialaphos (Supplemental Figure 1). No shoots were formed at 5 $mg.L^{-1}$ bialaphos. Only 5% of the explants had regenerated shoots at 4 $mg.L^{-1}$ bialaphos. In this study, a bialaphos concentration of 4 $mg.L^{-1}$ was used to select transformants.

Evaluation of Co-cultivation Period

Table 2. Effect of co-cultivation time on transient GUS expression.

Co-cultivation time (days)	No. of tested explants	No. of GUS^+ explants	No. of GUS^+ explants with dark blue region[a]	Percentage of GUS transient expression (%)[b]
2	50	4.7	0.0	9.3c*
3	50	23.3	8.3	46.7b
4	50	46.0	19.3	92.0a
5	50	45.7	16.7	91.3a

[a]The dark blue stained regions indicate GUS expression over more than 70% of the cotyledonary node, [b] The transient expression percentage of explants was calculated by [(No. of GUS^+ explants/No. of tested explants)×100%].

* The different small letters indicate significant difference within the same column by Tukey's test at $P< 0.05$.

Supplemental Figure 1. Effect of bialaphos concentrations on the differentiation of multiple shoots from cotyledonary nodes of 'NY-1001'. The numerals indicate the concentrations of bialaphos ($mg{\cdot}L^{-1}$).

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After infection with *A. tumefaciens*, GUS expression was detected after different co-cultivation times (Table 2). The transient GUS expression frequency increased from 2 to 5 days of co-cultivation. GUS expression efficiencies at 4-5 days reached 92%.

Both GUS-stained areas of the cotyledonary nodes and the number of regenerated shoots with GUS expression derived from cotyledonary nodes were influenced by co-cultivation time, and the extent of GUS expression increased with increased co-cultivation time (Figures 1A-C). Although the GUS expression frequencies were similar between 4 days and 5 days of co-cultivation, 5 days of co-cultivation resulted in overgrowth of *A. tumefaciens* and caused a high degree of browning and wrinkling of the explants (Figure 1-D).

After co-cultivating for different time periods, explants were cultured first on SIM without the selective agent for 7 days and then were cultured on fresh SIM containing 4 $mg.L^{-1}$ bialaphos for 2 weeks. After shoot induction for 3 weeks, the transformation status of the explants was re-examined by GUS assay (Figures 1E-H). The explants that were co-cultivated for 2 days exhibited a lower GUS expression after 3 weeks of shoot induction (Figure 1-E). The explants that were co-cultivated for 3 days only showed GUS expression at the base of the young shoot (Figure 1-F). The explants that were co-cultivated for 4 days had GUS expression throughout the entire young shoot (Figure 1-G). The explants that were co-cultivated for 5 days were unable to form shoots and only showed tiny GUS spots at the surface of the cotyledonary node (Figure 1-H). Thus, a co-cultivation period of 4 days was assumed to be the most favorable both for a higher transient GUS expression efficiency and for facilitating further regeneration of the transformed shoots.

Regeneration of Transformed Plants

The transformation process of cotyledonary nodes from the soybean cultivar 'NY-1001' using bialaphos as the selective agent is illustrated in Figure 2. Explants were obtained from 5-day-old aseptic seedlings (Figure 2-A). From three independent experiments, a total of 501 explants (Table 3) were infected with *A. tumefaciens* and were then co-cultured on CCM for 4 days (Figure 2-B). After co-cultivation, the explants were first cultured on SIM without bialaphos for 7 days and were then cultured on SIM containing 4 $mg.L^{-1}$ bialaphos for 3 weeks. After shoot induction for 4 weeks, the explants with shoot differentiation (Figure 2-C) were transferred onto SEM with 2 $mg.L^{-1}$ bialaphos. After culturing for 4 weeks, shoots were elongated (Figures 2-D and -E). Twelve GUS positive shoots reached 3-4 cm in height after a culture period of 2 months for elongation (Figures 2-F and -G). The maximum of early transformation efficiency (Bleho *et al.*, 2012) reached 2.66% (Table 3). Seven GUS positive shoots were successfully rooted on rooting medium for at least 3 weeks (Figure 2-H). Four rooted

Table 3. Transformation efficiency of soybean cv. 'NY-1001' using cotyledonary nodes.

