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Genetic transformation of buckwheat (*Fagopyrum esculentum* M.) with *Agrobacterium rhizogenes* and production of rutin in transformed root cultures

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Abstract

We developed an efficient protocol for transformation of buckwheat (*Fagopyrum esculentum* Moench.) root cultures by using stem explants that were infected by *Agrobacterium rhizogenes* strain 15834, a strain with the binary vector pBI121. Four weeks after infection, kanamycin-resistant roots appeared on 90% of explants that were maintained on hormone-free medium. PCR analysis of the neomycin phosphotransferase (*NTPII*) gene confirmed transformation in 17 of 20 kanamycin-resistant hairy root cultures. Detection of high levels of ß-glucuronidase (GUS) transcripts and enzyme activity, and GUS histochemical localization also confirmed the stable genetic transformation. We propagated isolated hairy roots in liquid medium to promote rapid growth and production of rutin, an important flavonol glycoside. The amount of hairy root clone 2 (12.6 g dry weight L^{-1}) was around 2.4 times more than that of wild type root (5.3 g dry weight L^{-1}). The content of rutin was found in hairy root clone 2 (1.3 mg/g dry weight) which was 2.6 times more than that of wild type root where the amount of rutin was 0.5 mg/g dry weight. Transgenic root cultures of *F. esculentum* will allow investigation of the molecular and metabolic regulation of rutin biosynthesis and evaluation of the genetic engineering potential of this species.

Keywords: *Agrobacterium rhizogenes*, *Fagopyrum esculentum* Moench, Hairy root, Rutin, Transformation

Abbreviations: GUS-ß-glucuronidase; MS-Murashige and Skoog; *NPT II*-neomycin phosphotransferase; PCR-polymerase chain reaction; HPLC-high performance liquid layer chromatography; CaMV-cauliflower mosaic virus

Introduction

Fagopyrum esculentum Moench (buckwheat) is a dicotyledonous crop plant whose seeds are used to make pancakes, bread, noodles, tea, and other foods. Buckwheat is a rich source of vitamins, essential amino acids, and a potentially important industrial source of rutin, a flavonid that is also present in other plants (Hinneburg and Neubert, 2004; Kalinova et al., 2006). Rutin is an antioxidant and has other interesting pharmacological properties. Previous research has documented its anti-inflammatory, anticarcino- genic, antithrombotic, cytoprotective, and vasoprotective effects (Kreft et al., 2002; Li and Zhang, 2001). Over the last decade, hairy root cultures have attracted considerable attention because of their genetic and biochemical stability, rapid growth rate and ability to synthesize secondary products at levels comparable to wild type roots (Giri and Narasu, 2000; Signs and Flores, 1990). Hairy root cultures have been used as a useful model system to study the production of flavonoid and a variety of other secondary metabolites. For example, hairy root cultures of *Pueraria candollei* were established for isoflavonoid production (Medina-Bolivar et al., 2007). In another study, peanut hairy root cultures were developed and tested as a bioproduction system for resveratrol and associated derivatives (Udomsuk et al., 2009). A third interesting example involves the production of pyrrolizidine alkaloids in hairy root cultures of *Echium rauwolfii* (Abd El-Mawla, 2010). Finally, transformed *Taxus media* hairy roots accumulate the taxane (Syklowska-Baranek et al., 2009). In vitro production of phenolic compounds in hairy root cultures of common buckwheat and tartary buckwheat has been reported (Kim et al., 2009; Lee et al., 2007; Trotin et al., 1993). However, to understand the molecular mechanisms that regulate the synthesis of rutin and other buckwheat flavonoids, it is necessary to establish an efficient protocol for stable genetic transformation. *Agrobacterium*-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. In this paper, we describe an efficient protocol to introduce foreign genes into buckwheat hairy root cultures using *A. rhizogenes*.

Materials and methods

Seed sterilization and germination

Dehulled seeds of *F. esculentum* were surface-sterilized with 70% (*v/v*) ethanol for 30 s and 2% (*v/v*) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Six 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, 1962) medium and solidified with 0.8% (*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 *A. rhizogenes* 15834 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 (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing kanamycin (50 mg L^{-1}), to mid-log phase (OD600 = 0.5). The *A. rhizogenes* cells were collected by centrifugation for 10min at 1500 rpm and resuspended in liquid inoculation medium (MS salts and vitamins containing 30g sucrose per liter). The *A. rhizogenes* cell density was adjusted to give an A_{600} of 1.0 for inoculation.

