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Natural Product Communications, 10, 11, pp. 1919-1922, 2015-11

NPC Natural Product Communications

2013
Vol. 8
No. 0
1 - 2

Production of Triterpenoid Sapogenins in Hairy Root Cultures of *Silene vulgaris*

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Received: April XX, 2013; Accepted: XX, 2013

Silene vulgaris (Moench) Garcke (Caryophyllaceae) is widely distributed in North America and contains bioactive oleanane-type saponins. In order to investigate in vitro production of triterpenoid saponins, hairy root cultures of *S. vulgaris* were established by infecting leaf explants with five strains of *Agrobacterium rhizogenes* (LBA9402, R1000, A4, 13333, and 15834). The *A. rhizogenes* strain LBA9402 had infection 100% frequency and induced the most hairy roots per plant. Methyl jasmonate (MeJA)-induced changes in triterpenoid saponins in *S. vulgaris* hairy roots were analyzed. Accumulation of segetalic acid and gypsogenic acid after MeJA treatment was 5-and 2-fold higher, respectively, than that of control root. We suggest that hairy root cultures of *S. vulgaris* could be an important alternative approach to the production of saponins.

Keywords: *Agrobacterium rhizogenes*, Hairy root culture, Methyl jasmonate, Triterpenoid saponins, *Silene vulgaris*

Silene vulgaris, a member of the Caryophyllaceae family, is native to Europe and widely distributed in North America. This plant is used to treat anemia. Its rhizomes contain saponins, alkaloids and tannins [1]. The young shoots and the leaves of this plant are used as food in Spain. Saponins, a vast group of glycosides, widely distributed in higher plants, can be classified into two groups based on the nature of their aglycone skeleton (sapogenin). The first group comprises the steroidal saponins, which are almost exclusively present in the monocotyledonous angiosperms. The second group comprises the triterpenoid saponins, which are the most common and occur mainly in the dicotyledonous angiosperms [2]. Figure 1 shows example of triterpenoids sapogenins which are typically found in the Caryophyllaceae.

Given the chemical, physical, and physiological characteristics of these saponins which make them important starting material for industrial applications [3-5], there has been an increase in interest in the biosynthesis and production of saponins in the recent years. For example, Glensk et al. [1] reported three new triterpenoid saponins named silenosides A-C from the roots of *S. vulgaris*. Hairy root cultures of many plant species produce secondary metabolites that are useful as pharmaceuticals, cosmetics, and food additives [6]. Moreover, hairy roots can grow well in hormone-free medium and produce many genetically stable lateral roots. T-DNAs of various *Agrobacterium rhizogenes* wild type strains are responsible for the induction of hairy root development.

A highly efficient and convenient method for the *A. rhizogenes*-dependent production of transformed roots of *Saponaria vaccaria* of the Caryophyllaceae family has been described [7]. To date, there are no reports on the genetic transformation of *S. vulgaris*. In this paper, we described the development of a highly efficient system for *A. rhizogenes*-mediated transformation of *S. vulgaris* and the analysis of triterpenoid sapogenins from hairy root cultures after treatment with methyl jasmonate (MeJA).

For the production of natural products in hairy root cultures, the optimization of A. rhizogenes can play an important role in the growth of the roots as well as natural product production.

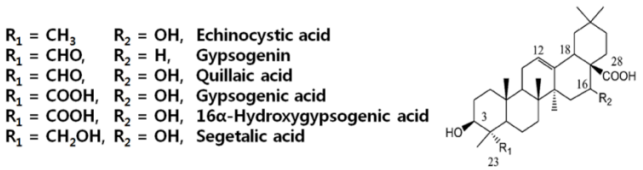

Figure 1: Aglycone skeletons of triterpenoid saponins.

Hairy roots of *S. vulgaris* were generated from leaf tissue by five strains of *A. rhizogenes*. After 1 week of culture, root formation was initiated from the leaf explants on MS medium supplemented with 3% sucrose, 0.7% phyto agar and 300 mg/l cefotaxime. In general, hairy root formation was evident after 2-4 weeks of culture. The hairy roots were formed mainly on the midrib of the infected leaf explants. No root formation was observed in control explants. Different strains of *A. rhizogenes* had varying influence on the induction of hairy roots.

