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Ginsenoside production in different phenotypes of *Panax ginseng* transformed roots

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Abstract

Transformed roots were obtained after the inoculation of sterile root discs of *Panax ginseng* C.A. Meyer with *Agrobacterium rhizogenes* A4. The established hairy root lines displayed three morphological phenotypes when cultured on hormone-free liquid Schenk and Hildebrandt medium. Most of the cultures showed the characteristic traits of hairy roots (HR-M), while others were either callus-like (C-M) or thin (T-M) without branching. The growth rate of the transformed root lines was always higher than that of untransformed roots, showing that the genetic changes caused by the *A. rhizogenes* transformation conditioned a higher biomass formation. When considering the different transformed root phenotypes, we can observe that the highest ginsenoside production was achieved by HR-M root lines, closely followed by C-M ones, whereas the lowest yield was reached by T-M root phenotype. The study of the integration of the TL-DNA and TR-DNA fragments of the pRiA4 in the root genome showed that the *aux1* gene was always detected in HR-M and C-M root phenotypes which presented the highest biomass and ginsenoside productions. This fact suggests a significant role of *aux* genes in the morphology of *Panax ginseng* transformed roots. The ginsenoside pattern of transformed roots varied according to their morphology, although the ginsenoside contents of the Rg group was always higher than that of the Rb group. From our results, we can infer the potential of some root phenotypes of *Panax ginseng* hairy root cultures for an improved ginsenoside production.

Keywords: *Panax ginseng* C.A. Meyer; *Araliaceae*; Ginseng; Hairy root cultures; *rol* Genes; *aux* Genes; Opines; Ginsenosides

1. Introduction

Panax ginseng roots have been widely used in Chinese traditional medicine since ancient times owing to their stimulating and tonic properties. The pharmacological activities of ginseng or its crude extracts are based on the presence of a mixture of triterpenic saponins referred to as ginsenosides. The two major groups of ginsenosides are the Rb and Rg groups, which have 20 (S) protopanaxadiol and 20 (S) protopanaxatriol, respectively, as the sapogenines. Rb group includes the ginsenosides Rb1, Rb2, Rc and Rd, while Rg group includes the Re, Rf and Rg1 ones as the main compounds. Among all these ginsenosides, Rb1 and Rg1 are the most effective compounds (Tanaka and Kasai, 1984).

Ginsenosides accumulate in the root of the plant, but the agricultural production of roots is expensive. Therefore, the production of ginsenosides, by means of different biotechnological alternatives has been extensively studied by a number of researchers, using callus tissues (Furuya et al., 1983a; Odnevall et al., 1989; Asaka et al., 1993; Mathur et al., 1994), cell suspensions (Furuya et al., 1983b; Mathur et al., 1994) and root cultures (Yoshikawa and Furuya, 1987); nevertheless, the productivity obtained so far has been low because of the low growth rates of cultures. The induction and establishment of hairy roots after the infection of *Panax ginseng* rhizomes with *Agrobacterium rhizogenes* has been successfully performed (Yoshikawa and Furuya, 1987; Ko et al., 1989; Inomata et al., 1993; Bulgakov et al., 1998; Washida et al., 1998). These roots grow more rapidly and produce higher levels of saponins than the ordinary cultured roots obtained by hormonal control.

Hairy root formation is a consequence of the integration of the T-DNA of Ri-plasmid of *Agrobacterium*

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rhizogenes A4 into the plant genome. In the case of agropine type strains (such as *A. rhizogenes* A4), two T-DNA fragments (TL-DNA and TR-DNA) are separately transferred into the plant material (Huffmann et al., 1984; Jouanin, 1984). The integration of the TL-DNA into the plant genome is essential for developing transformed roots; three genes of this fragment, known as *rol*A, *rol*B and *rol*C are responsible for the full hairy root syndrome (Carderelli et al., 1987; Palazón et al., 1997, 1998a). Although the TR-DNA is not essential for hairy root formation, it has been shown that the *aux1* genes harboured in this T-DNA segment provide to the transformed cells with an additional source of auxin. Recently, we have found that *aux* genes play a significant role in the morphology and alkaloid production of transformed roots of *Datura metel* and *Duboisia hybrid* (Moyano et al., 1999).

