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THE IMPACT OF PLANT *rolC* ONCOGENE ON GINSENOSIDE PRODUCTION BY GINSENG HAIRY ROOT CULTURES

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Key Word Index—*Panax ginseng*; Araliaceae; transgenic culture; *rol* genes; ginsenosides; hairy roots.

Abstract—Plasmid constructions containing *rolA*, *rolB* and *rolC* genes, isolated earlier from the TL-DNA of *Agrobacterium rhizogenes* were used to transform a cell culture (strain 1c) of *Panax ginseng*. The levels of ginsenosides were measured in the resulting transgenic tissues to evaluate the possible role of *rol* genes in ginsenoside formation. The ginsenoside content of the hairy root culture of *P. ginseng*, transformed by wild type A4 plasmid DNA and containing all *rol* loci, was higher than that of the control 1c culture (5.12–8.92 mg g^{-1} dry wt), being in the range of 13.23–21.27 mg g^{-1} dry wt. Ginseng tissue, transgenic for the *rolA* gene appeared to lose the ability to synthesize ginsenosides since only a trace amount of Re ginsenoside was found in 1c-*rolA* tissue. 1c-*rolB* cultures contained at least five times lower ginsenoside levels compared to the initial 1c culture. The ginsenoside content of *rolC* transgenic roots was about three times higher than that of the respective control. Taking into account the differences in cell differentiation levels in tissues transformed by *rol* genes, we compared the ginsenoside levels in *rolC* roots and tumours. It was found that ginsenoside production in tissues with different levels of differentiation is nearly the same. We have concluded that the plant oncogene *rolC* is responsible for increased ginsenoside formation in ginseng hairy root cultures.

INTRODUCTION

RolA, *rolB* and *rolC* oncogenes isolated from T-DNA of the *Agrobacterium rhizogenes* A4-Ri plasmids are known to be involved in the induction of hairy roots in transformed plants. The expression of individual *rol* genes in transgenic plants not only induces rhizogenesis [1], but also affects the development and physiology of the entire plant [2].

It was shown that hairy root cultures of a number of plants had higher levels of secondary products (e.g. nicotine, anabasine, cytisine, anagyrine, hyoscyamine, scopolamine, ginsenosides, thiarubrin and polyacetylenes) than wild type plants [for review see Refs [3] and [4]]. It has been proposed that increased biosynthesis of secondary metabolites in hairy root cultures is correlated with rhizogenesis caused by T-DNA integration [5–7]. In contrast to this conception, some data reveals that the production of secondary metabolites in hairy roots exceeds that in ordinary cultured roots. Thus, Yoshikawa and Furuya [8] performed ginsenoside determinations on several ginseng lines transformed by A4 *A. rhizogenes* strain and found the amount of ginsenosides was two times higher than that of untransformed cultured roots. Further, *Coreopsis tinctoria* hairy roots synthesized up to three times as much 1′-acetoxy-eugenol-isobutyrate as ordinary cultured roots [9], and transformed culture roots of *Chaenactis douglasii* contained two times higher thiarubrine levels compared to ordinary cultured roots [10]. However, the physiological relevance of the level of cell differentiation with respect to secondary metabolite formation has never been demonstrated, nor have the genes responsible for the increases in biosynthesis been identified.

To assess the possible role of *rol* genes in the phenomenon of secondary metabolite overproduction in hairy root cultures, we investigated the effects of these genes on ginsenoside levels by inoculating cultivated ginseng cells with bacteria harbouring *rol* genes. We chose to use a ginseng cell culture for these studies, since ginseng cultures have been well studied [11–13]. The results of this work

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Table 1. Changes in properties of ginseng cells after transformation

Tissue	Kanamycin resistance*	Hormonal requirements	Opine test	NPT assay	Dot blot hybridization
1c	—	auxins	—	—	—
1c-A4 roots	—	none	+	nd	nd
1c-*rolA*	+	none	nd	nd	+
1c-*rolB*-I	+	none	nd	+	+
1c-*rolC* tumors†	+	none	nd	+	+
1c-*rolC* roots‡	+	none	nd	+	+