The infection frequency (100%) of LBA 9402, A4, and 15834 was greater than that of R1000 (64%) and 13333 (73%). LBA9402 strain induced the maximal number (6.9) of hairy roots per explant, followed by the A4 strain (Table 1). The root length induced by strain 15834 at 3 weeks was the longest at 9.1 cm, but number of hairy roots was the lowest at 1.4 ± 1.1. Brillanceau et al. [8] and Bonhomme et al. [9] reported that 15834 strain was the most virulent and efficient for hairy root development in *Catharanthus roseus* and *Atropa belladonna*. In contrast, Schmidt et al. [7] reported that LBA9402 strain was highly effective for production of transformed roots of *S. vaccaria* L. (Caryophyllaceae). Among the various *A. rhizogenes* strains investigated in this study, LBA9402 was selected to be the most optimal for transformation frequency and transformed root elongation rate (Table 1). Hairy root lines were sub-cultured after 4 week intervals on MS medium containing 300 mg/l cefotaxime. After 2 months, transformed roots were transferred to fresh agar-solidified hormone-free MS medium (Figure 2). Rapidly growing hairy roots were transferred to MS liquid culture medium for elicitor treatment. The hairy root cultures established by *A. rhizogenes* are attractive for the production of

secondary metabolites and have the ability to synthesize useful natural products at levels comparable to those produced by whole plants [10].

Table 1. Effect of different strains of *A. rhizogenes* on the frequency of infection and growth of *S. vulgaris* hairy root cultures. Values represent the mean ± SD (n=20) 3 weeks after inoculation.

Agrobacterium strain	Infection frequency (%)	No. of hairy roots per explant	Root length (cm)
LBA 9402	100	6.9±1.9	8.4±1.2
R1000	64	1.8±0.9	5.8±1.6
A4	100	3.8±1.2	5.8±2.7
13333	73	1.8±1.4	2.7±2.1
15834	100	1.4±1.1	9.1±5.1
control	-	-	-

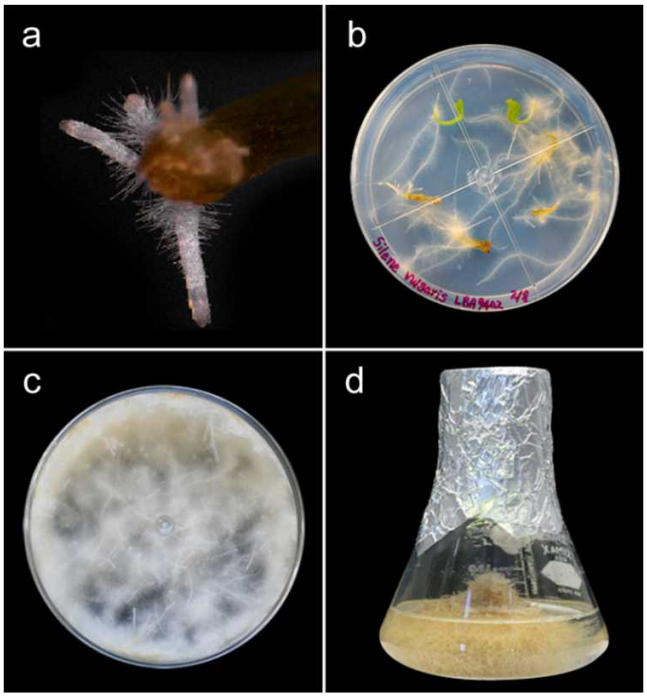

Figure 2: Developing hairy root from a cotyledon leaf of *S. vulgaris* after inoculation with LBA9402. a. Hairy roots after 7 days, b. 15 days, c. 90 days, d. hairy root culture in MS liquid culture medium.

To confirm the integration of *A. rhizogenes* plasmid T-DNA into the genome of *S. vulgaris*, we conducted PCR analysis using primers designed with the sequences of the *rolB* and *rolC* genes. The *rolA, B, C* genes play important roles in the production of secondary products in *A. belladonna* hairy roots and tobacco root cultures [11]. It can be clearly observed that the genomic DNAs of the hairy roots (T1-T7 lines) and Ri plasmid DNA (positive control) all showed a single PCR band, and all the bands were consistent with the predicted PCR product sizes (*rolB*-652 bp and *rolC*-490 bp). However, the non-transformed root (wild type) did not show any PCR band (Figure 3).

Triterpenoid saponins of the Caryophyllaceae family consist of a triterpenoid aglycone typically derived from β-amyrin (Figure 1). The most common sapogenins of this family are quillaic acid, gypsogenic acid and gypsogenin, which have hydroxy and carboxylate groups at C-3 and C-28, respectively [12]. The functional groups on these 2 carbons can be a wide variety of polysaccharide structures resulting in a large number of possible saponin structures. In order to simplify GC/MS analysis and more easily estimate levels of saponins present in the root samples, the saponins were hydrolyzed to their corresponding sapogenins.

