Agrobacterium rhizogenes-mediated genetic transformation of radish (*Raphanus sativus* L. cv. Valentine) for accumulation of anthocyanin

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Abstract

An efficient protocol for the transformation of radish (*Raphanus sativus* L. cv. Valentine) hairy root cultures was initiated using cotyledon explants and infected by *Agrobacterium rhizogenes* R1000, a strain with the binary vector pBI121. Kanamycin-resistant roots appeared on explants at 4 weeks after infection with *A. rhizogenes* and these roots were maintained on hormone-free selection medium. PCR analysis of the neomycin phosphotransferase (*NTPII*) gene confirmed transformation in 15 plants of kanamycin-resistant hairy root cultures from a total 20 plants. Detection of high levels of ß-glucuronidase (GUS) transcripts and enzyme activity, as well as GUS histochemical localization, also confirmed the stable genetic transformation. We inoculated isolated hairy roots in liquid medium to promote rapid growth and production of anthocyanin. Among the different auxin treatments, 1.0 mg/L 2,4-D treatment resulted the highest production of anthocyanin (30.9 mg/L) that was 26 times higher as compared to control (1.2 mg/L). Transgenic root cultures of *R. sativus* L. cv. Valentine will allow investigation of the molecular and metabolic regulation of anthocyanin biosynthesis and evaluation of the genetic engineering potential of this species.

Keywords: anthocyanin, hairy root culture, *Raphanus sativus* L. cv. Valentine, genetic transformation, 2,4 –D.
Abbreviations: *NPTII,* neomycin phosphotransferase; GUS, ß-glucuronidase; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA , and naphthalene acetic acid.

Introduction

Hairy roots from any plants through genetically transformed could obtaine by infection of plants with *A. rhizogenes*, a gram-negative soil bacterium, offers a promising system for secondary metabolite production (Hamill et al., 1987). It is mentionable that the fast growing hairy roots are unique in their genetic and biosynthetic stability. These fast growing hairy roots can be used as a continuous source for the production of valuable secondary metabolites. Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further subculturing and plant regeneration. Generally, root cultures need an exogenous phytohormone supply and grow very slowly, resulting in poor or negligible secondary metabolite synthesis. Moreover, the use of hairy root cultures has changed the role of plant tissue culture for secondary metabolite synthesis. Transformed roots of many plant species have been widely studied for the *in vitro* production of secondary metabolites (Hamill et al., 1986; Mano et al., 1987; Signs and Flores, 1990; Benjamin et al., 1994; Mukundan et al., 1998). Hairy root cultures produce secondary metabolites over successive generations without losing genetic or biosynthetic stability. This property can be utilized by

genetic manipulations to increase biosynthetic capacity. Sevon et al. 1997 characterized transgenic plants derived from hairy root cultures of *Hyoscyamus muticus* and concluded that a single hairy root that arises from the explant tissue is a clone.
The radish (*Raphanus sativus* L.), which belongs to the Brassicaceae family, is an edible root vegetable that is grown and consumed throughout the world. Radishes have numerous varieties, varying in size, color, and duration of required cultivation time (Gutierrez and Perez, 2004; Hara et al., 2009). The root phenotype of *R. sativus* L. cv. Valentine, a red radish, is unique in that it has white- and green-colored skin and red interior flesh at maturity. The red radish contains significant amounts of anthocyanins, predominantly pelargonidin and more than 12 differently acylated pelargonidin glycosides. Anthocyanins from red radishes are widely used as natural food colorings, because of the high stability and similarity to synthetic Food Red No. 40 (Otsuki et al., 2002; Rodriguez-Saona et al., 1999). To study the functional genomics that regulate anthocyanin biosynthesis in radishes, it is necessary to establish a protocol for efficient and stable genetic transformation. However, plant transformation of radishes is

not reported yet for the production of secondary metabolites. *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. Anthocyanin production by hairy root culture of radish has never been reported. In this paper, we describe first time a protocol for introducing foreign genes into hairy roots of *R. sativus* L. cv. Valentine by inoculation with *Agrobacterium rhizogenes* and quantify anthocyanin content in transformed hairy roots.