* Growth on the medium containing 100 mg l^{-1} kanamycin sulphate.
† Five primary tumour cultures (1c-*rolC*-I... 1c-*rolC*-V).
‡ Five root cultures (1c-*rolC*-I... 1c-*rolC*-V).
nd: not determined.

demonstrate that increased ginsenoside formation caused by *A. rhizogenes*, strain A4, can be explained as a result of *rolC* gene expression.

RESULTS

Transformation of ginseng cells by A. rhizogenes *and* A. tumefaciens *GV* 3101 *and regeneration of transformed roots*

Ginseng cell clusters were transformed by co-cultivation with *A. tumefaciens* GV 3101 strains harbouring *rol* genes, as well as with *A. rhizogenes* strain A4. Of the initial several hundred primary tumours established, one *rolA* gene line, two *rolB* gene lines and five *rolC* gene lines were resistant to high kanamycin concentrations on subculturing and were thus confirmed to be transformed (Table 1). Several hairy root cultures were established by wild-type *Agrobacterium* transformation.

The *rolA* calli grew very slowly (Table 2) as compact yellow globular aggregates on hormone-free medium as well as on W_{4CPA} medium. These calli were incapable of forming roots.

The *rolB* calli grew well as friable white-yellow tissue on hormone-free medium and on W_{4CPA} medium containing 0.1 mg l^{-1} of 4-chlorophenoxyacetic acid. No roots were observed on these calli and our attempts to trigger rhizogenesis in this culture using hormones failed.

The primary *rolC*-tumors grew rapidly on W_{4CPA} medium supplemented with kanamycin on which they displayed a friable, almost watery phenotype. Within the first or subsequent passages, all established primary tumours transformed with pPCV002-CaMVC had formed adventitious roots. Transgenic calli produced roots on the medium containing 4-CPA as well as on hormone-free medium. Although the efficiency of root formation was different in different tumour lines, none of the cultures of primary tumours showed decreased formation of roots from one subculture to the next. However, it was easy to select a non-root-forming tumour line by selection for non-root-forming calli.

Table 2. Biomass accumulation by initial and transgenic cultures growing in W_{4CPA} medium for 28 days

Tissue	Biomass accumulation*	
	Fr. wt (g l^{-1})	Dry wt (g l^{-1})
1c	223 ± 32	9.1 ± 0.9
1c-A4 roots	193 ± 21	11.1 ± 1.6
1c-*rolA*	36 ± 1	1.5 ± 0.1
1c-*rolB*-I	242 ± 24	16.9 ± 1.9
1c-*rolC*-I roots	199 ± 39	12.2 ± 2.7
1c-*rolC*-II roots	224 ± 27	14.4 ± 1.7
1c-*rolC*-III roots	202 ± 34	13.1 ± 2.1
1c-*rolC*-IV roots	186 ± 25	11.0 ± 1.6
1c-*rolC*-V roots	214 ± 26	12.4 ± 1.7

* Mean values ± s.e. based on five separate replicate samples.

Adventitious roots emerging on tumours were excised and transferred into liquid media. In the absence of phytohormones, as well as in the presence of indole-3-acetic acid, root cultures were characterized by slow growth and reduced lateral branching. In the presence of 4-CPA, roots grew vigorously but had a tendency to swell and form callus-like structures over several subcultures. Fast growing *rolC* root cultures with abundant lateral branching were established using indole-3-butyric acid and these cultures were used for further experiments.

Ginseng cell lines transformed with *A. rhizogenes* strain A4 were also established which grew as primary tumours and roots. One root clone showing rapid growth in W_{IBA} medium for several subcultures was selected and used for analysis of ginsenoside production.

