An efficient protocol for peanut (*Arachis hypogaea* L.) transformation mediated by *Agrobacterium rhizogenes*

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Abstract

The Agrobacterium-mediated transformation of hairy roots creates a rapid and simple means to introduce and express foreign genes in plant cells that are capable of synthesizing specific natural products. We describe a method for the production of transgenic hairy root cultures of peanut (Arachis hypogaea L.) using Agrobacterium rhizogenes R1000, a strain with the binary vector pBI121. We evaluated the stable transformation of kanamycin-resistant hairy roots by determining the integration of the NPTII gene into the plant genome, the histochemical localization of GUS activity, the presence of GUS mRNA, and the level of GUS enzyme activity. Transgenic root culture of A. hypogaea is a simple, reliable and well-defined model system to investigate the molecular and metabolic regulation of resveratrol biosynthesis, and to evaluate the metabolic engineering potential of peanut.

Introduction

Peanut (*Arachis hypogaea* L.) is an annual oil seed legume that is native to South America, but is currently grown in diverse environments world-wide [22]. Peanuts, grapes, and Japanese knotweed are important sources of resveratrol (trans-3,5,4'-trihydroxystilbene) [4; 24]. Resveratrol has anti-inflammatory, antioxidant, and anti-infective properties and also has potential therapeutic efficacy against breast cancer, prostate cancer, and neuroblastoma [1; 15; 12; 2].

In many plants, hairy root cultures have proven to be an efficient production system for secondary metabolites. Such cultures have genetic and biochemical stability, rapid growth rate, and the ability to synthesize natural compounds at levels comparable to those of intact plants [9; 23; 7; 8].

Investigation of the molecular mechanisms that regulate resveratrol biosynthetic enzymes in peanut requires establishment of protocols for its efficient and stable genetic transformation. *Agrobacterium rhizogenes*-mediated transformation of hairy roots provides a rapid and simple means to introduce and express foreign genes in plant cells that are capable of synthesizing specific secondary metabolites, such as resveratrol. Recently we reported five different strains of *Agrobacterium rhizogenes* differing in their ability to induce peanut hairy

roots and also showed varying effects on the growth and resveratrol production in hairy root cultures [11]. In this paper, we describe a protocol for introducing foreign genes into peanut hairy root cultures using *A. rhizogenes*.

Materials and Methods

Plant material

Seeds of *A. hypogaea* were surface-sterilized with 70 % (v/v) ethanol for 1 min and 2 % (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Four seeds were placed on 25 ml of agar-solidified culture medium in Petri dishes (100 x 15 mm). The basal medium consisted of salts and vitamins of MS (Murashige and Skoog) [18] medium and solidified with 0.7 % (w/v) agar. The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121 °C for 20 min. The seeds were germinated in a growth chamber at 25 °C under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod.

Preparation of *Agrobacterium rhizogenes*

This experiment was conducted using the binary vector pBI121. The pBI121 plasmid has a CaMV 35S promoter-GUS gene fusion and the neomycin phosphotransferase (NPT II) gene as a selectable marker. This binary plasmid was transferred into *Agrobacterium rhizogenes* R1000 by electroporation. The culture of *A. rhizogenes* was initiated from glycerol stock and grown overnight at 28 °C with shaking (180 rpm) in liquid Luria-Bertani medium containing 50mg/L kanamycin, to mid-log phase (OD600 = 0.5). The *A. rhizogenes* cells were collected by centrifugation for 10min at 250g and resuspended in liquid inoculation medium (MS salts and vitamins containing 30 g/L sucrose). The *A. rhizogenes* cell density was adjusted to given an A600 of 1.0 for inoculation.

Establishment of transgenic hairy root cultures

Excised leaves of *A. hypogaea* from 20-day-old seedlings were used as an explant material for co-cultivation with *A. rhizogenes* R1000. The excised leaves were dipped into the *A. rhizogenes* culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on Phytagar-solidified MS medium. After two days of co-cultivation, the explant tissues were transferred to a hormone-free MS medium containing salts and vitamins, 30 g/L sucrose, 500 mg/L carbenicillin, 50 mg/L kanamycin and 8 g/L agar. Putative transgenic hairy roots were observed emerging from the wound sites within two weeks.

Isolated putative transgenic roots (100 mg) were transferred to 30 ml of MS liquid medium, containing 30 g/L sucrose, in 100 ml flasks. Root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod. After 3 weeks of culture, hairy roots were harvested and the dry weight and resveratrol content were determined. Each experiment was carried out with 3 flasks per culture condition and repeated twice.

