Rapid And Efficient Selection For Transgenic Soybean Plants With The Improved Glufosinate Selection System*

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Abstract A rapid and efficient glufosinate selection system for obtaining high frequency of transformants insoybean [*Glycine max* (L.) Merrill] was developed. The cotyledonary node cells were wounded and inoculated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pCAMBIA3201 that contained a selectable *bar* gene and a *gus* reporter gene. The present standard selection based on glufosinate was performed at 5 mg/L glufosinate during shoot induction and at 3~5 mg/L glufosinate during shoot elongation. Many non-transformants were escaped with this standard selection system which resulted in low transformation efficiency of 1.6%, and a delayed shoot elongation with majority of transformed shoots elongation occurred from 21 to 41 weeks after *Agrobacterium* inoculation, and thus an improved glufosinate selection system was provided in this paper. After 3 weeks on shoot induction medium without glufosinate, the explants were transferred to shoot elongation medium containing 4 mg/L glufosinate for the first selection. The elongation of majority of transformed shoots occurred only from 7 to 12 weeks without glufosinate selection during shoot induction. The transgenic shoots were effectively screened by placing the excised shoots on the root induction medium (RIM containing 3 mg/L glufosinate to facilitate direct uptake of the selective agent that resulted in the high transformation efficiency of 6.7%. Shoots on the RIM rapidly responded to the selective agent applied, all the glufosinate sensitive shoots were completely necrotic within 10 days after selection. The majority of transgenic plantlets were obtained only 8~16 weeks under the improved selection system. Genomic Southern blot analysis confirmed stable integration of the transgenes in the genome of soybean. Stable expression was confirmed by GUS expression and herbicide application.

Key words *Agrobacterium tumefaciens*; Cotyledonary node; Glufosinate; Soybean; Transformation

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The increases in soybean transformation efficiency are required to improve selection of transgenic cells[1]. Transgenic soybean plants have been recovered using various selective agents kanamycin[2~4], glyphosate[5], and hygromycin B[1,6,7], respectively. Successful transformation techniques of soybean were also established using glufosinate as a selective agent, but the transformation efficiency was low due to long selection and nontransgenic escapes[8,9]. Recently, transformation effi-

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ciency has been improved to 5.9%[10], 6.3%[11] by refining glufosinate selection. However, the glufosinate selection experiments were performed during shoot induction and shoot elongation stages that resulted in greatly delaying the procedures of shoot elongation and a long time to achieve transgenic soybean plants[1,12]. Glufosinate has been extensively used as a plant selective agent in transformation events, and development of a more rapid and effective glufosinate selection system should significantly increase the production of transgenic soybean plants.

In this study, we describe an improved glufosinate selection system in the *Agrobacterium* mediated cotyledonary-node method that results in rapid and efficient selection for transgenic soybean shoots with a very low frequency of selection escapes.

1 Materials and Methods

1.1 *Agrobacterium* culture

A. tumefaciens strain LBA4404 carrying a binary vector pCAMBIA3201 was used for the transformation of soybean. The T-DNA region (Fig. 3 A) of the pCAMBIA3201 contains a *gus* reporter gene and a selectable *bar* gene. A single colony of *A. tumefaciens* strain LBA4404 was inoculated with 5 mL of liquid LB medium containing 20 mg/L chloramphenicol (filter-sterilized) and grown at 28℃ at 200 rpm until the OD_{600} reached 0.5~1.0. 3 mL of the *Agrobacterium* cells were added to 200 mL of liquid LB medium and shaken at 28℃ at 200 rpm until the OD_{600} reached 0.8~1.0. The bacterial culture was centrifuged at 5000 rpm for 5 min, and the pellet was then resuspended in a liquid cocultivation medium containing 1/2 MS salts[13], Gamborg's B_5 vitamins[14], 1.5 mg/L 6-benzylaminopurine (BAP), 1000mg/L L-cysteine (filter-sterilized), 200 umol/L acetosyringone (filter-sterilized), and 3% sucrose, and finally the OD_{600} was adjusted to 0.5.

