The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells

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Abstract

Resveratrol is a stilbene, which prevents carcinogenesis at stages of tumor initiation, promotion and progression. In the present investigation, we developed cell cultures of wild-growing grape (*Vitis amurensis* Rupr.). The cultures produced low levels of resveratrol, up to 0.026% dry wt., i.e., comparable to levels reported for other plant cell cultures previously established. Different methods commonly used to increase secondary metabolite production (cell selection, elicitor treatments and addition of a biosynthetic precursor) only slightly enhanced cell productivity. Transformation of *V. amurensis* V2 callus culture by the *rolB* gene of *Agrobacterium rhizogenes* resulted in more than a 100-fold increase in resveratrol production in transformed calli. The *rolB*-transformed calli are capable of producing up to 3.15% dry wt. of resveratrol. We show that the capability to resveratrol biosynthesis is tightly correlated with the abundance of *rolB* mRNA transcripts. Tyrosine phosphatase inhibitors abolished the *rolB*-gene-mediated stimulatory effect, thus documenting for the first time the involvement of tyrosine phosphorylation in plant secondary metabolism.

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Keywords: *Vitis amurensis*; Resveratrol; *rolB* gene; Callus culture

1. Introduction

Stilbenes play an important role in protecting plants against fungal infections (Dixon and Harrison, 1990) and constitute the main group of phytoalexins within the *Vitaceae* (Jeamet et al., 2002). In recent years, much attention has been emphasized to the effects of stilbenes, especially resveratrol, on human health. Although resveratrol synthesis has been reported in
several plants such as peanut, lily, mulberries, eucalyptus, spruce and pine, grapevine is the main source of this compound (Pervaiz, 2003). Multiple lines of compelling evidence indicate its beneficial effects on neurological, hepatic, and cardiovascular systems. One of the most striking biological activities of resveratrol investigated during the late years has been its cancer chemopreventive potential (Pervaiz, 2003). More recent data provide interesting insights into the effect of this compound on the lifespan of yeast and flies, implicating the potential of resveratrol as an anti-aging agent in treating age-related human diseases (Lastra and Villegas, 2005).

Only small amounts of resveratrol (less than 0.01% DW) have been reported for plant cell cultures of different species (Ku et al., 2005; Tassoni et al., 2005). Therefore, efforts have been made to overcome the inability of cultured plant cells to synthesize high levels of resveratrol. *Vitis vinifera* suspension cultures responded to MeJA and sodium orthovanadate by a five- to six-fold increase in resveratrol production (Tassoni et al., 2005). Likewise, cell cultures of *Arachis hypogaea* responded to ultraviolet (UV) irradiation with a 40–50-fold increase in resveratrol production, but the effect disappeared after 24 h of the treatment (Ku et al., 2005). After these treatments, the resulting levels of resveratrol in plant cell cultures did not exceed 0.02–0.03% cell dry wt. (Ku et al., 2005; Tassoni et al., 2005).

Genetic transformation by single Agrobacterium rol genes has emerged as a powerful tool for secondary metabolite manipulation in cultured plant cells (Bulgakov et al., 2002, 2003). One of the rol genes, rolB, is essential for plant (Altamura et al., 1994) and animal (Bulgakov et al., 2006) cell growth and development. In *Rubia cordifolia* transformed cells, the rolB gene was shown to be a more powerful inducer of anthraquinone biosynthesis than the rolC gene (Bulgakov et al., 2002). The development of additional rolB-transformed plant model systems is important to exploit this new biotechnological tool for genetic engineering of plant secondary metabolism. The RolB protein was shown to exhibit tyrosine phosphatase activity (Filippini et al., 1996) and to interact with 14-3-3 proteins (Morichi et al., 2004). Protein tyrosine phosphatases or 14-3-3 proteins have not been considered as components of signaling pathways regulating secondary metabolism in plants. However, taking into account the more pronounced stimulatory effect of the rolB gene on anthraquinone biosynthesis than that of MeJA or SA, one can suppose a potential significance of these proteins in plant secondary metabolism.

