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Transgenic Note

Agrobacterium-mediated genetic transformation of radish (*Raphanus sativus* L.)

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Abstract In order to generate transgenic radish (*Raphanus sativus* L., cv. Jin Ju Dae Pyong), hypocotyl explants were cultured on Murashige and Skoog medium containing 4 mg l^{-1} $AgNO_3$, 5 mg l^{-1} acetosyringone, 4 mg l^{-1} 6-benzyladenine, and 3 mg l^{-1} α-naphthaleneacetic acid in addition to either 10 mg l^{-1} hygromycin or 100 mg l^{-1} paromomycin after co-cultivation with disarmed *Agrobacterium tumefaciens* harboring a plant expression binary vector. Explants co-cultivated with *A. tumefaciens* GV3101 harboring pCAMBIA1301 and *A. tumefaciens* EHA101 harboring pPTN290 produced putative transgenic adventitious shoots at frequencies of 0.26% and 0.18%, respectively. Northern blot analysis revealed the *gus* gene transcript was detected in 8 regenerated plants which confirmed their genetic transformation. The transgenic plants were grown to maturity after vernalization in a greenhouse and appeared morphologically normal. Progeny analysis of independent transgenic plants demonstrated that the *gus* gene was transmitted in a Mendelian pattern in 3 lines, indicating a single copied gene was incorporated into the genome.

Key words: *Agrobacterium*, β-glucuronidase (GUS), hygromycin, paromomycin, transgenic radish.

Radish (*Raphanus sativus* L.) belongs to the family *Brassicaceae*, and is an important plant that is cultivated throughout Asia. Korean varieties are rich in vitamins *B* and C and have important medicinal properties. However, many of these varieties are susceptible to various pathogens and conventional breeding is not successful in introducing resistance genes into the varieties because of limited availability of germplasm. Alternatively, it is required to establish a genetic transformation system of this species to transfer resistance genes from various sources rather than radish germplasm. The importance and needs for the genetic improvement of radish have recently been reviewed by Curtis (2003). A floral-dip method previously developed for genetic transformation of Arabidopsis (Clough and Bent 1998) was used for radish, resulting in genetic transformation frequencies of 1.1%–1.4% (Curtis and Nam 2001; Curtis et al. 2002). However, transgenic plants produced only 50–100 seeds per individual. Furthermore, many of the plants were siblings (Curtis et al. 2004). Therefore, *Agrobacterium*-mediated transformation might be more appropriate to this species as demonstrated in a vast array of other crops. A few efficient adventitious shoot regeneration systems were developed for Japanese (Matsubara and Hegazi 1990), Chinese (Pua et al. 1996), and Korean ecotypes of radish (Curtis et al. 2004). However, there have been no reports on production of transgenic radish by *Agrobacterium*-mediated transformation. This paper describes *Agrobacterium*-mediated genetic transformation of radish using adventitious shoot formation on hypocotyl explants.

Zygotic embryos of F1 hybrid radish (*Raphanus sativus* L. cv. Jin Ju Dae Pyong) were used. Seeds were surface disinfected with 70% ethanol for 1 min and then with 0.4% sodium hypochlorite solution for 15 min. They were rinsed three times with sterile deionized-distilled water before being placed on MS medium (Murashige and Skoog 1962) without growth regulators contained in Petri dishes. The pH of all media was adjusted before autoclaving. Twenty-five ml of medium was dispensed into 90×15-mm plastic Petri dishes. After 5 to 7 days of incubation in the dark, hypocotyls, 4 to 6-cm long, were decapitated from 5 to 7 day old seedlings and then cut into 5 to 7-mm long segments (hypocotyl explants). To determine the optimum concentration and combination of growth regulators for adventitious shoot formation, hypocotyl explants were cultured on MS