Ginsenoside content of hairy roots, rolA, rolB *and* rolC *cultures*

Ginsenosides were extracted from transformed tissues and separated by HPLC. Transformed roots produced a set of ginsenosides which did not differ significantly from those produced by the parent culture (Table 3). However, compared to the control culture, ginsenoside levels were nearly doubled in 1c-A4 roots, in accordance with the observations of Yoshikawa and Furuya [8]. Surprisingly, we found that 1c-*rolA* calli derived from transformed *rolA* gene cells of *P. ginseng* lost the ability to synthesize ginsenosides whereas the ginsenoside content of *rolB* cultures was low, in contrast to 1c cells. Over 1.5 years of analysis, the average amount of ginsenosides in the 1c-*rolB*-I culture was 1.23 ± 0.14 mg g^{-1} dry wt; that is 5.7 times lower

Table 3. Production of ginsenosides by the initial and transformed cultures grown on liquid W_{4CPA} medium for 28 days

Cell line	Ginsenoside content (mg g^{-1} dry wt)*								
	Rg_1	Re	Rg_2	Rb_1	Rc	Rb_2	Rd	Total	Rb/Rg index
1c	2.25 ± 0.3	3.11 ± 0.59	0.53 ± 0.06	0.80 ± 0.17	0.13 ± 0.02	0.13 ± 0.03	0.17 ± 0.02	7.10 ± 0.74	0.21 ± 0.05
1c-A4	1.44 ± 0.25	8.04 ± 3.10	1.23 ± 0.42	3.00 ± 1.02	1.19 ± 0.42	1.33 ± 0.26	1.03 ± 0.12	17.25 ± 4.02	0.61 ± 0.12
1c-*rolA*	—†	tr‡	—	—	—	—	—		
1c-*rolB*-I	0.14 ± 0.06	0.36 ± 0.02	0.13 ± 0.06	0.10 ± 0.01	—	—	0.54 ± 0.05	1.27 ± 0.04	1.02 ± 0.05
1c-*rolC*-I	1.05 ± 0.14	2.68 ± 0.45	0.03 ± 0.005	1.29 ± 0.27	0.12 ± 0.04	0.55 ± 0.12	0.35 ± 0.04	6.07 ± 0.82	0.61 ± 0.10
1c-*rolC*-II	2.85 ± 0.67	8.92 ± 2.37	1.83 ± 0.64	4.78 ± 1.10	0.58 ± 0.11	0.92 ± 0.19	0.75 ± 0.20	20.63 ± 5.19	0.52 ± 0.14
1c-*rolC*-III	3.09 ± 0.55	7.14 ± 2.12	1.65 ± 0.70	4.13 ± 0.67	0.77 ± 0.22	2.06 ± 0.22	0.82 ± 0.10	19.66 ± 4.06	0.65 ± 0.12
1c-*rolC*-IV	2.10 ± 0.42	6.50 ± 1.65	0.66 ± 0.17	2.65 ± 0.21	0.62 ± 0.16	1.70 ± 0.20	0.55 ± 0.09	14.78 ± 2.25	0.55 ± 0.08
1c-*rolC*-V	2.68 ± 0.26	4.80 ± 1.25	0.22 ± 0.06	2.45 ± 0.55	0.59 ± 0.08	1.49 ± 0.26	0.59 ± 0.13	12.82 ± 2.12	0.61 ± 0.06

* Mean values ± s.e. based on at least five separate replicate samples.
† Ginsenoside not found.
‡ Trace amount of ginsenoside.

than in the 1c culture. During this time, the 1c-*rolA* line did not produce ginsenosides.

As indicated in Table 3, all *rolC*-root lines except for 1c-*rolC*-I contained ginsenosides concentrations that exceeded 1.8–3 fold those of the untransformed control culture. To determine whether this pattern of ginsenoside accumulation is stable between subcultures, we monitored ginsenoside levels in all root lines for 6 months. The results showed that differences in glycoside accumulation reported in Table 3 within the cultures remained constant during this time (data not shown). The 1c-*rolC*-II root culture possessing the highest growth rate (Table 2) was chosen for further investigation.

