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Agrobacterium rhizogenes-mediated transformation of β-glucuronidase reporter gene in hairy roots of *Angelica gigas* Nakai

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Abstract

Korean Angelica (*Angelica gigas*) produces pyranocoumarins (decursin and decursinol angelate), which have neuroprotective, anticancer, and anti-androgen receptor-signaling activities. An efficient protocol for the establishment of transgenic *Angelica gigas* root cultures using *Agrobacterium rhizogenes* is reported. To further characterise the putative transgenic roots, explant tissues were co-cultivated with *A. rhizogenes* strain R1601 carrying the pK2GW7-GUS binary vector. Except for the co-cultivation medium, all formulations included 50 mg L^{-1} kanamycin to select for transformants and 250 mg L^{-1} cefotaxime to eliminate the *Agrobacterium*. After 6 to 8 weeks co-cultivation with *Agrobacterium rhizogenes*, kanamycin-resistant roots appeared on 50% of explants maintained on hormone-free medium. Isolated hairy roots were transferred in liquid medium containing half-strength Schenk and Hildebrandt (SH) salt and 30 mg L^{-1} sugar. Detection of the neomycin phosphotransferase gene, high levels of β--glucuronidase (GUS) transcripts, and GUS histochemical localisation confirmed the integrative transformation. In the future, these protocols should facilitate the extraction and study of valuable metabolites, such as decursin and decursinol angelate from *A. gigas* hairy root cultures.

Keywords: *Angelica gigas*, hairy root culture, *Agrobacterium rhizogenes*, Metabolic engineering, Decursin, Decursinol angelate

Abbreviations: Ri-root inducing; HPLC-high performance liquid chromatography; CaMV-cauliflower mosaic virus; *NPTII*-neomycin phosphotransferase; MS-Murashige and Skoog; SH- Schenk and Hildebrandt;

Introduction

Angelica gigas Nakai, commonly known as Korean Angelica, is one of the most popular medicinal plants in Korea. *A. gigas* is a member of the Umbelliferae, and is easily identified by its dark purple flowers; the Chinese and Japanese Angelica varieties have white flowers (Ahn et al., 2008). The roots of *A. gigas* are widely used in traditional oriental herbal therapies for abdominal pain, injuries, migraine, and arthritis, anemia (in women), and dysmenorrhea (Chi and Kim, 1970; Choi et al., 2003). *A. gigas* produces a variety of compounds, including coumarins (Ryu et al., 1990). Specifically, the roots of *A. gigas* contain decursin, decursinol, nodakenin, nodakenetin, umbelliferone, and imperatorin, while the fruits largely contain decursinol, decursidin, and imperatorin (Choi, et al., 2003). Decursin and decursinol angelate (Fig. 1), pyranocoumarins, are the main constituents of *A. gigas* root isolates. Decursin and decursinol angelate exhibit significant neuroprotective, anticancer, and anti-androgen receptor-signaling activities (Guo et al., 2007; Kang et al., 2005; Yim et al., 2005). Three different *Angelica* species, Korean angelica (*A. gigas*), Chinese angelica (*A. sinensis*), and Japanese angelica (*A. acutiloba*), produce different compound profiles; Korean angelica produces decursin and decursinol angelate in greater quantity than the Chinese and Japanese species (Kim et al., 2006; Piao et al., 2007). Hairy root cultures can be produced by infection with *Agrobacterium rhizogenes*, a gram-negative soil bacterium that transfers DNA from its root inducing (Ri) plasmid into the genome of the infected plant. Hairy roots are genetically and biochemically stable, have a rapid growth rate, and synthesize natural compounds at levels comparable to intact plants (Christey and Braun, 2005; Georgiev et al., 2007; Srivastava and Srivastava, 2007). Hairy root cultures of medicinal plants may therefore provide a useful system for the production of valuable medicinal compounds. Methods for hairy root culture of *A. gigas* for decursin production have recently been developed (Xu et al., 2008; Xu et al., 2009). To understand the molecular biological mechanisms that regulate the production of phytochemical biosynthetic metabolites in plants, methods for efficient and stable genetic transformation are required. Here, we describe a method for transformation of *A. gigas* hairy root cultures using leaves infected by *Agrobacterium rhizogenes* R1601 containing the binary vector pK7FWG-GUS for generating CaMV 35S: GUS. We compared the concentrations of pyranocoumarins in different transgenic *A. gigas* hairy root samples by using high-performance liquid chromatography (HPLC). The protocol described here will be useful for the extraction and study of valuable metabolites such as decursin and decursinol angelate from *A. gigas* hairy root cultures.

