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RolB gene-induced production of isoflavonoids in transformed *Maackia amurensis* cells

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Abstract *Maackia amurensis* Rupr. et Maxim is a valuable leguminous tree grown in the Russian Far East, in China, and in Korea. Polyphenols from the heartwood of this species (primarily stilbenes and isoflavonoids) possess strong hepatoprotective activity. Callus culture of *M. amurensis* produced isoflavonoids and their derivatives. In pharmacological experiments, the callus complex was at least as effective, as the plant complex. To increase the yield of isoflavonoids, calli were transformed with the *rolB* gene of *Agrobacterium rhizogenes*. Neomycin phosphotransferase (*nptII*) gene was used for transgenic cell selection. Three *rolB* transgenic callus lines with different levels of the *rolB* gene expression were established. Insertion of the *rolB* gene caused alterations in callus structure, growth, and isoflavonoid production, and stronger alterations were observed with higher expression levels. MB1, MB2, and MB4 cultures accumulated 1.4, 1.5, and 2.1 % of dry weight (DW) isoflavonoids, respectively. In contrast, the empty vector-transformed MV culture accumulated 1.22 % DW. Isoflavonoid productivity of the obtained MB1, MB2, and MB4 cultures was equal to 117, 112, and 199 mg/L of medium, respectively, comparing to 106 mg/L for the MV culture. High level of expression of the *rolB* gene in MB4 culture led to a 2-fold increase in the isoflavonoid content and productivity and reliably increased dry biomass accumulation. Lower expression levels of the *rolB* gene in MB1 and MB2 calli did not significantly enhance biomass accumulation and isoflavonoid content, although the *rolB* gene activated isoflavonoid biosynthesis during the early growth stages and caused the increased content of several distinct compounds.

Keywords *Maackia amurensis* · *rolB* · Isoflavonoids · Callus culture · *Agrobacterium rhizogenes* · Secondary metabolism

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Introduction

Maackia amurensis Rupr. et Maxim is well known due to its hepatoprotective and choleretic properties, which are caused by the polyphenolic complexes accumulating in the heartwood (Vengerovsky et al. 1993; Vlasova and Vengerovsky 1994). It is widely used in folk medicine to cure liver diseases. The *M. amurensis* complex contains isoflavonoids and stilbenes. The drug Maxar was developed in 2004 based on a polyphenolic complex from *M. amurensis*. This drug was highly effective in the treatment of various liver diseases and was registered in the Russian Federation as a hepatoprotective drug (Fedoreyev et al. 2004). Maxar consists of the following compounds: the isoflavones genistein, daidzein, retuzin, afromozin, formononetin, orobol, tectorigenine, and 3-hidroxivestiton; the pterocarpans maackiain and medicarpin; large amounts of the stilbenes piceatannol and resveratrol; the isoflavonostilbene maackiasin; the dimeric stilbenes scirpusin A, scirpusin B, and maackin; and the stilbenolignan maackolin (Fedoreyev et al. 2004, 2008). *M. amurensis* is also the only aboriginal woody species of the *Fabaceae* family in

the Russian Far East and a relict from the Tertiary flora (Maksimov et al. 1992). Together, these facts make *M. amurensis* a valuable biological object.

Callus culture of the *M. amurensis* was obtained previously as a possible alternative source of secondary metabolites. The calli produced a polyphenolic complex consisting of isoflavonoids and their derivatives but no stilbenes (Fedoreyev et al. 2008). The therapeutic effect of the complex from the calli was more evident than that of Maxar, which could possibly be explained by the different compositions of the polyphenolic complexes produced by the plant and the calli (Saratikov et al. 2005). *M. amurensis* culture produced a complex of numerous isoflavonoids (20 identified) with a total content of up to 1.34 % of the cell dry weight (DW) (Fedoreyev et al. 2008), whereas callus cultures often produce a low quantity of secondary metabolites: *Vitis amurensis* produced resveratrol and a trace amount of two phenolic substances (Kiselev et al. 2007); *Rubia cordifolia* produced the antraquinones purpurin and munjistin (Mischenko et al. 1999); *Pueraria tuberosa* produced four isoflavonoids, namely, puerarin, genistin, genistein, and daidzein (Vaishnav et al. 2006); *Echium italicum* produced shikonin acetate (Zare et al. 2011); *Hypericum perforatum* produced hypericin, pseudohypericin, and an unknown compound classified as an isoflavonoid (Gadzovska et al. 2005). Despite this, attempts were made to increase the isoflavonoid content in the established culture.

Agrobacterium rhizogenes is a phytopathogenic soil bacterium known for the ability to insert part of its root-inducing (Ri) plasmid, the transferred DNA (T-DNA), into plant cell genomes. T-DNA carries the *rol* genes (*rolA*, *rolB*, *rolC*, and *rolD*), which together or separately cause the hairy root syndrome in host plants at sites of infection (White et al. 1985). Transgenic hairy roots were shown to stably produce secondary metabolites at levels comparable to or greater than those of the intact plants (Swain et al. 2012; Oksman-Caldentey and Hiltunen 1996; Giri and Narasu 2000; Grzegorczyk et al. 2006; Tiwari et al. 2008; Sheela 2012; Jiao et al. 2014; Bansal et al. 2014; Gai et al. 2015).

The method of transformation using a single *Agrobacterium rhizogenes rol* gene was recently shown to be effective for the induction of secondary metabolism in plant cell cultures (Bulgakov 2008). Previously, we established *rolC* transgenic calli of *M. amurensis* and showed that the *rolC* gene slightly increased isoflavonoid production in the calli, primarily via induction of cell growth (Grishchenko et al. 2013). The gene *rolB* from *A. rhizogenes* was shown to alter the morphology, development, and pathogen tolerance of plants and tissue cultures of different species (Schmülling et al. 1988; Spena et al. 1987; Cardarelli et al. 1987; Capone et al. 1989; Costantino et al. 1994; Altamura et al. 1994; Arshad et al. 2014). The gene was reported to strongly promote the formation of meristems of all types of organs (flowers, roots, and vegetative shoots), thus acting in general as a meristem-inducing gene (Altamura 2004). Some authors described the use of the *rolB* gene for the transformation of valuable crop plants (e.g., wheat) in order to obtain plants with improved yields (Manan et al. 2012). Casanova et al. (2005) enlightened the prospects for the application of the *rol* genes, and the *rolB* gene in particular, for improving ornamental plants. Those authors noted an unpredictable effect of the *rolB* gene, including the induction of numerous abnormalities in various plant species (Casanova et al. 2005, and references therein).