In this work, we report the establishment of three different phenotypes of *Panax ginseng* C.V. Meyer transformed roots in which the role of the TL-DNA and TR-DNA fragments of the pRiA4 on the root morphology is considered. At the same time, the relationship between the different root phenotypes and their capacity to produce biomass and ginsenosides is also studied.

2. Results

2.1. Study of growth and ginsenoside productivity

Root discs developed both callus tissues and roots 4–8 weeks after infection with *A. rhizogenes* A4. At the same time, numerous roots began to emerge from the surface of these callus pieces. Sixty five per cent of the inoculated root discs developed roots either directly from them or through callus formation.

These hairy root lines showed 3 different morphologycal phenotypes: 50% presented the characteristic traits of hairy roots described by Carderelli et al. (1987) with primary roots with extensive lateral branching and a profusion of root hairs (HR-M); 35% showed callus-like appearance with very thick primary roots and several secondary roots (C-M), and 15% were rather long and thin without branching points, tending to become brown except for newly developing yellowish parts (T-M) (Fig. 1). These three root phenotypes were maintained stable over successive subcultures for 2 years.

Hairy root lines started to grow without any lag phase (data not shown) and growth pattern varied according to observed phenotypes. When considering the growth rate

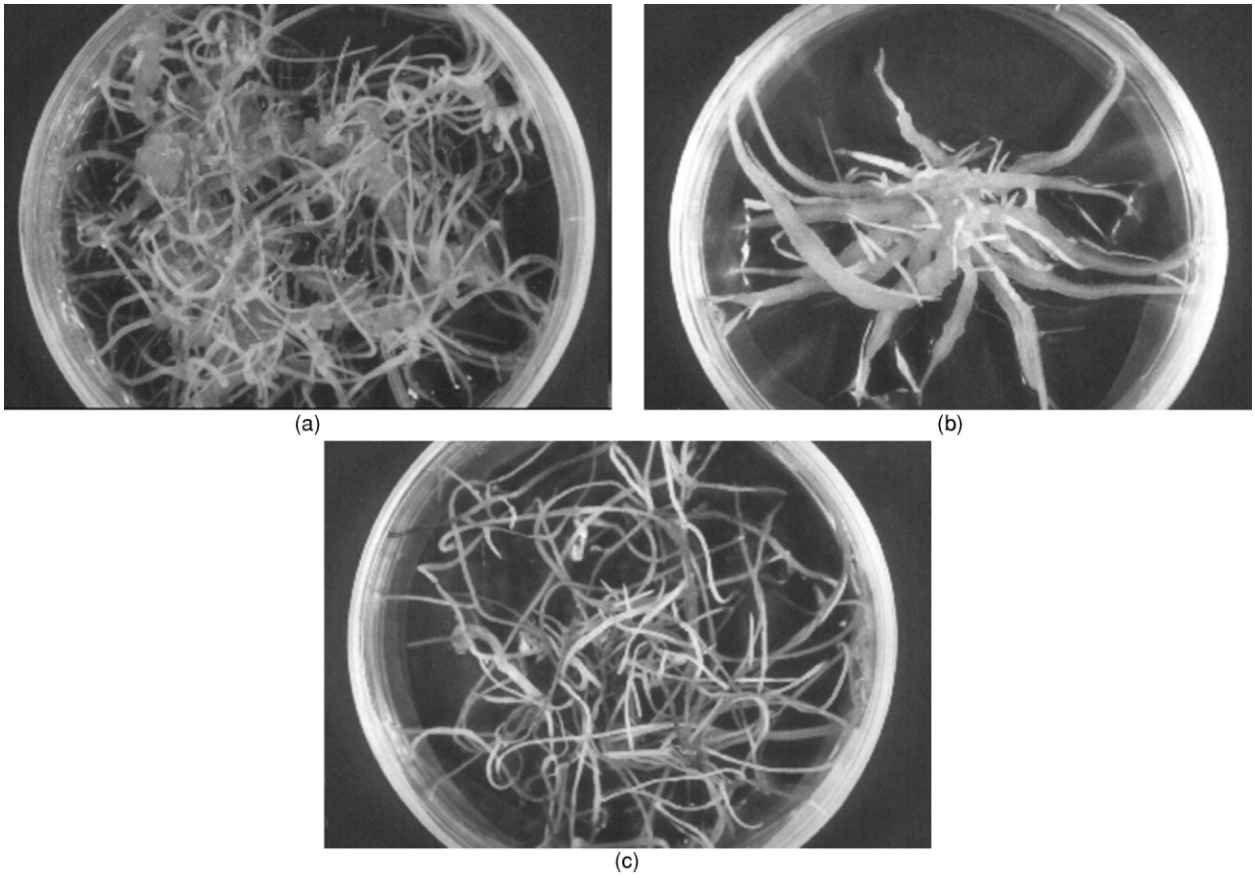

Fig. 1. Four week old *Panax* ginseng hairy root cultures on SH solid medium, showing three different phenotypes. (a) Roots with the characteristic traits of hairy roots (HR-M). (b) Roots with callus-like morphology (C-M). (c) Roots with thin morphology (T-M).

average of the transformed root lines studied (Fig. 2), it was possible to observe that the C-M and HR-M root lines achieved the highest values; the growth rate (final fresh weight/fresh inoculum weight) obtained after 4 weeks of culture, by C-M, HR-M and T-M roots was 7.9, 7.1 and 3.2, respectilvely, while in control roots it was 1.5. From these results we can infer that the genetic changes