Korean J. Medicinal Crop Sci. 15(2) : 100 - 104 (2007)

Factors Affecting *Agrobacterium tumefaciens*-mediated Transformation of *Panax ginseng* C.A. Meyer

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ABSTRACT : A protocol for the production of transgenic *Panax ginseng* C.A. Meyer was established via *Agrobacterium tumefaciens*-mediated genetic transformation of direct somatic embryos. A number of conditions related to the co-cultivation were tested with respect to maximizing transformation efficiency. The results showed that pH of the co-cultivation medium (5.7), the bacterial growth phase (optical density; OD_{600} = 0.8), co-cultivation period (3 days), and acetosyringone concentration (100 μM) had positive effects on transformation. Selected plantlets were cultured on the medium at an elevated hygromycin level (30 ㎎/ℓ). Integration of the transgenes into the *P. ginseng* nuclear genome was confirmed by PCR analysis using *hpt* primers and by Southern hybridization using *hpt*-specific probe. The transgenic plantlets were obtained after 3-month cultivation and did not show any detectable variation in morphology or growth characteristics compared to wild-type plants.

Key words **:** *Agrobacterium tumefaciens*, *Panax ginseng*, transformation, hygromycin

INTRODUCTION

Ginseng (*Panax ginseng* C.A. Meyer) is one of the most important plantation crops yielding a great variety of pharmacological activities. The cultivation of ginseng is troublesome and a period of 4-6 years is required to harvest the roots. Also, root rot disease pose serious problem to this plant. Application of plant biotechnology may be possible to overcome these difficulties caused by conventional breeding of ginseng.

To obtain transformants of plants, there are many factors which may be important, including the pH of co-cultivation medium, temperature, bacteria concentration, the period of co-cultivation with bacteria, the use of liquid or agar-solidified media, phenolic compounds. etc. There are no general rules about these factors (Komari *et al.*, 2004). Therefore, empirical optimization for species of interest is necessary.

Until now, the study has been conducted on the genetic transformation of ginseng plants through the introduction of herbicide-resistant gene (Choi *et al.*, 2001), and Lee *et al.*, (2004) has reported the *Agrobacterium*-mediated transformation of ginseng with *PgSS* (*P. ginseng* squalene synthase) gene to enhance the triterpene compounds. To more easily introduce the useful genes in ginseng genome, an established protocol of *Agrobacterium*-mediated transformation of ginseng has been required.

Although, there are reports to date on the genetic transformation of *P. ginseng* using *A. tumefaciens* (Choi *et al.*, 2001; Choi *et al.*, 2003), optimization of the co-cultivation conditions has been the lack of a protocol for direct somatic embryos from explants. Therefore, the present study was aimed at optimizing several key factors to utilize cotyledon explants in *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Plant material

Stratified *P. ginseng* seeds were immersed in 70% ethanol for 1 min, then in 3% sodium hypochlorite solution for 20 min, and followed by three washes in sterile distilled water. Carefully dissection of the zygotic embryos from seeds was performed as described by Choi *et al.* (1998). The cotyledons were prepared for *Agrobacterium* transformation.

Conditions of Co-cultivation

A single clone of the disarmed *A. tumefaciens* strain GV3101 containing the binary vector pCAMBIA1300 consisting of hygromycin phosphotransferase (*hpt*) gene as a selectable marker controlled under CaMV 35S promoter was selected and cultured into a petridish (89 × 15 ㎜) containing YEP medium supplemented with 1.5% agar and 50 ㎎/L kanamycin sulfate (Sigma-Aldrich, St. Louis, MO) in the dark at 28 ℃ After the cultivation, the *Agrobacterium* in a petridish

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Received March 3, 2007 / Accepted March 30, 2007

were collected by suspension in 1 mℓ liquid a half-strength MS medium (Murashige & Skoog, 1962) supplemented with 3% sucrose into a petridish. Explants were submerged in the bacterial suspension for 15 min, washed with sterilized water and blot-dried on sterile filter paper. The experiments of optimization of co-cultivation for transformation included the length of co-culture period (0, 1, 3, 5, 7 days), bacteria growth phase (OD values of 0.2, 0.4, 0.8, 1.0 at 600 nm), pH of the co-cultivation medium (5.4, 5.7, 6.0), agar (0, 1.0%), and acetosyringone concentration (50, 100, 200 μM). The phenolic compound acetosyringone (Sigma-Aldrich) was dissolved in ethanol, and the stock volume made up in autoclaved distilled water.