The *rolB* gene is also known as the most powerful activator of secondary metabolism in plant cells. In 2007, Kiselev et al. reported about the striking success in the application of the *rolB* gene transformation method for the induction of resveratrol production by *V. amurensis* callus culture. Transformation led to a 100-fold increase in the resveratrol content in comparison to a control culture. However, in addition to the observed biosynthesis activation, high levels of *rolB* gene expression were also reported to strongly inhibit cell growth (Kiselev et al. 2007).

Though mechanisms of the *rol* genes action in plants are not fully understood, the technology of *Agrobacterium*-mediated improvement of secondary metabolite production in cells is widely employed. The aim of the present work was to determine the effect of the *rolB* gene transformation on isoflavonoid production in *M. amurensis* callus culture, which has not been studied previously. Several *rolB* transgenic lines with different levels of gene expression were established. The effect of the *rolB* gene on growth and isoflavonoid biosynthesis was shown to correlate positively with the level of gene expression in the *M. amurensis* cells.

Materials and methods

Plant material and callus cultures

Seeds of *M. amurensis* were collected in the southern part of the Primorsky region of Russia (Chuguevsky District) and identified in the Botany Department of the Institute of Biology and Soil Sciences. The *M. amurensis* callus culture MK was obtained in 2001 from sterile seedlings. The seeds were surface-sterilized and germinated in vitro. Three- to 5-mm explants were cut from the seedlings and placed onto solid $W_{B/A}$ medium (Grishchenko et al. 2013). The medium included 0.5 mg/L 6-benzyladenine (BA) and 2 mg/L α-naphthaleneacetic acid (NAA). The calli were cultivated in 100-mL Erlenmeyer flasks containing 40 mL of the medium in the dark at 25 ± 1 °C, with subculture intervals of 30–40 days.

For transformation, callus was placed in the liquid $W_{B/A}$ medium and was cultivated in 250-mL flasks with 50 mL of

medium, in the dark, at 25 ± 1 °C, for 1 week. After transformation, suspension culture was transferred onto $W_{B/A}$ solid medium and was cultivated in the dark at 25 ± 1 °C. Test tubes with 20-mm diameter were used for the experiments. The inoculum mass was 0.2–0.22 g.

Genetic transformation

M. amurensis callus culture was transferred to suspension, which was then transformed with the GV3101/pMP90RK strain of *Agrobacterium tumefaciens* (Koncz and Shell 1986) (available at http://www.dna-cloning.com). The strain contains plasmid vector pPCV002-CaMVBT, carrying the *rolB* gene under cauliflower mosaic virus (CaMV) promoter (Spena et al. 1987). The plasmid vector also carries the *nptII* gene for kanamycin resistance, under control of the nopaline synthase promoter. The construct was provided by Angelo Spena (Max-Planck-Institut für Züchtungsforschung, Köln, Germany) and was used without changes. Agrobacterium strains were cultivated on a Triptose agar (Ferak, Germany) supplemented with 50 mg/L kanamycin sulfate (Km; Sintez, Russia) and 50 mg/L carbenicillin (Shawnee Mission, KS, USA) at 28 °C. One-day-old bacterial cells were dissolved in 1 mL of the liquid $W_{B/A}$ medium, and 60 and 150 μL was added to a *M. amurensis* suspension culture. Four days later, 250 mg/L of cefotaxim (Cf) was added to suppress the bacterial growth. After 1 month of cultivation *M. amurensis* cells were transferred into a solid $W_{B/A}$ medium that contained 250 mg/L Cf. *RolB*-transgenic cells were selected during 8 months in the presence of 5, 10, and 15 mg/L Km.

DNA and RNA isolation and analysis

After transformation and transgenic cell selection, callus cultures of *M. amurensis* were cultivated for 24 months without antibiotics. DNA from 45-day-old dried callus was isolated using the CTAB-method described earlier (Kiselev et al. 2015). The DNA concentrations were measured using a spectrophotometer UV mini 1240 (Shimadzu, Tokyo, Japan). Primers 5′GGG TTA GGT CTG GCT CCG GT and 5′CGA GGG GAT CCG ATT TGC TT were used to amplify a 623-bp fragment of the *rolB* gene (GenBank accession number K03313) at an annealing temperature of 57 °C with an elongation time of 25 s. Primers 5′GAG GCT ATT CGG CTA TGA CTG и 5′ATC GGG AGC GGC GAT ACC GTA were used to amplify a 700-bp fragment of the *nptII* gene (GenBank accession number AJ414108) at an annealing temperature of 58 °C with an elongation time of 50 s. The amplification reactions were performed in a total volume of 25 μL in a solution containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 0.01 % gelatin, 0.1 mM Triton X-100, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1 unit of *Taq* DNA polymerase (Sileks M, Moscow, Russia). Approximately 100 ng of DNA was used as a template. The analysis was performed in an UNO Thermoblock thermal cycler (Bio-Rad, Hercules, CA).

Total RNA isolation was performed using hexadecyltrimethylammonium bromide (CTAB)-based extraction protocol developed by Bekesiova et al. (1999) with some modifications (Kiselev et al. 2012). A complementary DNA (cDNA) was obtained using a reverse transcription kit (Sileks M); 1.5 μg of a total RNA was used. Reverse transcription polymerase chain reaction (RT-PCR) was performed in a 50-μL solution containing 1× RT buffer, 0.2 mM of each of the dNTP, 0.2 μM of oligo-(dT)15 primer, and 200 U of M-MLV polymerase. The reaction was performed during 1 h at 37 °C. Samples of RT-PCR products (0.5 μL) were then amplified using the PCR method. The actin gene of *M. amurensis* was used as an internal control. Primers 5′CAT GAT TGG GAT GGA ATC TC and 5′TTT TCA TGC TAC TTG GAG C were used to amplify a 185-bp fragment of the *M. amurensis* actin 1 gene (GenBank accession no. GU074380) at a temperature of 55 °C with an elongation time of 25 s.