Ginsenoside production by the 1c-*rol*C-II culture over long-term cultivation (two years) was found to vary from one subculture to another. The total ginsenosides levels in this line ranged from 6.76 to 65.83 mg g^{-1} dry wt whereas those of the original culture 1c varied between 5.12 and 8.92 mg g^{-1} dry wt. On average, 1c-*rolC*-II roots contained three times more ginsenosides than control 1c cells. The ginsenoside content of wild-growing and plantation plants were reported previously [14]. It was found that roots of 19 *P. ginseng* plants collected in the 12 regions of the Russian Far East accumulated ginsenosides ranging from 6.49 to 42.36 mg g^{-1} dry wt (an average value was 16.77 ± 2.24 mg g^{-1} dry wt). While maximum amounts of ginsenosides in the 1c-*rolC*-II roots exceeded those reported for natural ginseng roots, the average content of ginsenosides found corresponds to those occurring in natural roots. In the transgenic 1c-*rolC*-II root culture, the accumulation of all ginsenosides correlated with the increase in biomass during the log phase of growth and maximum values were attained during the stationary phase of growth (data not shown).

Rol genes change the Rb group/Rg group ratio

The Rb/Rg index, which is often used to clarify peculiarities in ginsenoside biosynthesis, indicates the ratio between protopanaxadiol glycosides (Rg_1, Re, Rg_2, Rf) and protopanaxatriol glycosides (Rb_1, Rc, Rb_2, Rd) in ginseng tissue. As a rule, aboveground ginseng parts have an index of less than 1, and underground parts more than 1 [11]. In the 1c callus culture, the Rb/Rg index is always less than 1 (0.16–0.26); this is not surprising since the culture originated from a stem. In contrast, the level of ginsenosides of Rb group increased in the all transgenic tissues (Table 3) which indicates that *rol* genes strongly influence ginsenoside biosynthesis.

Dependence of ginsenoside formation on the levels of cell differentiation

We explored whether increased ginsenoside production in *rolC* cultures is a phenomenon associated with rhizogenesis caused by T-DNA integration. A single root tip was isolated from the 1c-*rolC*-II culture and cultivated in liquid W_{IBA} medium for 3.5 months with 21 day subculture intervals. The resulting tissue, which consisted of young lateral roots (about 1 month old), mature lateral roots (2–2.5 month old), the main root (3–3.5 month old), and secondary tumour tissue originating from the base of the main root (3.5 month old) was analysed for ginsenoside content (Table 4). All tissues contained 14–16 mg g^{-1} dry wt of ginsenosides except for young roots which accumulated slightly decreased ginsenoside levels.

The *rolC*-II tumour was grown separately and a culture of secondary tumours was established. As shown in Fig. 1, the ginsenoside content of secondary tumour and *rolC*-II root cultures varied over several subcultures but on average they contained similar levels of ginsenosides.

DISCUSSION

The results presented in this report provide the first evidence that *rol* genes may have a strong effect on secondary metabolism in plants. To determine the effects of *rol* genes on ginsenoside production, we transformed cell culture of *P. ginseng* using plasmid DNA containing the individual *rol* genes from the TL-DNA of *A. rhizogenes*, A4 strain. The use

Table 4. Ginsenoside content at different stages of development and dedifferentiation of the *rolC*-II roots