caused by the *A. rhizogenes* transformation conditioned a higher capacity for the biomass formation affecting branching, as shown in cucumber (Amselem and Tepfer, 1992), *Catharanthus roseus* (Palazón et al., 1998b) and in *Duboisia*, *Datura* and tobacco transformed root cultures (Moyano et al., 1999). At the same time, as described by Rhodes et al. (1990), the growth rate of transformed roots depends, to a large extent, on linear extension, on the formation of a large number of new growing points as lateral branches and on a secondary increase in root diameter as the root cells undergo cell expansion and differentiation.

Four ginsenosides (Rb1, Rb2, Rc, Rd) of Rb group and three (Re, Rf and Rg1) of Rg group were extracted and quantified from the root lines studied. HR-M root lines achieved the highest total ginsenoside level (5.4 mg/gDW) followed by C-M (4.8 mg/gDW) and control roots (4.5 mg/gDW). Roots with thin morphology produced less than half of the ginsenoside production achieved by the HR phenotype (Table 1 and Fig. 2).

In the three root phenotypes considered, as well as in the untransformed roots, the RbT/RgT ratio was lower than 1, indicating that the production of Rg group ginsenosides was always higher than that of the Rb group (Table 1). Similar results were obtained by Bulgakov et al. (1998) with *P. ginseng* transgenic roots for the *rol*C gene of *A. rhizogenes*. As indicated by Ko et al. (1989) and Inomata et al. (1993), the maximum production of the different ginsenosides occurs at different growth stages. Maximum levels of Rb1 are usually found in the early growth stages, while maximum amounts of Rg1 appear later; these facts could explain the ginsenoside patterns of transformed root cultures after 4 weeks of growth.

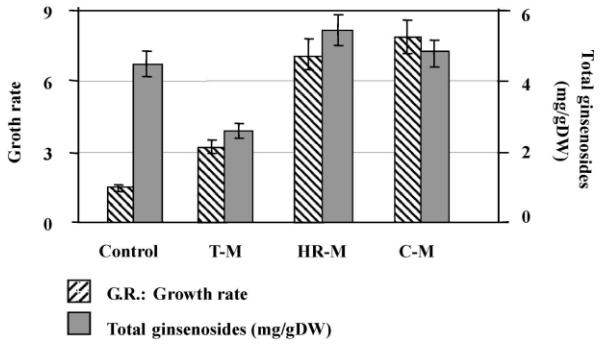

Fig. 2. Growth rate (final fresh weight/inoculum fresh weight) and ginsenoside contents (mg/g DW) of the different root phenotypes. Roots were cultured for 4 weeks in hormone-free SH liquid medium. In all cases the inoculum fresh weight was 0.5±0.05 g. Each value was the mean of five replicates of each one of the 15 root lines considered, belonging to each respective phenotype studied.