For TaqMan real-time RT-PCR, cDNAs were amplified in 20-μL reaction mixtures containing 1× TaqMan Buffer B, 2.5 mM $MgCl_2$, 250 μM of each deoxynucleotide, 1 U Taq DNA polymerase, 0.5 μL cDNA sample, and 0.25 μM of each primer and probe (Real-time PCR kit, Syntol, Russia). Quantitative real-time PCR was performed using an established protocol (Giulietti et al. 2001). The amplification conditions consisted of one cycle of 2 min at 95 °C followed by 50 cycles of 10 s at 95 °C and 25 s at 62 °C. The TaqMan PCR essays were performed in an iCycler thermocycler supplied with iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Inc., USA), and data were analyzed with the iQ5 Optical system software v.2.0 according to the manufacturer's instructions (Dubrovina et al. 2013). Expression was normalized (relative quantification by the $2^{-\Delta\Delta CT}$ method). Scaling options: highest (the highest expressing sample accrued the value 1 in the relative mRNA calculation). *M. amurensis* actin 1 gene was used as endogenous control to normalize variance in the quality and the amount of cDNA used in each real-time RT-PCR experiment. A no-template control was included in every assay, and no-cycle threshold (Ct) values were consistently obtained after 50 cycles of PCR. Each TaqMan probe for *M. amurensis* actin 1 gene was labeled with an FAM reporter dye at the 5′-end and a RTQ-1 quencher dye at the 3′-end, and TaqMan probe for *rolB* gene was labeled with an ROX reporter dye at the 5′-end and a BHQ-2 quencher dye at the 3′-end (Syntol, Russia). The data were summarized from five independent experiments. The primers 5′ACA TCA TAG GGG CGG TTT TCA GT, 5′TTT CGC AAG TTC CTT GTT CAT TC, and probe 5′CTA CCT CTC TCC CGT AAA CGT TGG TCA CTT were used for expression analysis of a *rolB* gene.

Analytical high-performance liquid chromatography

Sample preparation and determination of isoflavonoids from *M. amurensis* dry cells was performed using the methods described earlier (Fedoreyev et al. 2008; Grishchenko et al. 2013). Analytical HPLC was carried out using an Agilent Technologies 1100 Series HPLC system equipped with a VWD detector (λ = 280 nm). For the HPLC analysis, dry pounded cells were extracted with 96 % ethanol for 2 h in a 55–60 °C water bath. Dihydroquercetin, used as an internal standard, was added to the samples before assay. The sample solutions were membrane filtered (0.45 μm, Agilent) and 5-μL aliquots were used for analysis. Extracts were separated using a Hypersil BDS-C-18 column (5 μm, 250 × 5 mm) thermostated at 30 °C. The mobile phase consisted of 1 % aqueous acetic acid (A) and acetonitrile containing 1 % of acetic acid (B). The following seven gradient steps were programmed: 0–5 min, 5–10 % B; 5–10 min, 10–20 % B; 10–30 min, 20–30 % B; 30–35 min, 30–40 % B; 40–45 min, 50–90 % B; 45–50 min, 90–50 % B; and 50–60 min, 5 % B. The flow rate was 1 mL/min. The individual compounds extracted and identified previously were used for identification and quantification of the isoflavonoids in the established cell cultures (Fedoreyev et al. 2008). The retention time and correlation coefficient relative to the internal standard for each compound were refined before every experiment and were found to be equal to those described in Fedoreyev et al. (2008). The data were analyzed by the ChemStation program, var. 09 (Agilent Technologies, Waldbronn, Germany).

Statistical analysis

The data were processed using Statistica, version 10. The results are represented as the means ± standard errors of the means and were tested by a paired Student's *t* test. A level of 0.05 was selected as the minimum point required for statistical significance in all analyses.

Results

Transformation with the rolB gene and selection of the transformed callus cultures

Control callus culture MK was transferred into suspension for transformation. One-week suspension culture MK was cultivated simultaneously with *A. tumefaciens* carrying the construction pPCV002-CaMVBT, containing the *rolB* gene under the control of the 35S CaMV promoter. Cf was added to the culture medium 4 days after transformation to suppress bacterial growth. After an eight-month selection of actively growing aggregates in the presence of Km, four *rolB* transgenic callus lines MB1, MB2, MB3, and MB4 were obtained. Previously, we tested Km sensitivity of the parental MK culture. A two-phase experiment was conducted. In the first phase, Km concentrations of 0, 20, 50, 150, and 1000 mg/L were investigated. The results showed that 20 mg/L of Km caused significant growth inhibition in *M. amurensis* cells, while 50, 150, and 1000 mg/L totally inhibited cell growth. We repeated the experiment with Km concentrations of 0, 20, and 50 mg/L of medium. On the second passage, 20 and 50 mg/L of Km totally inhibited the growth of nontransgenic *M. amurensis* cells. The semilethal dose (LD50) of Km for *M. amurensis* was determined to be 9.5 mg/L of medium. Therefore, Km concentrations of 5–15 mg/L appeared to be adequate for the selection of the transformed cultures. After 2 years of cultivation, required for establishing stable actively growing callus cultures, three independently transformed lines—MB1, MB2, and MB4—were selected for further investigations. The vector callus culture MV, obtained previously, was used as a control in all experiments, as it reproduced the characteristics of the untransformed MK culture (Grishchenko et al. 2013).

The established *rolB*-transformed callus cultures displayed morphological variety (Fig. 1). MB1 calli produced a friable globular callus of a yellow-whitish and light-brown color (Fig. 1b). In contrast, MB2 and MB4 cultures produced a compact, nonwatery yellow-brown and brown callus with active growth; the MB4 calli were more compact (Fig. 1c,d). The phenotypes of the *rolB*-transformed lines differed significantly from that of the MV calli (Fig. 1a).

Gene-specific PCR analysis confirmed the presence of the *nptII* gene sequences in the DNA of all the obtained callus lines (Fig. 2a). The presence of the *nptII* gene sequence in the DNA of the MV calli has been detected previously (Grishchenko et al. 2013) and was confirmed in the present study (Fig. 2a). The absence of *A. tumefaciens* was confirmed using PCR to control the presence or absence of the *virB2* gene (Fig. 2a). Real-time RT-PCR analysis demonstrated that the *rolB* gene was transcribed at reliably different levels in the established cultures. The lowest level of transcription was shown for the MB1 culture, the moderate level of transcription was observed for the MB2 culture, and the highest level of transcription was observed for the MB4 culture (Fig. 2b).