Experiment no.	No. of tested explants (A)	GUS^+ shoots[a] (B)	Early transformation efficiency (B/A, %)	$Southern^+$ plants[b] (C)	Final transformation efficiency (C/A, %)
1	163	4	2.45	1	0.61
2	150	3	2.00	1	0.67
3	188	5	2.66	2	1.06

[a] GUS^+ shoots were based on the half-leaf GUS assay method, [b] Southern blotting was based on T_0 plants with pod-setting.

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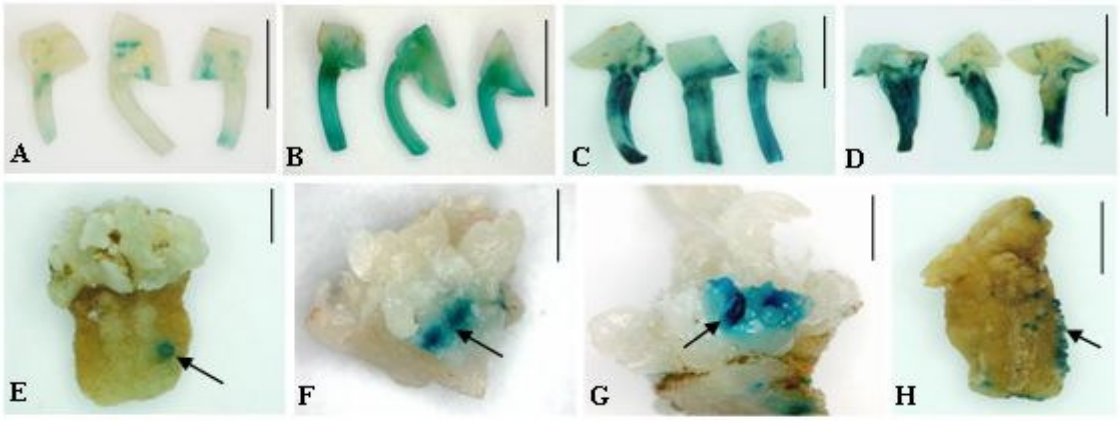

Figure 1. Transient GUS expression patterns on the cotyledonary node explants. After co-cultivation for 2 days (A), 3 days (B), 4 days (C), 5 days (D), and (E-H) after 3 weeks of shoot induction for the previous co-cultivation periods, respectively. *Bar* = 0.5 cm.

plantlets eventually survived in pots in the greenhouse to mature for about 2 months (Figure 2-I). The maximum of final transformation efficiency was 1.06% (Table 3).

GUS Expression of Transgenic Plants

GUS expression was observed in various tissues of transgenic plants by histochemical analysis (Figure 3). Stable GUS expression was detected in leaves (Figure 3-A) and stems (Figures 3-B,-C) excised from the elongated shoots. When the GUS-positive shoots were rooted and grown up in the greenhouse, GUS expression could be detected in the flowers (Figures 3-D and -E), pericarps (Figure 3-F), and seeds (Figure 3-G). In addition, the *gus* gene was inherited by the T_1 generation because stable GUS expression could be detected in the cotyledons (Figure 3-H) and roots (Figure 3-I) from the geminated T_1 seeds.

