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Full Length Research

Transformation of multiple soybean cultivars by infecting cotyledonary-node with *Agrobacterium tumefaciens*

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Transformation of four soybean cultivars (Nannong88-1, Nannong18-6, Yu23 and Nannong 87C-38) by infecting cotyledonary-node with *Agrobacterium tumefaciens* strain EHA105 harboring pBI121 containing GFP reporter gene was conducted. The results indicated that the addition of thiol compounds (L-cysteine, dithiothreitol and sodium thiosulfate) in co-cultivation period increased the transformation efficiency of all four soybean cultivars, with Nannong 88-1 most increased up to 2.20%. Detection of GFP expression in the rooted plants was an effective selection system for the confirmation of soybean transformation. And most GFP-positive plants were confirmed to be positive by Southern blot analysis, which showed that transformation of cotyledonary-node explants mediated by *Agrobacterium* delivered T-DNA with one or two copies into soybean genome. In our study, the combination of Nannong88-1 with EHA105 is the optimum selection for explant and bacterial inoculum in soybean transformation, which could be applied in future functional study of soybean genes.

Key words: Soybean, cotyledonary–node, *Agrobacterium*, transformation, GFP.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill.] is one of the most important leguminous seed crops as it is an economic source of both oil and protein. Developing an efficient transformation system should be helpful to investigating the functional soybean genes, as well as to improving soybean cultivars. Transgenic soybean plants have been produced using various DNA deliveries and plant tissue cultivation, including microprojectile bombardment of shoot meristems (McCabe et al., 1988), embryogenic suspension cultures (Finer and McMullen, 1991; Trick and Finer, 1998), *Agrobacterium tumefaciens* mediated T-DNA delivery into immature cotyledons (Parrott et al., 1989; Yan et al., 2000; Ko et al., 2003) and axillary meristematic tissue located in seedling cotyledonary nodes (Hinchee et al., 1988). But soybeans have been proven to be extremely resistant to transformation, which must be overcome in order to establish an ideal soybean transformation system (Trick et al., 1997).

One somewhat successful method is the cotyledonary-node soybean transformation system, which is based on *Agrobacterium*-mediated transferred DNA (T-DNA) delivery of genes into regenerable cells in the axillary meristems of the cotyledonary node (Hinchee et al., 1988). Recently, some improvements have been reported for this transformation system. Olhoft and Somers reported that increased T-DNA delivery and the production of transgenic shoots by adding thiol compounds (L-cysteine, dithiothreitol, and sodium thiosulfate) to the solid cocultivation medium (CCM) were observed, the

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Abbreviations: GFP, green fluorescent protein; 6-BAP, 6-benzylaminopurine; DTT, dithiolthreitol; IBA, indole-3-butyric acid.

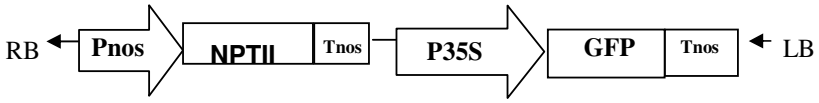

Figure 1. A linear map of the T-DNA region of pBI121 LB: Left T-DNA border. Pnos: NOS promoter; NPTII: Neomycin photransferaseII-coding region; Tnos: NOS terminator; P35S: Cauliflower mosaic virus 35S promoter; GFP: green fuorescent protein; RB: Right border.

transformation efficiency of which could reach 16.4% (Olhoft and Somers, 2001). In addition, an efficient hygromycin B based selection system for rapid screening of transgenic shoots was developped, with a very low frequency of non-transformants escape (Olhoft et al., 2001; Olhoft et al., 2003).

However, current transformation protocols with a high transformation frequency for soybean were only limited to a few cultivars. Based on the cotyledonary-node method originally reported by Hinchee et al.(1988) and recently improved by Olhoft (2003), we here attempt to select more different acceptors with high transformation frequency from Chinese soybean cultivars in order to be used in future soybean transformation.

MATERIALS AND METHODS

Soybean cultivars

Four soybean [*Glycine max* (L.) Merrill.] cultivars; Nannong88-1, Nannong18-6, Yu23 and Nannong87C-38, were used in our experiments.