Establishment of hairy root cultures

Excised stems of *F. esculentum* from 14-day-old seedlings were used as the explant material for co-cultivation with *A. rhizogenes*. The excised explants 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 agar-solidified MS medium. After two days of co-cultivation, the explant tissues were transferred to a hormone-free MS medium containing salts, vitamins, sucrose (30 g L^{-1}), timentin (250 mg L^{-1}), kanamycin (50 mg L^{-1}) and agar (8 g L^{-1}). Putative transgenic hairy roots were observed emerging from the wound sites within two weeks. Isolated putative transgenic roots (200 mg) were transferred to 30 ml of MS liquid medium in 100 ml flasks. Wild type root cultures were established by inoculating MS liquid medium, with excised roots from buckwheat seedlings grown *in vitro*. 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. Each experiment was carried out with 3 flasks per culture condition and repeated twice.

PCR analysis for NTPII.

Plant genomic DNA of buckwheat hairy roots for polymerase chain reaction (PCR) analysis was extracted as described by Edwards et al. (1991). 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 12,000g 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 12,000g. 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 *NTPII* gene were 5'-TATGTTATGTATG-TGCAGATGATT-3' and 5'-GTCGACTCACCCGAAGA-ACT. CGTC-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

Transgenic hairy roots collected and ground 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^{-1}. Assays were performed on 50 L of transgenic 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 (1976) 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. (1987), and 15 µg was fractionated on 1.0% formaldehyde agarose gels before transfer to nylon membrane (Sambrook et al., 1989). RNA gel blot was hybridized with random-primer ^{32}P-labeled (Feinberg and Vogelstein, 1984) 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 (Sambrook, et al., 1989); 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 (Jefferson, 1987) for fixation and the modified method recommended by Kosugi et al. (1990) for staining. Hairy roots 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 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.

HPLC analysis of rutin

Harvested buckwheat hairy roots (2 g) were frozen in liquid N_2, ground to a fine powder using a mortar and pestle, and extracted twice with methanol (50 ml) for 24 hr at 5 °C. Extracts were reduced to dryness under vacuum dried, and dissolved in methanol. The extracts analyzed by high performance liquid layer chromatography (HPLC) on a C_{18}-column (Hypersil ODS, 250 x 4.6 mm) at room temperature. The solvent gradient used in this study was formed through with an initial proportion of mix of 70% solvent A (3% acetic acid in water) and to 30% solvent B (methanol). After 50 minutes, the solvent gradient had reached 100% solvent B. The flow rate of the solvent was kept constant held at 1.0 ml/min. Samples (20 µl) were detected at wavelengths of 360 nm. We identified the rutin in standard solution A by matching its retention times and spectral characteristics to those from single HPLC runs of authentic a known rutin standard.