Table 1. List of oligonucleotide primers

	Name	Sequence (5′ to 3′)	Use
rol genes	*rol* A F	CATGTTTCAGAATGGAATTA	Genomic DNA PCR
	rol A R	AGCCACGTGCGTATTAATCC	
	rol B F	TCACAATGGATCCCAAATTG	
	rol B R	TTCAAGTCGGCTTTAGGCTT	
	rol C F	ATGGCTGAAGACGACCTGTGT	
	rol C R	TTAGCCGATTGCAAACTTGCA	
	rol D F	ATGGCCAAACAACTTTGCGA	
	rol D R	TTAATGCCCGTGTTCCATCG	
GUS	GUS F	ATCGGTCGACATGTTACGTCCTGTAGAAAC	
	GUS R	CGATCCATGGTCATTGTTTGCCTCCCTGCT	
GUS	GUS RT-F	TTACCCTTACGCTGAAGAGATGC	Real-time PCR
	GUS RT-R	GCTGTACAGTTCTTTCGGCTTGT	

Materials & methods

Seed sterilization and germination

A. gigas seeds were surface-sterilized with 70% (*v/v*) ethanol for 30 s and 4.5% (*v/v*) sodium hypochlorite solution for 10 min, then rinsed 3 times in sterilized water. The seeds were placed on MS medium (Murashige and Skoog, 1962) solidified with 0.8% (*w/v*) agar. Prior to the addition of agar, the medium was adjusted to pH 5.8, 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

The pK2GW7-GUS vector contains a cauliflower mosaic virus (CaMV) 35S promoter-GUS fusion sequence, and the neomycin phosphotransferase (*NPTII*) selectable marker. The binary vector (pK2GW7) was obtained from the Functional Genomics Unit (Department of Plant Systems Biology, VIB-Ghent University). The GUS gene was cloned into pK2GW7 using the Gateway cloning system (Invitrogen, USA). This vector was transferred into *Agrobacterium rhizogenes* strain R1601 by electroporation. Cultures of *Agrobacterium rhizogenes* were initiated from a 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) and spectinomycin (50 mg/L), to mid-log phase (OD600 = 0.5). Cells were collected by centrifugation for 10 min at 2000 rpm and resuspended in liquid inoculation medium (MS medium containing 30 g sucrose per liter). Cell density was adjusted to an A_{600} of 1.0 for inoculation.

Establishment of hairy root transformation

The establishment and maintenance of hairy root culture was performed as a modification of the procedures described by Xu et al. (2008). Hairy root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber. Excised leaves and stems of *A. gigas* from 30-day-old seedlings were used as the explant material for co-cultivation with *Agrobacterium rhizogenes.* Explants were dipped into the *Agrobacterium rhizogenes* culture in liquid inoculation medium for 10 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified half strength (1/2) MS medium. After 2 d of co-cultivation, the explant tissues were transferred to hormone-free 1/2 MS medium containing cefotaxime (250 mg/L) and kanamycin (50 mg/L). Putative transgenic hairy roots were observed emerging from the wound sites within 3 weeks. Isolated putative transgenic roots (200 mg) were transferred to 30 mL of 1/2 Schenk and Hildebrandt (SH) liquid medium 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.

PCR analysis for GUS and rol genes

Genomic DNA was extracted from *A. gigas* hairy roots for PCR analysis as described by Edwards et al. (1991). The oligonucleotide sequences used to amplify a fragment of the GUS and (root locus) *rol* genes are described in Table 1.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from 100 mg *A. gigas* hairy roots by grinding the tissue with a mortar and pestle under liquid nitrogen, then adding 1 mL TRIZOL (Invitrogen, USA). After incubation for 15 min at room temperature, 200 µL of chloroform per 1 mL of TRIZOL was added and the sample vortexed for 15 s, followed by room temperature incubation for 10 min. Supernatants were transferred to new tubes after 15 min centrifugation at 12,000 rpm, 4°C; 500 µL of isopropanol was added, mixed gently, and incubated for 10 min at room temperature. Total RNA was precipitated after centrifugation at 12,000 rpm for 10 min at 4°C, the RNA forming a gel-like pellet at the bottom of the tube. RNA pellets were washed twice with 1 mL of ice cold 70% ethanol, and dissolved in diethylpyrocarbonate-treated water. The quality and concentration of the RNA samples were assessed by agarose gel electrophoresis and spectrophotometric analysis. Reverse transcription of 1 µg RNA was performed according to the manufacturer's protocol (ReverTra Ace-α-, Toyobo, Japan) using an oligo$(dT)_{20}$ primer. The resulting cDNA mixtures were used as templates for real-time PCR.

