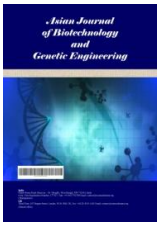

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Refined Factors in Multi-needle-assisted Transformation of Soybean

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Authors' contributions

This work was carried out in collaboration between both authors. Author BZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author RGX managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Previous studies have improved *Agrobacterium*-mediated soybean [*Glycine max* (L.) Merrill] cotyledonary node method by the development of a simple multi-needle-assisted wounding method using cotyledonary node cells of 1-day-old half seeds as target tissue. The goal of this study was to investigate the factors affecting the efficiency of the multi-needle-assisted transformation of soybean cotyledonary node cells (MNAT). The factors were studied by the GUS activity using a binary vector pCAMBIA1301 containing both a *gus*-intron gene and a *hpt* (hygromycin phosphotransferase) selectable marker. All of the factors affecting the transformation efficiency were determined after the 1-day-old half seeds punctured 2 times with the multi-needle. The transformation efficiency based on transient expression of the *gus* gene was significantly affected by the concentration of antioxidants, density of *Agrobacterium* suspension, infection time and the concentration of acetosyringone (AS). The frequency of the transformed cotyledonary node cells was also affected by soybean genotypes.

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Keywords: Agrobacterium tumefaciens; multi-needle; soybean; transformation; transient expression.

1. INTRODUCTION

Establishment of an efficient transformation system is required for production of transgenic plants and functional genomics research in soybean [*Glycine max* (L.) Merrill] [1]. Two soybean transformation methods that are currently utilized by most researchers are biolistic-mediated transformation of somatic embryo [2-5] and *Agrobacterium*-mediated transformation of cotyledonary node [6-12]. In the cotyledonary node method, the transgenic soybean plants were obtained from the explants derived from 5-7-day-old seedlings. An improved cotyledonary node method was developed by using the half seed as a target tissue [13-15]. The 1-day-germinated half seed was wounded 2 times with a multi-needle that result in a significant increase in soybean transformation efficiency [14]. Recently, the efficiency of soybean shoot regeneration and transformation is improved by glutamine and asparagine [16] or 5-azacytidine [17].

In order to enhance the transformation efficiency, the factors affecting the efficiency of the multi-needle-assisted transformation of soybean cotyledonary node cells (MNAT) were investigated in this study.

2. MATERIALS AND METHODS

2.1 Soybean Cultivars

Soybean seeds of the cultivars Hefeng 25, Hefeng 35, Hefeng 39, Dongnong 40, Dongnong 42, Junsery, Kennong18, Jilinxiaoli1 and K06-82 were used for transformation events.

2.2 Binary Vector and *Agrobacterium* Strain

A. tumefaciens strain LBA4404 carrying a plant expression vector pCAMBIA1301 (CAMBIA, Canberra, Australia) was used for the transformation of soybean in this study. The T-DNA region of the pCAMBIA1301 contains a *gus*-intron reporter gene driven by a CaMV 35S promoter and a selectable *htp* gene driven by a CaMV 35S promoter. The *gus*-intron cassette prevents background GUS expression derived from *Agrobacterium* cells contaminating in plant tissue.

2.3 *Agrobacterium* Culture

A single colony of *A. tumefaciens* strain LBA4404 was inoculated with 5 mL of liquid LB medium containing 20 mg L^{-1} 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 co-cultivation medium containing 1/ 2 MS salts, B5 vitamins, 100 or 200 μM acetosyringone (AS), and 3 % sucrose, and finally the OD_{600} was adjusted to 0.5.

2.4 Preparation of Multi-needle and Half Seed

The multi-needle containing 30 metal wires and the half seeds were prepared according to the procedure of Xue et al. [14].