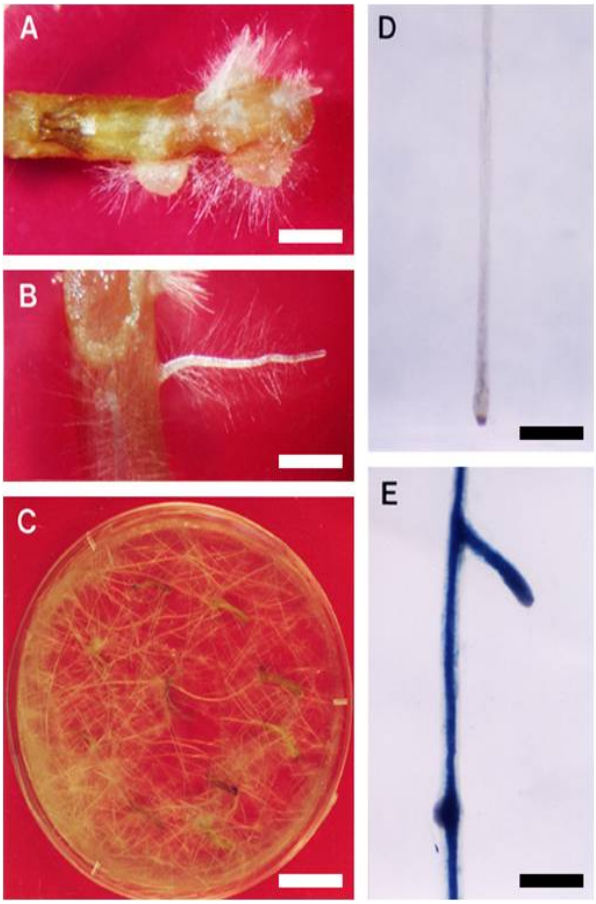

Fig. 1. Development of hairy roots from buckwheat stem after inoculation with *A. rhizogenes* strain 15834. Five days (A), 10 days (B), and 14 days (C) after inoculation. Histochemical staining of buckwheat wild type root (D) and hairy root tissue transformed with the GUS gene (E). Kanamycin-resistant roots were induced on the surface of explant tissue within 14 days after inoculation (E). The bars in A and B represent 2 mm, in C represent 10 mm, and in D and E represent 5 mm.

Results

Transformation preparation

An important prerequisite for the development of a plant transformation system is the demonstration of susceptibility to *Agrobacterium*. We tested *A. rhizogenes* 15834 for its ability to induce hairy root formation in buckwheat explants. *A. rhizogenes* 15834 successfully infected more than 90% of buckwheat explants and induced an average of four to five hairy root initials per stem explants within two weeks. Except for the co-cultivation medium, all formulations used in subsequent steps included kanamycin for selection of transformed plant tissues and timentin to eliminate *Agrobacterium* after co-culturing. In preliminary experiments, we examined the effects of kanamycin, an aminoglycoside antibiotic that is inactivated by the *NPTII* gene product, on the growth of buckwheat hairy roots transformed with wild type *A. rhizogenes* 15834. At concentrations between 10 and 100 mg L^{-1}, kanamycin progressively inhibited root growth. Kanamycin at concentrations of 50 mg/L completely inhibited the induction of hairy roots from explant tissues (data not shown). Therefore, we used 50 mg L^{-1} kanamycin for the selection of transformed hairy roots in subsequent steps.

Transformation for transgenic hairy root induction

After 2 days of co-cultivation with *A. rhizogenes* strain 15834, we transferred explants to agar-solidified hormone-free selection medium. Wounded explants were highly susceptible to infection by *A. rhizogenes* 15834. Three to 5 days after inoculation, hairy root initials emerged from wound sites on the stem (Fig. 1A). After 7 to 10 days, putative transgenic hairy roots began to grow more rapidly (Fig. 1B). *A. rhizogenes* 15834 infected more than 90% of the explants and induced an average of four to five hairy root initials per explant within 2 weeks (Fig. 1C). About 3 to 4 weeks after co-cultivation with *A. rhizogenes*, we excised hairy roots from the necrotic explant tissues and subcultured them on fresh agar-solidified selection medium. Mature hairy roots were generally thicker and exhibited more prolific branching. After repeated transfer to fresh selection medium for 2 to 3 months, we transferred rapidly growing hairy roots to a liquid culture medium that contained kanamycin (50 mg L^{-1}) and timentin (250 mg L^{-1}).

PCR analysis of NPTII gene

We evaluated the complete and stable transformation of kanamycin-resistant hairy roots by checking for integration of the *NPTII* gene into the genome, and then determining the histochemical localization of GUS activity in various tissues, the presence of GUS mRNAs, and the level of GUS enzyme activity. In 17 of 20 kanamycin-resistant hairy root cultures, PCR using primers specific for *NTPII* sequences resulted in the amplification of a single amplicon with the expected size of 823 bp (data not shown).

Cytohistochemical staining for GUS activity

Cytohistochemical staining for GUS activity can determine whether the transformation resulted in completely transgenic hairy roots, or chimeras that were composed of transgenic and wild type tissues. The cauliflower mosaic virus (CMV) 35S promoter-GUS fusion sequence contained in the pBI 121 binary vector should result in constitutive GUS activity in all cells of kanamycin-resistant tissues. We observed strong GUS staining in the hairy root vascular tissues of *NPTII*-positive hairy roots after co-cultivation with *A. rhizogenes* strain 15834 (Fig. 1E), but not in any wild type roots (Fig. 1D).