Qualitative and quantitative analysis of the isoflavonoid content in the established *M. amurensis* callus lines

Figure 3 shows the typical HPLC chromatograms for the extracts of the MV, MB1, MB2, and MB4 cultures. The data on the composition and content of the compounds produced by the established callus lines are shown in Table 1. The *rolB* transgenic cultures produced 20 isoflavonoids isolated previously from the *M. amurensis* control MK, vector MV, and *rolC*-transformed MC calli (Fedoreyev et al. 2008;

Fig. 1 Habitus of the established callus lines of *Maackia amurensis*. Vector calli MV (**a**); *rolB* transgenic calli MB1 (**b**); MB2 (**c**); MB4 (**d**). Bar 1 cm. Calli were grown on the $W_{B/A}$ medium for 45 days in the dark at 25 ± 1 °C

Grishchenko et al. 2013). A total of 15 isoflavones and their derivatives (daidzein, daidzin, 4′-*O*-β-glucopyranosyldaidzin, 3′-methoxydaidzin, genistein, genistin, 4′-*O*-β-glucopyranosylgenistin, 6″-*O*-malonylgenistin, calycosin, 7-*O*-β-D-glucopyranosylcalycosin, formononetin, ononin, 6″-*O*-malonylononin, pseudobaptigenin, and derrone) and 5 pterocarpans and their derivatives (maackiain, medicarpin, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosyl-6, 6a-dehydromaackiain, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain, and 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin) were identified.

Thus, transformation by the *rolB* gene did not affect the composition of the isoflavonoid complexes accumulated by the *M. amurensis* cells. However, transformation caused alterations in the quantities of distinct compounds. We studied isoflavonoid accumulation for 3 years, collecting samples at 45 days of culturing (the data are represented as the mean of seven independent measurements, Table 1), as this period of cultivation was previously shown to be optimal for biomass and isoflavonoid accumulation (Grishchenko et al. 2013). The total isoflavonoid content did not change significantly comparing to the MV culture, except for the MB4 culture, which accumulated 2-fold more isoflavonoids than the vector culture. The MB4 culture also produced higher levels of isoflavonoids than the previously established *rolC*-transformed culture MC (Grishchenko et al. 2013). With respect to individual compounds, the *rolB* gene caused a reliable increase in the accumulation of several substances in each of the obtained cultures (four in MB1, five in MB2, and nine in MB4 calli; bold type in Table 1). The levels of daidzein, maackiain, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain, and 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin were increased in the MB1 culture. The levels of daidzein, genistein, formononetin, maackiain, and 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain were increased in the MB2 culture. The levels of daidzein, genistein, formononetin, calycosin, ononin, 7-*O*-β-D-glucopyranosylcalycosin, maackiain, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain, and 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin were increased in the MB4 cells. According to our results, high *rolB* gene expression induced the most evident alterations in the MB4 culture, causing a significant increase in the content of nine substances and a 2-fold increase in the total isoflavonoid content compared to the MV culture.

Productivity of the MB1, MB2, and MB4 callus cultures

To determine the productivity of the obtained callus cultures, we conducted experiments on their growth and isoflavonoid biosynthesis dynamics. Calli were grown for 60 days. We measured fresh and dry biomass accumulation every 10 days and analyzed the content of isoflavonoids every 10 days starting from day 20 (Fig. 4). The MB1 calli exhibited growth and biosynthetic properties similar to those of the MV culture, described previously (Grishchenko et al. 2013). Those calli were characterized by an elongated lag period, with active growth starting after 30 days of cultivation and continuing until the end of the experiment (Fig. 4a, b). Isoflavonoid accumulation is performed with a classic curve with relatively low (0.5 % DW) levels at 20 days of cultivation, an active

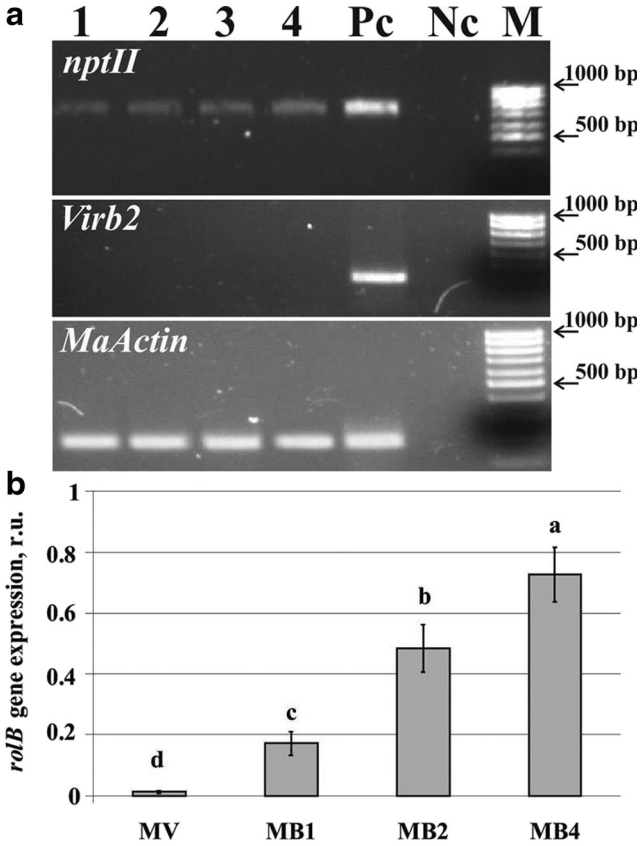

Fig. 2 **a** The gene-specific PCR analysis of the *nptII*, *Virb2*, and *MaActin* genes on the DNA isolated from the MV (*1*), MB1 (*2*), MB2 (*3*), and MB4 (*4*) callus cultures. *Pc* positive control (plasmid pPCV002-CaMVBT for the *nptII*, plasmid pPCV002-CaMVBT for the *Virb2*, and genomic DNA isolated from the MV callus culture for the *MaActin gene*), *Nc* negative control (PCR mixture without DNA), *M* 100-bp DNA ladder (SibEnzyme, Russia, Novosibirsk). **b** *rolB* expression levels detected by real-time RT-PCR in *M. amurensis* MV, MB1, MB2, and MB4 calli. Means followed by the *different letter* were different using Student's *t* test. $p < 0.05$ was considered to be statistically significant

increase of the biosynthetic activity after 30 days and a maximum level at 40 days (1.38 % DW), followed by a gradual decrease until the end of the experiment (1.14 % DW) (Fig. 4c). The productivity levels of the total isoflavonoids in the MB1 culture were equal to those of the MV calli: 10–17 mg/L of the nutrient medium on days 20–30, increased to a maximum level of 107 mg/L of medium on day 50, and slightly decreased to 106.3 mg/L of medium on day 60 (Fig. 4d).