1.2 Transformation, selection and regeneration

Soybean (*Glycine max* L. Merrill) seeds from cultivar *Jungery* were sterilized by soaking in 70% (v/v) ethanol for 1 min. and in 1% sodium hypochlorite for 20 min, and rinsed four times with sterile distilled water. The sterilized seeds were germinated on germination medium (GM) [1/2 MS salts, Gamborg's B_5 vitamins, 1.0 mg/L BAP, 3% sucrose, and 0.65% agar (Sigma), pH 5.8] at 26℃ in the 16/8 h photoperiod for 4~7 days. The preparation of explants and the transformation of soybean followed the procedure of Zhang et al. (1999) with a slight modifications: After co-cultivation, the explants were placed on shoot induction medium (SIM) [1/2 MS salts, Gamborg's B_5 vitamins, 1.5 mg/L BAP, 200 mg/L cefotaxime, 250 mg/L carbenicillin, 3% sucrose, and 0.65% agar, pH 5.8] with or without 5 mg/L glufosinate to induce shoots formation. After 3 weeks on SIM, the explants were transferred to shoot elongation medium (SEM) [1/2 MS salts, Gamborg's B_5 vitamins, 0.5 mg/L gibberellic acid (GA), 0.1mg/L indole-3-acetic acid (IAA), 50mg/L asparagines (filter-sterilized), 50mg/L glutamine (filter-sterilized), 4 mg/L glufosinate (filter-sterilized), 200 mg/L cefotaxime and 250 mg/L carbenicillin, 3% sucrose, and 0.65% agar, pH 5.8]. The elongated shoots (3~5 cm) were cut from the cotyledonary explants and placed on the root induction medium (RIM) containing 1/2 MS salt, Gamborg's B_5 vitamins, 0~5 mg/L glufosinate, 2% sucrose, and 0.65% agar, pH 5.8 for the efficient selection. After rooting, the glufosinate-resistant plantlets were transferred to soil and grown into adult plants.

1.3 GUS assay

A piece of leaf tissue was removed from the rooted plantlets and placed in GUS histochemical staining buffer [50 mM $NaPO_4$ (pH 8.0), 10 mM Na_2EDTA, 0.1% (v/v) Triton-X, 20% (v/v) methanol, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 500 mg/L X-Gluc] for 1 day at 37℃, and then the leaf tissue was cleared in 70% ethanol[15].

1.4 Herbicide resistance test

Herbicide resistance soybean tissues were estimated by applying 150 mg/L glufosinate solution

on leaves of 1 month-old plants using a small paint brush until droplets run off. The treated leaf tissue was examined for herbicide tolerance at 5 ~ 7 days after the herbicide application.

1.5 Southern blot analysis

Genomic DNA of soybean plants was prepared using a Genomic DNA purification kit (Nucleo-Gen). Approximately 10 μg DNA was digested with *Hind* Ⅲ that cut once between the *bar* and *gus* coding sequence within the T-DNA region and separated on a 0.8% agarose gel. DNAs on the gels were transferred onto nylon membrane Gene Screen®. The blots were hybridized with a *bar* probe following a standard procedure [16].

2 Results and Discussion

2.1 Establishment of rapid and efficient selection system

Five independent experiments were conducted for evaluation of transformation efficiencies from two replicates. All treatments included 1000 mg/L L-cysteine in the solid co-cultivation medium.

Table 1 Optimization of glufosinate selection scheme for rapid and efficient transformation of soybean cotyledonary node cells

Selection scheme[a] (mg/L)	Total No. of explants	Recovered plants	GUS$^+$ plants	Transformation efficiency (%[b])
5.0 to 4.0 to 0.0	125	11	2	1.6a
0.0 to 4.0 to 1.0	112	8	2	1.8a
0.0 to 4.0 to 2.0	98	5	4	4.1ab
0.0 to 4.0 to 3.0	105	7	7	6.7b
0.0 to 4.0 to 5.0	90	0	0	0.0

a The numbers in the first column for each selection scheme represent glufosinate levels (mg/L) during the shoot induction, shoot elongation, and root formation stages, respectively.

b Efficiency (%) = (No. of independent GUS$^+$ plants / total number of explants inoculated) × 100