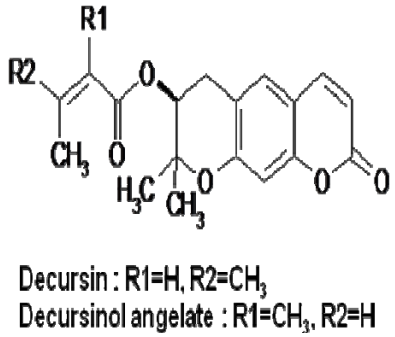

Fig 1. Chemical structure of decursin and decursinol angelate

Fig 2. Development of hairy root from a leaf of *A. gigas* after inoculation with *Agrobacterium rhizogenes* R1601 containing GUS (a). Rapidly growing hairy root culture on solid (b) and in liquid (c) culture media. Scale bar: 0.1 cm (a), 1 cm (b, c).

Quantitative real-time PCR

Transcriptional level analysis of GUS expression in *A. gigas* hairy roots was performed by quantitative real-time PCR using a Mini Opticon Real-time PCR system (BioRad). Real-time PCR was carried out in a 20 µL reaction volume containing 0.4 µM of each primer and 10 µL of SYBR Green Real time PCR master mix (Toyobo, Japan). Amplification was conducted as recommended by the manufacturer cycling parameters. Triplicate quantitative real-time PCR experiments were performed for each sample.

GUS staining analysis and microscopic analysis

Hairy roots were stained for GUS activity in staining solution (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg/mL X-gluc, 2 mM potassium ferricyanide, and 2 mM potassium ferrocyanide) (Sundaresan et al., 1995). Hairy roots were soaked directly in the staining solution, placed under vacuum for 10 min, and incubated overnight at 37°C. After staining, the solution was repeatedly exchanged with 70% ethanol until the tissue was cleared.

Hairy roots stained for GUS activity were mounted on a microscope slide in 10% glycerol, covered with a coverglass, and observed using a stereoscopic microscope (SZ2-ILST, Olympus, Japan) equipped with a moticam 2000 digital camera (Motic). The images were assembled using Adobe Photoshop software (version 8.0, Adobe Systems Inc., USA).

HPLC analysis of decursin and decursinol angelate

HPLC analysis was carried out on a Futecs model NS-4000 HPLC system (Daejeon, Korea) equipped with an autosampler and connected to a UV detector. Peak area integration was performed using the chromatographic data (Multichro 2000). The column was a reversed-phase C_{18}, Optimapak C_{18} (5 µm, 250 × 4.6 mm); column oven temperature was 35°C. The mobile phase was 40% acetonitrile, 50% water, and 10% tetrahydrofuran. The flow rate was 0.8 mL/min, detection wavelength 280 nm, and injection volume 10 µL. Decursin and decursinol angelate standards were provided by the Plant Natural Products Lab at the Korea Research Institute of Chemical Technology (Deajon, Korea). The components of the mobile phase, including distilled water, acetonitrile, and tetrahydrofuran, were purchased from Burdick & Jackson (Honeywell, USA). Samples were dried in a freeze dryer for 48 h at -80°C. Dried samples were ground into a fine powder using a mortar and pestle. Samples (0.5 g) were extracted with 30 mL 70% (v/v) ethanol at 50°C in a water bath for 1h (repeated 3 times). After centrifugation, the supernatant (25 mL) was concentrated under vacuum and 3-fold methylene chloride separation. The dried methylene chloride fraction was dissolved in 1 mL acetonitrile. Samples were filtered through 0.45 µm poly filters.