In MB4 culture, active growth started after 10 days of cultivation and continued until 50 days of cultivation. After 50 days, a stationary growth phase was observed (Fig. 4a, b). The isoflavonoid content in the MB4 culture reached the level of 1.6 % DW on 20 days of cultivation, increased to 2 % DW on 40 days, and remained stable until the end of the experiment (Fig. 4c). The productivity of the MB4 calli reached the level of 74 mg/L of medium on day 20, increased to 192 mg/L medium on day 50, and was equal to 200 mg/L medium on 60 days (Fig. 4d).

The MB2 culture demonstrated a lag period of 20 days, then actively accumulated fresh and dry biomass until the end of cultivation, with a phase of stationary growth on 40–50 days (Fig. 4a, b). The isoflavonoid content reached 0.84 % DW on day 20, and increased to 1.7 % DW on 60 days, with a period of slight decrease from 40 to 50 days (1.41–1.24 % DW) (Fig. 4c). The productivity of the MB2 calli increased from a low level of 15 mg/L medium at 20 days to 177 mg/L medium on 60 days, with a period of decrease from 40 to 50 days (93–86 mg/L medium) (Fig. 4d). However, these periods of decrease on 40–50 days were statistically unreliable. Thus, the MB2 calli displayed features intermediate between those of the MB4 and MB1 cultures. The MB2 calli accumulated biomass and secondary metabolites from low levels, like the MB1 culture, and showed no tendency to reduce the isoflavonoids accumulation by the end of cultivation, like the MB4 culture.

The MB4 calli represented the strongest alterations in growth and secondary metabolite production comparing to the MV culture. The MB4 culture exhibited features similar to those of the *rolC*-transformed culture MC, established previously: active growth from the start of the experiment, a high isoflavonoid content and productivity at 20 days of culturing and a lack of evident growth and isoflavonoid accumulation decay by the end of cultivation. These calli differ from the MC culture with the callus appearance and higher levels of isoflavonoids, reaching 2 % DW. In addition, our results from the biomass and isoflavonoid accumulation dynamics experiments allowed us to conclude that a cultivation period of 40–50 days is optimal for the *rolB*-transformed cultures of *M. amurensis*.

We monitored the growth, isoflavonoid content, and productivity of the obtained cultures for more than 3 years. The cultures were cultivated in 100-mL Erlenmeyer flasks containing 40 mL of $W_{B/A}$ medium, with 45-day subculture intervals in the dark at 25 ± 1 °C. The average data are presented in Table 2. The data showed that the *rolB* gene reliably decreased fresh callus growth, as the vector culture MV accumulated fresh biomass 1.5–2 times more actively than the transgenic cultures. However, the levels of dry biomass accumulation were equal in the MV, MB1, and MB2 cultures and were authentically higher in the MB4 culture, as that culture produced a dense callus. The isoflavonoid content did not differ significantly in the established MB1 and MB2 cultures but was reliably increased in the MB4 culture compared to the MV calli (Table 2). The isoflavonoid productivity of the MB1 and MB2 cultures did not differ significantly from that of the MV calli, whereas in the MB4 culture the isoflavonoid productivity appeared to be nearly two times higher than that observed in the vector culture. Characteristics of the established cultures (fresh and dry biomass accumulation,