Table 1
Ginsenoside contents (expressed as percentage of DW) of the different hairy root phenotypes studied. Roots were cultured for 4 weeks in hormone-free SH liquid medium. In all cases the inoculum fresh weight was 0.5±0.05 g. Each value is the mean of five replicates of each one of the 15 root lines considered, belonging to each respective phenotype studied. Total ginsenoside contents is expressed as mg ginsenosides/gDW

Ginsenosides	Control	T-M	HR-M	C-M
Rb1	11.3	8.0	14.8	21.2
Rb2	2.8	5.3	4.8	3.5
Rc	0.5	4.1	4.5	3.7
Rd	0.1	2.6	7.6	3.6
Re	68.3	57.7	40.1	38.1
Rf	2.0	5.6	2.1	2.0
Rg1	15.0	16.8	28.5	27.9
RbT	14.7	20.0	29.1	31.9
RgT	85.3	80.0	70.8	68.1
Total (mg/gDW)	4.55	2.58	5.44	4.84
RbT/RgT	0.17	0.25	0.41	0.47

The ginsenoside pattern also varied depending on the root phenotypes. The main ginsenoside found in T-M, HR-M and C-M root lines was the Re ginsenoside followed by the Rg1 one (Table 1). Whereas in T-M roots the Re and Rg1 represented 57.7 and 16.8%, respectively, of the total ginsenoside contents, in HR-M and C-M root lines, the Re constituted 40.1 and 38.1%, and the ginsenoside Rg1, represented 28.5 and 27.9%, respectively of the total. As it is known (Gubar et al., 1997), the Re ginsenoside is formed after the addition of two rhamnose residues to the Rg1. These results suggest a higher activity of enzymes involved in the incorporation of rhamnose to the Rg1 compound in the T-M root phenotype than on the other phenotypes studied. The main ginsenoside of the Rb group in T-M, HR-M and C-M root lines was the Rb1, which represented 8, 14.8 and 21.2% of the total ginsenosides determined. Because the Rd ginsenoside represented at most 2.6% of the total, and from it Rb1, Rb2 and Rc are formed by the action of different enzymes involved in the addition of sugar residues, our results suggest that the activity of these enzymes was high and dependent on the root phenotypes studied. But in order to clarify the ginsenoside metabolism, further assays are requested.

The control roots presented a different ginsenoside pattern from that achieved by HR-M and C-M roots, but more similar to that of T-M roots. The Re, Rg1 and Rb1 constituted 68, 15 and 11.3% of the total ginsenoside contents.

When considering the ginsenoside yield of the *P. ginseng* transformed root cultures after 4 weeks of growth (Fig. 3), we can observe that the highest yield was achieved by HR-M root lines (72.9 mg/l), followed by

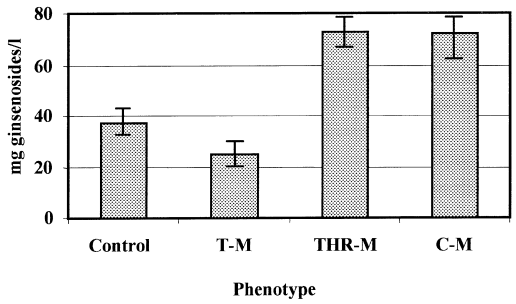

Fig. 3. Ginsenoside yield, expressed as mg total ginsenosides/l, in the different *Panax ginseng* hairy root phenotypes studied. Roots were cultured for 4 weeks in hormone-free SH liquid medium. In all cases the inoculum fresh weight was 0.5±0.05 g. Each value is the mean of five replicates of each one of the 15 root lines considered, belonging to each respective phenotype studied.

the C-M ones (71.6 mg/l). The lowest ginsenoside yield of the transformed root phenotypes studied corresponded to the T-M roots (24.8 mg/l), which also presented low ginsenoside contents and a limited growth. The ginsenoside production of untransformed roots (36.8 mg/l), was always lower than that obtained by HR-M and C-M root lines studied.

As it is known, the ginsenoside yield of the cultures not only depends on the accumulation of these compounds but also on the biomass formation. In our case, although the ginsenoside contents of HR and C root phenotypes were only 1.2 and 1.1 times higher than that achieved by control, in both cases their productivity was twice as high as that of the controls. These results show that in our *P. ginseng* transformed root cultures, the capacity to produce and accumulate ginsenosides is associated with biomass formation because the highest productivity was achieved by the root phenotypes with the highest growth rate.

From our results we can infer that the transformed nature of *P. ginseng* roots conditioned an important increase of biomass and productivity, especially in roots displaying the full hairy root morphology, corroborating the suitability of transformed root cultures for an improved secondary metabolite production.