Results

Hairy root transformation with Agrobacterium rhizogenes R1601

Previously, Xu et al. (2008) described the production of decursin by hairy root cultures of *A. gigas* transformed with *Agrobacterium rhizogenes* R1000. In this study, another *Agrobacterium rhizogenes* strain, R1601, was used to produce transgenic hairy roots. To determine the appropriate kanamycin concentration for plant selection, explants were initially grown on media without kanamycin and were shifted to selection media containing 1 of 4 different kanamycin concentrations (0, 10, 30, 50, and 100mg/L) after transformation by *Agrobacterium rhizogenes* R1601. Hairy root infection frequency was approximately 70% on kanamycin-free medium and approximately 20% on medium containing 30 mg/L of kanamycin (data not shown). However, the 50 and 100 mg/L kanamycin media completely inhibited the development of hairy roots from explants with wild-type *Agrobacterium rhizogenes* R1601. Therefore, for the remainder of the study, we used 50 mg/L kanamycin media to select for transformed hairy roots.

Hairy roots of *A. gigas* were initiated with leaf explants inoculated with *Agrobacterium rhizogenes* R1601 containing the binary vector pK7FWG2-GUS for generating CaMV 35S::GUS. This vector contains the cauliflower mosaic virus (CaMV) 35S promoter-GUS fusion sequence, which ensures constitutive GUS activity in all cells of kanamycin-resistant tissues. Two weeks after inoculation, hairy root emerged from wound sites on the leaves and stems (Fig. 2a). After 6 to 8 weeks co-cultivation with *Agrobacterium rhizogenes*, putative transgenic hairy roots began to grow more rapidly on kanamycin-containing medium (Fig. 2b). *Agrobacterium rhizogenes* R1601 infected more than 50% of the explants and induced an average of 2 to 3 hairy roots per explant within 2 weeks. Mature hairy roots were generally thinner and exhibited a long branching habit. After repeated transfer

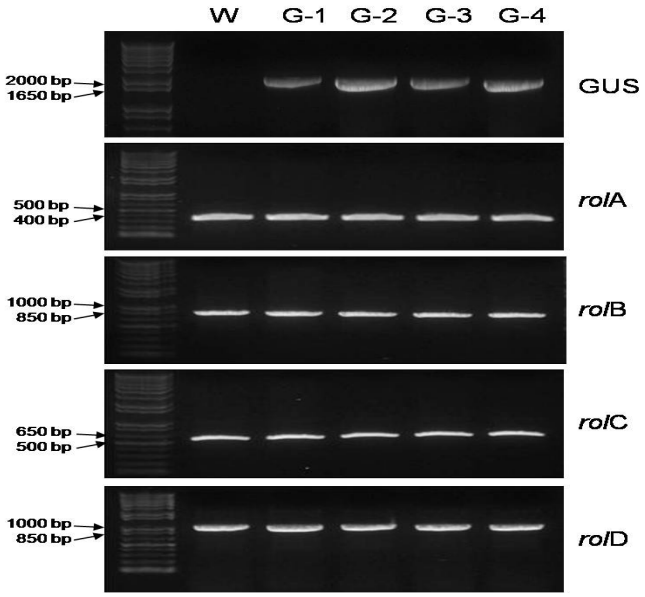

Fig 3. PCR analyses of the GUS, *rol*A, *rol*B, *rol*C, and *rol*D genes in transgenic hairy root lines. W, wild-type hairy root induced by *Agrobacterium rhizogenes* R1601; Gn, transgenic hairy root lines induced by *Agrobacterium rhizogenes* R1601 (pK7FWG2-GUS), "n" indicates the line number; M, DNA marker.

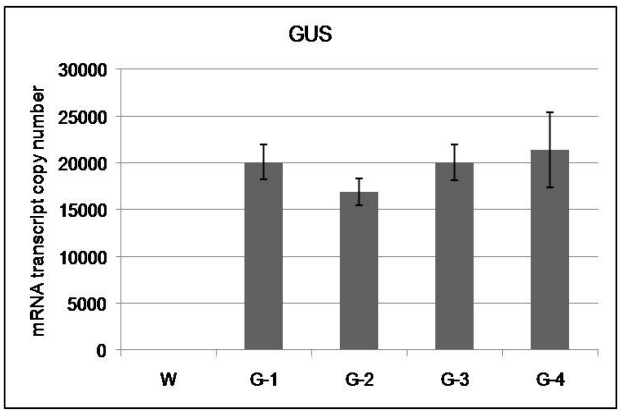

Fig 4. Quantitative RNA analysis of GUS in transgenic hairy root lines. W, wild-type hairy root induced by *Agrobacterium rhizogenes* R1601; Gn, transgenic hairy root lines induced by *Agrobacterium rhizogenes* R1601 (pK7FWG2-GUS), "n" indicates the line number.

to fresh selection medium over the course of 2–3 months, rapidly growing hairy roots were transferred to a liquid culture medium that contained kanamycin (50 mg/L) and cefotaxime (250 mg/L) (Fig. 2c). Four independent transgenic hairy root (G1–4) sets were produced.