Cell line	Ginsenoside content (mg g^{-1} dry wt)*								
	Rg_1	Re	Rg_2	Rf	Rb_1	Rc	Rb_2	Rd	Total
Young lateral roots	1.35 ± 0.20	3.37 ± 0.22	0.51 ± 0.08	1.68 ± 0.20	3.37 ± 0.34	0.37 ± 0.06	0.51 ± 0.08	0.67 ± 0.10	11.8 ± 1.15
Mature lateral roots	1.76 ± 0.12	4.48 ± 0.67	0.96 ± 0.11	0.32 ± 0.04	4.48 ± 0.50	0.64 ± 0.09	0.80 ± 0.12	0.48 ± 0.07	13.92 ± 1.69
Main root	2.18 ± 0.18	6.25 ± 0.55	1.00 ± 0.16	0.20 ± 0.03	4.66 ± 0.25	0.69 ± 0.09	1.09 ± 0.21	0.59 ± 0.16	16.65 ± 2.03
Tumour tissue originated from the main root	1.94 ± 0.35	8.27 ± 1.11	0.64 ± 0.06	0.51 ± 0.08	2.07 ± 0.19	—†	0.35 ± 0.03	0.27 ± 0.09	14.05 ± 1.50

* Mean values $\pm$ s.e. based on three independent determinations.
† Ginsenoside not found.

of callus culture was considered necessary in order to ensure the homogeneity of the material for transformation. While only a few tissues transgenic for *rol* genes were obtained and analyzed, significant differences were found with respect to their growth, morphology and ginsenoside accumulation. Ginsenoside content in the hairy root culture established after transformation of the 1c cell lines by A4 *A. rhizogenes* was 2-fold higher then in the control culture (Table 3). We have shown that there is much variation between *rolA*, *rolB* and *rolC* cultures in terms of their ability to produce ginsenosides. Cultures of *rolC* roots accumulated generally 1.8–3-fold more ginsenosides than control culture. In contrast, minute amounts of ginsenosides were detected in *rolA* and *rolB* tissues (Table 3). Our results suggest that the *rolC* gene alone may play an important role in stimulating the biosynthetic activity of ginseng hairy root cultures.

In addition to the determination of glycoside production by the various *rol* cultures, preliminary experiments were undertaken in an attempt to understand the mechanisms by which transformed plant cells increase secondary metabolite production. To assess whether stimulation of ginsenoside production in *rolC* roots is due to the direct action of *rolC* gene or is a phenomenon associated with rhizogenesis caused by *rolC* gene integration, we investigated the ginsenoside content of roots and tumours of the same origin. As equal amounts of ginsenosides in tumour and root cultures were observed (Table 4, Fig. 1), it is difficult to accept the suggestion that increased ginsenoside production in hairy roots could be attributed to root formation. This result is in agreement with the observation that pRiA4-derived calli of *P. ginseng* produce larger amounts of ginsenosides than non-transformed calli obtained from the same plant [15].

EXPERIMENTAL

Agrobacterium and plasmids

Plasmid DNA samples (pPCV002-A, pPCV002-CaMVBT and pPCV002-CaMVC) were kindly provided by Angelo Spena (Max-Planck-Institute für Züchtungsforschung, Germany). These plasmids contain the plant cassette vector pPCV002 [16] containing the gene of interest: *rolC* and *rolB* under cauliflower mosaic virus (CaMV) 35S promoter control and *rolA* under the control of its own 5′ promoter [1]. Constructions also carry a gene for