Northern hybridization and enzyme activity analysis of GUS

Next, we tested six randomly selected *NTPII*-positive and rapidly growing hairy root lines to confirm the presence of GUS transcripts. RNA gel blot hybridization analysis revealed high levels of GUS transcripts in each of the putative transgenic hairy root cultures, but no signal in wild type roots of buckwheat (Fig. 2A). We also tested six transgenic hairy root cultures for GUS enzyme activity levels. Transgenic hairy roots had much higher GUS activity than non-transformed roots, which exhibited only background activity (Fig. 2B). Individual transformants expressed a wide range of GUS activities, from 784 to 1284 MU $min^{-1}mg^{-1}$ protein. This variation in GUS transcript and enzyme activity level is typical in transformed plant tissues due to variations in transgene copy number, location of chromosomal insertion, and other post-translational effects.

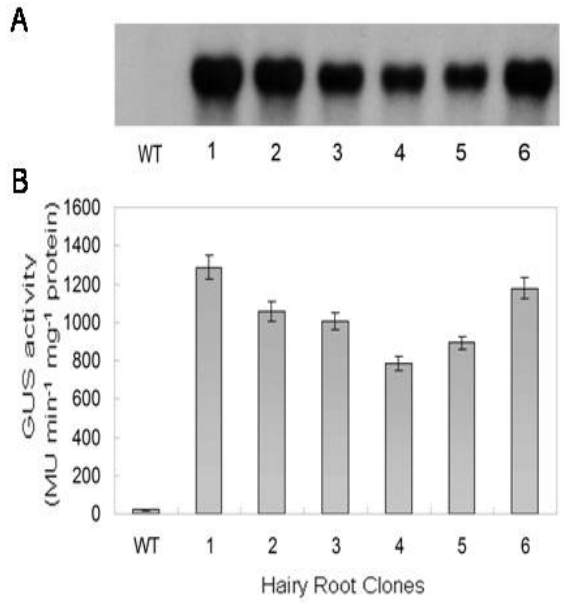

Fig 2. RNA gel blot hybridization analysis for the β-glucuronidase (GUS) reporter gene in wild type (WT) and kanamycin-resistant (1-6) buckwheat agarose gel, transferred to a nylon membrane, and hybridised at high stringency with a ^{32}P-labelled full-length probe for GUS (A). GUS activity in wild type (WT) and kanamycin-resistant (1-6) buckwheat hairy root cultures using 4-methylumbelliferyl-β-D-glucuronide (MUG) as the substrate. Bars represent the mean ± SD of three independent measurements (B).

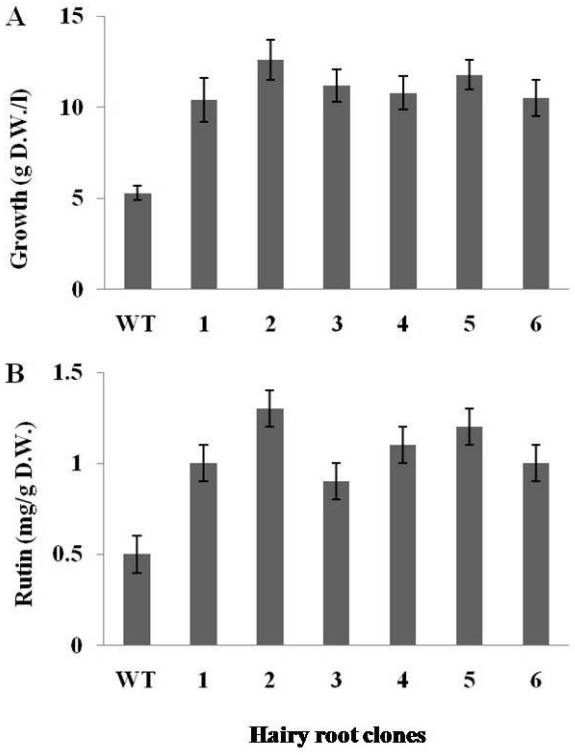

Fig 3. The growth (A) and rutin production (B) of buckwheat wild type (WT) and transgenic hairy root clones (1-6) in MS liquid medium after 21 days in culture. Bars represent the mean ± SD of three independent measurements.