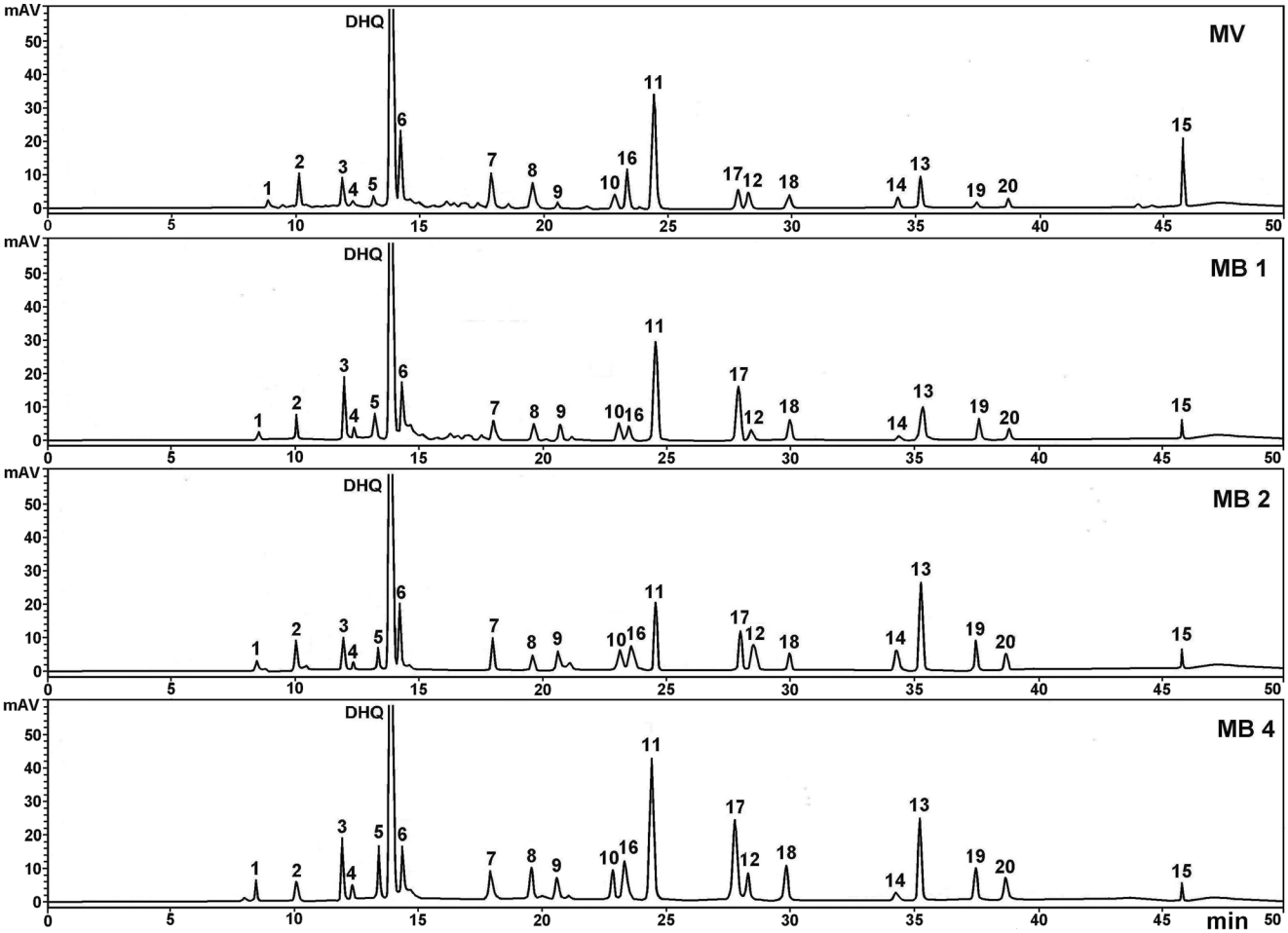

Fig. 3 Representative HPLC-UV profiles (λ = 280 nm) for the extracts obtained from the established callus cultures MV, MB1, MB2, and MB4 of *Maackia amurensis*. *DHQ* dihydroquercetin. Numbers of peaks correspond to numbers of compounds shown in Table 1: *1*, 4′-*O*-β-glucopyranosyldaidzin; *2*, 4′-*O*-β-glucopyranosylgenistin; *3*, daidzin; *4*, 3′-methoxydaidzin; *5*, 7-*O*-β-D-glucopyranosylcalycosin; *6*, genistin; *7*, 6″-*O*-malonylgenistin; *8*, ononin; *9*, daidzein; *10*, calycosin; *11*, 6″-*O*-malonylononin; *12*, genistein; *13*, formononetin; *14*, pseudobaptigenin; *15*, derrone; *16*, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosyl-6,6a-dehydromaackiain; *17*, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain; *18*, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin; *19*, maackiain; *20*, medicarpin

isoflavonoid content, and productivity) were stable during the observation period, which is important in case of practical application.

Thus, the MB4 callus culture displayed the most striking effect of the *rolB* gene on growth and isoflavonoid biosynthesis. We compared the characteristics of the MB4 culture and the previously established MC calli (Grishchenko et al. 2013) and found that dry biomass accumulation reached the same levels in the MB4 and MC calli, although the productivity levels were reliably higher in the MB4 culture. It is obvious that high levels of expression of the *rolB* gene induced secondary metabolism in the *M. amurensis* calli more effectively than the *rolC* gene (Table 2). Similar results were reported for *Rubia cordifolia* cells, in which the *rolB* gene was more effective than the *rolC* gene, in the activation of antraquinone biosynthesis (Bulgakov et al. 2002). In *V. amurensis* calli, the *rolB* gene was also shown to be more effective in the stimulation of resveratrol biosynthesis than the *rolC* gene, as the *rolB* gene insertion led to a more than 100-fold increase in resveratrol production, whereas the *rolC* gene caused a 4- to 12-fold increase in the resveratrol accumulation (Kiselev et al. 2007; Dubrovina et al. 2010).

Discussion

We successfully established *rolB*-transformed calli of the *M. amurensis* for the first time on our third attempt. Previous efforts with seedlings were undertaken in 2001 and 2003, but the obtained cultures were nonviable and died soon after callus initiation. Trees and some leguminous plants are known to be difficult to transform using *Agrobacterium* (Somers et al. 2003; Dhakulkar et al. 2005). Nevertheless, we established three transgenic cultures with low, medium, and high levels of *rolB* gene expression. The insertion of the *rolB* gene (as well as the *rolC* gene) significantly modified the basic

Table 1 Isoflavonoid content in *Maackia amurensis rolB*-transformed cell cultures. Samples were taken at 45 days of culture

	Substances\cell cultures	MV	MB-1	MB-2	MB-4	MC
	Isoflavones and their derivatives					
1	4′-*O*-β-Glucopyranosyldaidzin	0.04 ± 0.008	0.032 ± 0.006	0.044 ± 0.008	0.082 ± 0.031	**0.082** ± 0.017*
2	4′-*O*-β-Glucopyranosylgenistin	0.066 ± 0.012	***0.021*** ± 0.003*	0.038 ± 0.008	***0.029*** ± 0.004*	0.038 ± 0.011
3	Daidzin	0.064 ± 0.008	0.087 ± 0.017	0.046 ± 0.009	0.104 ± 0.025	0.073 ± 0.009
4	3′-Methoxydaidzin	0.016 ± 0.002	0.014 ± 0.004	0.016 ± 0.002	0.025 ± 0.006	0.013 ± 0.004
5	7-*O*-β-D-Glucopyranosylcalycosin	0.038 ± 0.006	0.055 ± 0.012	0.059 ± 0.019	**0.073** ± 0.009**	0.043 ± 0.008
6	Genistin	0.103 ± 0.012	0.064 ± 0.004	0.104 ± 0.031	0.083 ± 0.006	***0.06*** ± 0.013*
7	6″-*O*-Malonylgenistin	0.121 ± 0.011	***0.054*** ± 0.006*	0.093 ± 0.021	0.091 ± 0.014	0.087 ± 0.015
8	Ononin	0.046 ± 0.007	0.035 ± 0.005	***0.022*** ± 0.002*	**0.073** ± 0.007*	0.046 ± 0.007
9 +	Daidzein	tr	**0.021** ± 0.006*	**0.025** ± 0.006**	**0.036** ± 0.004**	**0.045** ± 0.009**
10+	Calycosin	0.018 ± 0.003	0.03 ± 0.005	0.029 ± 0.008	**0.042** ± 0.003**	**0.032** ± 0.005*
11	6″-*O*-Malonylononin	0.214 ± 0.026	0.188 ± 0.02	0.135 ± 0.011	0.292 ± 0.018	0.207 ± 0.03
12+	Genistein	0.012 ± 0.002	0.014 ± 0.005	**0.057** ± 0.023**	**0.031** ± 0.008*	0.022 ± 0.006
13+	Formononetin	0.026 ± 0.006	0.041 ± 0.007	**0.077** ± 0.017**	**0.075** ± 0.022*	**0.079** ± 0.014**
14+	Pseudobaptigenin	0.013 ± 0.004	tr	0.028 ± 0.005	0.02 ± 0.006	0.021 ± 0.003
15+	Derrone	0.018 ± 0.006	tr	tr	0.01 ± 0.002	0.013 ± 0.002
	Pterocarpans and their derivatives					
16	6′-*O*-Malonyl-3-*O*-β-D-glucopyranosyl-6,6a-dehydromaackiain	0.144 ± 0.016	0.094 ± 0.014	0.092 ± 0.019	0.169 ± 0.015	0.112 ± 0.021
17	6′-*O*-Malonyl-3-*O*-β-D-glucopyranosylmaackiain	0.184 ± 0.024	**0.395** ± 0.048**	**0.389** ± 0.059**	**0.571** ± 0.042**	**0.308** ± 0.036**
18	6′-*O*-Malonyl-3-*O*-β-D-glucopyranosylmedicarpin	0.076 ± 0.012	**0.163** ± 0.038*	0.081 ± 0.014	**0.183** ± 0.024**	0.054 ± 0.014
19+	Maackiain	0.013 ± 0.004	**0.058** ± 0.014**	**0.134** ± 0.044**	**0.099** ± 0.025**	**0.048** ± 0.011**
20+	Medicarpin	0.015 ± 0.005	0.025 ± 0.005	0.03 ± 0.012	0.04 ± 0.015	0.017 ± 0.005
	Total	1.22 ± 0.121	1.399 ± 0.082	1.503 ± 0.208	2.1 ± 0.089**	1.392 ± 0.141

