Int. J. Forest, Soil and Erosion, 2014 4 (4): 127-131
ISSN 2251-6387

Research Paper

Agrobacterium rhizogenes-mediated genetic transformation of Hypericum perforatum L.

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Abstract: Polyphenols constitute the major portion of dietary antioxidants and a dietary rich in these compounds reduces the risk of various diseases. As a valuable polyphenol, resveratrol possesses different biological activities and has the potential to prevent or slow the progression of a wide range of disorders such as cancer and cardiovascular disease. Considering growing need to resveratrol and advantages of *in vitro* production of secondary metabolites especially hairy root cultures, in this study was aimed to isolate and transfer resveratrol synthase gene into *Hypericum perforatum* L. via *Agrobacterium rhizogenes*-mediated transformation method. To do this, a resveratrol synthase gene was isolated from grapevine and after cloning in binary vector pBI121 introduced into *A. rhizogenes* strain ATCC 15834. Transformed bacterium was then served for induction of hairy roots in shoots from pot plants and *in vitro* grown plants. After emergence and confirmation of transformation, transformed hairy roots were grown in half-strength B5 medium for four weeks in which they were able to accumulate 0.63% resveratrol based on the dry weight.

Keywords: *Hypericum perforatum* L.; hairy root; *Agrobacterium rhizogenes*; resveratrol

Introduction:

Polyphenols are ubiquitous components of plants and, beside providing color to leaves, flowers and fruits, increase plant survival towards environmental threats with their various properties such as antimicrobial, antioxidant, insect feeding deterrence and UV screening activities (Negrao and Faria 2009). These compounds are common ingredients of foods of plant origin and constitute the major antioxidants of our diet (Scalbert 2005). In fact, together with other dietary reducing agents such as vitamin C, vitamin E and carotenoids, polyphenols protect the body's tissues against oxidative stress (Neyestani 2008). Epidemiological studies has been suggested a relationship between the high dietary intake of polyphenol compounds and reduced risk of diseases such as cardiovascular disease, specific forms of cancer and neurodegenerative disorders (Vauzour et al. 2010).

Stilbenes are a group of polyphenol compounds and occur in the members of different plant families including Dipterocarpaceae, Cyperaceae, Gnetaceae, Pinaceae, Leguminosae, Myrtaceae, Moraceae, Fagaceae, Liliaceae and Vitaceae. They are commonly found in the roots, barks, rhizomes and leaves and often occur in plants that are not routinely consumed for food or in the nonedible tissues (Cassidy et al. 2000). Due to potential of these metabolites in therapeutic or preventive applications, stilbenes and their derivatives have gained significant interest for drug research and development (Shen et al. 2009). Plant stilbenes are mostly derivatives of the basic unit *trans*-resveratrol (Chong et al. 2009).

Resveratrol is found in some edible plant species including peanuts and grapes but the highest amount of this compound occurs in the roots of Japanese Knotweed (*Poligonum cuspidatum*) (Giovinazzo et al. 2012). This compound is one of the best known plant secondary metabolites and has the powerful antioxidant activity (Kieselv 2011). Resveratrol also exhibits other properties including anti-inflammatory, antiaggregatory, chemopreventive, modulation of lipoprotein metabolism and reducing cholesterol levels and has the potential to prevent or slow the progression of a wide range of disorders such as cancer, cardiovascular disease, ischaemic injuries, diabetes and neurodegenerative disorders. Moreover, this compound can improve stress resistance and extend the lifespan of different organisms (Baur and Sinclair 2006, Halls and Yu 2008, Giovinazzo et al. 2012). Due to its beneficial effects, *trans*-resveratrol is now commercially available as a dietary supplement in the United States and Europe and it is approved to be a potentially long-term medicine (Su 2011). Resveratrol is biosynthesized from the phenylpropanoid pathway with the resveratrol synthase (RS) enzyme as the ultimate ones. This enzyme is a member of the type III polyketide synthases and catalyzes the condensation of resveratrol from one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA (Hasan et al. 2013).

