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

S. C. Liu¹,², G. C. Zhang², L. F. Yang², M. Mii³, J. Y. Gai¹, and Y. L. Zhu¹,²*

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 T₀ 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,

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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 cotyledonal 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 cotyledonal 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 cotyledonal-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 °C under cool white fluorescent lamps (90 µmol.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
Bialaphos-resistant Transgenic Soybeans Produced

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

<table>
<thead>
<tr>
<th>Bialaphos concentration (mg.L⁻¹)</th>
<th>Total no. of treated explants</th>
<th>No. of differentiated shoots</th>
<th>Regeneration efficiency (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>31</td>
<td>51.7a</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>25</td>
<td>41.7b</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>8</td>
<td>13.3c</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>3</td>
<td>5.0d</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>0</td>
<td>0.0e</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>0</td>
<td>0.0e</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

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 ºC were streaked on solidified YEP medium containing 50 mg.L⁻¹ rifampicin and 50 mg.L⁻¹ kanamycin and were incubated at 28 ºC 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 ºC 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 ºC until the OD<sub>650</sub> 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<sub>3</sub>, 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<sub>650</sub> of 0.8 before infection.

Bialaphos Response Test

et al. (2012), 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.
A range of selection concentrations was evaluated using sixty explants for each experiment. The experiment was conducted with three replications. The cotyledonal 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 cotyledonal 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 cotyledonal 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\(_2\)S\(_2\)O\(_3\), 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 cotyledonal 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 cotyledonal 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.
(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 Na₂HPO₄ (pH 8.0), 8 mM Na₂EDTA, 0.8% (v/v) Triton X-100, 1.6% (v/v) dimethyl sulfoxide, 20% (v/v) methanol, 0.38 mM K₄Fe(CN)₆, 1 mM X-gluc (Goldbio, USA)] for overnight at 37 °C (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 T₀ putative transgenic plants was identified by painting 0.5% Basta₀ (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'-TGGTACGACATGTCCGCAGAC-3' and reverse 5'-GGTGATGATATCGCTGATGCAG-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 MgCl₂ (25 mM), 1.0 µl of each primer (20 µM), 0.2 µl of Taq DNA polymerase (5U µl⁻¹) (Takara, Japan), and 1 µl of template DNA (50 ng µl⁻¹) in a 50 µl final volume. The amplification program for the bar primers was as follows: 95 °C for 5 minutes for initial denaturation, then 30 cycles at 95 °C for 2 minutes for denaturation, 55 °C for 1 minute for primer annealing, and extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 10 minutes. For the gus fragment, the PCR cycle was as follows: 95 °C for 5 minutes for initial denaturation, then 30 cycles at 95 °C for 45 seconds for denaturation, 55 °C for 45 sec for annealing, and extension at 72 °C for 45 seconds, followed by a final extension at 72 °C for 10 minutes. The amplified products were separated by electrophoresis in a 1% agarose gel and were stained with 0.5 µg 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 EcoR I (Promega, USA). Digested DNA fragments were separated by electrophoresis in a 1% 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
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 T₀ 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 ($\chi^2$) analysis was conducted to determine if the observed segregation ratio was consistent with Mendelian segregation in the T₁ 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 explants 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.**

<table>
<thead>
<tr>
<th>Co-cultivation time (days)</th>
<th>No. of tested explants</th>
<th>No. of GUS⁺ explants</th>
<th>No. of GUS⁺ explants with dark blue region</th>
<th>Percentage of GUS transient expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>50</td>
<td>4.7</td>
<td>0.0</td>
<td>9.3c*</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>23.3</td>
<td>8.3</td>
<td>46.7b</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>46.0</td>
<td>19.3</td>
<td>92.0a</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>45.7</td>
<td>16.7</td>
<td>91.3a</td>
</tr>
</tbody>
</table>

*The dark blue stained regions indicate GUS expression over more than 70% of the cotyledonary node. **The transient expression percentage of explants was calculated by [(No. of GUS⁺ explants/No. of tested explants) x 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.L⁻¹).
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 cotyledonal 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 cotyledonal 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 cotyledonal nodes.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>No. of tested explants (^{(A)})</th>
<th>GUS(^{a}) shoots (^{(B)})</th>
<th>Early transformation efficiency (^{(B/A, %)})</th>
<th>Southern(^{a}) plants (^{(C)})</th>
<th>Final transformation efficiency (^{(C/A, %)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>163</td>
<td>4</td>
<td>2.45</td>
<td>1</td>
<td>0.61</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>3</td>
<td>2.00</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>188</td>
<td>5</td>
<td>2.66</td>
<td>2</td>
<td>1.06</td>
</tr>
</tbody>
</table>