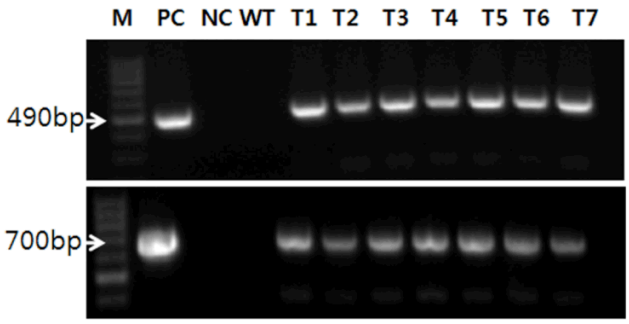

Figure 3: Detection of *rolB* and *rolC* gene by PCR showing amplified products from genomic DNA isolated from hairy roots of *S. vulgaris* (PC-positive control, NC-negative control, WT-wild type, T-transgenic)

The triterpenoid aglycones of *S. vulgaris* hairy root were compared with normal root aglycones. The level of sapogenins in wild type root was 0.55 µg/mg, while sapogenin levels in hairy roots was 0.19 µg/mg. The native roots contained 2.9 to 29 times more triterpene sapogenins than hairy roots. In native roots, four major sapogenins - echinocystic acid, 16-hydroxygypsogenic acid, gypsogenic acid and quillaic acid were detected. As well, minor amounts of segetalic acid, oleanolic acid, and gypsogenin were present (data not shown). However, for hairy roots, the major sapogenin identifiable peak corresponded to 16-hydroxygypsogenic acid, while the other six sapogenins found in the native root were present in low levels. For the MeJA experiment, the level of sapogenins found was 0.02 µg/mg, which was than the initial analysis (data not shown). Segetalic and gypsogenic acid accumulation increased 5- and 2-fold higher, respectively, after MeJA treatment compared to control root. After treatment with MeJA, except for echinocystic acid, all sapogenin levels increased gradually for 4 days and then decreased (Figure 4). These results suggest that MeJA treatment might be an effective approach to induce higher production of saponins.

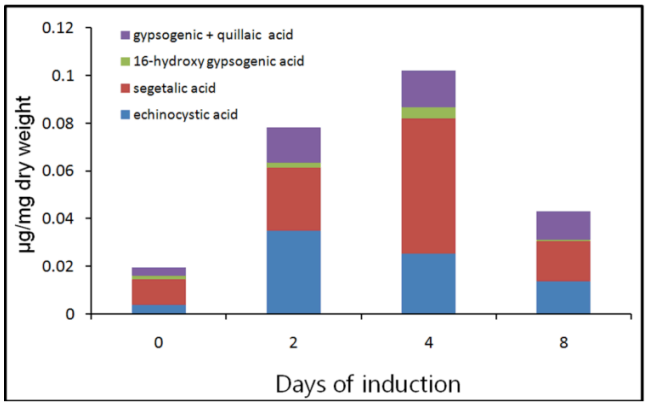

Figure 4: Sapogenin analysis after treatment with 100 µM MeJA

MeJA has also been used to increase ginsenoside content in cell suspension cultures [13] and root cultures of *P. ginseng* [14]. Overproduction of triterpenes by metabolic engineering might be an attractive strategy to produce higher quantities and better quality medicinal compounds. In this study, in order to obtain hairy root cultures of S. vulgaris transformed by A. rhizogenes, various A. rhizogenes strains were used. The results obtained in this study indicate that S. vulgaris hairy root culture might be a useful means of saponin production. Even though triterpenoid sapogenin amounts in *S. vulgaris* hairy roots were less than those of normal roots, the observation of triterpenoid sapogenin formation of *S. vulgaris* in hairy roots demonstrates the potential of the production of valuable

plant secondary products. Using a selected optimization of *A. rhizogenes* and MeJA treatment, improved root growth and a relatively high saponin production can be achieved. In the future, optimal hormone condition and culture media investigations for the improvement of saponin production in hairy root cultures of *S. vulgaris* will be carried out. The hairy root culture will be helpful to vital model system to understand molecular regulation and evaluate the potential of metabolic engineering.

Experimental

Plant materials: Seeds of *S. vulgaris* were purchased from B & T World Seeds company (France). Seeds were sterilized with 70% (v/v) ethanol for 1 min, then 2% (v/v) sodium hypochlorite solution for 10 min, and washed four times in sterile water. The seeds were placed on MS medium [15] pH 5.8, with 3% sucrose and 0.7% (w/v) Phyto Agar. The seeds were incubated for germination in a culture room at 23°C with 16 h light photoperiod. Seedlings with 3-4 leaves were transferred into sterilized magenta boxes.

Preparation of* A. rhizogenes*: *A. rhizogenes* wild type strains 15834, R1000, 13333, A4, and LBA9402 were each used to determine the transformation efficiency. Prior to infection, the strains 15834, R1000, and 13333 were grown for 2 days on 50 ml YEP liquid medium [16] containing 100 µM acetosyringone (Sigma-Aldrich), while A4 and LBA9402 were grown for 3 days on 50 ml YMB medium [17] containing 100 µM acetosyringone at 28°C on a rotary shaker at 220 rpm in the dark. The bacterial cells were collected by centrifugation for 5 min at 5000 rpm, and resuspended in liquid inoculation MS medium containing 3% sucrose.