In the present study, we tested different approaches to increase resveratrol production in cultured *Vitaceae* cells. Wild-growing plants of *Vitis amurensis* were used as a source of explants, because this species was previously characterized as a rich source of stilbenes (Huang et al., 2001). We show that genetic transformation of *V. amurensis* cells with the rolB gene was efficient for high resveratrol production while aggregate cell selection, metabolite selection, precursor addition and elicitor treatments were not effective.

2. Materials and methods

2.1. Plant material and cell cultures

Wild-growing plants of *Vitis amurensis* Rupr. (*Vitaceae*) were collected from the southern Primorsky Region of the Russian Far East and identified in the Botany Department of the Institute of Biology and Soil Science. The V1, V2 and V3 callus cultures were established in 2002 from young stems of the mature plants. Cultures were cultivated in 100 ml Erlenmeyer flasks using *W*$_{B/A}$ medium (Bulgakov et al., 2002) supplemented with 0.5 mg/l 6-benzylaminopurine and 2.0 mg/l α-naphthaleneacetic acid in the dark, at 25°C. The inoculum mass for all cultures was 0.2 g. The calli were grown for 35–40 days.

2.2. Elicitor and effector treatments

Sterile solutions of methyl jasmonate (MeJA), and salicylic acid (SA) were added to the culture medium aseptically in desired concentrations as described (Bulgakov et al., 2002). Phenylalanine and sodium nitroprusside were dissolved in sterile hot water. Stock solutions of phenylalanine and sodium nitroprusside (100 and 40 mg/ml, respectively) were used. Phenylarsonic oxide (PAO) was dissolved in dimethyl sulfoxide (10 mM stock solution) and supplied at the final concentrations indicated in the figure legends. All reagents were added to the medium after autoclaving. The chemicals were obtained from Sigma and ICN Pharmaceuticals.
2.3. Selection of fluoro-phenylalanine-resistant cells

*p*-Fluoro-<i>dL</i>-phenylalanine (PFP, Sigma) was dissolved in hot water (10 mg/ml), sterilized by Millipore filtration and added into autoclaved media. Cell aggregates (2–3 mm) of V2 culture were transferred to the media containing PFP. Well-growing aggregates were selected on media containing gradually increasing concentrations (10, 30 and 100 mg/l) of the inhibitor as described (Bulgakov et al., 2001).

2.4. Genetic transformation

The transgenic <i>rolB</i> callus cultures (designated VB1 and VB2) were established by transformation of the V2 calli with <i>A. tumefaciens</i> strains GV3101/pMP90RK containing plasmid vector pPCV002-CaMVB (Spena et al., 1987). In the construction, the <i>rolB</i> gene was under the control of cauliflower mosaic virus (CaMV) 35S promoter (Spena et al., 1987). The construction also carried a gene for kanamycin resistance (nptII) under the control of the nopaline synthase promoter. The V2-vector culture (designated VV) was obtained by co-cultivation of the V2 calli with <i>A. tumefaciens</i> GV3101/pPCV002 (empty vector) according to the procedure described (Bulgakov et al., 1998). After transformation, the calli were cultivated for a 3-month period in the presence of 250 mg/l of cefotaxim to suppress the bacteria. Selection of transgenic aggregates was carried out for 5 months using 15–20 mg/l of kanamycin sulphate.

2.5. DNA and RNA analyses

DNA samples were isolated as described (Bulgakov et al., 2005) from the pPCV002 and pPCV002-CaMVB-transformed calli, which were grown for 12 months in the absence of antibiotics. The primers 5′-GGG TTA GGT CTG GCT CCG GT and 5′-CGA GGG GAT CCG ATT TGC TT allowed the amplification of a 623 bp <i>rolB</i> gene fragment (accession no. K03313). The nptII gene 700-bp fragment was amplified by using primers 5′-GAG GCT ATT CGG GTA GAG GTG CCG ATT TGC TT and 5′-AGG AGC TGC TTT TGT TTT CC designed to the <i>V. vinifera</i> actin gene (accession no. AY680701). A 392-bp fragment of actin gene was amplified using DNA as a template and a 301-bp fragment was amplified using cDNA as a template (the difference of the 91 bp was due to the presence of an intron in the actin gene). Quantitative mRNA analysis was performed by using a DNA 1000 LabChip® kit (Agilent 2100 Bioanalyzer, Agilent Technologies, Germany) following the manufacturer’s protocol and recommendations.