Regarding advantages of *in vitro* cultures over the conventional methods of production of industrially important metabolites such as production in controlled conditions independent of environmental instabilities, considerable efforts have been paid so far to exploit these systems for production of a wide variety of secondary metabolites. However, with respect to this fact that cultured cells are genetically unstable and tend to accumulate low levels of secondary metabolites (Giri and Narasu 2000), there has been a growing interest to use *in vitro* cultures based on differentiated cells especially hairy roots cultures. Owing to attractive properties such as fast growth, lack of geotropism, ability to grow in hormone-free media, the high biosynthetic capacity and genetic and biosynthetic stability, these genetically transformed roots, resulted from the infection of plants with the

gram-negative bacterium *Agrobacterium rhizogenes*, are considered promising for production of secondary metabolites (Giri and Narasu 2000, Guillon et al. 2006, Kim et al. 2002, Zhou et al. 2011).

Genetic engineering of medicinal plants with the aim of altering their metabolic pattern to increase or decrease specific metabolites or production of novel compounds has been an interest field of research in recent years. Although plants can be engineered via several ways, *Agrobacterium*-mediated transformation has been the most abundant method of transformation. Genetic engineering of secondary metabolites of interest by introducing useful genes is another interesting strategy of hairy root cultures (Kang 2006). In fact, beside T-DNA from the root-inducing (Ri) plasmid, *A. rhizogenes* also has the ability to transfer the T-DNA of binary vector present and therefore produce transgenic hairy roots containing foreign genes inserted on a binary vector (Seki 2005).

Considering growing need to resveratrol and advantages of *in vitro* production of secondary metabolites especially hairy root cultures, in this study a resveratrol synthase gene was isolated from grapevine and transferred to *H. perforatum* L. via *A. rhizogenes*-mediated transformation method to establish a transformed hairy root culture of this medicinal plant.

Materials and methods

Gene cloning and induction of transformed hairy roots

To induce transformed hairy roots of *H. perforatum*, in this study shoots of pot and *in vitro* grown plants (obtained by culturing sterilized seeds in the solid MS medium) were infected with the *A. rhizogenes* strain ATCC 15834 carrying recombinant binary vector pB121*RS*. To construct mentioned recombinant binary vector, in brief, a resveratrol synthase gene (*RS*) from grapevine (*Vitis vinifera* L.) was isolated using a pair of degenerated primers with the sequences 5'-CCTCTAGAATGGCTTCAGTYGAGGAAWTTAG-3' (Forward primer) and 5'-CCGAGCTCTTAATTTGTAACYRTAGGAATGC-3' (reverse primer). Highlighted sequences represent restriction sites related to *XbaI* and *SacI* restriction enzymes, respectively. The full length grapevine *RS* gene (1179 bp) was isolated using polymerase chain reaction (PCR) with *pfu* polymerase enzyme on its cDNA prepared from DNaseI treated total RNA of mature leaves. PCR was performed at 94 °C for 4 min (initial denaturation), followed by 35 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 150 sec, and the final extension at 72 °C for 7 min. Isolated gene was firstly cloned into pGEM-T Easy vector and recombinant plasmid digested with *XbaI* and *SacI* restriction enzymes from recombinant vector. Afterwards, cloning vector pBI121 was digested with the same enzymes and *RS* gene inserted on it. Ligation was performed using T4 ligase enzyme. In this study, constructed plasmids were introduced into competent cells of *E. coli* (strain DH5α). To select transformed colonies, bacterium was grown after transformation on solid LB medium containing ampicillin or kanamycin (in the case of pGEM-T Easy and pBI121 vectors, respectively). Transformed colonies were identified with colony-PCR and digestion assays. Recombinant pBI121*RS* vector was finally extracted from transformed *E. coli* bacterium and introduced into competent *A. rhizogenes* strain ATCC 15834 cells using freeze-thaw method. After transformation, bacterium was grown on solid LB medium containing rifampicin and kanamycin to select transformed colonies. For induction of hairy roots, transformed *A. rhizogenes* strain ATCC 15834 (carrying pBI121*RS*) was grown on solid LB medium containing rifampicin and kanamycin and kept at 28 °C for one day.

