Biotechnology Letters **24:** 547–550, 2002.

Agrobacterium-mediated genetic transformation of Chinese foxglove, *Rehmannia glutinosa* L.

Sang-Un Park[1,*], Haeng-Hoon Kim[2], Chang-Yeon Yu[3], Cheol-Ho Park[3] & Young-Am Chae[2]
[1]*GenePark Inc., 855-3 Toegye, Chunchon, Kangwon 200-190, Korea*
[2]*Research Center for New Biomaterials in Agriculture (RCNBA) and Department of Agronomy, Seoul National University, Suwon 441-744, Korea*
[3]*College of Agriculture and Life Sciences, Kangwon National University, Chunchon 200-701, Korea*
[*]*Present address and author for correspondence: Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, Alberta T2N 1N4, Canada (Fax: 403-289-9311; E-mail: spark@ucalgary.ca)*

Received 13 December 2001; Revisions requested 18 December 2001; Revisions received 28 January 2002; Accepted 1 February 2002

Key words: Agrobacterium, Chinese foxglove, genetic transformation, *Rehmannia glutinosa* L., shoot organogenesis

Abstract

An efficient transformation protocol was established for Chinese foxglove, *Rehmannia glutinosa* L., using *Agrobacterium tumefaciens.* Putative transgenic plants were induced from leaf explants co-cultivated with *Agrobacterium*. Detection of the neomycin phosphotransferase gene, high activity of β-glucuronidase (GUS) transcripts and histochemical localization of GUS confirmed the integrative transformation of Chinese foxglove. This result demonstrates the potential for using *A. tumefaciens* to transfer important genes into commercial Chinese foxglove cultivars.

Introduction

Chinese foxglove, *Rehmannia glutinosa* L. is an important medicinal plant in Korea, Japan, and China. Rehmanniae radix (the dried root of *Rehmannia glutinosa* L.) is a popular herbal medicine and is commonly used in clinics. This herbal medicine stops bleeding, provides energy and helps strengthen the immune system (Oshio *et al.* 1981, Hasegawa *et al.* 1982).

At the present time various methods and different approaches for the genetic transformation of plants have been developed. However, *Agrobacterium*-mediated gene transfer is one of the major techniques which has allowed the routine production of transgenic plants. More than 100 species have been transformed by this method.

Although many examples have been reported of *Agrobacterium*-mediated transformation of medicinal plants, there are relatively few reports of *Agrobacterium*-mediated gene delivery to oriental medicinal plants. In this paper, we describe a stable *Agrobacterium*-mediated transformation protocol for one of the important oriental medicinal plants, Chinese foxglove.

Materials and methods

Preparation of Agrobacterium *culture*

The binary vector, pBI121, which has a CaMV 35S promoter-GUS (β-glucuronidase) gene fusion and the neomycin phosphotransferase (*NPTII*) gene as a selectable marker, was used. It was transferred into *Agrobacterium tumefaciens* LAB 4404 by electroporation. The culture of *A. tumefaciens* was grown overnight at 28 $\pm$ 1 °C with shaking (180 rpm) in liquid Luria–Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, pH 7] containing 50 mg kanamycin l^{-1}, to mid-growth phase. The *A. tumefaciens* cells were collected by centrifugation for 10 min at 1500 *g* and resuspended in liquid

inoculation medium (Murashige & Skoog salt with 20 g sucrose l^{-1}). The *A. tumefaciens* cell density was adjusted to OD_{600nm} 1 for inoculation.

Production of transgenic plants

Young leaves of *Rehmannia glutinosa* were taken from greenhouse grown plants. Explants were surface-sterilized by a 10 s immersion in 70% (v/v) ethanol and for 10 min in aqueous solution of 1% (v/v) $NaClO_3$ containing a few drops of Tween 20. After three washes in sterilized water, leaves were cut aseptically at the ends, in sections 7 × 7 mm^2. Excised leaves were dipped into the *Agrobacterium* suspension in liquid inoculation medium for 10–15 min, blotted dry on sterile filter paper and incubated on shoot induction medium in dark treatment. Previously, we established shoot induction medium consisting of MS (Murashige & Skoog 1962) salts and vitamins, 30 g sucrose l^{-1}, 8 g Phytagar l^{-1}, 4 mg 6-benzylaminopurine l^{-1}, 0.1 mg 1-naphthalene acetic acid l^{-1}, and 7 mg $AgNO_3$ l^{-1}. After 2 days co-cultivation, explants were transferred to shoot induction medium containing 50 mg kanamycin l^{-1} and 200 mg Timentin l^{-1} and were transferred to fresh selection medium every 2 weeks. After 6 weeks on the shoot induction medium, putative transgenic shoots were transferred to root induction medium consisting of MS salts and vitamins, 30 g sucrose l^{-1}, 8 g Phytagar l^{-1}, 0.1 mg indole 3-butyric acid l^{-1}, 50 mg kanamycin l^{-1} and 200 mg Timentin l^{-1}. Putative transgenic shoot were incubated at 25 ± 1 °C in a growth chamber with a 16 h photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}) for 30 days. Putative transgenic plantlets were then transferred to the pots containing autoclaved vermiculite, covered with polythene bags to maintain high humidity and kept at 25 ± 1 °C in a growth chamber for 1 week. After 1 week the bags were perforated and these plants were then transferred to the green house.