2.6. DNA sequencing

RT-PCR and PCR products were sequenced with the same primers and Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Biosystems, Forster City, CA) following the manufacturer’s protocol and recommendations. After purification with ethanol the sequences were identified on an ABI 310 Genetic Analyzer (Perkin-Elmer Biosystems).

2.7. Resveratrol isolation and identification

Dry <i>V. amurensis</i> calli were exhausted extracted with EtOH. The extract was evaporated under reduced pressure and separated by low-pressure column chromatography (silica gel, 40–60 mesh; CHCl<sub>3</sub>–acetone 9:1) with monitoring fractions by HPLC. The fractions containing resveratrol were combined and resveratrol was re-crystallized from ethanol. Resveratrol was identified by 1H and 13C NMR using a Bruker NMR Avance.
DPX-500 instrument. Samples were run in deuterated chloroform with tetramethylsilane as the internal standard.

2.8. High-performance liquid chromatography

The dried and powdered callus culture sample (100 mg) was extracted with 96% EtOH (3 ml) for 24 h at 40–45 °C. An ethanolic solution of dihydroquercetin (I) (0.050 ml, 1 mg/ml) was added to 0.450 ml of the extract as the internal standard. The sample solution was membrane filtered (0.45 μm, Agilent) and 5 μl aliquots were used for analysis. The analytical HPLC was carried out using an Agilent Technologies 1100 Series HPLC system equipped with VWD detector (λ = 280 nm). Extracts and fractions were analyzed using a HYPERSIL BDS-C-18 column (5 μm, 250 mm x 5 mm) thermostated at 30 °C. The mobile phase consisted of 1% aqueous acetic acid (A) and acetonitrile containing 1% acetic acid (B). For the analyses 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. The flow rate was 1 ml/min. The data were analyzed with the ChemStation® program var. 09 (Agilent Technologies, Germany).

3. Results

3.1. Callus growth and resveratrol production in V. amurensis non-transformed cultures

Several callus cultures were established from young stems of wild-growing plants of V. amurensis. These calli represented friable vigorously growing homogenous tissues, which did not appear to have undergone differentiation. We analyzed growth and stilbene production in three callus cultures designated V1, V2

Fig. 1. HPLC profiles of polyphenol fractions from V. amurensis cell cultures: (A) V2 and (B) VB2 culture. 1, dihydroquercetin and 2, resveratrol.
and V3. The HPLC and NMR-determinations revealed that trans-resveratrol (3,4′,5-trihydroxystilbene) was exclusively produced by these calli. A trace amount of two phenolic substances (Fig. 1), presumably ampelopsin and piceatannol, was also detected by comparing HPLC profiles of polyphenolic fractions isolated from the calli and wild-growing V. amurensis plants (data not shown). Resveratrol in the normal calli of V. amurensis was produced up to 0.004–0.026% dry wt. (Table 1), i.e. comparable to levels reported for V. vinifera calluses (Ku et al., 2005; Tassoni et al., 2005).

As we observed the appearance of light-brownish tissue aggregates within the V3 calli, we expected that selection of these aggregates would result in establishment of a more productive culture. The selected line (V3-1) was sub-cultivated for 3 months and analyzed for stilbene production. However, this approach appears to be ineffective, because stilbenes were produced at low levels (Table 1). During prolonged periods of the V2 calli cultivation, maximal resveratrol yield was observed at 30–40-day cultivation. Some decrease of resveratrol content was observed thereafter, although the culture continued to grow vigorously. Similar growth and production parameters were observed also for the V1, V3 and V3-1 calluses (data not shown).

3.2. Elicitor treatments

To increase stilbene production, we cultivated V. amurensis calli in the presence of inducers of secondary metabolism, such as MeJA and salicylic acid. These plant defense hormones caused a 2–3-fold increase in resveratrol accumulation in the V2 culture (Table 2). However, resveratrol content did not exceed 0.058%. Na-orthovanadate, an inhibitor of plant H+-ATPase, was shown to increase resveratrol content in V. vinifera suspension cells (Tassoni et al., 2005). When used in our experiments, the inhibitor did not significantly affect resveratrol production and substantially inhibited callus growth (Table 2).