Shoots of pot and *in vitro* plants were used as explants for induction of hairy roots. Before inoculation, shoots of pot plants were surface sterilized by immersing in 75% ethanol for 30 sec and immediately in 1% sodium hypochlorite for 10 min. Treated shoots were afterwards rinsed three times in sterile water. For inoculation, explants were wounded with inoculated scalpel, placed on MS medium supplemented with 7 g/L agar and 30 g/L sucrose. After 48 h co-cultivation in the dark at 25 °C, explants were transferred to the solid MS medium containing 500 mg/L cefotaxime to eliminate *Agrobacterium*. In the subsequent subcultures, the level of cefotaxime was gradually decreased. After emergence, each hairy root was separately excised and cultured to establish hairy root lines which were subsequently subjected to molecular analyses. Ultimately, one fast growing transformed hairy root line was selected and grown in Erlenmeyer flasks containing 70 ml half-strength B5 medium supplemented with 30 g/L sucrose for four weeks in the dark at 25 °C on shaker at 120 rpm.

Confirmation of transformation

Total genomic DNA from hairy roots was extracted (Murray and Thompson 1980) and subjected to PCR analyses with specific primers of *RS*, *rolC* and *virD* genes. Sequences of *rolC* primers used for amplification by PCR were 5'-CTCCTGACATCAAACTCGTC-3' (forward) and 5'-TGCTTCGAGTTATGGGTACA-3' (reverse) and a primer pair with sequences 5'-ATGTCGCAAGGACGTAAGCCGA-3' (forward) and 5'-GGAGTCTTTCAGCATGGAGCAA-3' (reverse) was used for amplification of *virD* gene. PCR conditions for these genes were initial denaturation at 94 °C for 4 min, 35 cycles including 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and the final extension at 72 °C for 7 min. Additionally, transcription of *RS* and *rolC* genes in selected hairy root line was evaluated by conducting reverse transcription PCR (RT-PCR). To do this, the first strand of DNA (cDNA) was synthesized from DNaseI treated total RNA by using the reverse transcriptase enzyme and reverse primers of these genes. Prepared cDNA was served as template for PCR amplification with gene specific primers and conditions which mentioned above. Fragments amplified by PCR were firstly separated by electrophoresis in 1% agarose gel and after staining with ethidium bromide visualized under UV.

Extraction and analysis of resveratrol

To extract and analyze resveratrol content of hairy roots, they were lyophilized and extraction was performed using 80% ethanol according to the method described by Pan et al. (2012). Analysis of resveratrol was carried out using an ion mobility spectrometer (IMS-200) system with the drift tube length of 11 cm and N_2 as the drift gas. The cell and injection temperatures were 180 and 230 °C, respectively and corona voltage was adjusted on 2 kV. For analysis, different concentrations of standard resveratrol were prepared freshly and injected to construct a calibration curve. Quantification of resveratrol concentration in tested samples was done based on constructed standard calibration curve.

Data analysis

In this study, collected data was subjected to statistical analysis using the statistical MSTAT-C software.

Results and discussion:

Induction of transformed hairy roots

The present study was aimed to transfer a resveratrol synthase gene from grapevine into *H. perforatum* plant via *A. rhizogenes*-mediated method. To induce transformed hairy roots, shoots of pot and *in vitro* plants were infected with the *A. rhizogenes* strain ATCC 15834 carrying constructed recombinant binary vector pB121*RS*. About three to four weeks after inoculation, hairy roots were appeared (Fig. 1). According to the results, there were significant differences ($P<0.05$) between potential of tested explants for induction of hairy root. Shoot explants from *in vitro* grown plants has the more potential to induce hairy root than those of pot plants with a frequency of 35.3% (Fig. 2).