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(Murashige and Skoog 1962) medium supplemented with 1.0, 2.0, 4.0, or 8.0 mg l^{-1} BA (6-benzyladenine) in combination with 1.0, 2.0, or 3.0 mg l^{-1} NAA (α-naphthaleneacetic acid). The highest frequency (>55%) of adventitious shoot formation was obtained at the combination of 4.0 mg l^{-1} BA and 3.0 mg l^{-1} NAA (data not shown). Hypocotyl explants were co-cultivated with either disarmed *Agrobacterium tumefaciens* GV3101 harboring the binary vector pPTN290 or *A. tumefaciens* EHA101 harboring pCAMBIA1301. The binary vectors comprise the neomycin phosphotransferase gene (*nptII*) and hygromycin resistance gene (*hpt*) as selective markers, respectively. The freeze-thaw method was used according to An et al. (1987) to introduce a binary vector to *A. tumefaciens*. The bacterial cells were grown in YEP medium supplemented with an appropriate antibiotic to an OD_{650}=0.6 to 0.8 at 27°C. Bacterial cells were pelleted and then resuspended to the same OD_{650} value in MS medium containing 200 μM acetosyringone, 3% sucrose, and 20 mM MES at pH 5.4 (co-cultivation medium). Hypocotyl explants were inoculated with *Agrobacterium* suspensions for 30 min and then incubated on co-cultivation medium. Fifty inoculated explants were placed on a single filter paper laid over medium contained in a 90×15 mm petri-dish. After co-cultivation for 3 days, the explants were washed three times with a sterilized distilled water and then cultured for 2 weeks on shoot induction medium (MS inorganic salts and vitamins, 4 mg l^{-1} $AgNO_3$, 5 mg l^{-1} acetosyringone, 4 mg l^{-1} 6-benzyladenine, 3 mg l^{-1} α-naphthaleneacetic acid, 250 mg l^{-1} cefotaxime, pH 5.6) supplemented with an selection agent. In the infection of pCAMBIA1301 and pPTN290, 10 mg l^{-1} hygromycin and 100 mg l^{-1} paromomycin were used for selection agent, respectively. Explants were incubated in 16 h photoperiods at 46 μmol m^{-2} s^{-1} at 24°C. Explants were subcultured to fresh medium every 2 weeks. After 4 to 10 weeks of culture, the number of adventitious shoots formed on explants was determined. Regenerated shoots were transferred to solidified shoot elongation medium (MS inorganic salts and vitamins, 150 mg l^{-1} cefotaxime, pH 5.8) including either 10 mg l^{-1} hygromycin for *A. tumefaciens* GV3101 harboring pCAMBIA1301 or 100 mg l^{-1} paromomycin for *A. tumefaciens* EHA101 harboring pPTN290. Elongated shoots were transferred to rooting medium comprising 1/2MS inorganic salts and vitamins, 3% sucrose, 3 mM MES, 50 mg l^{-1} cefotaxime, and 0.8% agar at pH 5.8. Regenerated plantlets were transplanted to soil after acclimation.

To verify T-DNA transfer (Jefferson et al. 1987), a histochemical β-Glucuronidase assay was conducted using leaf tissues from putative transgenic plants (T_0) by immersing into a 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) solution overnight at 37°C. The leaf tissues were subsequently cleared in 70% ethanol before visualization. Total RNA was prepared using Tri-Reagent (Invitrogen), following the manufacturer's instructions. Approximately 30 μg total RNA was electrophoresed on 1% agarose gel containing 5.1% (v/v) formaldehyde and then blotted onto nylon membrane (Zeta-Probe GT genomic tested blotting membranes; Bio-Rad) in 20X SSC. The 622-bp GUS PCR product was used and then labeled with [α-^{32}P]dCTP using the Random Primed DNA Labeling kit (Boehringer Mannheim). Eight independent transgenic T_0 radish plants were vernalized at 4°C for 2 months. Plants were self-pollinated to produce seeds. To analyze the transgene segregation ratio in progenies, 6 to 28 seeds in each line were placed on a wet bandage. After 5 to 7 days of incubation in the dark, they germinated to 4 to 6-cm long seedlings, which were subjected to GUS assay. The segregation ratio of GUS^+ plants to that of GUS^- was 3 : 1 ($P<0.05$). After 6 weeks of culture, *Agrobacterium*-infected hypocotyl explants on shoot induction medium containing antibiotic (selection medium) formed yellowish-green calluses (Figure 1A). After 10 weeks of culture, the callus gave rise to shoot primordia (Figure 1B), which then elongated into shoots. As results, 10 and 4 putative transgenic shoots were generated at frequencies of 0.44% and 0.36%, respectively (Table 1). These shoots were rooted on rooting medium (Figures 1C, D). GUS positive response in leaf was obtained from 6 plants out of 10 plants and 2 plants out of 4 plants plantlets (Figure 1E). Transgenic