Agrobacterium strain and transformation vector

The plasmid, pBI121, contained NPTII gene, CaMV35S promoter, NOS promoter, NOS terminator, and GFP gene (Figure 1). The construct pBI121 was mobilized into the *A. tumefaciens* strain EHA105 by freezing-melting. *Agrobacterium* cultures harboring pBI121 were grown on the plate of YEB medium containing 50 mg l-1 Kanamycin at 28°C until colony formation. Then, 50 ml liquid YEB medium containing 50 mg l-1 Kanamycin was inoculated with a single colony and shaken at 28□C and 180 rpm until OD650 of 0.6-0.8. *Agrobacterium* cultures were pelleted at 3,000 rpm for 10 min, and resuspended in liquid cocultivation medium (see below) to OD650 of 0.6 for use in the inoculations. Cocultivation medium (CCM): 1/10 Gamborg's B5 medium supplemented with 3% sucrose, 1.67 mg l-16-BAP, 200 µM acetosyringone, 20 mM MES , 0.25 mg l-1GA3, 3.3 mM L-cysteine, 1.0 mM DTT, 1.0 mM Sodium thiosulfate, pH 5.4.

System sensitivity to kanamycin

Cotyledonary-node used as explants, cultured from 90 mature seeds, were cultured on the regeneration medium supplemented with a gradient level of kanamycin (10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 mg l-1), respectively. After 3 weeks, the adventitious shoots were counted to determine regeneration frequencies.

Explant preparation and inoculation

Soybean seeds were disinfected with 0.1% $HgCl_2$ with 1 drop of Tween-20 for 6 to 7 min, followed by a 3 to 5 times wash in distilled water. The sterilized seeds were placed on germination medium (GM, Gamborg's B5 medium supplemented with 3% sucrose, 1 mg l-16-BAP, 0.75% agar, pH 5.8) and allowed to germinate for 4 to 5 days at 25°C, with a 18/6 h light/dark regime. For each germinating seed (seedling), two explants were obtained, first by making a cut through the hypocotyl region approximately 3 to 5 mm below the cotyledonary node, and then by cutting the seed vertically through the hypocotyls region, as previously described (Hinchee et al., 1988). After the epicotyl region, axial shoots/buds were all removed, cotyledonary node region of explant was wounded by slicing about 7 to 8 times, perpendicular to the hypocotyls, with a scalpel blade. The wounded explants were soaked in *Agrobacterium* inoculum suspension for 30 min. The explants inoculated were randomly placed on a piece of sterile filter paper on top of plate of co-cultivation medium (CCM). Plates were incubated in the dark for 5 days at 25℃.

Selection and plant regeneration

Following co-cultivation, explants were briefly washed in the liquid shoot induction medium (SIM, Gamborg's B5 medium supplemented with 1.67 mg l-16-BAP, 3% sucrose, 100 mg l-1 timentin, 250 mg l-1/L cefotaxime, 3 mM MES, 0.75%agar, pH 5.6) containing 75 mg l^{-1} kanamycin and were then placed on the solid SIM. After the first shoot induction cultivation for 14 days, the explants were trimmed and then transferred to the fresh SIM containing 100 mg l^{-1} kanamycin for an additional 14 days under the same cultivation condition. At the end of the shoot induction period, the differentiating explants isolated from cotyledons were transferred to shoot elongation medium (SEM, MS medium supplemented with 3% sucrose, 0.5 mg l^{-1} GA3, 100 mg l^{-1} timentin, 250 mg l^{-1} cefotaxime, Zeatin-R 1mg l^{-1}, asparagine 50mg l^{-1} and glutamine 50mg l^{-1}, 100mg l^{-1} kanamycin, 0.75% agar, pH5.6) for over 4 weeks at 25°C, with a 18/6 h light/dark regime. During shoot elongation cultivation, the tissues were transferred to fresh SEM every two weeks.

At each transfer, fresh horizontal slices at the base of the tissue were made. Once the shoots have elongated to approximately 4 cm in length, the isolated shoots were rooted on rooting medium (RM, 1/2 Gamborg's B5 salts plus 3% sucrose, 0.8mg l-1 IBA, 20 mg l-1 kanamycin and 0.75% agar) for 2 to 3 weeks. After transplanted to the pot with vermiculite for 15 days, the rooted shoots were grown in the greenhouse, with a 16/8 h light/dark regime.

GFP detection

The roots of transformed soybean plant were used to identify GFP expression, using the fluorescent microscope (Leica DM IRB, Germany).