\(^{a}\) GUS\(^{a}\) shoots were based on the half-leaf GUS assay method, \(^{b}\) Southern blotting was based on T\(_{0}\) plants with pod-setting.
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**
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 T₀ 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 T₀ 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 T₀ (H) and (I) from T₁. (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: T₀ transgenic plants.
Figure 5. Southern blotting analysis of four individual transgenic T₀ plants. Lane P: pCAMBIA3301 digested with EcoRI (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 (T₁ lines) of four independent T₀ plants with one copy of the transgene. The Chi-squared (χ²) analysis indicated that four T₁ generations segregated in a Mendelian fashion (3:1) (P < 0.05) (Table 4).

The inheritance of gus gene in T₂ 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 T₂ 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 T₁ transgenic lines.

<table>
<thead>
<tr>
<th>T₀ line</th>
<th>No. of T₁ plants</th>
<th>χ² valueᵃ (3:1)</th>
<th>P-valueᵇ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GUS⁺</td>
<td>GUS⁻</td>
<td>Bar⁺</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
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<td>13</td>
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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. χ²₀.₀₅.₁ = 3.84.
Bialaphos-resistant Transgenic Soybeans Produced

Figure 6. The inheritance of gus gene in T2 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: T2 progeny samples derived from four independent T0 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 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 cotyledonal node method was the inefficient targeting of competent cells in the cotyledonal node and not poor susceptibility to Agrobacterium. A strict selection
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 mgL\(^{-1}\) 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
breeding of herbicide-resistant soybean cultivars.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Yan-Ming Zhu (College of Life Sciences, Northeast Agricultural University, Harbin, China) for donating the soybean cultivar ‘NY-1001’. This work was supported by the National Key Project of GMO Crops (2009ZX08004-011B) of China. S. C. Liu and G. C. Zhang contributed equally to this work.

Abbreviations

BAP: 6-Benzylaminopurine; GA₃: 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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Bialaphos-resistant Transgenic Soybeans Produced


تولید سویا تراکمته مقاوم به بیالافوس با روشه لبه ای به میانجی اگروبیکریوم

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چکیده

در این پژوهش، یک سیستم پایدار تراکمته به میانجی‌گیری اگروبیکریوم ایجاد شد که در آن بیالافوس به عنوان عامل انتخاب سویا [Glycine max (L.) Merr.] برداشت شد و با ریسه EHA105 اگروبیکریوم تومفماسینس که حاوی ناقل gus به عنوان گزارشگر و ذهن برای انتقال مقاومت به بیالافوس بود، تلفیق شدند. پیشترین فراوانی (پس‌آمد) تظاهر گذشته GUS به میزان 92% بعد از تلفیق و 4 روز هم اگروبیکریوم تومفماسینس به دست آمد. کارآمدی بالایی تظاهر گذشته برای های پاسیفای ساقد به بیالافوس از دست رفت به تعداد 7 روز و به دنبال آن دو فته بیالافوس به مقدار 4 میلی‌گرم در لیتر به دست آمد. در انتخاب تراکمته های بیالافوس (به مقدار 4 میلی‌گرم در لیتر در محیط SIM و 2 میلی‌گرم در لیتر در محیط SIM سیستم تراکمته به گونه ای موتر عمل کرد. با استفاده از روش به دقت فلزی مشاهده شد. گونه ای دیمقی قابل تشخیص با 4 روز و این امر نشان دهنده به کوتاهی شدن دوره کشت برای شناسایی زودهنگام ساقه های تراکمته شد. کارآمدی تراکمته در این روش برابر 1.06% بود. گیاهان تراکمته با کار برده و اکتشین زنجبیل ای پیشرفت (CR) به کار گذاری سریالی، و پاسخ مقاومت به علیک کشف ایستوند شده.

در نتیجه این مطالعه گزارش به دو قیمت gus به نسبت 3:1 به فرندان با ترکیب 0.75 گیاه حجمی چهار گیاه bar تراکمته بارور بوده و روند به دو روز و همچنین به خود منقل کردن. این نتایج حاکی از آن است که میانجی و روشه به کار رفته در این مطالعه برای به نزدیکی تکثیره برای مقاومت به علیک کشف و نیز متغیرات ذهن نامه نویسی (زنویکس) عملیاتی سویا مناسب است.