PCR analysis for GUS and root locus (rol) genes

To determine whether the root tissues had been successfully transformed by pK2GW7-GUS, we assayed for integration of the GUS gene into the plant genome. In 4 hairy root cultures (G1–4), GUS-specific PCR yielded an amplified product, whereas the wild-type hairy root (W) infected with wild type *Agrobacterium rhizogenes* R1601 did not yield a GUS PCR product. In all 4 kanamycin-resistant hairy root cultures (G1–4), including wild-type hairy roots (W), *rol* A, B, C, and D genes were amplified by PCR, each producing a single band of the expected size (Fig. 3). The *rol* genes of the Ri-plasmid are responsible for the induction of hairy roots by *Agrobacterium rhizogenes*. The amplification of *rol* genes results revealed that wild-type and transgenic hairy roots contained *rol* genes from the Ri-plasmid.

Transcript analysis and cytohistochemical staining for GUS

Quantitative real-time PCR was used to quantify GUS transcription levels in hairy roots. After using PCR to verify the insertion, GUS transcription in the hairy roots was assessed by quantitative real-time PCR. Quantitative real-time PCR analysis revealed high levels of GUS transcripts in each of the putative transgenic hairy root cultures (G1–4), but no signal was observed in wild type hairy roots (W) (Fig. 4). Cytohistochemical staining for GUS activity can reveal whether transformation resulted in completely transgenic hairy roots, or chimeras, composed of both transgenic and wild type tissues. Although PCR analysis can determine only whether the introduced gene inserted in genome, cytohistochemical techniques can confirm the inserted gene into genome is expressed. Strong GUS staining was observed in hairy root vascular tissues after co-cultivation with *Agrobacterium rhizogenes* strain R1601 containing pK2GW7-GUS (Fig. 5a–c), but not in hairy roots cultivated with the unaltered *Agrobacterium rhizogenes* strain R1601 (Fig. 5d).

Analysis of decursinol angelate and decursin compounds

To analyze pyranocoumarins from *A. gigas* hairy roots, we maximized the volume of wild type hairy roots (W) and GUS hairy root lines (G1–4) in liquid culture for HPLC analysis. Decursin and decursinol angelate were identified from all wild type (W) and GUS (G1–4) hairy root lines (Fig. 6). Although GUS hairy root lines (G1–4) showed the level of GUS gene expression increased, overexpressed GUS gene have no effect to produce the secondary metabolites. Decursinol angelate content ranged from 0.18 to 0.25 mg/g dry weight (DW); decursin content was 0.022 to 0.026 mg/g DW.

Discussion

Plant transformation has become a core research tool for crop improvement and for the study of gene function in plants. Over the past 3 decades, various plant transformation methodologies have been developed to increase the efficiency of transformation and to achieve stable transgene expression (Gelvin, 2009; Rao et al., 2009). The transgenic hairy roots and *Agrobacterium rhizogenes* vector systems have been used for Ri plasmid introduction of foreign genes. *Agrobacterium rhizogenes*-mediated root transformation is a valuable tool for basic plant research, and a useful technique for metabolic engineering in the biosynthesis of secondary metabolites (Giri and Narasu, 2000; Hu and Du, 2006). 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. For example, overexpression of a cDNA encoding *Panax ginseng* Farnesyl diphosphate synthase (FPS) in hairy roots of *Centella asiatica* caused an increase in the content of phytosterol and triterpene (Kim et al., 2010). In another study, the efficiency of the licorice chalcone isomerase (CHI) gene at regulating flavonoid biosynthesis in *Glycyrrhiza uralensis* was tested using transformed root cultures (Zhang et al., 2009). The CHI gene was capable of enhancing