PCR analysis for NTPII

Plant genomic DNA for polymerase chain reaction (PCR) analysis was extracted as described by Edwards *et al.* (1991). The leaf tissue (50 mg fresh weight) was homogenized in 200 μl extraction buffer [0.5% (w/v) SDS, 250 mM NaCl, 100 mM Tris/HCl, pH 8, and 25 mM EDTA pH 8] and centrifuged at 10 000 *g* for 5 min. The supernatant was transferred to a new tube and an equal volume of 2-propanol was added. The sample was incubated on ice for 5 min and then centrifuged for 10 min at 10 000 *g*. The pellet was dried at 60 °C for 5–10 min and then resuspended in 100 μl of TE (10 mM Tris/HCl, pH 7.4 and 1 mM EDTA, pH 8).

The sequences of the two primers used to amplify a fragment of the *NPTII* gene were 5′-TATGTTATGTATGTGCAGATGATT-3′ and 5′-GTC GACTCACCCGAAGAACTCGTC-3′. The amplification cycle consisted of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 1 min. After 30 repeats of the thermal cycle and final extension 72 °C for 5 min, amplification products were analyzed on 1% (w/v) agarose gels. Gels were stained with ethidium bromide and visualized with UV light.

Assay of GUS activity

Putative transgenic young leaf tissues were collected and ground with extraction buffer consisting of 50 mM KPO_4 buffer, pH 7, 1 mM EDTA, and 10 mM β-mercaptoethanol. The GUS flurometric assay buffer consisted of 50 mM $NaPO_4$ buffer, pH 7, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 0.1% (w/v) Triton X-100. 4-Methylumbelliferyl-β-D-glucuronide was added to give 0.44 mg ml^{-1}. Assays were performed on 50 μl transgenic shoot tissue extract for 3 h at 37 °C and stopped with a 10× volume of 0.2 M Na_2CO_3. A fluorescence spectrophotometer was used to quantify the amount of 4-methylumbelliferone cleaved from 4-methylumbelliferyl-β-D-glucuronide. The protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Northern blot hybridization

Total RNA for gel-blot analysis was isolated from putative transgenic plant tissue according to the method of Logemann *et al.* (1987), and 15 μg was fractionated on 1% (v/v) formaldehyde agarose gels before transfer to nylon membrane (Sambrook *et al.* 1989). RNA gel blot was hybridized with random-primer ^{32}P-labeled (Feinberg & Vogelstein 1984) full-length GUS-intron. Hybridization was performed at 65 °C in 0.25 mM sodium phosphate buffer, pH 8, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. Blot was washed at 65 °C, twice with 2× SSC and 0.1% (w/v) SDS and twice with 0.2× SSC and 0.1% (w/v) SDS (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7), and au-

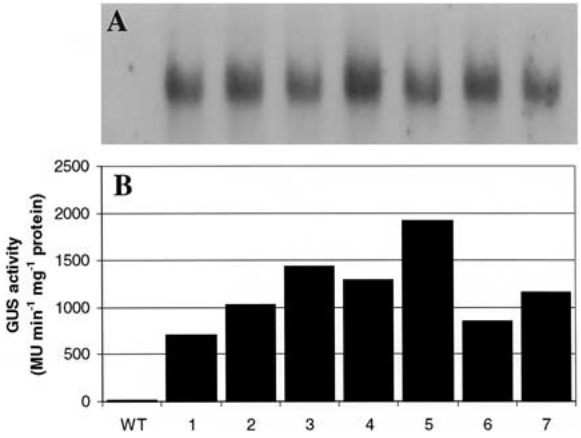

Fig. 1. (A) RNA gel blot hybridization analysis for GUS reporter gene in wild-type (WT) and randomly selected kanamycin-resistant (1–7) Chinese foxglove, *Rehmannia glutinosa* L. (B) GUS activity in wild-type (WT) and kanamycin-resistant (1–7) Chinese foxglove.

toradiographed with an intensifying screen at −80 °C for 24 h.