Results

Establishment of hairy root cultures

A. rhizogenes which was harboring binary vector was co-cultivated with cotyledons of *R. sativus* cv. Valentine for its ability to induce transgenic hairy root formation. After 2 days of co-cultivation with *A. rhizogenes*, cotyledons were transferred to agar-solidified, hormone-free selection medium containing 50 mg/L kanamycin and 200 mg/L timentin. All formulations used in subsequent steps included kanamycin for selection of transformed hairy roots 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 neomycin phosphotransferase (*NPTII*) gene product, on the growth of red radish hairy roots transformed with wild-type *A. rhizogenes* R1000. At concentrations between 10 and 100 mg/L, 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 kanamycin for the selection of transformed hairy roots in subsequent steps.

Hairy root emerged from wound sites on red radish cotyledons (Fig. 1A) within 2 weeks after inoculation. After 4 weeks, putative transgenic hairy roots of red radishes (Fig. 1B) began to grow more rapidly. About 4–5 weeks after co-cultivation with *A. rhizogenes*, hairy roots from red radish cotyledons were excised from the necrotic explant tissues and subcultured on fresh agar-solidified selection medium. After repeated transfer to fresh selection medium for 2–3 months (Fig. 1C), rapidly growing hairy roots were transferred to liquid culture medium.

Molecular analysis of transgenic hairy roots

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 ß-glucuronidase (GUS) activity in various tissues, the presence of GUS mRNAs, and the level of GUS enzyme activity. In 15 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 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 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 R1000, but not in any wild-type roots (Fig. 2A and B). Next, we tested 5 randomly selected *NTPII*-positive and rapidly growing hairy root lines to confirm the presence of GUS transcripts. After using PCR to verify the insertion, GUS transcription in the hairy roots was assessed by quantitative real-time PCR, which revealed high levels of GUS transcripts in each of the *NTPII*-positive hairy root cultures (T1–5), but no signal in wild-type hairy roots (Fig. 2C). We also tested 5 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. 2D). Individual transformants expressed a wide range of GUS activities, from 589 to 1230 MU/min/mg 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.

Effect of auxins on growth and production of anthocyanin in hairy roots of R. sativus

The effects of different auxins on growth and anthocyanin production in *R. sativus* hairy root cultures are presented in Table 1. Four different auxins, i.e., 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA), at 3 different concentrations, i.e., 0.1, 0.5, and 1 mg/L, were used. Results revealed that all tested auxins treatments increased the growth rates of the hairy roots and increased the production of anthocyanin under our experimental conditions (Table 1 and Fig. 3). Among the different auxin treatments, hairy roots treated with 2,4-D showed the greatest increase in the production of anthocyanin. Increasing the concentration of 2,4-D increased the production of anthocyanin, and at a concentration of 1.0 mg/L, the production of anthocyanin was 26 times higher compared to control. For all auxin treatments, the highest concentration (1.0 mg/L) was associated with the greatest anthocyanin production. IAA at 1.0 mg/L resulted in 5 times higher production of anthocyanin compared to control. Hairy root production did not differ much among the different auxin treatments. But IBA at 0.5 mg/L performed the highest hairy root growth among the auxin treatments (Table 1).

Discussion

Plant transformation has become a core research tool for crop improvement, as well as for studying gene function in plants. Various methodologies of plant transformation have been developed to increase the efficiency of transformation and to achieve stable expression of transgenes in plants (Gelvin, 2009; Rao et al., 2009). Over the last decade, transformed root cultures from plants 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 (Christey and Braun, 2005; Georgiev et al., 2007; Srivastava and Srivastava, 2007). Recently, transgenic hairy root systems have shown potential for the introduction of foreign genes along with the Ri-plasmid into plant cells with *A. rhizogenes* vector systems. *A. rhizogenes*-mediated root transformation is a valuable tool for basic plant research, as well as a useful technique for metabolic engineering in the biosynthesis of secondary metabolites (Giri and Narasu, 2000; Hu and Du, 2006). Our results also in conagree with our results indicate an efficient *A. rhizogenes*-mediated transformation protocol for the establishment of *R. sativus* L. cv. Valentine hairy root cultures and also provide a valuable alternative approach for the production of anthocyanin from red radishes which are in agreement with others i.e., Gelvin, 2009; Rao et al., 2000; Christey and Braun, 2005; Georgiev et al., 2007; Srivastava and Srivastava, 2007 those are successfully achieved hairy root culture and transgenic plants