Rutin analysis

Finally, we compared the growth and rutin content of transformed root cultures with wild type root culture. To obtain baseline values, we cultured these hairy root clones in MS liquid medium for 21 days, and then investigated their growth rates (Fig. 3A) and rutin contents (Fig. 3B). Among the six transformed root cultures, clone 2 exhibited a highest growth rate and rutin contents. The amount of hairy root clone 2 (12.6 g dry weight L^{-1}) was around 2.4 times more than that of wild type root (5.3 g dry weight L^{-1}). The content of rutin was found in hairy root clone 2 (1.3 mg g^{-1} dry weight) which was 2.6 times more than that of wild type root where the amount of rutin was 0.5 mg g^{-1} dry weight.

Discussion

Various species of bacteria can transfer genes to higher plants (Broothaerts et al., 2005). *Agrobacterium rhizogenes,* a gram-negative soil bacterium, is one of the most widely studied among them. It infects the plant cell and leads to the formation of hairy roots (Guillon et al., 2006; Hamill et al., 1987). There are some reports that suggest the successful use of *A. rhizogenes* harboring binary vectors with desired gene constructs for plant genetic transformation (Christey, 2001). *A. rhizogenes* 15834 is one of the most common strains used for hairy root induction. Recently, several other studies have also reported the production of secondary metabolites from hairy root cultures induced by this strain. For example, this strain was used for production of isoliquiritigenin from *Genista tinctoria* (Luczkiewicz and Kokotkiewicz, 2005), podophyllotoxin from *Podophyllum hexandrum* (Giri et al., 2001), solasodine glycoside from *Physalis minima* L. (Putalun et al., 2004), coniferin from *Linum flavum* (Lin et al., 2003), dicentrine from *Stephania suberosa* (Putalun et al., 2009), and catapol from *Rehmannia glutinosa* (Hwang, 2009). Genetically engineered root cultures have been used as a model system to study various aspects of the metabolic and molecular regulation of several secondary metabolite pathways. There are several reports of the metebolic engineering of flavonoid biosynthesis in transgenic root cultures. For example, hairy root cultures of *S. involucrate* transformed with chalcone isomerase gene with produced higher levels of apigenin and total flavonoids than wild-type hairy roots did (Li et al., 2006). Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch was achieved by the over-expression of chalcone isomerase gene (Zhang et al., 2009). 2-hydroxyisoflavanone dehydratase overexpressing transgenic hairy root cultures of *Lotus japonicus* accumulate isoflavones (Shimamura et al., 2007). In contrast, chalcone synthase gene silencing transgenic hairy roots of *Medicago truncatula* showed reduced levels of flavonoids (Wasson et al., 2006). Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase was reported. The isoflavone and coumestrol concentrations were decreased by about 90% in most transgenic hairy root lines apparently due to gene silencing (Lozovaya et al., 2007). There is also an increasing availability of genes that encode flavonoid biosynthetic enzymes. The protocol that we have developed for the production of rapidly growing transgenic root cultures of buckwheat provides a powerful and versatile model system that allows investigation of the molecular regulation of flavonoid biosynthesis and evaluation of the potential to metabolically engineer high rutin production in this species.

References

Abd El-Mawla AMA (2010) Effect of certain elicitors on production of pyrrolizidine alkaloids in hairy root cultures of *Echium rauwolfii*. Pharmazie 65:224-226

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254

Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LMA, Yang W, Mayer JE, Roa-Rodriguez C, Jefferson RA (2005) Gene transfer to plants by diverse species of bacteria. Nature 433:629-633

Christey MC (2001) Use of ri-mediated transformation for production of transgenic plants. In Vitro Cellular & Developmental Biology - Plant 37:687

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

Feinberg AP, Vogelstein BA (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 137:266-269

Giri A, Giri CC, Dhingra V, Narasu ML (2001) Enhanced podophyllotoxin production from *Agrobacterium rhizogenes* transformed cultures of *Podophyllum hexandrum*. Natural Product Research: Formerly Natural Product Letters 15:229 - 235

Giri A, Narasu ML (2000) Transgenic hairy roots: recent trends and applications. Biotechnology Advances 18:1-22

Guillon Sh, Tremouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Harnessing the potential of hairy roots: dawn of a new era. Trends in Biotechnology 24:403-409

Hamill JD, Parr AJ, Rhodes MJC, Robins RJ, Walton NJ (1987) New routes to plant secondary products. Nat Biotech 5:800-804

Hinneburg I, Neubert RHH (2004) Influence of extraction parameters on the phytochemical characteristics of extracts from buckwheat (*Fagopyrum esculentum*) herb. Journal of Agricultural and Food Chemistry 53:3-7