DNA analysis in transgenic plants

Genomic DNAs of the non-transformed leaves and transformed plants were isolated with the cetyldimethylethylammonium bromide method (Doyle & Doyle, 1987). DNA was dissolved in autoclaved water and then stored at −20 ℃ until used. PCR was performed to detect the *hpt* and *VirC* genes in both the T-DNA of the *Agrobacterium* plasmid and genomic DNAs of transgenic plants. The plasmid DNA of *A. tumefaciens* GV3101 strain containing pCAMBIA1300 was used as a positive control. Primers for PCR were designed according to the DNA sequence of the *hpt* gene. The primer pair used for *hpt* gene amplification of an 700-bp fragment was: 5'-GCGTGACCTATTGCATCTCC-3' and 5'-TTCTACACAGCCATCGGTCC-3'; for the *VirC* gene amplification of an 730-bp fragment was: 5'-ATCATTTGTAGCGACT-3' and 5'-AGATCAAACCTGCTTC-3'. The PCR mixture consisted of DNA, 0.4 mM of each primer, 0.2 mM of each dNTP, 2.5U Taq DNA polymerase (5U/μℓ, Takara, Shiga, Japan), and 10 μℓ 10× buffer in a final volume of 100 μℓ. The PCR was run under the following conditions: an initial denaturation at 94 ℃ for 5 min; denaturation at 94 ℃ for 1 min; primer annealing at 58 ℃ for 1 min; elongation at 72 ℃ for 1 min, 30 cycles; a final extension at 72 ℃ for 10 min. The PCR products were examined by electrophoresis on a 1.2% (w/v) agarose gel. For Southern analysis, genomic DNAs (10 μg) were digested with *Hin*dIII, electrophoresed on an 0.8% agarose gel, and then transferred to a positively charged nylon membrane (Boehringer, Mannheim, Indianapolis, Ind.) by capillary blotting (Sambrook *et al.*, 1989). For hybridization, PCR-amplified *hpt* gene products labeled using digoxigenin (DIG)-dUTP (PCR DIG Probe Synthesis Kit, Roche, Basel, Switzerland) was used as the probe. Hybridization and detection of the DIG-labeled nucleic acid was performed using the DIG Easy Hyb solution and DIG Nucleic Acid Detection Kits (Roche). DNA cross-linked positively nylon membranes were incubated at 48 ℃ for 12 h with the probe for hybridization and then washed two times at 25 ℃, 5 min each wash, in 2 × SSC, 0.1% SDS, followed by two washes, 15 min each wash, at 68 ℃ in 0.5 × SSC, 0.1% SDS.

Selection and plant regeneration

After co-cultivation at 20 ℃ in the dark, the segments were transferred to a selection medium composed of MS medium containing 5% sucrose, 1.5% agar, and 400 mg/ℓ cefotaxim. To avoid necrosis of explants at the early stage of transformation by hygromycin, the segments were cultured without hygromcyin for 9 weeks. For hygromycin selection, cotyledon explants with somatic embryos were cultured on the medium containing 20 mg/ℓ hygromycin. The cultures were maintained at about 25 ℃ under a 16 h photoperiod and were subcultured every 3 weeks. The regenerated plants from somatic embryos were induced as described by Choi *et al.* (1998). The morphologically normal plantlets were transferred to square plastic pots containing soil, sand, and peat 4 : 4 : 3 (v/v) in a greenhouse.