For optimization of selection based on glufosinate, a total five different selection schemes were evaluated at level 5-4-0, 0-4-1, 0-4-2, 0-4-3, and 0-4-5 mg/L glufosinate during the shoot induction, shoot elongation, and root induction stages, respectively (Table 1). After 3 weeks on SIM supplemented with 5 mg/L glufosinate, approximately 80% of the explants survived and then was transferred to SEM for shoot elongation under 4 mg/L glufosinate selection pressure. Of the shoots resistant to glufosinate, 70% ~ 83% were nontransgenic escapes when the selection was performed only during the shoot initiation and shoot elongation, and thus resulted in a low efficiency of 1.6% (Table 1). This result was consistent with the observation made by Olhoft and Somers (2001). In addition, the selection for 3 weeks during the shoot induction greatly delayed the procedures of 'Jungery' shoot elongation. The elongation of majority of transformed shoots occurred from 21 to 41 weeks after *Agrobacterium* inoculation under the glufosinate selection during shoot induction while only 7 to 12 weeks without glufosinate selection (Fig. 1). Thus further investigation on the selection of transformed shoots was focused on root formation stage in the presence of glufosinate. After the shoot induction on the glufosinate free SIM for 3 weeks, the regeneration of transformed shoots was greatly stimulated (data not shown), and the explants were placed on SEM containing 4 mg/L glufosinate for shoot elongation. The elongated shoots (3 ~ 5 cm) were excised and placed into root formation medium containing glufosinate ranged from 1 to 5 mg/L. None of nontransformed shoots survived on the RIM containing 3 mg/L glufosinate whereas transformed shoots survived under this selection pressure (Fig. 2A, B). Shoots rapidly responded to the selective agent applied on RIM, all the glufosinate-sensitive shoots were completely necrotic only within 10 days after selection on RIM containing 3 mg/L glufosinate. The majority of transgenic plantlets were obtained between 8 to 16 weeks after *A. tumefaciens* inoculation under this improved selection pressure. At 5 mg/L glufosinate on the RIM, no transgenic plants were obtained. Although most of the transformed shoots survived under the 5 mg/L glufosinate selection pressure, further growth of those shoots stopped at this stage. The optimal selection scheme was glufosinate at 4 mg/L during the shoot elongation and at 3 mg/L during root formation that was also referred to as tight glufosinate selection pressure.

The transformation efficiency based on GUS assay ranged from 1.6% to 6.7%, depending on different selection pressure (Table 1). This indicated proper selection was very important for efficient transformation. The transformation efficiency was higher than previously reported in the glufosinate selection system[8,10,11,17]. In this study, as an improved approach, the shoots excised from the explants were placed into the root induction medium containing glufosinate, thereby facilitating direct uptake and rapid translocation of the herbicide through xylem and phloem[18]. It took only 1 to 2 weeks to select PPT-resistant plantlets and gave the high transformation efficiency (6.7%).