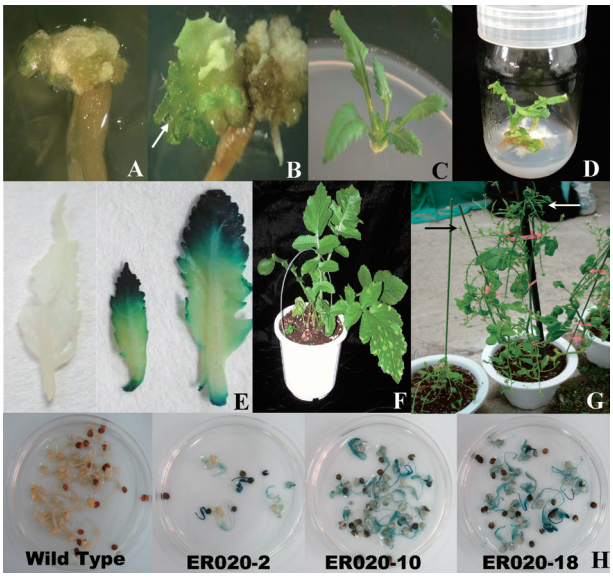

Figure 1. Plant regeneration from hypocotyl explants of radish transformed with *gus* gene. A: Yellowish-green callus formation on selection medium with 10 mg l^{-1} hygromycin. B: Hygromycin-resistant shoot primordia. C, D: Shoot elongated from the hygromycin-resistance primordia. E: GUS negative response in leaf of non-transgenic plant (left), GUS positive response in the leaf of transgenic plant (right). F: Transgenic radish grown in soil. G: Flowering plants. H: GUS assay for transgenic radish events (T_1).

Table 1. Frequency (%) of GUS positive response in leaf of putative transgenic radish formed from hypocotyl explants mediated by *Agrobacterium tumefaciens* strains.

Agrobacterium strains/vectors	No. of explants cocultured	No. of shoots formed on hypocotyl explants (%)	No. of GUS positive response in leaf (%)
GV3101/pCAMBIA1301	2,281	10 (0.44)	6 (0.26)
EHA101/pPTN290	1,090	4 (0.36)	2 (0.18)

Table 2. Progeny analysis of transgenic radish by histochemical GUS assay.

Vector	Transgenic events	GUS assay in progeny			Chi-square[a] (3:1 ratio)
		Total seedlings (T_1)	positive	negative	
pCAMBIA1301	ER020-2	6	6	0	2.00
	ER020-10	26	22	4	1.28
	ER020-18	24	21	3	2.00

[a] A 3:1 ratio was used for chi-square ($p=0.05$ with d. of f.$=1$).

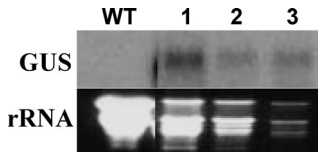

Figure 2. Northern blot analyses of total RNA extracted from transgenic radish plants (T_0 generation). The RNA (30 μg) was separated in 1% agarose gel in each lane and subjected to Northern hybridization. The 622-bp GUS PCR product was labeled with [^{32}P]dCTP and then used as probe. WT: Wild type, 1: ER020-2, 2: ER020-10, 3: ER020-18.

plants were transplanted to soil and grown in a greenhouse (Figure 1F). After vernalization, transgenic plants flowered. Self-pollinated flowers produced seeds (T_1) (Figure 1G). Regenerated plants with GUS positive response were obtained from explants that had been co-cultivated with *A. tumefaciens* GV3101 harboring pCAMBIA1301 or *A. tumefaciens* EHA101 harboring pPTN290 (Table 1). The former seemed better than the latter because of higher frequency of genetic transformation. However, no plants showed GUS positive response that were derived from explants co-cultivated with *A. tumefaciens* LBA4404 harboring pCAMBIA1301 (data not shown). The *gus* gene expression at the transcription level was examined in 8 transgenic lines with GUS positive response. Total RNA was extracted from leaf tissues and subjected to Northern hybridization assay. All of the 8 lines exhibited the *gus* gene transcript (Figure 2). The 7 lines were subjected to progeny analysis for the *gus* gene by chi-square analysis (Figure 1H; Table 2). It was revealed that the *gus* gene was transmitted in a Mendelian fashion (3 : 1) in 3 lines, indicating that the *gus* gene was incorporated into the genome as a single copy gene in these lines (Table 2), which was further confirmed by Southern blot analysis (data not shown). In conclusion, we demonstrated that *Agrobacterium*-mediated genetic transformation in radish for the first time. The frequency of genetic transformation conducted by the system established in this study was lower than the reported frequency by floral-dip method (Curtis and Nam 2001; Curtis et al. 2002). However, *Agrobacterium* method is more popular than floral-dip method for higher plants so as to enable this method to be more rapidly improved. Therefore, genetic transformation of radish by *Agrobacterium* method could also be further developed. *Agrobacterium*-mediated genetic transformation would be applicable to improvement of this crop including development of pathogen-resistant cultivars.

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