The data are represented as means in % of dry weight (DW) ± standard error based on seven independent measurements. The + symbol indicates free compounds. The bold type indicates a significant increase in the substance content; italic type indicates a significant decrease in the substance content, compared with the MV calli. Data for the MC calli were taken from Grishchenko et al. 2013

tr trace content (less than 0.01 % DW)

*$P < 0.05$; **$P < 0.01$, versus values of the content in the MV calli

characteristics of the *M. amurensis* cells. The manifestation of these alterations depended on the levels of *rolB* gene expression. Higher expression levels caused earlier growth initiation and secondary metabolite accumulation and nullified the lowering of biosynthetic activity that occurred after 40–50 days of culturing, relevant for the MV culture. The strongest transformation effect was observed for the MB4 calli, characterized by the highest level of the *rolB* gene expression, as shown in Fig. 2b. The MB4 calli exhibited rapid biomass increment after 10 days of culturing, whereas the MB1 and MB2 calli accumulated biomass actively after 30 and 20 days of culturing, respectively. High *rolB* gene expression activated isoflavonoid biosynthesis at the early stages of cultivation; thereby, the MB4 culture accumulated 1.6 % DW isoflavonoids on 20 days and reached the maximum level on 35–40 days. The MB2 culture with the moderate level of *rolB* gene expression accumulated lower levels of isoflavonoids on 20 days and exhibited high biosynthetic activity on days 20–60. The production of isoflavonoids in the MB1 culture (with the lowest level of *rolB* gene expression) was low until 30 days, increased from 30 to 40 days followed by the gradual decrease, thus providing similarity with the MV culture. This result indicated the activation of isoflavonoid degradation mechanisms during the final cultivation stage. It is interesting that the *rolB* gene acts differently in different plant cultures. For example, *rolB* transgenic cultures of *Panax ginseng* produced 5.7 times lower ginsenoside levels than the control culture (Bulgakov et al. 1998). In contrast, we achieved a 2-fold increase in the isoflavonoid content and isoflavonoid productivity of *M. amurensis* calli. The observed effect was not as strong as the effect observed for *V. amurensis* culture, as reported by Kiselev et al. (2007). Nevertheless, the findings of this study represent the most prominent results for *M. amurensis* culture. Based on this study, we can confirm the status of the *rolB* gene as the most powerful activator of the secondary metabolism in *M. amurensis* calli among the *A. rhizogenes rol* genes.