Table 2
Effect of different effectors on growth and resveratrol production in V. amurensis V2 callus culture

<table>
<thead>
<tr>
<th>Effector</th>
<th>Effector concentrations</th>
<th>Fresh biomass (g)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Resveratrol content (% dry wt.)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeJA (μM)</td>
<td>0</td>
<td>2.8 ± 0.2</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.3 ± 0.1</td>
<td>0.058</td>
</tr>
<tr>
<td>SA (μM)</td>
<td>0</td>
<td>4.0 ± 0.2</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.5 ± 0.2</td>
<td>0.045</td>
</tr>
<tr>
<td>Na-orthovanadate (mM)</td>
<td>0</td>
<td>2.4 ± 0.1</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.4 ± 0.2</td>
<td>0.036</td>
</tr>
<tr>
<td>SNP (mM)</td>
<td>0</td>
<td>3.4 ± 0.2</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.2 ± 0.1</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.5 ± 0.1</td>
<td>0.050</td>
</tr>
<tr>
<td>Phe (mM)</td>
<td>0</td>
<td>2.0 ± 0.2</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.6 ± 0.2</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>1.5 ± 0.1</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.4 ± 0.1</td>
<td>0.037</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean values ± S.E. based on 10 replicate samples obtained in a single experiment.

<sup>b</sup> Average values from the 10 replicate flasks.
Since MeJA, salicylic acid and Na-orthovanadate were not efficient inductors of resveratrol production in *V. vinifera* cultures, we proposed that stilbene biosynthesis is regulated by another signal. The role for nitric oxide (NO) in plant defense and, in particular, in activation of secondary metabolism is now evident from several investigations (Zhao et al., 2005). Sodium nitroprusside, a commonly used NO donor, was used in the next experiment. A four-fold increase of resveratrol content was detected in the Na nitroprusside-treated cells (Table 2), thus indicating the importance of NO signaling in resveratrol production. However, the maximal resveratrol accumulation was still low, not exceeding 0.15% callus dry wt.

### 3.3. Effect of phenylalanine and selection for cells resistant to fluorophenylalanine

Resveratrol is synthesized via the phenylpropanoid pathway there the phenylalanine ammonia-lyase (PAL), the first enzyme in this pathway, catalyzes the monooxidative deamination of phenylalanine to produce cinnamylate, the first structure with a phenylpropanoid skeleton (Jeandet et al., 2002). Therefore, availability of phenylalanine is an important factor for stilbene biosynthesis. Exogenously added phenylalanine, however, had little effect on resveratrol production in our cultures (Table 2).

We proposed that increased resveratrol production by *V. amurensis* cells could potentially be achieved by stimulation of the metabolite flux via the phenylpropanoid pathway. It is known that selection for resistance to the phenylalanine analogue para-fluorophenylalanine (PFP) can result in hyper-accumulation of aromatic compounds in cultured plant cells because the selected cells produce high amounts of Phe (Quesnel and Ellis, 1989).

The selection of PFP-resistant lines of *V. amurensis* was conducted by the step-wise exposure of the parent line to increased levels of the analogue. Sequential selection yielded the V2F callus line capable of growing normally on the medium containing concentrations of PFP which significantly inhibited growth of the parent line (Fig. 2). The dose of PFP which decreases cell growth by half (LD$_{50}$) was found to be 55 mg/l PFP. This value is in the same order as those reported for *Lithospermum erythrorhizon* and *Nicotiana tabacum* PFP-resistant cells and exceeds the value reported for *Cathamntus roseus* cells (Quesnel and Ellis, 1989; Bulgakov et al., 2001).

The examination of resveratrol content in the V2F line was started after 5–6 subcultures of the calluses had been grown on the media without PFP. The analyses revealed more than a 10-fold increase of resveratrol in the V2F culture, compared with the initial culture (Table 3).