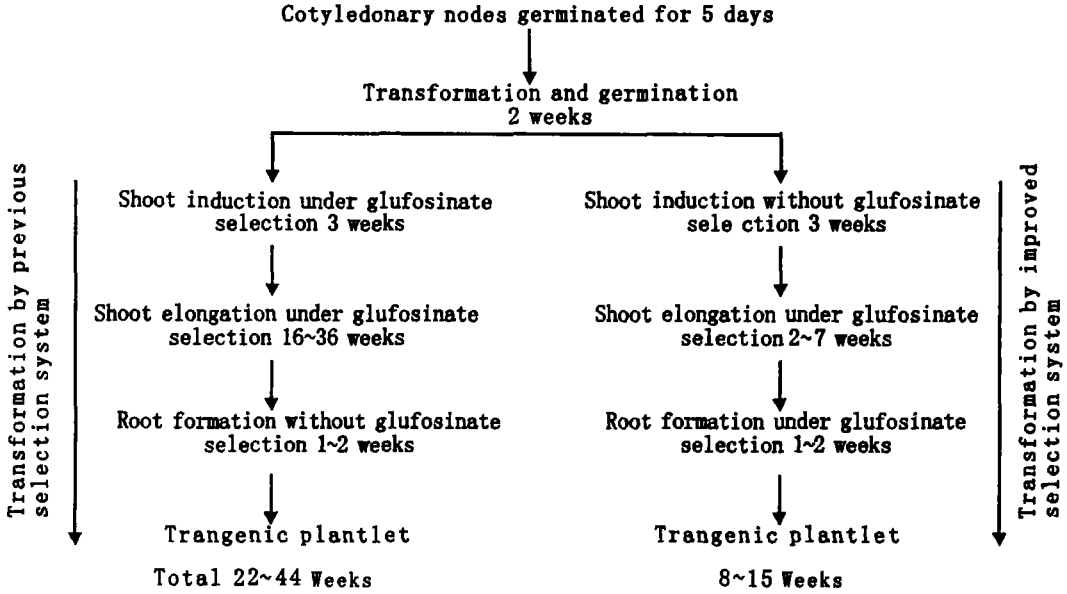

Fig. 1 Flow chart comparing the transformation by previous selection system with the transformation by improved slection system

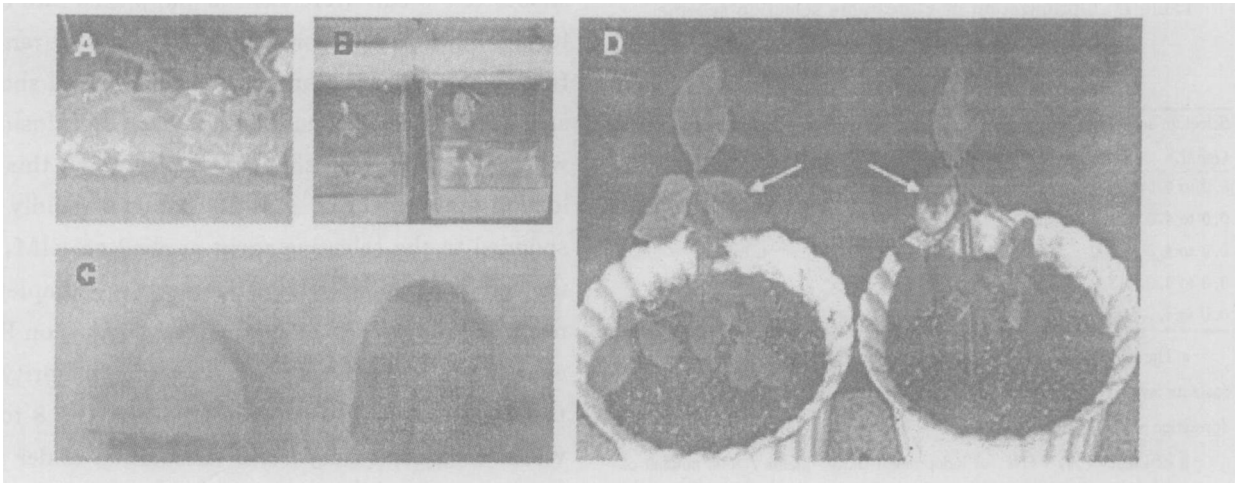

Fig. 2 Selection of transformed shoots and expression. A. All the non transformed shoots died on the RIM containing 3 mg/L glufosinate. B. Transformed plantlets were grown on the RIM containing 3 mg/L glufosinate. C. Expression of GUS in 4 day germinated cotyledons from non transformed (left) and transformed (right) seeds. D. Herbicide resistance of transgenic soybean plants was assayed by applying 150 mg/L PPT solution on leaves of 1 month old plants using a small paint brush. Photographs of the plants were taken 7 days after the treatment, the non transformed plant was shown on the right.

2.2 Integration of transgenes

Stable integration of the transgenes in the genome of glufosinate resistant T_0 plants was confirmed by genomic Southern blot analysis (Fig. 3B). The genomic DNAs isolated from the GUS^+ plants were digested with *Hind* III, and hybridized

with a [^{32}P]- labeled *bar* probe. *Hind* Ⅲ recognizes unique site between the *bar* and *gus* coding region within the T - DNA. All the transformation events tested had one to two copies of the *bar* gene (Fig. 3B). These results confirmed that the transgenes were stably integrated into the genome of the transgenic plants.