RESULTS AND DISCUSSION

To examine optimization of *P. ginseng* transformation, we investigated several factors related to co-cultivation. In the present study, when pH of co-cultivation medium is 5.7, the highest number of transformation events was observed (Table 1). During co-cultivation, acidic pH as one of several factors has been reported to be important for the expression of *vir* genes (Alt-Moerbe *et al.*, 1988; Turk *et al.*, 1991). An acidic pH of 5.4 is generally considered to be suitable as acidic pHs because of more induction the *vir* (virulence) genes (Stachel *et al.*, 1986). But, our results showed pH of 5.7 is proved to be the best formation of transformation.

At a late log phase corresponding to OD_{600} = 0.8, the highest transformation was detected by PCR analysis (Table 2). At a high OD (over 1.0), the formation of somatic embryos from ginseng explants was dramatically decreased by bacterial induced stress, and it also became difficult to eliminate the

Table 1. The effect of pH in the medium on the formation of transgenic *P. ginseng* plants

pH	n (A)†	Hm^r (B)‡	PCR positive/negative	Efficiency (%) (B/A)
5.4	60	5	4/1	8.3
5.7	82	9	8/1	10.9
6.0	56	2	2/0	3.6

†Numbers of explants after infection with *Agrobacterium*
‡Numbers of hygromycin-resistant hairy roots after 2-month culture on selection medium containing 20 mg/ℓ hygromycin

Table 2. The effect of bacteria growth phase on the formation of transgenic *P. ginseng* plants

Bacteria growth phase (OD = 600 ㎚)	n (A)†	Hm^r (B)‡	PCR positive/negative	Efficiency (%) (B/A)
0.2	67	3	2/1	4.5
0.4	85	6	6/0	7.1
0.8	91	12	11/0	13.2
1.0	76	–	–	–

†Numbers of explants after infection with *Agrobacterium*
‡Numbers of hygromycin-resistant hairy roots after 2-month culture on selection medium containing 20 ㎎/ℓ hygromycin
–, Contamination caused by over-growth of bacteria

Table 3. The effect of the co-cultivation period with *A. tumefaciens* on induction of transgenic *P. ginseng* plants

The period of co-cultivation (days)	n (A)†	Hm^r (B)‡	PCR positive/negative	Efficiency (%) (B/A)
0	67	0	0	0
1	56	3	3/0	5.3
3	71	7	7/0	9.9
5	60	–	–	–
7	60	–	–	–

†Numbers of explants after infection with *Agrobacterium*
‡Numbers of hygromycin-resistant hairy roots after 2-month culture on selection medium containing 20 ㎎/ℓ hygromycin
–, Contamination caused by over-growth of bacteria

bacteria following co-cultivation. Our similar results showed that a late log phase corresponding to an OD of 0.8 was the most effective for obtaining high rates of transformation in *Citrus* (Pena *et al.*, 1995).

Table 4. The effect of acetosyringone concentration in the medium on the formation of transgenic *P. ginseng* plants

Acetosyringone concentration (μM)	n (A)†	Hm^r (B)‡	PCR positive/negative	Efficiency (%) (B/A)
0	56	6	6/0	10.7
50	54	5	5/0	9.3
100	60	9	8/1	15
200	55	7	7/0	12.7

†Numbers of explants after infection with *Agrobacterium*
‡Numbers of hygromycin-resistant transgenic plants after 2-month culture on selection medium containing 20 ㎎/ℓ hygromycin

Table 5. The effect of agar and liquid of the medium on the formation of transgenic *P. ginseng* plants

Agar (%)	n (A)†	Hm^r (B)‡	PCR positive/negative	Efficiency (%) (B/A)
0	55	6	6/0	10.9
1.0	78	5	5/0	6.5

†Numbers of explants after infection with *Agrobacterium*
‡Numbers of hygromycin-resistant transgenic plants after 2-month culture on selection medium containing 20 ㎎/ℓ hygromycin