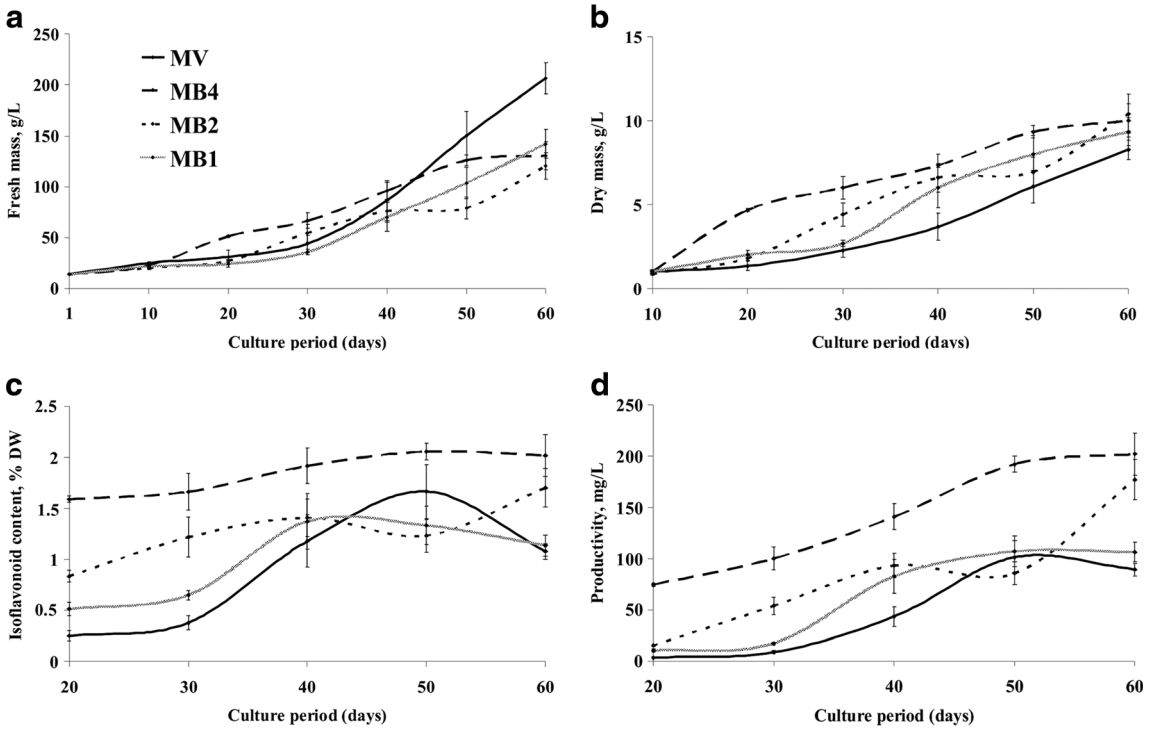

Fig. 4 The dynamics of fresh (**a**) and dry (**b**) biomass accumulation, isoflavonoid accumulation (**c**), and isoflavonoid productivity (**d**) of the *Maackia amurensis* MV, MB4, MB2, and MB1 callus cultures. We considered each of the 20 isoflavonoids produced by callus cultures of *M. amurensis*: daidzein, daidzin, 4′-*O*-β-glucopyranosyldaidzin, 3′-methoxydaidzin, genistein, genistin, 4′-*O*-β-glucopyranosylgenistin, calycosin, 7-*O*-β-D-glucopyranosylcalycosin, 6″-*O*-malonylgenistin, ononin, 6″-*O*-malonylononin, formononetin, pseudobaptigenin, derrone, maackiain, medicarpin, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosyl-6,6a-dehydromaackiain, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain, and 6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin

As demonstrated previously, high levels of expression of the *rolB* gene cause high secondary metabolite content in the transgenic cells but also significantly inhibit cell growth (Kiselev et al. 2007). In attempts to overcome this effect of the *rolB* gene, the authors used tyrosine phosphatase inhibitors, as the rolB protein was shown to possess tyrosine phosphatase activity (Filippini et al. 1996). Those authors succeeded in partially preventing the toxic effects of *rolB* on cell growth, but the production of secondary metabolites was also decreased. In our case, a high level of *rolB* gene expression did not inhibit dry biomass accumulation and increased the isoflavonoid content. We may not have observed the growth suppression due to the moderate effect of the gene on isoflavonoid biosynthesis, comparing to the discussed studies.

The *rolB* gene mainly activated the accumulation of the free isoflavones daidzein, genistein, formononetin, and calycosin (in MB4 calli), thus indicating the promoting effect of the *rolB* on the initial reactions of the isoflavonoid biosynthesis pathway in *M. amurensis* cells. The content of isoflavone glycosides did not change reliably and even decreased (4′-*O*-β-glucopyranosylgenistin), except for 7-*O*-β-D-glucopyranosylcalycosin, levels of which were reliably higher in the MB4 calli. In addition, the *rolB* gene promoted the accumulation of the pterocarpan maackiain and more actively, malonylglycosides of maackiain and medicarpin. The

Table 2 The average growth, isoflavonoid content, and isoflavonoid productivity of the MV, MB1, MB2, and MB4 cell cultures of *Maackia amurensis* over 3 years of observation

Culture	Fresh biomass (g/L)	Dry biomass (g/L)	Isoflavonoid content (%DW)	Isoflavonoid productivity (mg/L)
MV	210.44 ± 9.67	8.58 ± 0.49	1.22 ± 0.121	106.1 ± 8.04
MB1	115.47 ± 8.32**	8.72 ± 0.63	1.399 ± 0.082	117.39 ± 10.73
MB2	149.84 ± 9.56**	9.37 ± 0.65	1.503 ± 0.208	112.97 ± 16.98
MB4	154.63 ± 6.04**	10.72 ± 0.47**	2.10 ± 0.089**	199.53 ± 14.85**
MC	*173.60 ± 5.50*	*11.21 ± 0.54***	*1.392 ± 0.141*	*145.9 ± 17.2**

Samples were taken at 45 days of culture. The data for the MC culture were taken from Grishchenko et al. 2013

*$P < 0.05$; **$P < 0.01$, versus values of the MV calli

conversion of isoflavonoids to their glycosides and glucomalonate conjugates is the final step in flavonoid biosynthesis in many plants, leading to an increase in water solubility and the protection of glycosides from enzymatic degradation by glycosidases (Heller and Forkmann 1994). This conversion is catalyzed by glycosyltransferase and malonyltransferase enzymes (Dixon 2004). We suggest that the *rolB* gene could enhance the activity of the glycosyltransferases and malonyltransferases with high substrate specificity for pterocarpans and maackiain in particular.

In a previous study (Grishchenko et al. 2013), the *rolC* gene activated the accumulation of the isoflavone aglycones daidzein, calycosin, and formononetin, daidzein glycoside 4′-*O*-β-D-glucopyranosyldaidzin, as well as the pterocarpans maackiain and its 6′-O-malonyl-3-*O*-β-D-glucopyranosyl. Taking into account the evidence reported in the present study, we can conclude that both genes activate the first steps of secondary metabolite biosynthesis, leading to the enhanced isoflavone accumulation, but also stimulate the production of pterocarpans, which represent the latter steps of the biosynthetic chain of the isoflavonoid compounds. This observation could possibly be explained by the high importance of pterocarpans in the defensive reactions of *M. amurensis*.

The *rolB* gene was also shown to influence the isoflavone/pterocarpan ratio in the established cultures. The pterocarpan content attained a level of 50 % of the total isoflavonoid content, equal to the isoflavone content. The isoflavone/pterocarpan ratios were equal to 0.9, 1.1, and 1 in MB1, MB2, and MB4 calli, respectively. In contrast, the pterocarpan content was 1.8 times lower than the isoflavone content in the MV calli and 1.6 times lower than the isoflavone content in the MC calli. Thus, the *rolB* gene seems to activate the branch of the biosynthesis leading to the pterocarpan formation.

Fedoreyev et al. (2008) reported about high stilbene content in the heartwood of *M. amurensis*. Insertion of the *rolB* gene was shown to strongly activate stilbene accumulation in a cell culture of *V. amurensis* (Kiselev et al. 2007). Despite this, we did not detect stilbenes in the control and *rolB* transgenic cultures of *M. amurensis*.

In conclusion, the stimulating effect of the *rolB* gene from *A. rhizogenes* on isoflavonoid production in *M. amurensis* calli was shown for the first time in the present study. These secondary metabolites provide a wide range of biological activities, including hepatoprotective properties, and the established cultures could be used as an alternative source of these valuable compounds.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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