Molecular Analyses of Transgenic Plants

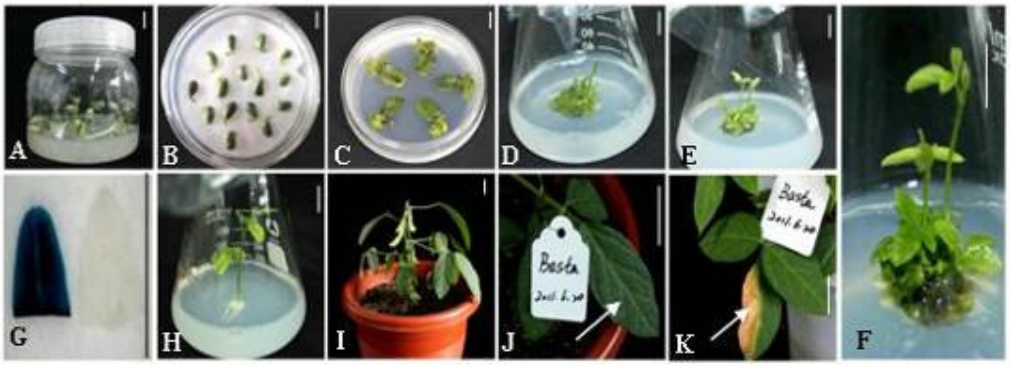

Figure 2. *Agrobacterium*-mediated transformation system of soybean using cotyledonary nodes and bialaphos for selection: (A) The 5-day-old aseptic seedlings; (B) Co-cultivation; (C) Shoot induction; (D) and (E) Shoot elongation; (F) The elongated shoots reached 3-4 cm in height; (G) GUS detection; (H) Rooting; (I) Maturity, (J) and (K) Basta resistance test. *Bar*= 1.0 cm.

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PCR analysis was conducted to confirm the integration of the alien genes into the genome of the transgenic soybean plants. The expected 413-bp band (for the *bar* gene) and 944-bp band (for the *gus* gene) were detected in the soybean genome (Figure 4). These PCR results confirmed that the *bar* and *gus* genes had been successfully introduced into the genome of the transgenic plants.

Southern blotting analysis was carried out to further confirm the transgenic nature of the PCR-positive plants. *Bar* was detected in the four T_0 transgenic plants (Table 3), whereas no hybridization signal was observed in the non-transformed control plants (Figure 5, Lane C). The number of hybridization signals showed that the four T_0 plants all had a single copy (Figure 5) in their genomes. The results also indicated that these plants were derived from independent transformation events. Thus, Southern analysis confirmed the integration of the *bar* gene in the transformants.

In addition to the molecular confirmation of the transgenic plants by PCR and

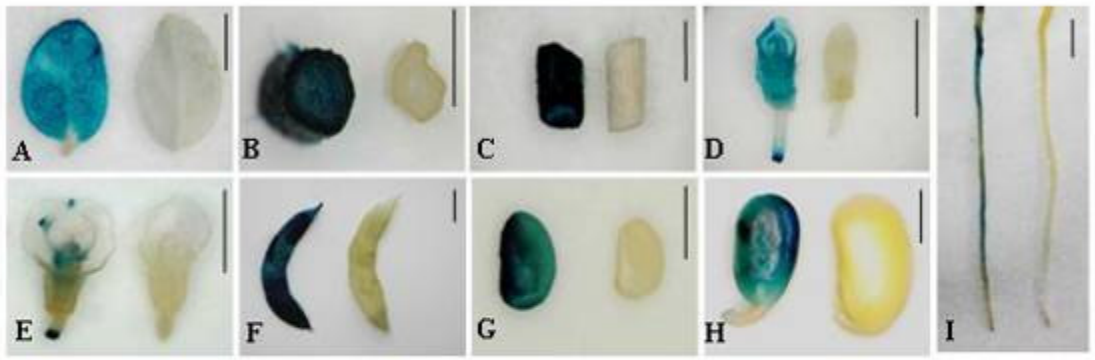

Figure 3. Stable GUS expression in various organs of transgenic plants (the left from each slide) in contrast to non-transformed plants (right), (A-G) from T_0, (H) and (I) from T_1. (A) Leaflet; (B) Transverse stem section; (C) Longitudinal part of the stem; (D) and (E) Flowers at different development stages; (F) Pericarp; (G) Seed; (H) Cotyledon, (I) Root from 7-day old seedling. *Bar*= 0.5 cm.