2.2. Study of the fragments of Agrobacterium T-DNA integrated into the transformed root genome

The presence of the *rolC* gene of the TL-DNA of the pRiA4 as well as that of the *aux1*, *ags* and *mas1* genes of the TR-DNA has been studied in the three different transformed root phenotypes established. The transformed nature of the all selected root lines of each morphology was confirmed by the presence of the *rolC* gene in the plant genome. The products of *aux* genes are involved in the additional route for indole acetic acid in transformed cells (Nilsson and Olsson, 1997), whereas *ags* and *mas1* genes code the enzymes responsible for agropine and manopine biosyntheses, respectively. 100% of the root lines displaying callus-like (C-M) or hairy root morphology (HR-M) but only 25–45% of root lines showing a thin structure (T-M) presented this gene in their genome. Previously, we reported that in *D. metel*, *Duboisia* and tobacco transformed roots, the presence of *aux1* gene in the plant genome conditioned the callus-like morphology (Moyano et al., 1999). We could observe that 100% of roots displaying this structure included this gene in their genome, whereas only 25–60% of roots with typical hairy root morphology presented this gene in their cells. From our results obtained in *P. ginseng* transformed roots, we can infer that the *aux1* gene is related to root morphology, especially to lateral branching development, since thin roots did not form secondary root, whereas C-M and HR-M roots presented a very high degree of secondary root formation. In our case, the additional source of auxin induced by *aux1* gene in the root genomes, was not the only factor responsible for the disorganization of root tissues (Jung et al., 1995), since roots displaying the full hairy root morphology also harboured the *aux1* gene in their genome.

The presence of the opines agropine and manopine and the genes which codify for enzymes involved in their formation, *ags* and *mas1* genes in the root genomes was also studied (Table 2). The results obtained show that 25% of thin root lines, 75% of hairy roots and 100% of callus-like ones included the *ags* gene in their genome whereas *mas1* gene was present in 100% of HR and C roots but only in 50% of thin root lines. When considering the presence of both opines, agropine and manopine, in the selected root lines it can be seen that the root lines with thin morphology did not include these compounds in their cells, but that they were present in all root lines with callus-like structure. On the other hand in the HR-M root lines studied, it was possible to observe that roots which lacked the *ags* gene did not include agropine in their composition and manopine was hardly detected. When studying the relationship between the presence of opines in the transformed root phenotypes established with their ginsenoside production it is possible to

Table 2
PCR study of the presence of different fragments of *Agrobacterium rhizogenes* T-DNA inserted in the genome of the transformed root phenotypes. Percentage of transformed root lines of each phenotype studied in which the presence of opines was detected

Root morphology	Genes				Opines	
	rolC	*aux1*	*ags*	*mas1*	Agropine	Manopine
HR-M	100%	100%	75%	100%	75%	100%
C-M	100%	100%	100%	100%	100%	100%
T-M	100%	25–45%	25%	50%	0%	50%

observe that the less productive root lines (T-M ones) also lacked the *ags* and *mas1* genes and consequently the capacity to produce opines. Nevertheless, further studies are needed in order to know the effect of the presence of the genes responsible for the opine biosynthesis in ginsenoside production.

Taking all these results together, we can observe that the transformed root phenotypes, which presented the highest growth rate (HR-M and C-M phenotypes) were also those with the highest ginsenoside production. At the same time, together with the *rol* genes of the TL-DNA segment of *A. rhizogenes*, 100% of root lines belonging to these two phenotypes harboured the *aux1* gene of the TR-DNA fragment. On the contrary, the phenotype with very slow growth and ginsenoside production (T-M) never included this gene in its genome, although the *rol* genes were always present. From these results we can infer that *aux1* gene, although not essential for the root formation, plays an important role in the root morphology by increasing lateral branching resulting in higher biomass and ginsenoside production. Our results also show that in *P. ginseng*, the capacity of transformed roots to produce opines does not interfere with the ginsenoside production. To our knowledge, this is the first report that a relationship between growth and ginsenoside production, and the presence of different genes of the TL-DNA and TR-DNA of the Ri plasmid of *A. rhizogenes* has been shown in *P. ginseng* hairy root cultures.