Table 1. Effect of auxins on growth and production of anthocyanin in hairy roots of *Raphanus sativus* L. cv. Valentine after 3 weeks in culture. Data are presented as mean ± SE.

Auxin concentration (mg/L)		Dry weight (g/L)	Anthocyanin / Dry weight(mg/L)
Control		10.4 bc[a]	1.2 e[a]
2,4-D	0.1	11.7 ab	17.3 c
	0.5	9.5 c	28.2 b
	1.0	7.8 d	30.9 a
IAA	0.1	11.3 ab	2.6 e
	0.5	11.9 ab	4.3 d
	1.0	12.3 a	5.8 d
IBA	0.1	11.6 ab	1.5 e
	0.5	12.5 a	2.1 e
	1.0	11.6 ab	2.3 e
NAA	0.1	11.8 ab	1.1 e
	0.5	10.5 bc	1.2 e
	1.0	9.4 c	1.4 e

[a]Mean values indicated by the same letter in a column do not differ significantly at the 5% level by Duncan's Multiple Range Test
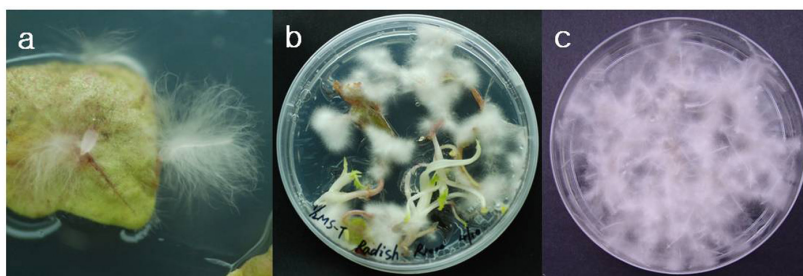
Fig 1. Development of hairy roots from cotyledons of *Raphanus sativus* L. cv. Valentine after inoculation with *A. rhizogenes* strain R1000. Hairy roots within 2 weeks (a: 3x), 4 weeks (b: 0.3x), and 2 months (c: 0.3x) after inoculation.

by *Agrobacterium rhizogenes* –mediated transformation for producing secondary metabolites. 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.

The availability of an expanding collection of genes encoding anthocyanin biosynthetic enzymes, coupled with a protocol for the production of rapidly growing transgenic root cultures of radishes, provide a powerful and versatile model system to investigate the molecular regulation of anthocyanin biosynthesis in this plant

Materials and methods

Plant materials

For preparing plant materials, Seeds of *R. sativus* cv. Valentine were purchased from Stokes Seeds Ltd., St. Catharines, Canada and) stored at 4°C. The seeds were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite (Sigma, St. Louis, Mo. USA) solution for 10 min, then rinsed three times in sterilized water. Seven seeds were placed on 25 mL of agar-solidified culture medium in Petri dishes (100 × 15 mm). The basal medium consisted of MS (Murashige and Skoog, 1962) salt and vitamin medium (Sigma, St. Louis, Mo. USA) solidified with 0.7% (w/v) agar. The MS salt and vitamin medium was adjusted to pH 5.8 before adding the agar and was then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated after 2 weeks culture in a growth chamber about 70-80% humidity 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 A. rhizogenes

The binary vector pBI121was purchased from Clontech.