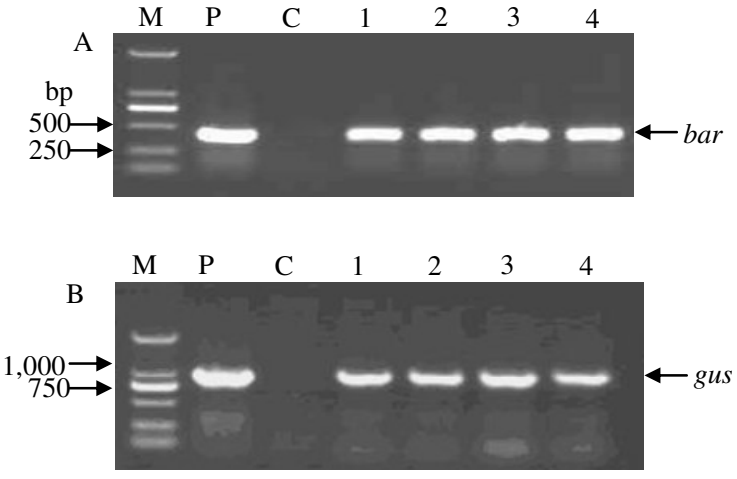

Figure 4. PCR analysis of transgenic soybean plants. (A) The 413-bp fragment of the *bar* gene. (B) The 944-bp fragment of the *gus* gene. Lane M: DL2000 Marker; Lane P: pCAMBIA3301 plasmid (positive control); Lane C: Wild-type plant (negative control), Lanes 1-4: T_0 transgenic plants.

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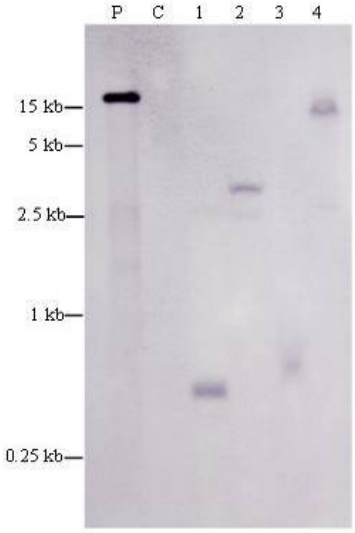

Figure 5. Southern blotting analysis of four individual transgenic T_0 plants. Lane P: pCAMBIA3301 digested with *Eco*R I (positive control); Lane C: Genomic DNA from wild-type plant (negative control), Lanes 1-4: Genomic DNA from transgenic plants.

Southern hybridization, a functional analysis of the *bar* gene was also conducted by Basta painting to verify the herbicide resistance of the transgenic plants. Healthy leaves from transgenic plants and non-transgenic plants (control) were painted with a 0.5% Basta solution. The transgenic plants showed resistance to Basta, while the control plants showed necrosis (Figures 2-J and -K). This result verified the functional expression of the *bar* gene in the transgenic plants.

Progeny Analysis

Segregation analyses of *gus* and *bar* genes were performed on the progenies (T_1 lines) of four independent T_0 plants with one copy of the transgene. The Chi-squared (χ^2) analysis indicated that four T_1 generations segregated in a Mendelian fashion (3:1) ($P< 0.05$) (Table 4).

The inheritance of *gus* gene in T_2 transgenic progenies was further confirmed (Figure 6). GUS expression was detected both in germinating seeds (Figure 6-A) and leaves (Figure 6-B). PCR analysis of *gus* gene from the leaf samples indicated that a 944-bp fragment was amplified (Figure 6-C). These results verified the transgenes were inherited into T_2 generation.

DISCUSSION

A successful method of *Agrobacterium*-mediated transformation was established for producing herbicide-resistant transgenic soybean plants using cotyledonary nodes as explants and bialaphos as the selective agent. Bialaphos was effective for selecting transformants with the genomic integration of *bar* gene in the soybean cultivar 'NY-1001'.