Previously, induction of hairy roots in *H. perforatum* plant has been reported in different studies. For example, inoculation of plant shoots with the *A. rhizogenes* strain A4M70GUS has been resulted in hairy root formation in 21% inoculated explants (Vinterhalter et al. 2006). Also, 33% of inoculated root segments of this plant with *A. rhizogenes* strain A4 were able to develop hairy roots (Tusevski et al. 2013) and hairy root formation was observed in 25 and 13% of infected leaves and root segments, respectively, with *A. rhizogenes* strain ATCC 15834 (Di Guardo et al. 2003). Factors such as explant genotype and structure, chemical and physical factors, bacterial strains and signal molecules influence *Agrobacterium*-mediated genetic transformation (Tao and Li 2006).

Molecular and chemical analyses of hairy roots

Due to factors such as differences in the number of inserted T-DNA and inserted sites, different lines of hairy roots are usually variable in terms of morphology, growing rate and ability of metabolite production. Therefore, in this study appeared hairy roots were separately excised and cultured to establish hairy root lines. To evaluate transformation status of hairy root lines, total genomic DNA was extracted from each and subjected to PCR analysis with *RS*, *rolC* and *virD* genes specific primers. Results of PCR analyses for two transformed hairy root lines can be seen Fig. 3. In these lines, PCR with primers of *RS* and *rolC* genes has been resulted in

Fig 1. Induction (left) and culture (right) of *H. perforatum* hairy roots.

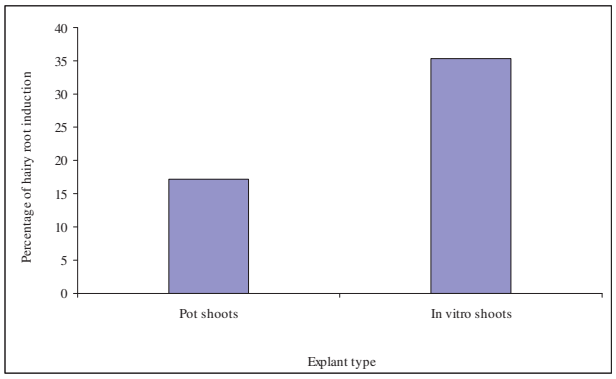

Fig 2. Effects of explant type on induction of hairy roots in *H. perforatum.*

in amplification of related fragments and no fragments were amplified with *virD* specific primers. Considering PCR with DNA extracted from natural *H. perforatum* plants produced no fragments, it can be concluded at least one copy of *RS* and *rolC* genes is present in the genome of mentioned hairy root lines. A fast growing transformed hairy root line was selected and subjected to further analyses. Transcription of *RS* and *rolC* genes were evaluated in this line with RT-PCR analysis and results are presented in Fig. 4. PCR with cDNA prepared from DNaseI treated total RNA of selected line as template and primers of *RS* and *rolC* genes amplified corresponding fragments. Therefore, inserted genes are transcribed in this transformed hairy root line. Based on the dry weight, this line accumulated 0.63% resveratrol in half-strength B5 medium.

So far, a wide number of plant species have been genetically engineered using *A. rhizogenes*-mediated method (Chattopadhyay et al. 2011, Jaggi et al. 2011, Matvieieva et al. 2012, Mehrotra et al. 2013, Park et al. 2012, Schmidt et al. 2007). In fact, this method of transformation can be served to meet different purposes including metabolic engineering of biosynthetic pathways with the aim of increasing production of valuable metabolites and production of novel compounds and recombinant proteins. With a number of advantages, hairy roots are considered as valuable tools for *in vitro* production of useful metabolites and, hence, transferring foreign genes into them