Southern blot analysis

Total genomic DNA was extracted from plant leaves following the protocol of CTAB. The DNA (15–20 µg) was digested with EcoRI,

Table 1. Kanamycine sensitivity of the cot-node from the four soybean cultivars. The regeneration medium was supplemented with various kanamycin concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and100 mgl^{-1}), and the number of adventitious shoots was counted 3 weeks later.

cultivars	Kanamycin concentration (mgl^{-1})				
	10	20	30	40	50
Nannong88-1	12	5	1	0	0
Nannong18-6	9	3	1	0	0
Yu23	8	2	1	0	0
Nannong87C-38	6	1	0	0	0

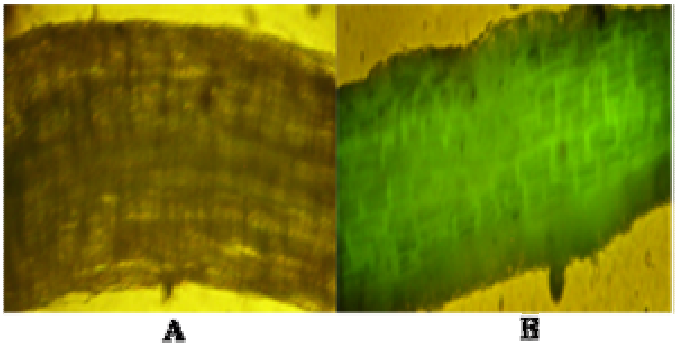

Figure 2. The expression of GFP in transformated roots under fluorescent microscope (20x). A: non-transformed root. B: transformed root.

overnight at 37°C. GFP specific probe (650 bp) was generated by PCR (Primers: 5'GTAAACGGCCACAAGTTCAG3' and 5'TACTTGTAC AGCTCGTCCATGC3'), and labeled and detected with DIG High Prime DNA Labeling and Detection Starter Kit (Roche Applied Science, Mannheim, Germany), respectively. The hybridization was conducted at 40°C. The hybridized membranes were exposed to Kodak X-film for signal detection.

RESULTS

Optimum kanamycin concentration

Of four cultivars tested, Nannong87C-38 was more sensitive to kanamycin than the other three cultivars, the kanamycin lethal concentration of which was 30 mg l^{-1} and 40 mg l^{-1}, respectively. That indicated that cotyledonary-node explants from all four cultivars were unable to form any shoots after treated with kanamycin of 40 mg l^{-1} level (Table 1). So, kanamycin at 40 mg l^{-1} or above can be used as selective agent for all four tested cultivars transformation. However, we used 100 mg l^{-1} kanamycin in our experiment to avoid the high frequency of non-transformed plant 'escapes' or the production of chimeric plants.

Analysis on transformation efficiency of multiple soybean cultivars

Two independent experiments were established by adding 3.3 mM L-cysteine and thiol compounds (3.3 mM L-cysteine, 1 mM DTT, 1 mM sodium thiosulfate) to the solid cocultivation medium, respectively, during the co-cultivation. The data showed that the GFP-positive frequency and transformation efficiency in the transformation system amended with thiol compounds were all increased among all cultivars tested, except for Nannong 87C-38, compared to that in L-cysteine alone (Table 2). It was suggested that the relative high transformation efficiency benefited from combination of L-cysteine, DTT and sodium thiosulfate. For example, the transformation efficiency of $Nannong88^{-1}$ was 1.39% in system only with L-cysteine, while 2.20% in system with thiol compounds.

However, the influence of thiol compounds on transformation efficiency was related to different genotype, too. In all four cultivars tested, $Nannong88^{-1}$ and Nannong18-6 were more highly responsive to thiol compounds than Yu23 and Nannong87C-38. In addition, we observed that the lower level of kanamycin (75 mg l^{-1}) was better for callus and de novo shoots in the cotyledonary-node region than the higher level of kanamycin (100 mg l^{-1}) in the first 14 days inducing shoot period. The abaxial side of explants orientated upwards on the first SIM was also helpful to stimulating the regeneration of transformated shoots.

GFP expression in transformed plants

Using green fluorescent protein (GFP) as a visual marker allowed us to identify the transformed plants. Before transferring to soil, some roots excised from the rooted planlets were identified for the GFP expressing under the fluorescent microscope, which showed that GFP strongly expressed in root cells of transgenic plants (Figure 2).

Southern blot analysis on transformed plants

Data of Southern-blot with GFP probe for putative transgenic plants showed that the transgenes were randomly integrated into the soybean genome with one or

Table 2. The effect of thiol compounds on soybean transformation.

Cultivars	No. of explants infected		No. of rooted plants GFP-positive		Transformation frequency (%)[a]	
	A treatment	B treatment	A treatment	B treatment	A treatment	B treatment
Nannong88-1	143	136	2	3	1.39	2.20
Nannong18-6	107	138	1	2	0.93	1.44
Yu23	119	95	1	1	0.84	1.05
Nannong87C-38	268	252	1	1	0.37	0.39

A treatment: add 3.3 mM L-cysteine to CCM.
B treatment: add 3.3mM L-Cysteine+1mM DTT +1mM Sodium thiosulfate to CCM.
[a]Transformation frequency (%) = (No.of rooted GFP-positive plants/ No. of explants infected)×100.