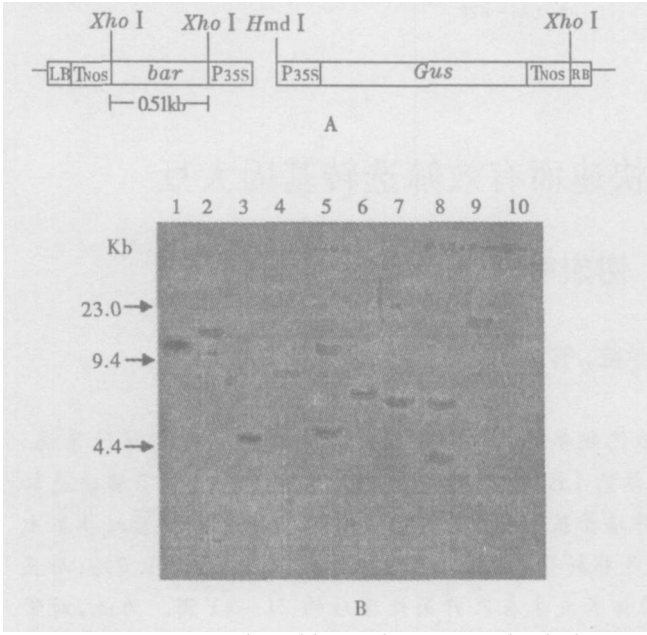

Fig. 3　Genomic Southern blot analysis of 8 individual soybean plants. A. The T - DNA region of the recombinant DNA (pCAMBIA3201) was shown. B. Genomic DNA (approximately 10μg) was digested with *Hind* Ⅲ which produces unique fragment for each integrated T - DNA and hybridized with the *bar* probe. Lane 1: pCAMBIA3201 DNA digested with *Hind* Ⅲ. Lane 2 to 9: DNAs from transformed plants resistant to glufosinate. Lane 10: a non transformed control plant.

2.3　Expression of transgenes

The expression of transgenes in soybean genome was confirmed by GUS assay and herbicide application. According to the GUS assay, most of the plantlets recovered from the glufosinate selection at 5 4 0, 0 4 1, 0 4 2 mg/L were GUS negative whereas all the plantlets recovered from the 0 4 3 mg/L selection pressure were GUS positive, indicated that this glufosinate selection was very tight (Table 1). The transformed *gus* gene was also highly expressed in the germinated T_0 seeds (Fig. 2C).

The results of herbicide resistance test showed that the leaves of non transformed control plants were wilted to death at five to seven days after the herbicide application, whereas those of transformed plants were not affected by this concentration of glufosinate (Fig. 2D). These results showed that both the genes *bar* and *gus* were stably integrated and constitutively expressed in the transgenic soybean plants.

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利用改良的草丁膦筛选系统快速而有效筛选转基因大豆

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摘要 为提高大豆(*Glycine max* (L.) Merrill)遗传转化效率建立了一种快速而有效的草 膦筛选系统。将刺伤的子叶节外植体接种于含有 pCAMBIA3201 载体的 LBA4404 农杆菌溶液。目前利用草 膦筛选转基因大豆的常规方法是在含有 5 mg/L 草 膦的芽诱导培养基和含有 3~5 mg/L 草 膦的芽伸长培养基上进行筛选。利用此常规筛选方法所获得的大多数植株是非转化体, 从而导致转化效率变低, 为 1.6%; 而且这种方法极大地延迟了芽的伸长过程, 使多数不定芽的伸长发生在农杆菌处理后的 21~41 周。为此, 对常规草 膦筛选方法进行了改良。首先将外植体放置在不含有除草剂的芽诱导培养基上培养 3 周, 然后转到含有 4 mg/L 草 膦的芽伸长培养基上进行筛选。此时, 多数不定芽仅在 7~12 周内便可伸长。当芽长到 3~5 cm 时, 从外植体上切下来转到含有 1~5 mg/L 草 膦的根诱导培养上进行进一步的筛选。结果表明, 在添加有 3 mg/L 草 膦根培养基上, 转化效率达到最大值为 6.7%。不定芽对根培养基中的除草剂反应迅速, 仅在 10d 天内所有非转化体都变枯死亡。利用这种改良的筛选系统, 大多数转基因植株仅在 8~16 周内便可获得。Southern 杂交结果证实了外源基因稳定地整合在大豆基因组中。GUS 检测和除草剂抗性分析结果表明, 被整合的外源基因在大豆细胞中得到了稳定的表达。

关键词 农杆菌; 子叶节; 草 膦; 大豆; 遗传转化