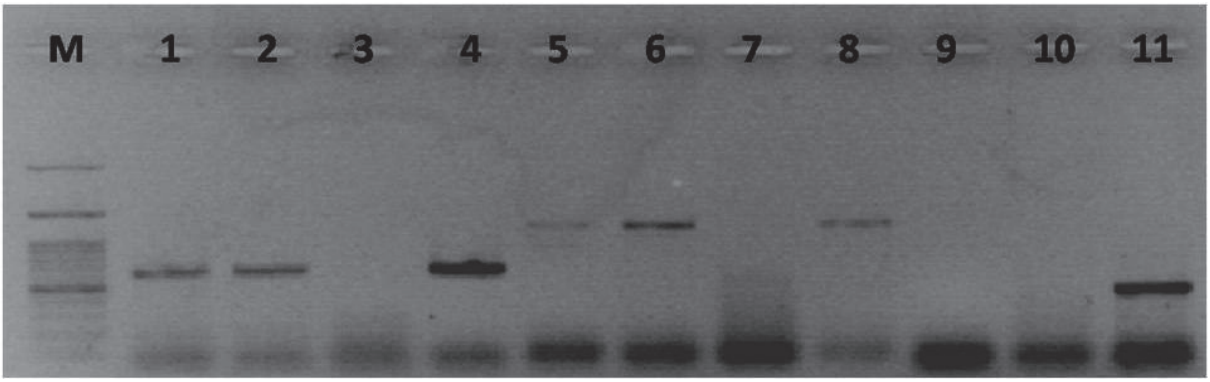

Fig 3. PCR analysis of two lines of hairy roots with *rolC* (1-4), *RS* (5-8) and *virD* (9-11) specific primers. 1, 5, 9 DNA of line 1; 2, 6, 10 DNA of line 2; 3, 7 DNA of natural plant; 4, 8, 11 *A. rhizogenes* strain ATCC 15834 carrying pBI121*RS*.

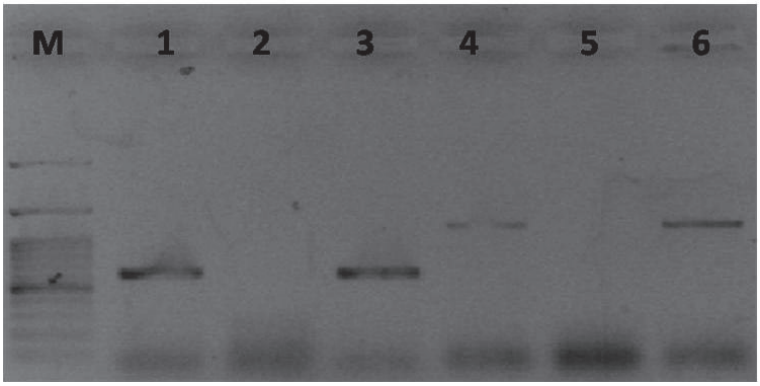

Fig 4. RT-PCR results with *rolC* (1-3) and *RS* (4-6) primers. 1, 4 DNA of selected transformed hairy root line, 3, 6 *A. rhizogenes* strain ATCC 15834 carrying pBI121*RS* (positive control); 2, 5 negative control

provide an opportunity to increase productivity of target metabolites. In this study, a resveratrol synthase gene was transferred into *H. perforatum* plant via *A. rhizogenes*-mediated method. In addition to the production of resveratrol, this method can also be served to increase efficiency of *in vitro* production of valuable *H. perforatum* metabolites with metabolic engineering of its biosynthetic pathways.

Conclusion:

A number of previous studies have been devoted to transfer stilbenes synthase genes mainly with the aim of increasing resistance against pathogens. Sweet potato (Pan et al. 2012), tobacco (Hain et al. 1990), rice (Stark-Lorenzen et al. 1997), tomato (Thomzik et al. 1997), hop (Schwekendiek et al. 2007) and lettuce (Liu et al. 2006) are among the engineered plants. However, the present study was aimed to transfer a resveratrol synthase gene into *H. perforatum* using *A. rhizogenes*-mediated method. In addition to production of resveratrol, this method can also be served to increase efficiency of *in vitro* production of valuable *H. perforatum* metabolites with metabolic engineering of its biosynthetic pathways.

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