Bialaphos, a tripeptide antibiotic produced by *Streptomyces hygroscopicus*, has been used as the selection agent for the *bar* gene in many transgenic plants, such as *Brassica* (De Block *et al.*, 1989), snapdragon (Hoshino and Mii, 1998), and alfalfa (Montague *et al.*, 2007). Paz *et al.* (2004) assessed the selection schemes using glufosinate and bialaphos as selective agents. Their results indicated that

Table 4. Segregation analysis of T_1 transgenic lines.

T_0 line	No. of T_1 plants				χ^2 value[a] (3:1)	*P*-value[b]
	GUS^+	GUS^-	Bar^+	Bar^-		
1	13	2	13	2	1.09	0.30
2	7	1	7	1	0.67	0.41
3	10	5	10	5	0.56	0.45
4	7	3	7	3	0.13	0.72

[a] Data were based on the GUS histochemical assay for the *gus* gene and the Basta-resistant analysis for the *bar* gene, [b] A single degree of freedom was used to obtain *P*-values. $\chi^2_{0.05,1}= 3.84$.

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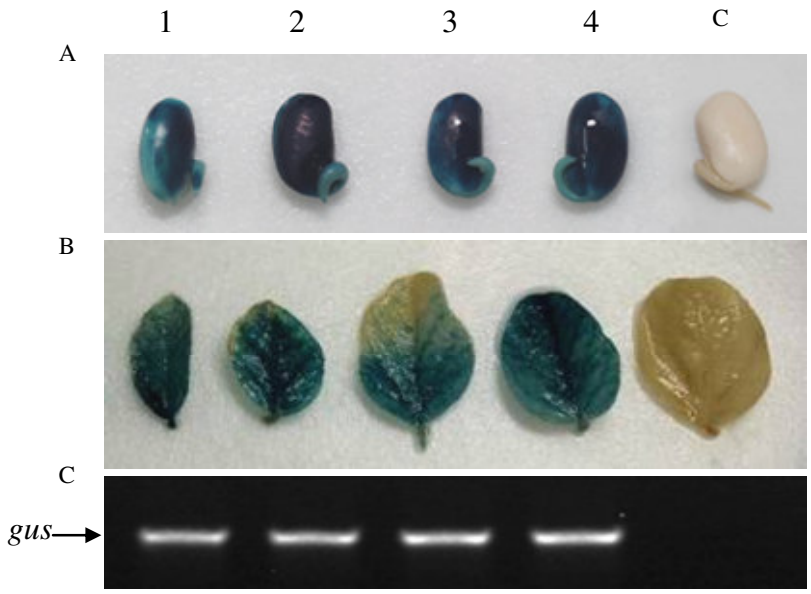

Figure 6. The inheritance of *gus* gene in T_2 transgenic progenies. (A) Seeds after germination for 24 hours; (B) Leaves of 3-week-old seedling were assayed, (C) PCR detection from leaf samples. Lanes 1-4: T_2 progeny samples derived from four independent T_0 transgenic plants; Lane C: wild-type control.

glufosinate selection was effective for certain kinds of soybean cultivars. However, our preliminary experiment showed that glufosinate might not be applicable to some genotypes of soybean since it caused severe tissue necrosis of explants even at a low concentration (2 $mg.L^{-1}$) and no shoot formation in the 'NY-1001' cultivar (data not shown), indicating that this cultivar was very sensitive to glufosinate. In contrast, bialaphos selection did not result in tissue necrosis of explants at concentrations of up to 8 $mg.L^{-1}$ in 'NY-1001' and suppressed shoot regeneration of the cotyledonary node explants at a concentration of 4 $mg.L^{-1}$ in the present study (Table 1) without tissue necrosis. Therefore, this concentration of bialaphos was used to select the transformed shoots. In addition, we also tried to use hygromycin as selective agent. Although the transformation system was established, vitrification and abnormality of transformed shoots were observed (Supplemental Figure 2).