After infection with *Agrobacterium*, the plant tissues and bacteria are cultivated together for a few days. Important events at this procedure are taking place; plant cells and bacteria are dividing further, and T-DNA is being transferred from bacteria to plant cells (Komari *et al.*, 2004). The length of co-cultivation also affects the efficiency of transformation (Mihaljevic *et al.*, 1996; Niu *et al.*, 2000; Tao & Li, 2006). Therefore, the effect of the length of co-cultivation on induction of transgenic plants was investigated. As shown in Table 3, statistically significant differences among co-culture periods of

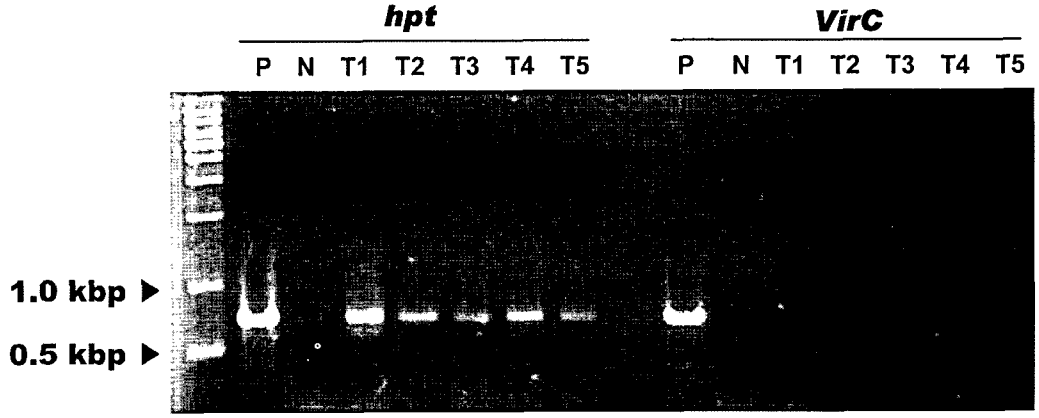

Fig. 1. PCR analysis of the *hpt* and *VirC* genes in transgenic *P. ginseng* plants. Lanes: P, plasmid DNA isolated from *A. tumefaciens* GV3101 strain as a positive control; T1, T2, T3, T4, and T5, DNA isolated from leaves of transgenic plants; N, DNA isolated from non-transformed leaves as a negative control.

different durations were detected. A 3-day co-cultivation with *Agrobacterium* significantly increased the number of transgenic plants, which are able to grow under hygromycin selection. The transformation was not showed over 5 days of co-cultivation length because of extensive tissue damage caused by overgrowth of bacteria. Thus, the optimum length of co-culture was concluded to be 3 days.

The induction of vir genes is initiated at an early stage of co-cultivation in the presence of acetosyringone. Several studies demonstrated that the levels of transient expression GUS after co-cultivation was dramatically high in the presence of acetosyringone (Chan *et al.*, 1993; Hiei *et al.*, 1994). While in wood plants, such as plum and poplars, acetosyringone did not help in increasing transformation efficiency (Mannie *et al.*, 1991; Confalonieri *et al.*, 1997). Among different concentrations (0-200 μM) of acetosyringone, 100 μM is optimal concentration to enhancement of ginseng transformation efficiency (Table 4). We also tested the use of liquid and agar-solidified media. The result revealed that the transformation efficiency was up to 10.9% increased at the agar-solidified media of co-cultivation than liquid media (Table 5).

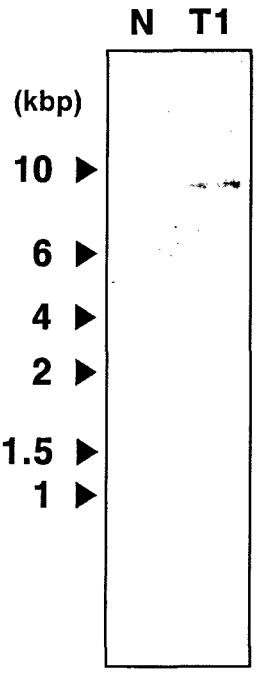

Fig. 2. Southern blot analysis of transgenic *P. ginseng* plants. Lanes: N, DNA isolated from non-transformed leaves as a negative control; T1, DNA isolated from leaves of transgenic plant.