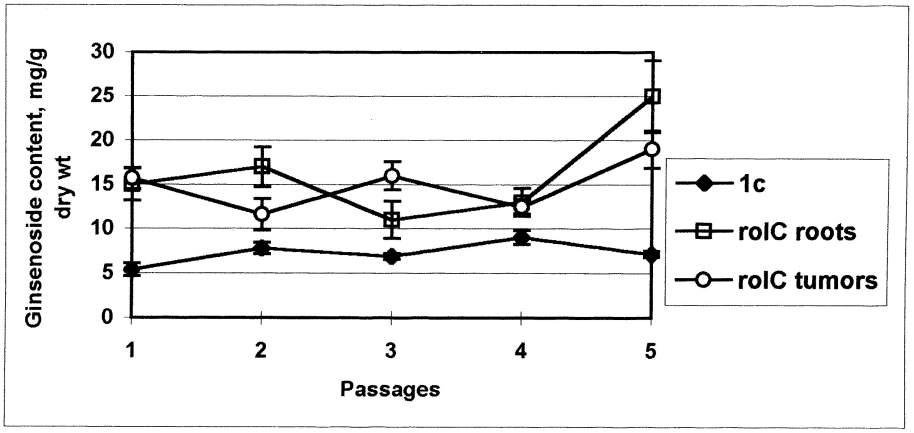

Fig. 1. Ginsenoside production in *Panax ginseng* 1c culture, 1c-*rolC*-II root culture and 1c-*rolC*-II secondary tumour culture grown in W_{4CPA} liquid medium for 4 weeks at 25°. Values are means $\pm$ s.e. for three independent determinations.

kanamycin resistance (NPT-II) under eukaryotic control sequences. Standard techniques were used for the construction of transformation systems, isolation and analysis of DNA [17]. *Esherchia coli* TG2 strain was transformed by pPCV002 vectors. Further constructions were transferred from *E. coli* strains TG2 to *A. tumefaciens* GV 3101 [16] as described [16]. The presence of *rol* genes in *Agrobacterium* strains was confirmed by restriction analysis and DNA hybridization.

Culture media and reagents

E. coli TG2/pPCV002 strains were grown at 37° in LB medium with the addition of tetracycline (15 mg l^{-1}) and ampicillin (50 mg l^{-1}), GV 3101-derived strains were grown in the LB medium containing 50 mg l^{-1} kanamycin sulphate and 100 mg l^{-1} carbenicillin at 28°. A4 *A. rhizogenes* strain was grown under the same conditions without antibiotics.

Murashige and Skoog [18] medium was modified by decreasing the NH_4NO_3 to 400 mg l^{-1}. This medium was supplemented with the following components (mg l^{-1}): thiamine HCl (0.2), nicotinic acid (0.5), pyridoxine HCl (0.5), meso-inositol (100), peptone (100), sucrose (25000) and agar (6000) (denoted as W_0 medium). W_0 medium supplemented with 0.4 mg l^{-1} 4-chloro-phenoxyacetic acid (4-CPA) was designated as W_{4CPA} medium and W_0 medium supplemented with 1.0 mg l^{-1} indole-3-butyric acid was designated as W_{IBA} medium.

Reagents were purchased from Sigma Chemical Co (MO, USA) and Serva Feinbiochemica GmbH & Co (Heidelberg, Germany).

Cell culture 1*c*

The callus culture 1c was established in 1988 from the stem of a two-month old plant of *Panax ginseng* var. Mimaki C.A. Meyer [11]. Culture 1c was deposited at the Russian Collection of Plant Cell Cultures (Moscow) as a source of ginsenosides [12]. Cultivation conditions, growth and hormonal requirements were as described previously [12–13]. The culture possessed cytokinin autonomy, and during the period of observation (more than 7 years), did not show any rhizogenic effects.

Establishment of transgenic cell lines

Calli of 1c culture (0.5 g) were transferred to the liquid W_{4CPA} medium (10 ml) in Petri dishes and cultured at 24° in the dark on a rotary shaker. A suspension of *A. tumefaciens* GV3101 cells diluted 1:10 with the W_{4CPA} medium was added to an 8-day-old ginseng cell culture. After 2 days, cefotaxime was added to a final concentration of 500 mg l^{-1}. After a 5 day interval, the cells were transferred to a fresh W_{4CPA} medium supplemented with 250 mg l^{-1} cefotaxim and 100 mg l^{-1} kanamycin sulfate. After 4–5 weeks of cultivation in the same liquid medium, small white aggregates were observed. These 1.5–2 mm aggregates were transferred to W_{4CPA} agarized medium with kanamycin to produce lines of primary kanamycin-resistant tumours designated as 1c-*rolA* (for the pPCV002-A construction), 1c-*rolB*-I, 1c-*rolB*-II (for pPCV002-CaMVBT construction) and 1c-*rolC*-I, 1c-*rolC*-II,..., 1c-*rolC*-V (for pPCV002-CaMVC construction). The 1c strain as well as the primary tumours were cultivated with 30 day subculture intervals in the dark at 24–25° in 100 ml Erlenmeyer flasks.