3. Materials and methods

3.1. Induction and culture of hairy roots

Transformed roots of *Panax ginseng* C.A. Meyer were induced from 4-year-old rhizomes after infection with *Agrobacterium rhizogenes* A4 strain. Sterilized root discs were wounded with a sterile needle loaded with *A.rhizogenes* suspension grown in liquid YEB medium (Vervliet et al., 1975) for 24 h at 28±2°C on a rotary shaker at 100 rpm. The inoculated root discs were placed on Schenk-Hildebrandt's medium (SH) (Schenk and Hildebrandt, 1972) containing 3% (w/v) sucrose, 0.1% (w/v) myo-inositol and 0.27% (w/v) Phytagel (Sigma) at 26°C. The medium was adjusted to pH 7.0 before autoclaving. After 2 days of cocultivation the explants were transferred to the fresh medium containing cefotaxime (500 mg/l) in order to eliminate the bacteria. After cultivation for one or two months, roots started to appear at the infection sites and, in order to obtain the root lines, single roots were picked off and placed onto new media together with cefotaxime, until the bacteria were eliminated. Hairy roots with no bacterial contamination were cultured on hormone-free SH solid medium, already described, in the dark at 26°C. After 6 months of subculturing the roots every two weeks on fresh solid medium, the root lines were transferred to SH liquid medium and kept in a rotary shaker at 100 rpm, 26°C in the dark. Subcultures were performed every two weeks in fresh medium. Untransformed roots (control roots) were obtained from sterile rhizome discs cultured in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.01% (w/v) myo-inositol, 2 mg/l indole butyric acid, 0.1 mg/l kinetin and 0.27% (w/v) Phytagel (Sigma) at 26°C in darkness. Roots 2–3 cm long were transferred separately to the same medium used for hairy roots and subcultured every 2–3 weeks.

In order to study the capacity of hairy roots to produce biomass and ginsenosides, from 80 hairy root lines established, 15 relatively quickly growing root lines showing each phenotype considered were selected and cultured for 4 weeks in the above mentioned medium and conditions. Biomass formation was expressed as growth rate (final fresh weight/inoculum fresh weight); the inocula fresh weight (0.5±0.05 g) were cultured in 100 ml Erlenmeyer flasks containing 10 ml of SH liquid medium.

3.2. PCR analysis

Total DNA was isolated from hairy roots by using "genomic Prep ™ Cells and Tissue DNA isolation Kit" (Pharmacia Biotech). PCR analyses were performed using the pre-formulated, predispensed single dose reaction beads "Ready to Go™" (Pharmacia Biotech). The complete mixture contained 200 ng of total DNA, 12.5 pmol/μl of each oligonucleotide primer, 200 μM dNTPs, 1.5 units Taq polymerase, and buffer supplied by the enzyme manufacturer (1/10 V) in a total volume of 25 μl. The oligonucleotide primers used that would anneal to *rol*C gene of the TL-DNA of pRi of *A. rhizogenes* A4, produced a DNA fragment of 534 bp.

3.3. Opine analyses

Opine analyses were performed by High Voltage Paper Electrophoresis according to a modification of the method indicated by Petit et al. (1983), as described in Moyano et al. (1999).

3.4. Extraction and analysis of ginsenosides

Extraction of ginsenosides was based on the method described by Samukawa et al. (1995). Freeze-dried powdered samples (50 mg) were washed in petroleum ether (3×8 ml) and centrifuged at 33,520 *g* for 10 min. The pellet was extracted and sonicated for 15 min with 15 ml of 70% methanol and centrifuged again at 33,520 *g* for 10 min. After evaporating the methanolic extract to dryness and dissolving it in 10 ml of acetonitrile: water (80:20), 20 μl of the ginsenoside extract was loaded onto a Hewlett-Packard Lichrospher® 100 Diol (5 μm) column

(250×4 mm) connected to a Pharmacia LKB HPLC system. The solvent mixture of acetonitrile-water (4.5:1 v/v, pH 2.5 with H_3PO_4) was eluted at a flow rate of 0.8 ml/min and ginsenosides were detected at 202 nm. The peak areas corresponding to ginsenosides from the samples, with the same retention time as authentic ginsenosides (Rf, Rg1, Rd, Re, Rc, Rb2, Rb1), were integrated by comparison with an external standard calibration curve. Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 (99.8%) were purchased from Karl Roth (Germany).

Ginsenoside contents of the analyzed samples were expressed as mg ginsenosides/g DW (dry weight). The total ginsenoside productivity, after 4 weeks of culture, of transformed roots was indicated as mg of total ginsenosides per liter of culture medium as described by Jouhikainen et al. (1999).

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