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.

Keywords: Arachis hypogaea L., Agrobacterium rhizogenes, hairy root, peanut, transformation

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 NPTII**

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 5 min 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'-GTCGACTCACCGAAGAACTCGTC-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 at 72°C for 5 min, 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 KPO4 buffer, pH 7.0, 1 mM EDTA, and 10 mM-mercaptoethanol. The GUS fluorometric assay buffer consisted of 50 mM NaPO4 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 Na2CO3. A fluorescence spectrophotometer (model F-2000, Hitachi, Tokyo, Japan) was used to quantify the amount of 4-Methylumbelliferoncleaved 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 32P-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 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 7.0, 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 \textit{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 \textit{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). \textit{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.

\textbf{Figure 1.} Development of hairy roots from leaf of \textit{Arachis hypogaea} after inoculation with \textit{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).

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 32P-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.

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References

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