PCR analysis for NTPII

Plant genomic DNA for polymerase chain reaction (PCR) analysis was extracted as described by Edwards et al. [5]. The tissue (50 mg fresh weight) was homogenized in 200 µl of extraction buffer (0.5% SDS, 250 mM NaCl, 100 mM Tris-HCl, pH 8, and 25 mM EDTA pH 8.0) and centrifuged at 13,200 rpm for 5 min. The supernatant was transferred to a new tube and

an equal volume of isopropanol was added. The sample was incubated on ice for 5min and then centrifuged for 10 min at 13,200 rpm. 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 8.0).

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

Assay of GUS activity

Putative transgenic peanut roots were collected and grounded with extraction buffer consisting of 50 mM KPO_4 buffer, pH 7.0, 1 mM EDTA, and 10mM-mercaptoethanol. The GUS flurometric assay buffer consisted of 50 mM $NaPO_4$ buffer, pH 7.0, 10 mM-mercaptoethanol, 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 0.1% (w/v) Triton X-100. 4-Methylumbelliferyl-D-glucuronide was added at a final concentration of 0.44 mg/ml. Assays were performed on 50 L of transgenic shoot tissue extract for 3 h at 37°C and stopped with a 10X volume of 0.2 M Na_2CO_3. A fluorescence spectrophotometer (model F-2000, Hitachi, Tokyo, Japan) was used to quantify the amount of 4-Methylumbelliferone cleaved from 4-Methylumbelliferyl-D-glucuronide. The protein concentration was determined by the method of Bradford [3] using BSA as a standard.

Northern blot hybridization

Total RNA for gel-blot analysis was isolated from putative transgenic root tissue according to the method of Logemann *et al*. [16], and 15 μg was fractionated on 1.0% formaldehyde agarose gels before transfer to nylon membrane [20]. RNA gel blot was hybridized with random-primer ^{32}P-labeled [6] full-length GUS-intron. Hybridization was performed at 65°C in 0.25 mM sodium phosphate buffer, pH 8.0, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. Blot was washed at 65°C, twice with 2X SSC and 0.1% (w/v) SDS and twice with 0.2X SSC and 0.1% (w/v) SDS; 1X SSC (= 0.15M NaCl, 0.015M sodium citrate, pH 7.0), and autoradiographed with an intensifying screen at -80°C for 24 h.

GUS histochemical staining

Histochemical staining for GUS activity was performed by standard protocol [10] for fixation and the modified method recommended by Kosugi *et al*. [13] for staining. Hairy roots were fixed in a 0.35% (w/v) formaldehyde solution containing 10 mM MES, pH 7.5, and 300 mM mannitol for 1 h at 20°C, rinsed three times in 50mM sodium phosphate, pH 7.5, and subsequently incubated in 50mM sodium phosphate, pH 7.5, 10 mM EDTA, 300 mM mannitol, pH 7.0, and 1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide cyclohexylammonium salt for 6 to 12 h at 37°C. Stained tissues were rinsed extensively in 70% ethanol to remove residual phenolic compounds.

Results

We used *A. rhizogenes* to develop an efficient protocol for creation of transgenic peanut root cultures. To establish selection conditions, we examined the effect of different concentrations of kanamycin (an aminoglycoside antibiotic that is inactivated by the *NPTII* gene product) on the induction of peanut hairy roots transformed with wild type *A. rhizogenes*

1000 contained no transformation vectors. Kanamycin at concentrations of 50, 70, and 100 mg/L completely inhibited the induction of hairy roots from explant tissues (data not shown). Therefore, we used 50 mg/L kanamycin for all subsequent procedures.