### 3.4. Transformation with the rolB gene and selection of transformed cultures

The V2 suspension culture was incubated with *Agrobacterium* strains, bearing constructs pPCV002 (empty vector) or pPCV002-CaMV (the *rolB* gene under the control of 35S CaMV promoter). We selected fast-growing callus aggregates in the presence of kanamycin and established several kanamycin-resistant transformants. The analyses revealed more than a 10-fold increase of resveratrol in the V2F culture, compared with the initial culture (Table 3).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Increased resveratrol production in PFP-selected <em>V. amurensis</em> calluses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus line</td>
<td>Fresh biomass (g)</td>
</tr>
<tr>
<td>V2 (initial culture)</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>V2F (selected culture)</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

Data presentation: see footnote to Table 2.
resistant lines. Previously, kanamycin (Km) sensitivity of the parent V2 culture was tested and it was shown that these calli ceased to grow at such low Km concentration as 10 mg/l (data not shown). Therefore, selection in the presence of 15–20 mg/l Km was adequate to establish transformed cultures. After the 5-month selection procedure, the transgenic calli were resistant to 20 mg/l concentration of Km. The vector culture (designated VV) reproduced morphological, growth and biosynthetic characteristics of the parent V2 culture, indicating that transformation by the empty vector did not cause significant perturbations in transformed cells.

The primary calli, transformed by the rolB gene, showed high variability in morphology and growth. Besides the normal (friable) callus phenotype, which is characteristic for the non-transformed and vector cultures, the rolB cells produced compact globular calli. Both compact and friable calli formed white-yellowish or olive-brown tissues with clear dark-brown zones of necrosis. Interestingly, the dark-brown clusters, when analyzed by semi-quantitative RT-PCR, showed a high level of rolB transcripts. This trait was obviously associated with the necrotic phenotype. In other words, the strong expression of rolB ultimately induced cell death. It was reported that constitutive rolB apparently induced cellular death (necrosis) in tobacco cells and spontaneous counter-selection against cells expressing high levels of rolB occurred after transformation (Schmulling et al., 1988). It is likely that similar processes determined callus differentiation in V. amurensis. Kanamycin selection was favorable to select rolB-transformed cells from the primary mixed cell population (presumably consisted of non-transformed and transformed cells) and, simultaneously, the counter-selection against cells expressing high levels of rolB occurred. Electrophoretic analysis of DNA samples from the dark-brown clusters revealed DNA laddering, thereby indicating possible events of apoptosis in the rolB-transformed cells.

The formation of stable callus phenotypes was observed only after a 12-month selection of friable actively grown cell clusters. Two characteristic cell lines, the fast-growing VB1 and moderately growing VB2 were chosen for further investigation. Gene-specific PCR analysis revealed that DNA of the VV culture contained the nptII gene sequence; DNA from the VB1 and VB2 calli contained the rolB and nptII gene sequences (data not shown). The semiquantitative RT-PCR analysis has shown that the rolB gene was transcribed in the VB1 culture at a low level, thus contrasting with the VB2 culture, where the gene was transcribed more actively (Fig. 3). The identity of the rolB RT-PCR products was confirmed by DNA sequencing.

3.5. Resveratrol production in rolB-transformed callus cultures

The HPLC determinations revealed low resveratrol values in the VB1 calli (Table 4). In contrast, the VB2 calli produced high amounts of resveratrol, up to 3.15%
Table 4
Biomass accumulation and resveratrol production by V. amurensis rolB-transgenic callus cultures

<table>
<thead>
<tr>
<th>Callus line</th>
<th>Fresh biomass (g)</th>
<th>Percentage of dry biomass</th>
<th>Dry callus mass (mg)</th>
<th>Resveratrol content (% dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV</td>
<td>4.2 ± 0.3</td>
<td>3.4</td>
<td>143</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>VB1</td>
<td>3.2 ± 0.1</td>
<td>4.4</td>
<td>141</td>
<td>0.006 ± 0.003</td>
</tr>
<tr>
<td>VB2</td>
<td>1.5 ± 0.2</td>
<td>6.2</td>
<td>93</td>
<td>3.150 ± 0.300</td>
</tr>
</tbody>
</table>

The data presented as mean ± S.E. from four independent experiments with 10 replicates each.