2.5 Transformation

Soybean seeds were soaked in 70% (v/ v) ethanol for 1 min, and in 0.1% $HgCl_2$ 15 min and rinsed 4 times with sterile distilled water. The sterilized seeds were germinated in sterile distilled water at 26℃ in the dark for 1 day. The cotyledonary node cells of the half seeds were wounded by puncturing 2 times with a multi-needle and infected with *Agrobacterium* for 0-60 min. The explants were rinsed 4 times with sterile distilled water and sucked dry on a sterile paper, and placed on a solid co-cultivation medium (as above with the addition of 6 mg L^{-1} agar) at 22℃ in the dark for 3 days. Experimental unit composed of 10 explants was placed in a single plate.

To evaluate the effects of antioxidants during co-cultivation period, transient expression assay was performed on the wounded cotyledonary nodes of the half seeds placed onto solid co-cultivation medium with or without 1.0 mM DTT, 1.0 mM sodium thiosulfate, 3.3 mM L-cysteine.

To determine the effects of AS during co-cultivation period, transient expression was scored on the wounded cotyledonary nodes of the half seeds placed onto solid co-cultivation medium with or without 0, 50, 100, 200 μM.

Soybean genotypes, concentration (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 OD_{600}) of *Agrobacterium* and infection time (10, 15, 20, 30, 45, 60 min) of *Agrobacterium* were also evaluated on the wounded cotyledonary nodes of the half seeds co-cultivated onto solid medium.

All factors were evaluated under the conditions that the cotyledonary node cells of 1-day-old half seeds were punctured 2 times with the multi-needle.

2.6 GUS Assay

After 3 days co-cultivation, the explants were placed in GUS histochemical staining buffer [50 mM $NaPO_4$ (pH 7.0), 10 mM Na_2EDTA, 0.1% (v/v) Triton-X, 0.5 mM K_3 [Fe (CN) $_6$], 0.5 mM $K_4[Fe(CN)_6]$, 500 mg L^{-1} X-Gluc] for 1 day at 37℃, and then the explants were washed in 70% ethanol and destained in 100% ethanol [18]. The transient expression of the *gus* gene was observed under a Zeiss SV8 dissecting scope and the blue spots that only fell within 2 mm of cotyledonary node were counted in all the experiments in this study (Fig. 1).

3. RESULTS AND DISCUSSION

3.1 Comparison of Genotypes on Transformation Efficiency

After 3 days of co-cultivation, histochemical GUS analysis revealed transient gene expression in the transformed half seeds in MNAT method. Transient GUS expression was observed on where both cotyledonary node punctured by the multi-needle and other parts of the half seeds (Fig. 1). The blue foci that only fell within 2 mm of cotyledonary node of the half seed were investigated in all the experiments in this study.

Fig. 1. Transient GUS expression in transformed half seed in MNAT method. GUS assay was performed on half seeds at 3 days after co-cultivation with *Agrobacterium* following the cotyledonary node cells of 1-day-old half seeds were punctured 2 times with the multi-needle. Blue foci that fell within circle were counted

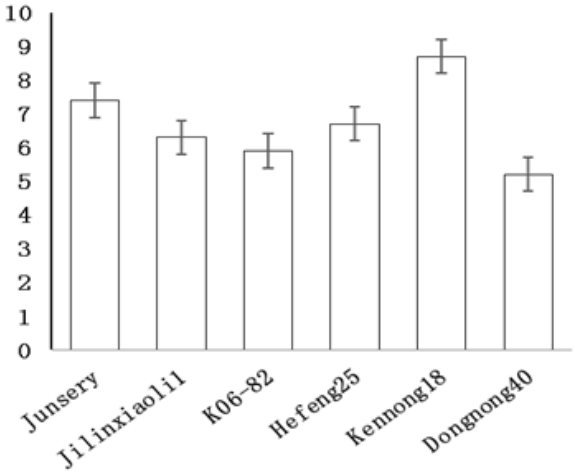

Fig. 2. Effects of the genotypes on transient GUS expression in MNAT method. The average number of blue foci per explant was investigated on 6 soybean genotypes after 3 days on solid co-cultivation medium containing 100 µM acetosyringone