Leaf explants from in vitro-grown peanut plants were infected with *A. rhizogenes* R1000 that contained the binary vector pBI 121. Two days after infection, we transferred explants to agar-solidified hormone-free selection medium. Wounded explants were highly susceptible to infection by *A. rhizogenes* R1000. Hairy root initials emerged from wound sites on peanut leaves 2 to 3 weeks after inoculation (Figure 1-A). After 4 weeks, putative transgenic hairy roots of peanut began to grow more rapidly (Figure 1-B). *A. rhizogenes* R1000 infected about 70% of the explants and induced an average of four to five hairy root initials per explant within 6 weeks.
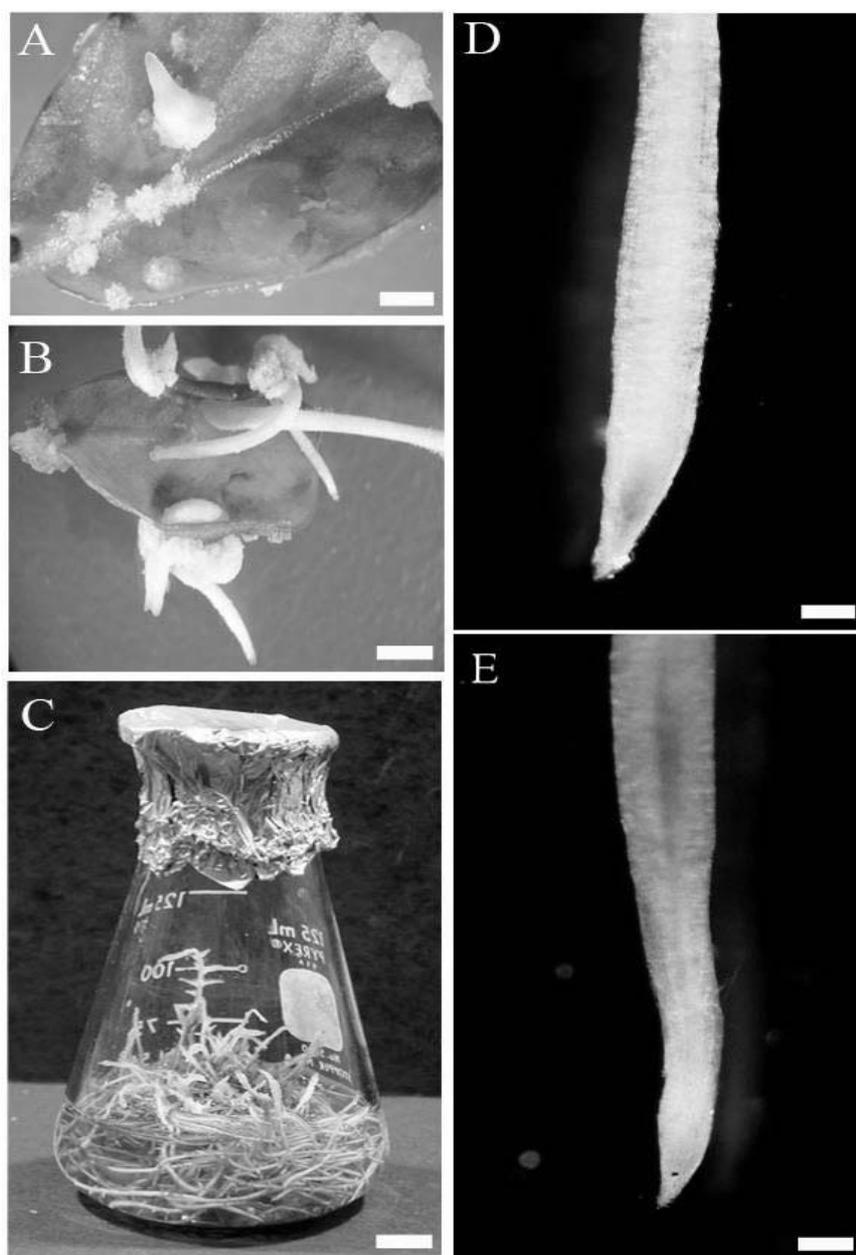

Figure 1. Development of hairy roots from leaf of *Arachis hypogaea* after inoculation with *Agrobacterium rhizogenes* strain R1000; Two weeks (A) and four weeks (B) after inoculation. Rapidly growing hairy root culture in MS liquid culture medium (C). Histochemical staining of peanut wild type root (D)

and hairy root tissue transformed with the GUS gene (E). Kanamycin-resistant roots were induced on the surface of explant tissue within 4 weeks after inoculation (E).

The bars in A, B and C represent 1 mm, 1.5 mm, and 15mm and in D and E represent 0.5 mm.

About 6 weeks after *A. rhizogenes* infection, we excised the hairy roots from the necrotic explant tissues and subcultured them on fresh agar-solidified selection medium. Mature hairy roots generally became thicker following subculture. After repeated transfer to fresh selection medium for 2 to 3 months, rapidly growing hairy root cultures of peanut were transferred to liquid culture medium containing 50 mg/L kanamycin and 250 mg/L Timentin (Figure 1-C).