Table 3. Transformation efficiency of soybean with *Agrobacterium* EHA105.

Cultivar	No. of total explants	No. of GFP positive plants	No. of southern positive plants	Transformation frequency (%)[a]
Nannong88-1	309	8	7	2.26
Nannong18-6	219	3	3	1.37
Yu23	165	2	2	1.21
Nannong87C-38	470	3	2	0.42

[a]Transformation frequency (%)=(No. of southern positive plants)/ (No. of total explants) × 100.

two copies (Figure 3). And the results of Southern blot were in good correlation to that of GFP detection, about 85% of GFP-positive plants being also positive in Southern blot analysis.

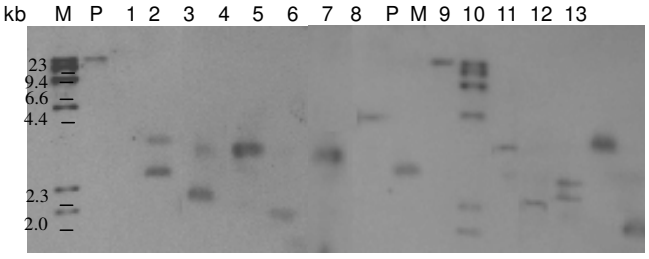

Figure 3. Southern blot analysis of the transgenic soybean plant. GFP-positive plants transformed with *A. tumefaciens* strain EHA105 (pBI121). Genomic DNA of non-transgenic (lane 1) and transgenic plants (lane2-13) were digested with restriction enzyme EcoRI, GFP-positive plasmid (lane p), Marker (Roche, lane M) fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the gfp probe.

DISCUSSION

The production of transgenic plants is fundamental to investigations of functional plant gene, as well as to the improvement of crops. However, soybean transformation is still tough now, with very low transformation efficiency. The increase in the frequency of transformed cells obtained by the addition of thiol compounds to the solid co-cultivation medium is almost independent of soybean genotypes and *Agrobacterium* strains, as well as of binary vectors, making this approach to improving *Agrobacterium*-mediated transformation of soybean cot-node cells generally useful (Olhoft, 2001). Our results confirmed that the thiol compounds were effective to increasing the frequency of transformed cells, especially for some genotypes like Nannong88-1.

Meurer et al. (1998) reported that strain EHA105 was effective in promoting cotyledonary node transformation. The hypervirulent strain EHA105 has been commonly used for soybean transformation (Trick and Finer, 1997, 1998; Simmonds and Donaldson, 2000; Yan et al., 2000). However, Ko et al. (2003) reported EHA105 was ineffective in producing transgenic somatic embryos. It is suggested that the transformation efficiency of different soybean cultivar is associated with specificity of *Agrobacterium*. Interestingly, the explants of Nannong18-6 and Yu 23 showed bacterial overgrowth during cocultivation with strain EHA105, while Nannong88-1 and Nannong 87C-38 did not. In general, the four soybean cultivars in our experiment are all compatible to strain EHA105, but combination of Nannong88-1 with EHA105 is the optimum (Table 3).

During the shoot induction cultivation, lower level of kanamycin (75mg l^{-1}) for first 14 days, different from the higher level of kanamycin (100 mg l^{-1}) for the second 14 days, greatly stimulated the regeneration of transformed shoots. In the cotyledonary-node transformation system, prolific callus growth occurred in the region of nodal, and

shoot elongation beginning 2 months after inoculated with *A. tumefaciens*, except for Nannong87C-38. It was difficult for the shoot of Nannong87C- 38 to elongate, which was probably imputed to high sensitive to kanamycin.

GFP has been widely used as a marker for convenient noninvasive detection without the introduction of cofactors or the destruction of the biological sample, in plant transformation (Jordan, 2000; Huber et al., 2002; El-Shemy et al., 2004). Detection of GFP expression in the rooted plants has become a primary selection system to avoid non-transformed plant 'escapes' with kanamycin. It was simple and inexpensive for GFP detection to detect transgenic plants by comparison with southern blot analysis.

Taken together, transformation mediated by *Agrobacterium* has generally resulted in the transfer of relatively low copy number of defined DNA fragments with little rearrangement, compared to direct DNA delivery, such as particle bombardment used in many plants. In addition, this kind of transformation system was simple and inexpensive, with a broad application of soybean transformation. At present, the methodology for soybean transformation from more cultivars and more different *Agrobacterium tumefaciens* strains are underway in our laboratory.

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