Table 1. Effects of AS concentrations on transformation efficiency in the multi-needle-assisted transformation of soybean cotyledonary node cells. Scores were average of three replications with ten half seeds each. Different letters show significant differences at the 5% level

Genotype	Average blue foci concentration of AS (μM)			
	0	50	100	200
Hefeng 25	2.5a	4.5b	7.4c	8.6c
Hefeng 35	2.1a	4.6b	6.5bc	7.9c
Hefeng 39	2.2a	4.5b	7.1c	8.2c
Dongnong 42	1.8a	3.9b	6.4c	7.6c

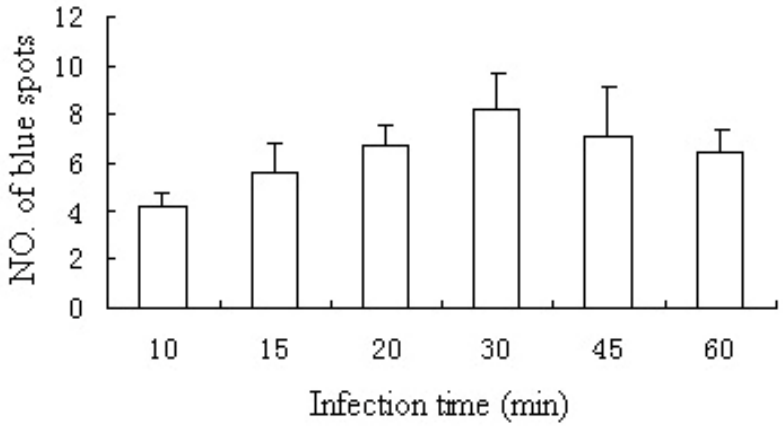

Fig. 3. Effects of *Agrobacterium* (LBA4404) infection time on transient GUS expression in MNAT method. The average number of blue foci per explant was investigated on soybean cv. Hefeng25 explants after 3 days on solid co-cultivation medium containing 200 μM acetosyringone

To determine the effects of genotypes on transformation efficiency in MNAT method, we conducted GUS assay on the half seeds of 6 soybean varieties following co-cultivation on medium containing 100 μM AS. All of the varieties tested responded to *Agrobacterium* infection and the differences in the number of GUS foci were observed (Fig. 2). The genotype Kennong18 gave the highest transformation efficiency of 8.7 GUS foci. The differences in susceptibility to *Agrobacterium* infection among genotypes were reported by Meurer et al. [19] and Olhoft et al. [9].

3.2 Effects of as on Transformation Efficiency

To determine the effects of AS on transient expression during the co-cultivation period, the different concentrations of AS (0, 50, 100, 200 μM) were supplemented in the co-cultivation medium and the effects of AS were conducted in genotypes Hefeng 25, Hefeng 35, Hefeng 39 and Dongnong 42. The addition of AS to the co-cultivation medium could significantly increase the transient expression (Table 1).

Although GUS foci were observed in controls (no AS) in all 4 genotypes tested, a significant increase in transient expression resulted from 100-200 μM AS. Although soybean is a suitable host for *Agrobacterium*, it is not susceptible to infection as many other dicot plants. The incompatibilities between *Agrobacterium* and soybean have been overcome by application of AS [20-22]. Our results also showed that application of AS on co-cultivation medium could significantly enhance the transient expression.

3.3 Effects of Infection Time on Transformation Efficiency

The effect of infection time (10, 15, 20, 30, 45 and 60 min) on transformation frequency was investigated. The number of blue foci increased with a prolonged infection period. The highest level of transient GUS expression was detected at 30 min after *Agrobacterium* infection and then

a slight decrease in transient expression was observed after 45 min of infection (Fig. 3).

3.4 Effects of Concentrations of *Agrobacterium* on Transformation Efficiency

To determine the best *Agrobacterium* concentration, 6 different densities of *Agrobacterium* suspension (0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 OD_{600}) were tested for the transient expression. Differences in the number of GUS foci were observed among different treatments. The highest level of GUS transient expression was obtained at OD_{600} = 0.6 (Fig. 4).