Co-cultivation time of 3 days (Clemente *et al.*, 2000; Zhang *et al.*, 1999) and 5 days (Olhoft *et al.*, 2003) has been previously reported for the successful transformation of soybean. In the present study, the highest frequency (92%) of transient GUS expression was found using a co-cultivation time of 4 days (Table 2), which initiated resistant shoots after 3 weeks of culture on shoot induction medium (Figure 1-G) and finally transgenic plants. However, after 5 days, the explants were under the state of prolonged co-cultivation and the *Agrobacterium* cells were in the status of overgrowth, which finally resulted in the softening, shrinking (Figure 1-D), and death of the explants and the survival was very poor. This result coincides with the reports of other researchers (Sumithra *et al.*, 2010; Menges and Murray, 2006).

The recovery of regenerated resistant shoots was influenced by the concentration of the selection agent. Donaldson and Simmonds (2000) reported that the most significant obstacles for creating transgenic soybean plants using the cotyledonary node method was the inefficient targeting of competent cells in the cotyledonary node and not poor susceptibility to *Agrobacterium*. A strict selection

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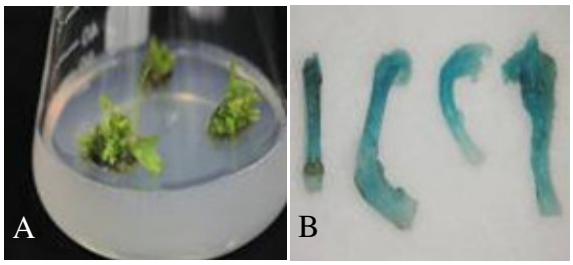

Supplemental Figure 2. Vitrification (A) and abnormality (B) of transformed shoots under hygromycin selection.

concentration was usually applied for plant transformation to reduce the frequency of escapes (Li *et al.*, 2004; Zeng *et al.*, 2004). However, Goodwin *et al.* (2005) suggested that if the transformation frequency of the targeted plant species was very low, a relatively mild selective pressure with a higher percentage of escapes was preferable to avoid losing rare transgenic plants. In general, soybeans have been considered to be recalcitrant to genetic transformation. In the present study, bialaphos concentration at 5 $mg.L^{-1}$ completely inhibited shoot regeneration in not only non-infected control explants but also *Agrobacterium*-infected ones (Table 1). Consequently, we used 4 $mg{\cdot}L^{-1}$ bialaphos as the primary selective concentration during the early stages (3 weeks) of induction for transformed shoots. Since the shoots were obtained at this bialaphos concentration (Figure 1-G), the half-leaf GUS assay method (Figure 2-G) was applied to identify the GUS positive shoots, which was beneficial not only to keeping the positive shoots growing normally but also to reducing the selective pressure toward the elimination of escapes. Additionally, the half-leaf GUS assay method resulted in the shortening of culture period for the early detection of transformed shoots. Selection regimes based on PPT often led both to slow death of non-transformed shoots and to a long time culture for the screening of putative transformants (Olhoft *et al.*, 2003). Unhealthy shoots which exhibited stunted growth and low surviving rate were caused by prolonged culture of soybean tissues in selective medium (Olhoft *et al.*, 2003; Jin *et al.*, 2006; Olhoft and Somers, 2007). In the present transformation system, the half-leaf GUS assay method was used to detect GUS expression in the resistant shoots at the early stage of shoot formation and identify the transformants with a short culture period.

T_1 seeds were successfully harvested from all the four T_0 transformed plants. The results of GUS staining and Basta painting indicated that the introduced alien genes were inherited to the T_1 generation. The *gus* and *bar* genes were segregated in a 3:1 ratio in the progeny of four T_0 transgenic plants (Table 4). All T_0 plants had a single copy of transgene (Figure 5) segregated in a 3:1 ratio suggesting that the transgene segregated as a single locus. The integration of the *bar* gene into the soybean genome was confirmed by Southern blotting analysis, suggesting that the transgenic plants contained a low copy number of *bar* genes (Figure 5), which is desirable for using the transgenic plants in a breeding program for herbicide resistance.