Laboratories (Palo Alto, CA). It has a CaMV 35S promoter-GUS gene fusion and the NPT II gene as a selectable marker. This binary plasmid was transferred into *A. 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 (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing 50 mg/L kanamycin, 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 30 g/L sucrose). The *A. rhizogenes* cell density was adjusted to give an A600 of 1.0 for inoculation.

Establishment of hairy root cultures

Excised cotyledons of *R. sativus* cv. Valentine 10-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 and vitamins, 30 g sucrose l^{-1}, 500 mg l^{-1} carbenicillin, 50 mg/L kanamycin and 8/L agar. Putative transgenic hairy roots were observed emerging from the wound sites within 4 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 and a 16-h photoperiod. Each experiment was carried out with 3 flasks per culture condition and repeated twice.

PCR analysis for NTPII gene

Plant genomic DNA for PCR analysis was extracted as
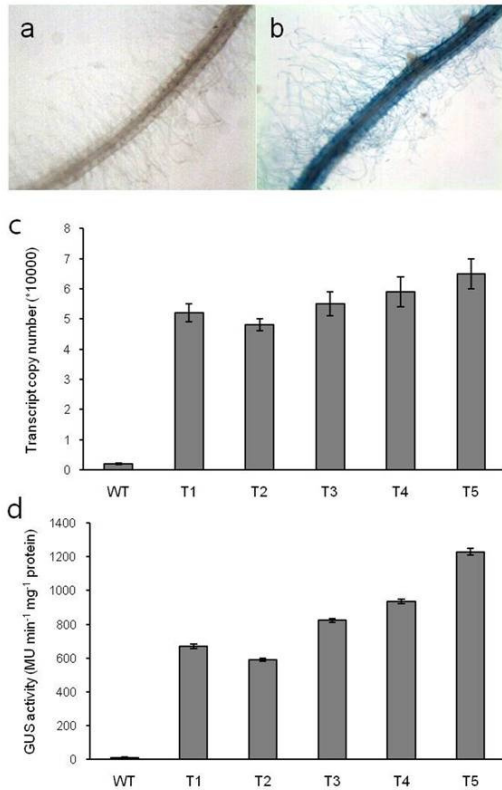
Fig 2. Histochemical analysis of GUS, quantitative RNA analysis of the GUS gene and GUS activity in transgenic hairy roots of *R. sativus* L. cv. Valentine. Transformed hairy roots with *A. rhizogenes* R1000 only (a: 7x), and transformed hairy roots with *A. rhizogenes* R1000 with pBI121 (b: 7x). Quantitative RNA analysis of the GUS gene in transgenic hairy root lines. d GUS activity in wild-type (WT) and kanamycin-resistant (T1–5) red radish hairy root cultures using 4-methylumbelliferyl-β-D-glucuronide as the substrate. WT, wild-type hairy root induced by *A. rhizogenes* R1000 only; Tn, transgenic hairy root lines induced by *A. rhizogenes* R1000 with the binary vector pBI121 ("n" indicates the line number) (c). GUS activity in wild type (WT) and kanamycin-resistant (T1–5) radish hairy root cultures using 4-methylumbelliferyl-β-D-glucuronide (MUG) as the substrate. Bars represent the mean ± SD of three independent measurements (d).
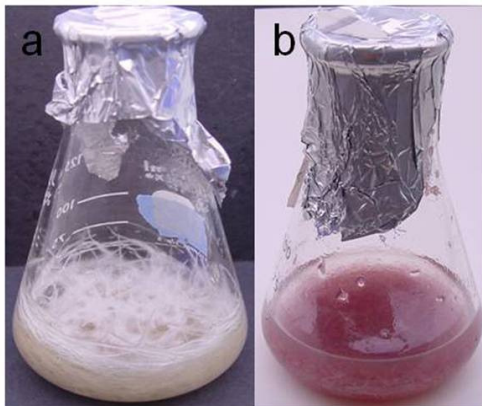
Fig 3. Effect of auxins on the production of anthocyanin in hairy roots of *R. sativus*. Hairy roots in liquid medium with hormone-free (a) and 1 mg/L 2,4-D treatment (b).