GUS histochemical staining

Histochemical staining for GUS activity was performed by standard protocol (Jefferson 1987) for fixation and the modified method recommended by Kosugi *et al.* (1990) for staining. Different transgenic plant organs were fixed in a 0.35% (v/v) formaldehyde solution containing 10 mM Mes, pH 7.5, and 300 mM mannitol for 1 h at 20 °C, rinsed three times in 50 mM sodium phosphate, pH 7.5, and subsequently incubated in 50 mM sodium phosphate, pH 7.5, 10 mM EDTA, 300 mM mannitol, pH 7, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt for 6 to 12 h at 37 °C. Stained tissues were rinsed extensively in 70% (v/v) ethanol to remove chlorophyll.

Results and discussion

Plant transformation

An efficient *Agrobacterium*-mediated transformation protocol developed to regenerate transgenic Chinese foxglove, *Rehmannia glutinosa* L. through shoot organogenesis is reported. We have previously studied the effects of kanamycin on the shoot regeneration of Chinese foxglove, which, up to 40 mg l^{-1}did not significantly inhibit callus growth or shoot regeneration. However, 50 mg kanamycin l^{-1} completely inhibited callus growth and shoot regeneration.

Agrobacterium-infected explants were cultured on shoot induction medium with 50 mg kanamycin l^{-1} for selection. After 3 weeks, kanamycin-resistant shoots emerged from leaf explants in more than 90% of the explants. Fully differentiated, putative transgenic shoots (1 cm) were transferred to root induction medium containing 50 mg kanamycin l^{-1}. After 30 days on the root induction medium, putative transgenic plants were transferred to soil and grown to maturity. Transgenic plants grew normally and flowered within 3 months.

Analysis of transformation

The stable and complete transformation of kanamycin-resistant Chinese foxglove plants was demonstrated by (1) the PCR of *NPTII* assay from regenerated plant tissue, (2) the presence of GUS mRNAs, (3) the presence of GUS enzyme activity, and the histochemical localization of GUS activity in all plant organs. PCR performed using primers specific for sequences in the *NPTII* gene resulted in the amplification of a single product with the expected size of 823 base pair in 95% (19 of 20 plants) of the regenerated plants.

The randomly selected 7 *NPTII*-positive regenerants were also evaluated for GUS enzyme activity and were compared to young leaf tissue from non-transformed plant. Transgenic plant showed higher GUS activity than non-transformed plant which demonstrated low-level background GUS activity. Individual transformants expressed a range of GUS activities, from 710 to 1850 pmol min^{-1} mg^{-1} protein. Varying activities may be due to 'positional effects' of the introduced gene within the nuclear chromosomes, and possibly also due to the presence of chimeric tissue in the leaf material used for assay.

Northern blot analysis showed signals corresponding to the GUS transcripts in the RNA of the seven transgenic plants and no signal was found in the non-transformed plant. The mRNA levels of GUS from seven transgenic plants were almost the same.

Histochemical staining for GUS activity was also performed to investigate whether the transformation process resulted in transgenic Chinese foxglove plants. GUS staining was present only in tissues transformed with pBI121 plasmid which has a CaMV 35S promoter-GUS gene fusion and the neomycin phosphotransferase. Staining revealed whole tissue from

transgenic plants whereas GUS was not detected in any wild type plant tissues.

The combined demonstration that the *NPTII* gene was present in kanamycin-resistant plant tissue, high levels of GUS mRNA and enzyme activity could be detected, and histochemical staining revealed the presence of GUS in all organs of the regenerated plants is proof that we have indeed achieved the stable genetic transformation of Chinese foxglove plants via shoot organogenesis.

Our protocol for the stable genetic transformation of Chinese foxglove is simple, efficient, and rapid. This transformation system allows new opportunities to investigate the molecular regulation and gene expression studies in this oriental medicinal plant.

References

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for preparation of plant genomic DNA for PCR analysis. *Nucl. Acids Res.* **19**: 1349.

Feinberg AP, Vogelstein B (1984) A technique for radiolabeling DNA restriction endonuclease fragments for high specific activity. *Anal. Biochem.* **137**: 266–269.

Hasegawa T, Koike K, Takahashi S, Ariyoshi U (1982) Constituents of leaves and roots of Kailei Jio (*Rehmammia glutinosa Libosch. Forma hueichingensis* Hsiao). *Shoyakugaku Zasshi.* **36**: 1–5.

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**: 387–405.

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. *Plant Sci.* **70**: 133–140.

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**: 16–20.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15**: 473–497.

Oshio H, Naruse Y, Inouye H (1981) Quantitative analysis of iridoid glycosides of *Rehmanniae radix. Shoyakugaku Zasshi.* **35**: 291–294.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.