Primary 1c-*rolC* tumors were observed to spontaneously form adventitious roots. A number of transgenic ginseng root cultures were established by placing root tips, isolated from adventitious roots, into liquid W_{IBA} medium. These cultures, designated as 1c-*rolC*-I roots, 1c-*rolC*-II roots, etc., were further subcultured at 28 day intervals. Root cultures were cultivated in the dark at 25° in 500 ml Erlenmeyer flasks in an orbital shaker (100 r.p.m.; amplitude 20 mm).

Transformation of 1c calli by *A. rhizogenes* A4 was carried out using the standard feeder layer technique [19]. Cell suspension culture 1c, containing 2–3 g of cells in 30 ml W_{4CPA} medium, was used as a feeder culture. Cells and bacteria were co-cultivated in W_{4CPA} liquid medium for 7 days at 18°. Further aggregates were cultivated in W_0 medium with the addition of 250 mg l^{-1} cefotaxim. Growth of primary tumours was observed after 5–6 weeks and root formation after 14 weeks from the beginning of the experiment. Establishment of the root culture (designated as 1c-A4) was made as described above.

Growth studies

Growth measurements were made on cultures which had been cultivated without kanamycin at least 6 months.

DNA analysis

DNA was isolated from callus and root tissues according to the method of Rogers and Bendich [20]. Ten micrograms of DNA digested with *Eco*RI/*Hin*dIII was separated by agarose gel electrophoresis. DNA was blotted to a Zeta-Probe (Bio-Rad) membrane and hybridized with probe DNA (*Eco*RI/*Hin*dIII 1488 bp fragment of pPCV002-CaMVC) which had been labeled with ^{33}P-dATP using the Prime-a-Gene Labeling System (Promega) according to Southern [17]. *Eco*RI 2225 bp fragment of pPCV002-CaMVBT was used as probe DNA for 1c-*rolB* culture. *Eco*RI 4480 bp fragment of pPCV002-ABC [1] was used as a probe for 1c-*rolA* culture.

Neomycin phosphotransferase II (NPT) assay

Enzyme activity was assayed following the protocol developed by Reiss *et al.* [21]. The assay was performed on crude extracts from tissues (200 mg fr wt) frozen in liquid N_2 and crushed in an Eppendorf centrifuge tube with 40 μl 20 mM Tris-HCl, 40 mM Na EDTA, 100 mM NH_4Cl, 150 mM NaCl, 15 mM dithiotreitol, and 2 mM phenylmethyl sulphonil fluoride, pH 7. The homogenate was cleared by centrifugation. Samples were fractionated on 10% polyacrylamide gel in non denaturating conditions. The position of enzymatically active NPT II proteins in the gel was determined by *in situ* phosphorylation of kanamycin using [γ^{32}-P] ATP as the substrate.

Opine detection in 1c-A4 roots

Agropine and mannopine synthesized in the hairy roots were extracted and analyzed by high voltage paper electrophoresis as described by Yoshikawa and Furuya [8].

Determination of ginsenosides

Isolation and determination of ginsenosides were carried out according to the procedure described [22, 23]. The lyophilized tissues were extracted with H_2O–MeOH. The extract was evaporated to dryness and sequentially extracted with pentane and with butanol with H_2O. The butanol residue was dissolved in MeOH (10 mg ml^{-1}) and 6 μl examined by HPLC [column: ODS 5 m, 64 × 2 mm; eluant: MeCN–H_2O (gradient from 1:4 to 3:2 at a flow rate of 100 μl min^{-1}; detection: 204 nm].

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