described by the manufacturer's protocol (DNeasy Plant mini kit; Qiagen, Valencia, CA). The sequences of the two primers used to amplify a fragment of the NTPII gene were 5'-TATGTTATGTATGTGCAGATGATT-3' and 5'-GTCGACTCACCCGAAGAACTCGTC-3'. The amplification cycle consisted of denaturation at 95 $^{\circ}$C for 1min, primer annealing at 55 $^{\circ}$C for 1min, and primer extension at 72 $^{\circ}$C for 1min. After 30 times repeats of the thermal cycle and final extension 72 $^{\circ}$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

Transgenic roots collected and were ground with extraction buffer consisting of 50 mM KPO_4 buffer, pH 7.0, 1mM EDTA, and 10mM -mercaptoethanol. The GUS fluorometric assay buffer consisted of 50 mM $NaPO_4$ buffer, pH 7.0, 10mM -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 0.44mg/ml. Assays were performed on 50 µL of transgenic tissue extract for 3 h at 37 $^{\circ}$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 Bradford method using BSA as a standard (Bradford, 1976).

Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from 100 mg red radish hairy roots by grinding the material with a mortar and pestle under liquid nitrogen, and adding 1 mL of TRIZOL (Invitrogen, Carlsbad, CA). Reverse transcription of 1 µg total RNA was performed according to the manufacturer's protocol (ReverTra Ace-α-; Toyobo, Osaka, Japan) using an oligo$(dT)_{20}$ primer. The resulting cDNAs were used as templates for real time-PCR.
Transcriptional level analysis of GUS expression in red radish hairy roots was performed by quantitative real-time PCR using a Mini Opticon Real-time PCR system (Bio-Rad Laboratories, Hercules, CA). Primers designed from full-length GUS cDNA sequences were highly specific for a gene confirmed by the online program (http://frodo.wi.mit.edu/primer3). The primer sequences used were as follows: 5'-CAACGTCTGCTATCAGCGCGAAGT-3' and 5'-TATCCGGTTCGTTGGCAATACTCC-3'. Real-time PCR was carried out in a 20 µl reaction volume containing 0.4 µM of each primer and 1× SYBR Green Real-Time PCR master mix (Toyobo). Amplification was conducted as recommended by the manufacturer cycling parameters. Triplicate experiments were performed for each sample.

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*. (Kosugi et al., 1990) for staining. Roots were fixed in a 0.35% (v/v) formaldehyde solution containing 10mM MES, pH 7.5, and 300mM mannitol for 1 h at 20 $^{\circ}$C, rinsed three times in 50mM sodium phosphate, pH 7.5, and subsequently incubated in 50mM sodium phosphate, pH 7.5, 10mM EDTA, 300mM mannitol, pH 7.0, and 1mM 5-bromo-4-chloro-3-indolyl--D-glucuronide cyclohexylammonium salt for 6 to 12 h at 37 $^{\circ}$C. Stained tissues were rinsed extensively in 70% ethanol to

remove residual phenolic compounds.

Anthocyanin measurements

Anthocyanin was extracted from 200 mg of hairy roots in 1.5 ml of acidified methanol (1% HCl v/v), for 24hrs in darkness at 4 ^{o}C with occasional shaking. A partitioning was performed by the addition of 1 ml H_2O and 2.4 ml chloroform to the extracts and centrifugation for 30 min at 5000 rpm. The absorption of the top phase was determined at 530 nm (λ max for anthocyanin) and 657 nm (peak of absorption for chlorophyll). The values for anthocyanin yield, A530 - 0.25 A657 were expressed as anthocyanin/g fresh wt (Mancinelli et al., 1991). Each value for anthocyanin reported in figures is a mean of 3 replicates.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) with sums of squares partitioned to reflect trial effects using the SAS Software release 9.2 (SAS, 2010) and means were separated via Duncan Multiple Range Test ($P < 0.05$).

Acknowledgments

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