Next, we tested for integration of the *NPTII* gene into the genome of the peanut hairy root cultures. Then, we performed histochemical localization of GUS activity in various tissues, and checked for the presence of GUS mRNA, and the level of GUS enzyme activity. PCR experiments, which used primers specific for sequences in the *NPTII* gene, resulted in the amplification of a single amplicon with the expected size of 823 bp in 16 of 20 (data not shown). We then performed cytohistochemical staining for GUS enzyme activity to confirm that we had created completely transgenic peanut hairy roots and not chimeric cells that had transgenic and wild type tissues. The cauliflower mosaic virus (CaMV) 35S promoter-GUS fusion gene contained in the pBI 121 binary vector should result in constitutive GUS activity in all cells of transformed tissues. Indeed, we found strong GUS activity in the peanut hairy root vascular tissues of *NPTII*-positive cells after infection by *A. rhizogenes* strain R1000 with pBI 121 (Fig. 1-E). There was no GUS activity in any of the wild type peanut hairy root (Fig. 1-D).
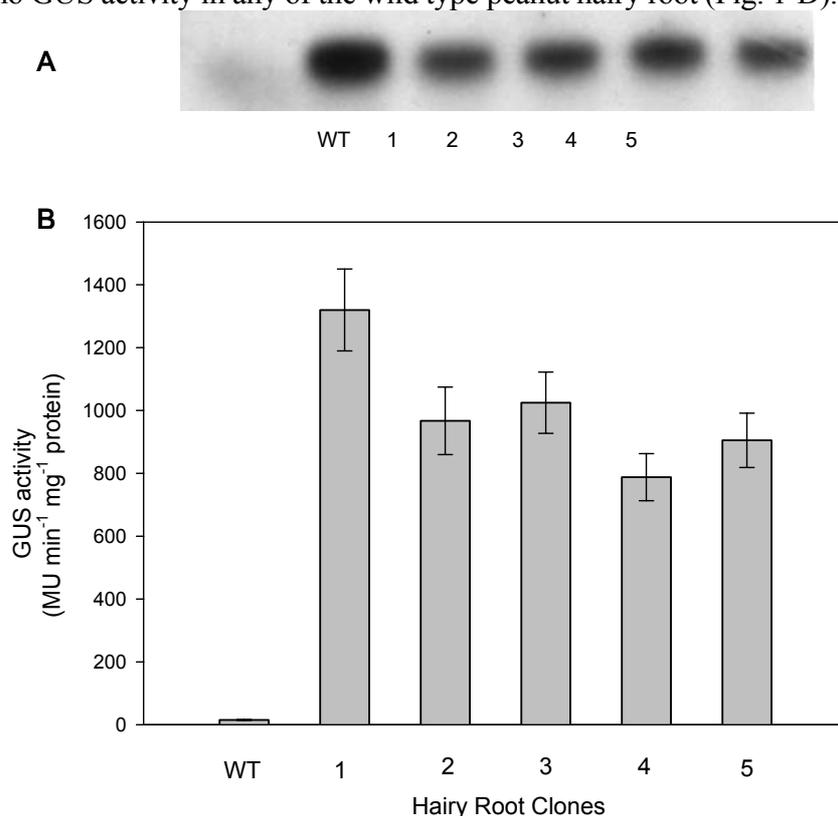

Figure 2. RNA gel blot hybridization analysis for the β-glucuronidase (GUS) reporter gene in wild type (WT) and kanamycin-resistant (1-5) peanut agarose gel, transferred to a nylon membrane, and hybridized at high stringency with a ^{32}P-labelled full-length probe for GUS (A). GUS activity in wild type (WT) and kanamycin-resistant (1-5) peanut hairy root cultures using 4-methylumbelliferyl-β-D-glucuronide (MUG) as the substrate. Bars represent the mean ± SD of three independent measurements (B).

Following these results, we examined five randomly selected *NPTII*-positive hairy root lines to confirm the presence of GUS mRNA. Northern blots revealed high levels of GUS transcripts in each of the five hairy root lines (Fig. 2-A), but not in peanut wild type roots. These five hairy root lines also had much higher GUS enzyme activity than non-transformed roots (Fig. 2-B). There was some variation in GUS activity among the hairy root lines (from 776 to 1315 MU $min^{-1}mg^{-1}$ protein), which is to be expected since there are likely to be differences in the five hairy root lines in the location of chromosomal insertion, the number of transgene copies, and in other post-translational effects.

Discussions

In vitro production of resveratrol from callus, cell, and hairy root cultures of *A. hypogaea* has been reported [19; 14; 17; 11]. However, we describe here for the first time an efficient *A. rhizogenes* R1000 mediated transformation protocol for the establishment of transgenic peanut hairy root cultures. Genetically transformed hairy root cultures are ideal for study of the regulation of metabolic pathways that produce resveratrol and other important natural products. With our development of transformed peanut hairy root cultures, we expect to be able to more easily study the metabolic regulation of resveratrol in this species.