Establishment of hairy root cultures: Leaf explants of S. vulgaris from 3 week old seedlings were immersed in bacterial suspension (OD600 = 0.7-1.0) separately for 20 min. The explants were blotted dry on sterile filter paper to remove excess bacteria, transferred to agar-solidified MS medium and incubated in the dark at 23°C. After 3 days of co-cultivation, the explants were transferred onto hormone-free medium supplemented with 3% sucrose, 0.7% Phyto Agar and 300 mg/l cefotaxime (Duchefa, Netherlands) to eliminate bacterial contamination. The hairy roots were sub-cultured into fresh medium of the same composition at 3 weeks intervals. For MeJA elicitation, the hairy roots were grown at 23°C on a rotary shaker (130 rpm) in darkness and pre-cultured for 4 weeks and then treated for 0, 2, 4, 8 days with 100 µM MeJA in 50 ml flasks.

PCR analysis: PCR was used to detect the integration of *rolB* and *rolC* genes of the Ri plasmid into root cultures. The genomic DNA was extracted from 100 to 130 mg sample of each root culture using an SP Plant DNA Kit (Omega Biotek, USA). The primers were designed according to agropine-type pRiA4b plasmids described by Piispanen et al. [18]. One microliter (80 ng) of gDNA template was used in 20 µl PCR containing 1 µl of 2.5 mM of each dNTP, 1 µl of 10 µM of each primer, 2 µl of 10X PCR buffer, 1 unit of Ex-Taq polymerase (Takara, Japan) and 13.8 µl RNase free-water. The PCR conditions were 94°C for 3 min for one cycle; 94°C for 30s, 49°C (*rolC* 52°C) for 30 s and 72°C for 45 s (*rolC* 30 s) for 35 cycles; 72°C for 10 min for 1 cycle in Stratagene Robocycler Gradient Thermocycler (USA). The polymerase chain reaction was used to confirm the presence of the *rolB* and *rolC*. The *rolB* primers of forward primer (5'-ACTATAGCAAACCCCTCCTGC-3') and reverse primer (5'-TTCAGGTTTACTGCAGCAGGC-3') used to amplify a 652 bp fragment. The *rolC* primers for the amplification of a 490 bp fragment were as follows: Forward primer: 5'-TGTGCAAGAGGATGAGC-3' and reverse primer: 5'-GATTGCA AACTTGCACTCGC-3'.

GC/MS analysis of triterpenoid sapogenins: Gypsogenic, quillaic, 16-hydroxygypsogenic, segetalic acids were isolated from *Saponaria vaccaria* seed and kindly provided by Dr. J. Balsevich. Echinocystic was purchased from Indofine Chemical Company Inc. Betulinic acid was purchased from Sigma-Aldrich. The freeze dried plant material (50 mg) was place in a round bottom glass culture tube with a Teflon seal. One milliliter of the extraction/acid hydrolysis solution (89% methanol, 9% water, 2% HCl) was added along with an internal standard. For qualitative analysis, 10 µg of cholesterol was used (10 µl of 1 mg/ml solution in methanol) and for quantitative analysis 5 µg betulinic acid was used (10 µl of 0.5 mg/ml solution in methanol) as the internal standard. The tube was sealed and heated to 80°C for 2 h. The tube was cooled to room temperature and approximately 200 mg KOH was added to the tube to create the basic hydrolysis solution. The tube was sealed, vortexed to mix and heated to 80°C for 2 h. The tube was cooled and 50% HCl was added to acidify the mixture. Two milliliters of water and 2 mL CH_2Cl_2 was added and the samples were vortexed and centrifuged. The bottom layer was recovered and concentrated to dryness; the residue was taken up in 20 µl of a 1:1 N,O-Bis (trimethylsilyl) trifluoroacetamide (10% trichloro-methyl silane)/ pyridine solution and placed in GC vial inserts for GC/MS analysis. GC/MS analysis was done using an Agilent 6890 Gas Chromatograph equipped with an auto-injector (30:1 split injection) and coupled via a DB-5MS column (30 m X 0.25 mm i.d., 0.25 micron film thickness, J & W Scientific; temperature programmed from 225°C to 325°C at 5°C/min) to an Agilent 5973N single quadrupole mass selective detector operating under standard EI+ conditions (70 eV). All samples were analyzed in triplicate.

Acknowledgments - The authors are grateful to John Balsevich for supplying sapogenin standards, Patricia Polowick and Alison Ferrie for reviewing the manuscript, and support of members of the PhytoMetaSyn project as funded by Genome Canada and affiliates, McGill Innovation Centre and the Saskatchewan Agriculture Development Fund.

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