3.5 Effects of Antioxidants on Transformation Efficiency

To investigate whether antioxidants increase the transformation efficiency in the MNAT method, the antioxidants (1.0 mM DTT, 1.0 mM sodium thiosulfate and 3.3 mM L-cysteine) were added to the solid co-cultivation medium containing 100 μM. The results showed that addition of the antioxidants to the co-cultivation medium resulted in more than 12 foci per cotyledonary node while the control was about 7 foci, indicating the antioxidants.could significantly increase the frequency of transformed cells than did the no addition (Fig. 5). Similar results were

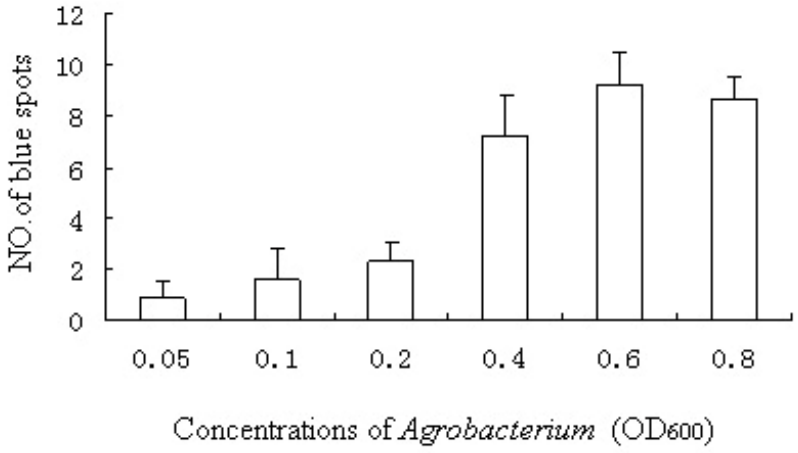

Fig. 4. Effects of densities of *Agrobacterium* (LBA4404) suspensions on transient GUS expression in MNAT method. Soybean cv. Hefeng25 explants were determined by GUS assay after 3 days on solid co-cultivation medium containing 200 μM acetosyringone

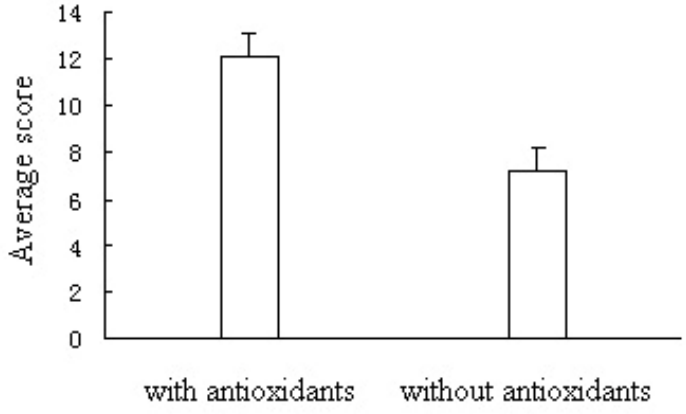

Fig. 5. Effects of antioxidants on transformation efficiency in MNAT method. Soybean cv. Hefeng25 explants were determined by GUS assay after 3 days on solid co-cultivation medium with or without 1.0 mM DTT, 1.0 mM sodium thiosulfate, 3.3 mM L-cysteine

reported by Olhoft et al. [9]. The antioxidants could inhibit the activity of the enzymes such as PODs and PPOs that cause browning in plant defense response mechanisms and thereby increase the frequency of transformed cells [23].

4. CONCLUSION

There are many factors influencing the efficiency of soybean transformation, here we enhanced the soybean transformation efficiency in the multi-needle-assisted method by refining some important factors such as genotypes, wounding treatments, the concentrations of AS, infection time, concentrations of *Agrobacterium* and antioxidants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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