In summary, fertile transgenic plants with bialaphos resistance were successfully obtained from the soybean cultivar 'NY-1001' by *Agrobacterium*-mediated transformation using cotyledonary node explants. Bialaphos was used to select transformants with genomic integration of the *bar* gene, which conferred herbicide resistance in this soybean cultivar. This stable transformation system can serve as a tool for introducing agronomically important genes into soybean plants with the aid of *bar* gene and lays the foundation for further

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breeding of herbicide-resistant soybean cultivars.

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Abbreviations

BAP: 6-Benzylaminopurine; GA_3: Gibberellic acid; GUS: β-Glucuronidase; IAA: Indole-3-acetic acid; MES: 2-(N-Morpholino) ethanesulfonic acid; PPT: Phosphinothricin, X-gluc: 5-Bromo-4-chloro-3-indolyl-β-glucuronide

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تولید سویای تراریخته مقاوم به بیالافوس با روش گره لپه ای به میانجی اگروباکتریوم

س. چ. لیو، گ. س. ژانگ، ل. ف. یانگ، م. می، ج. ی. گائی، و ی. ل. ژوو

چکیده

در این پژوهش, یک سیستم پایدار تراریختی به میانجیگری اگروباکتریوم ایجاد شد که در آن بیالافوس به عنوان عامل انتخاب سویا [*Glycine max* (L.) Merr.] بود. ریز نمونه ها ی سویا از گره لپه ای کالتیوار "NY-1001" برداشت شد و با ریسه EHA105 اگروباکتریوم تومفاسینس که حاوی ناقل pCAMBIA3301همراه با ژن *gus* به عنوان گزارشگر و ژن *bar* برای انتقال مقاومت به بیالافوس بود، تلقیح شدند. بیشترین فراوانی(بسآمد) تظاهر گذرای GUS به میزان 92٪ بعد از تلقیح و 4 روز هم کاشتی با ریسهEHA105 اگروباکتریوم تومفاسینس به دست آمد . کارآمدی بالای تظاهر GUS در ساقه های باززایی شده ریز نمونه ها بعد از 4 روز هم کاشتی همراه با کاشت روی محیط کشت القاگر ساقه(SIM) بدون بیالافوس به مدت 7 روز و به دنبال آن دو هفته بیافالوس به مقدار 4 میلیگرم در لیتر به دست آمد. در انتخاب تراریخته ها، بیالافوس (به مقدار 4 میلی گرم در لیتر در محیط SIMو 2 میلی گرم در لیتر در محیط توالی ساقهSEM) به گونه ای موثر عمل کرد. با استفاده از روش نیم قطعه سنجش گاس برای شناسایی تظاهر گاس در ساقه های رشد کرده مقاوم، تراریخته های مفروض و جا مانده به گونه ای دقیق قابل تمیز از هم بودند و این امر منجر به کوتاه شدن دوره کشت برای شناسایی زود هنگام ساقه های تراریخته شد. کارآیی تراریختن در این روش برابر 1.06٪ بود. گیاهان تراریخته با کار برد واکنش زنجیره ای پلیمراز(PCR) ، لکه گذاری ساترنی، و پاسخ مقاومت به علف کش اثبات شدند. همه چهار گیاه T_0 تراریخته بارور بوده و فنوتیپ هر دو ژن *gus* و *bar*را به نسبت 3:1 به فرزندان خود منتقل کردند.این نتایج حاکی از آن است که سیستم و روش به کار رفته در این مطالعه برای به نژادی کالتیوار های تراریخته برای مقاومت به علف کش و نیز مطالعات ژن نامه نویسی (ژنومیکس) عملیاتی سویا مناسب است.

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