We conducted PCR analysis with primers designed according to the sequence of the *hpt* gene. Using DNAs from the transgenic plantlets and the non-transformed leaves as templates, the fragments with lengths of 700 bp were detected from hairy roots but not detected from a non-transformed plant (Fig. 1). We also performed additional PCR reactions with primers designed to amplify regions outside the T-DNA (Hamill *et al.*, 1991). Negative results in the attempted amplification of the *VirC* gene in the root DNA suggested that there was no *Agrobacterium* contamination. Southern analysis was conducted to further confirm the introduction of the *hpt* gene into the ginseng genome (Fig. 2). Bands for *hpt* gene were detected in genomic DNA from a transgenic plantlet (in T1 lane), whereas there was no hybridization band from a non-transformed plant (in N lane). Southern blot analysis of *Hin*dIII-digested genomic DNA from ginseng transgenic plantlet confirmed the presence of the *hpt* gene.

To induce germination, transgenic somatic embryos were transferred to half-strength MS medium supplemented with 5 mg/ℓ GA_3 and 20 mg/ℓ hygromycin. Germination of somatic embryos was generally induced from explants on the medium containing high hygromycin concentration (Fig. 3A). Transgenic ginseng plantlets at 7 cm in height (Fig. 3B) were transferred in a mixture of autoclaved soil [soil: sand: peat (4 : 4 : 4, v/v)] in a greenhouse (Fig. 3C). A transgenic plant did not show any detectable variation in morphology or growth characteristics compared to wild-type plants.

We established an efficient transformation system of *P. gin-*

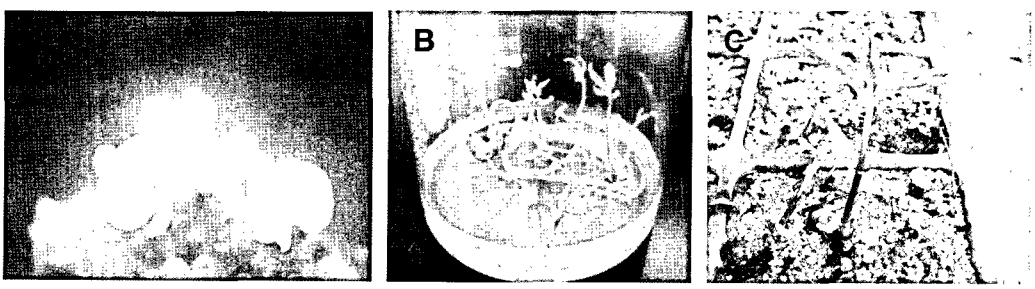

Fig. 3. Transgenic plants of *P. ginseng*. A, Germination of transformed somatic embryos on MS medium supplemented with 5 mg/ℓ GA_3, 3% sucrose, 1% agar, 400 mg/ℓ cefotaxim, and 20 mg/ℓ hygromycin; B, Transgenic plantlets on 1/2MS medium supplemented with 30 mg/ℓ hygromycin; C, A transgenic plant 8 weeks after transfer to the soil.

seng, using the GV3101 strain of *A. tumefaciens*. Our results indicated that the integration of T-DNA into the plant genome was verified via PCR analysis and Southern hybridization. The establishment of an efficient genetic transformation protocol will facilitate biotechnological applications for the enhancement of target compound yields or generating functional plants for environmental stress.

ACKNOWLEDGEMENT

This study was supported by the Post Doctoral Course Program of the National Institute of Crop Sciences, Rural Development Administration, Republic of Korea.

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