Acknowledgments

References

1. AGGARWAL B.B., BHARDWAJ A., AGGARWAL R.S., SEERAM N.P., SHISHODIA S., TAKADA Y., (2004). Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res. 24: 2783-2840.
2. BAUR J.A., PEARSON K.J., PRICE N.L., JAMIESON H.A., LERIN C., KALRA A., PRABHU V.V., ALLARD J.S., LOPEZ-LLUCH G., LEWIS K., PISTELL P.J., POOSALA S., BECKER K.G., BOSS O., GWINN D., WANG M., RAMASWAMY S., FISHBEIN K.W., SPENCER R.G., LAKATTA E.G., LE COUTEUR D., SHAW R.J., NAVAS P., PUIGSERVER P., INGRAM D.K., DE CABO R., SINCLAIR D.A., (2006). Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444: 337-342.
3. BRADFORD M.M. (1976). A rapid and sensitive method for quantization of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
4. BURNS J., YOKOTA T., ASHIHARA T., LEAN MEJ, CROZIER A., (2002). Plant foods and herbal sources of resveratrol. J. Agric. Food Chem. 50: 3337-3340.
5. EDWARDS K., JOHNSTONE C., THOMPSON C., (1991). A simple and rapid method for preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research 19: 1349.
6. FEINBERG A.P., VOGELSTEIN B.A., (1984). Technique for radiolabeling DNA restriction endonuclease fragments for high specific activity. Anal. Biochem. 137: 266-269.
7. GIRI A., NARASU M.J., (2000). Transgenic hairy roots: recent trends and applications. Biotechnology Advances 18: 1-22.
8. GUILLON S., TREMOUILLAUX-GUILLER J., PATI P.K., RIDEAU M., GANTET P., (2006). Harnessing the potential of hairy roots: dawn of a new era. Trends Biotechnol. 24: 403-409.
9. HAMILL J.D., PARR A.J., RHODES M.J.C., ROBINS R.J., WALTON N.J., (1987). New routes to plant secondary products. Biotechnology 5: 800-804.
10. JEFFERSON R.A., (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-405.
11. KIM J.S., LEE S.Y., PARK S.U., (2008). Resveratrol production in hairy root culture of peanut, *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains. African Journal of Biotechnology. 7: 3785-3787.

12. KING R.E., KENT K.D., BOMSER J.A., (2005). Resveratrol reduces oxidation and proliferation of human retinal pigment epithelial cells via extracellular signal-regulated kinase inhibition. Chem. Biol. Interact. 151: 143-149.

13. 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-glucuronidase activity. Plant Sci. 70: 133-140.

14. KU K.L., CHANG P.S., CHENG Y.C., LIEN C.Y., (2005). Production of stilbenoids from the callus of *Arachis hypogaea*: a novel source of the anticancer compound piceatannol. J. Agric. Food Chem. 53: 3877-3881.

15. LAUX M.T., AREGULLIN M., (2004). Identification of a p53-dependent pathway in the induction of apoptosis of human breast cancer cells by the natural product, resveratrol. J. Altern. Complement Med. 10: 235-239.

16. LOGEMANN J., SCHELL J., WILLMITZER L., (1987). Improved method for the isolation of RNA from plant tissues. Anal. Biochem. 163: 16-20..

17. MEDINA-BOLIVAR F., CONDORI J., RIMANDO A.M., HUBSTENBERGER J., SHELTON K., O'KEEFE S.F., BENNETT S., DOLAN M.C., (2007). Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68: 1992-2003.

18. MURASHIGE T., SKOOG F., (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

19. ROLFS C.H., SCHÖN H., STEFFENS M., KINDL H. (1987). Cell-suspension culture of *Arachis hypogaea* L.: model system of specific enzyme induction in secondary metabolism. Planta 172: 238-244.

20. SAMBROOK J., FRITSCH E.F., MANIATIS T., (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

22. SHARMA K.K., BHATNAGAR-MATHUR P., (2006). Peanut (Arachis hypogaea L.). Methods Mol. Biol. 343: 347-358.

23. SIGNS M., FLORES H.,(1990). The biosynthetic potential of plant roots. Bioessays 12: 7-13.

24. WENZEL E., SOMOZA V., (2005). Metabolism and bioavailability of trans-resveratrol. Mol. Nut. Food Res. 49: 472-481.