3.6. Effect of growth regulators on growth and resveratrol production

As the rolB gene product was shown to increase sensitivity to auxin (Maurel et al., 1991), we tested whether or not decreasing rates of auxin or synthetic auxin analogues could increase growth of rolB-transformed cells. As shown in Table 5, a cytokinin (6-benzylaminopurine) was required for callus growth of both cultures, in contrast to auxins, which did not significantly affect callus growth (with the exception of 2,4-D which in relatively high concentrations apparently blocked callus growth). However, resveratrol measurements revealed absolute requirement of BAP/NAA combination for resveratrol production in both vector and rolB-calli (data not shown). Increased concentrations of IAA and 2,4-D had a negative effect of resveratrol synthesis in both cultures. We conclude from these experiments that changes in hormone composition, although provoked marked perturbations in growth and biosynthetic characteristics of the cultures, caused similar effects on the vector and rolB-expressing cultures. Therefore, exogenous hormone signals scarcely interfere with the rolB-gene generated signals. These data are in agreement with the observations that the rolB gene does not increase the auxin sensitivity by modifying the intracellular auxin concentration (Delbarre et al., 1994), but affects auxin perception pathway (Filippini et al., 1996).

Table 5
Effect of growth regulators on dry biomass accumulation by V. amurensis vector and rolB-transformed callus cultures

<table>
<thead>
<tr>
<th>Composition of growth regulators (mg/l)</th>
<th>Dry callus weight (g)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vector culture (VV)</td>
</tr>
<tr>
<td>None growth regulators</td>
<td>0.01</td>
</tr>
<tr>
<td>BAP 0.5; NAA 2.0 (standard composition)</td>
<td>0.09</td>
</tr>
<tr>
<td>BAP 0.5</td>
<td>0.10</td>
</tr>
<tr>
<td>NAA 2.0</td>
<td>0.03</td>
</tr>
<tr>
<td>BAP 0.5; NAA 0.2</td>
<td>0.11</td>
</tr>
<tr>
<td>BAP 0.5; NAA 0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>BAP 0.5; 2,4-D 2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>BAP 0.5; 2,4-D 0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>BAP 0.5; 2,4-D 0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>BAP 0.5; IAA 2.0</td>
<td>0.12</td>
</tr>
<tr>
<td>BAP 0.5; IAA 0.2</td>
<td>0.11</td>
</tr>
<tr>
<td>BAP 0.5; IAA 0.02</td>
<td>0.12</td>
</tr>
</tbody>
</table>

\(^a\) Mean values based on 10 replicate samples obtained in a single experiment.
3.7. Tyrosine phosphatase inhibitors abolish the stimulatory effect of the rolB gene on resveratrol synthesis

The rolB gene product was shown to exhibit tyrosine phosphatase activity (Filippini et al., 1996). PAO, a powerful Tyr phosphatase inhibitor, is widely used in both plant and animal studies (Olivari et al., 2000; Reyes et al., 2006). If the inhibitor would have an effect on secondary metabolism in rolB-transformed cells, it would mean that Tyr dephosphorylation activity is indeed involved in the activator function of the rolB gene product. The vector and rolB calli were cultivated in the presence of the inhibitor. The data presented in Fig. 4 indicate that PAO suppresses the growth of the vector VV culture, but has a positive effect on growth of rolB-transformed calli. When the rolB gene was expressed at a high level (the VB2 culture), PAO partially restores normal callus growth inhibited by rolB transformation. An intermediate effect is observed for the low rolB-expressing VB1 culture, where the negative effect of the rolB-transformation was completely abolished by PAO. Such low expression of rolB, however, does not provide a sufficient signal to activate resveratrol biosynthesis. In contrast, the high-expressing VB2 culture actively produced resveratrol and, in this case, the rise of resveratrol synthesis was diminished by PAO treatment. Similar trends were also observed in the case of the Na-orthovanadate application, although the inhibitor had a less prominent effect (Fig. 4). This inhibitor is less specific than PAO, because Na-orthovanadate could interfere in plant cells not only with Tyr phosphatase activity, but also with the H+-ATPase activity (Giacometti et al., 2004). Summarizing these results, one can conclude that Tyr dephosphorylation process (mediated by expression of the RolB tyrosine phosphatase) is involved in growth and biosynthetic processes of the rolB-transformed cells.

4. Discussion

In the present investigation, we report high resveratrol production by V. amurensis callus culture transformed with the rolB gene of A. rhizogenes. The callus culture accumulates resveratrol up to 3.15% callus dry wt. (Table 4). This value is two orders of magnitude higher than that reported for other resveratrol-producing plant cell cultures (Ku et al., 2005; Tassoni et al., 2005).