Hwang SJ (2009) Catapol production in Chinese foxglove (*Rehmannia glutinosa* Libos.) hairy roots transformed with *Agrobacterium rhizogenes* ATCC15834. Methods Mol Biol 547:263-273

Jefferson R (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Molecular Biology Reporter 5:387-405

Kalinova J, Triska J, Vrchotova N (2006) Distribution of vitamin E, squalene, epicatechin, and rutin in common buckwheat plants (*Fagopyrum esculentum* Moench). Journal of Agricultural and Food Chemistry 54:5330-5335

Kim YK, Li X, Xu H, Park NI, Uddin MR, Pyon JY, Park SU (2009) Production of phenolic compounds in hairy root culture of tartary buckwheat (*Fagopyrum tataricum* Gaertn). Journal of Crop Science and Biotechnology 12:53-57

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 Science 70:133-140

Kreft S, Strukelj B, Gaberscik A, Kreft I (2002) Rutin in buckwheat herbs grown at different UV-B radiation levels: comparison of two UV spectrophotometric and an HPLC method. J Exp Bot 53:1801-1804

Lee S-Y, Cho S-I, Park M-H, Kim Y-K, Choi J-E, Park S-U (2007) Growth and rutin production in hairy root cultures of buckwheat (*Fagopyrum esculentum* M.). Preparative Biochemistry and Biotechnology 37:239-246

Li F-X, Jin Z-P, Zhao D-X, Cheng L-Q, Fu C-X, Ma F (2006) Overexpression of the *Saussurea medusa* chalcone isomerase gene in *S. involucrata* hairy root cultures enhances their biosynthesis of apigenin. Phytochemistry 67:553-560

Li S-q, Zhang QH (2001) Advances in the development of functional foods from buckwheat. Critical Reviews in Food Science and Nutrition 41:451 - 464

Lin H-w, Kwok KH, Doran PM (2003) Development of *Linum flavum* hairy root cultures for production of coniferin. Biotechnology Letters 25:521-525

Logemann Jg, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Analytical Biochemistry 163:16-20

Lozovaya V, Lygin A, Zernova O, Ulanov A, Li S, Hartman G, Widholm J (2007) Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. Planta 225:665-679

Luczkiewicz M, Kokotkiewicz A (2005) *Genista tinctoria* Hairy Root Cultures for Selective Production of Isoliquiritigenin. Zeitschrift für Naturforschung 60c:867-875

Medina-Bolivar F, Condori J, Rimando AM, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan MC (2007) Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992-2003

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15:473-497

Putalun W, Prasarnsiwamai P, Tanaka H, Shoyama Y (2004) Solasodine glycoside production by hairy root cultures of *Physalis minima* Linn. Biotechnology Letters 26:545-548

Putalun W, Yusakul G, Patanasethanont D (2009) Dicentrine production from a hairy roots culture of *Stephania suberosa*. Z Naturforsch C 64:692-696

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

Shimamura M, Akashi T, Sakurai N, Suzuki H, Saito K, Shibata D, Ayabe S-i, Aoki T (2007) 2-Hydroxyisoflavanone dehydratase is a critical determinant of isoflavone productivity in hairy root cultures of *Lotus japonicus*. Plant Cell Physiol 48:1652-1657

Signs M, W., Flores H, E. (1990) The biosynthetic potential of plant roots. BioEssays 12:7-13

Syklowska-Baranek K, Pietrosiuk A, Kokoszka A, Furmanowa M (2009) Enhancement of taxane production in hairy root culture of *Taxus x media var. Hicksii*. Journal of Plant Physiology 166:1950-1954

Trotin F, Moumou Y, Vasseur J (1993) Flavanol production by *Fagopyrum esculentum* hairy and normal root cultures. Phytochemistry 32:929-931

Udomsuk L, Jarukamjorn K, Tanaka H, Putalun W (2009) Isoflavonoid production in a hairy roots culture of *Pueraria candollei*. Z Naturforsch C 64:687-691

Wasson AP, Pellerone FI, Mathesius U (2006) Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by Rhizobia. Plant Cell 18:1617-1629

Zhang H-C, Liu J-M, Lu H-Y, Gao S-L (2009) Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the over-expression of chalcone isomerase gene with the elicitation treatment. Plant Cell Reports 28:1205-1213