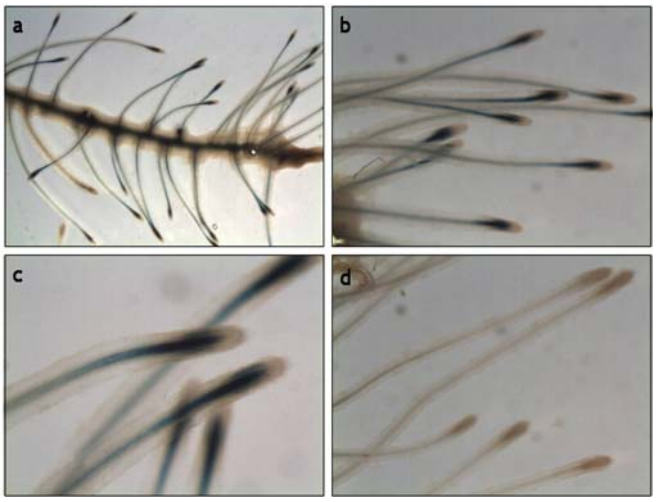

Fig 5. GUS histochemical analysis of transgenic hairy roots of *A. gigas* harboring *35S: GUS* (a–c) and hairy roots transformed with *Agrobacterium rhizogenes* R1601 (d). (a, × 10; b and d, × 25; c, × 50).

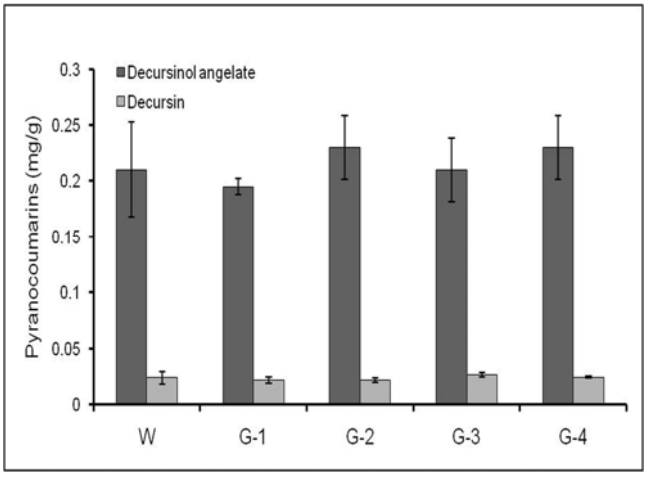

Fig 6. Production of decursinol angelate and decursin in GUS-transgenic hairy roots of *A. gigas*. W, wild-type hairy root induced by *Agrobacterium rhizogenes* R1601; Gn, transgenic hairy root lines induced by *Agrobacterium rhizogenes* R1601 (pK7FWG2-GUS), "n" indicates the line number

flavonoid biosynthesis in hairy roots of *Glycyrrhiza uralensis.* A third interesting example involves the introduction of a homologous tropinone reductase I (TRI) genes into hairy root cultures of *Anisodus acutangulus*, which accumulates tropane alkaloids (Kai et al., 2009). AaTRI-transformed hairy-root lines were accompanied by a mean 1.87-fold higher level of hyoscyamine and a mean 8-fold higher level of scopolamine compared with control roots, indicating that AaTRI is a promising target for genetic engineering to increase tropane alkaloid in *A. acutangulus.* Finally, transformed Beta vulgaris (sugar beet) root cultures expressing a bacterial gene for p-hydroxycinnamoyl-CoA hydratase/lyase (HCHL), which involved in phenylpropanoid biosynthesis (Rahman et al., 2009). These results suggest that the availability of HCHL yields a high accumulation of p-hydroxybenzoic acid (pHBA) glucose ester.

Conclusions

Genetically engineered root cultures have been used as a model system to study various aspects of the metabolic and molecular regulation of several natural product pathways. We have demonstrated an efficient *Agrobacterium rhizogenes*-mediated transformation protocol for the establishment of *A. gigas* hairy root cultures, and a valuable alternative approach for the production of pyranocoumarins from *A. gigas*. A tremendous amount of research remains to be done on the pyranocoumarin biosynthetic pathway. The first task will be the cloning of genes encoding the biosynthetic enzymes. This protocol will improve the production of decursin and decursinol angelate in *A. gigas* hairy root cultures in response to the introduction of important genes in the pyrocoumarin synthetic pathway.

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