Numerous investigations revealed high production rates of many groups of polyphenols by plant cell cultures. For example, rosmarinic acid was found to
accumulate in cultured plant cells as high as 36% of the cell dry wt., representing probably a record yield for secondary metabolites in vitro (Petersen and Simmonds, 2003). However, despite many efforts, no significant amount of resveratrol was obtained in cultured plant cells (Ku et al., 2005; Tassoni et al., 2005). Why were the attempts unsuccessful? Our experiments with MeJA, SA and a nitric oxide donor, sodium nitroprusside (Table 2), supports the results of other authors that high resveratrol production cannot be initiated by simple elicitor treatments. As elicitor treatments, including the treatment by the powerful elicitor MeJA, have a little effect on resveratrol production, the existence of a negative regulatory mechanism that prevents resveratrol biosynthesis may be proposed.

The possibility that a low pool of resveratrol precursors is a cause of the low resveratrol yield seems to be unlikely, because such manipulations as the addition of Phe to the culture medium and cell selection in the presence of Phe fluoro-analoque (PFP) were not effective (Table 3). Moreover, the high resveratrol production observed in the rolB-transformed V. amurensis cells shows that the cellular pool of the precursors was high enough to ensure normal biosynthesis of phenylpropanoid metabolites.

The rolB gene is thought to affect expression of a particular stilbene-biosynthetic gene, because only the production of resveratrol was specifically stimulated in the transformed cells. Recently, the stimulatory effect of the rolB gene on synthesis of shikimate-derived anthraquinone phytoalexins in the R. cordifolia transgenic callus cultures was reported (Bulgakov et al., 2002). MeJA and SA also stimulated anthraquinone production in the R. cordifolia cultures, but these inductors had an additive effect, indicating that RolB and the common defense hormones were involved in parallel signaling pathways (Bulgakov et al., 2002).

The rolB gene is known to encode a protein possessing a tyrosine phosphatase activity (Filippini et al., 1996). The stimulatory effect of the gene on stilbene production was abolished when the rolB-calli were cultivated in the presence of PAO, an inhibitor of Tyr phosphatases (Fig. 4). A similar result was obtained when we used another Tyr phosphatase inhibitor, Na-orthovanadate (Fig. 4). This result indicates that Tyr phosphorylation indeed is involved in the stimulatory function of the rolB gene. Thus, a component of the signaling network that controls stilbene biosynthesis in V. amurensis cells is negatively regulated by Tyr phosphorylation. The rolB signal seems sufficient to overcome this negative signal, thus switching the regulatory balance to pathway activation. In this context, it is interesting to note that although Tyr kinases/phosphatases play a critical role in cell growth and development in animals, none of the typical Tyr kinases have been characterized from higher plants (Gupta and Luan, 2003). The existence of Tyr phosphatases in plants has also been controversial until recently when several members of the protein Tyr phosphatase family were characterized from Arabidopsis (Gupta and Luan, 2003). Recent studies suggest that protein Tyr phosphorylation performs critical functions in plants, regulating activity of MAP kinases, transcription factors and ROS signaling (Laloi et al., 2004). Our results suggest an additional role of Tyr (de) phosphorylation in plant secondary metabolism.

The mechanism by which RolB affects secondary metabolite production is not fully understood. The nuclear localization of RolB, reported by Moriuchi et al. (2004), raises the possibility that RolB might function as a transcriptional coactivator/mediator. Our results indicate the involvement of the rolB-gene signal in the regulation of activity of calcium-dependent protein kinase genes (Bulgakov et al., submitted). Interestingly, the signaling pathway by which the rolB gene activates plant defense reactions does not depend on oxidative signals. Moreover, the gene seems to have an inhibitory effect on ROS production in transformed cells (Bulgakov et al., submitted). This uncommon signaling pathway mediated by the rolB gene is independent of plant defense hormones (MeJA, SA and ethylene) and NADPH oxidase activity (Bulgakov et al., 2002, 2003). The present investigation is the most prominent demonstration of the potential of rolB-gene transformation in cases where the success in secondary metabolite production cannot be achieved by commonly used methods.

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