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Effect of Plant Stilbene Precursors on the Biosynthesis of Resveratrol in *Vitis amurensis* Rupr. Cell Cultures

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Abstract—The biosynthesis of resveratrol after the application of a precursor for biosynthesis, i.e., phenylalanine (Phe), has been studied. The application of Phe has been shown to increase significantly the expression of the phenylalanine–ammonia–lyase (*PAL*) and stilbene synthase (*STS*) genes and enhance the production of resveratrol by 8.5 times. Data on resveratrol production after the addition of Phe and coumaric acid (CA) were compared with known analogs.

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INTRODUCTION

Currently, one of the promising and developing areas in biotechnology is the search for alternative sources to obtain biologically active compounds (BACs). The major part of these compounds is of considerable pharmacological value and, therefore, they are essential components of various drugs. An obstacle for the industrial production of BACs is the deficiency of quickly renewable sources. At present, cell cultures and genetically modified microorganisms are these sources for industrial BAC production. Nevertheless, the experience in cell culture growth shows that they most often contain target compounds in a lower quantity than is needed for efficient production [1]. Therefore, it is currently urgent to increase the content of BACs in plant cells in vitro using various biotechnological approaches.

Grapes contain a number of BACs, which have a favorable effect on the human body. Among such compounds, resveratrol, i.e., 3,5,4'-trihydroxystilbene, is the most known one [1]. Resveratrol is found in many plants, such as mulberries, peanuts, cranberries, and blueberries. Grapes, including *Vitis amurensis* Rupr., are major sources of resveratrol [1]. Resveratrol is known to exert a protective action against some cancer types and have a positive effect on the cardiovascular system, as well as it has a considerable pharmacological potential for the treatment of neurodegenerative diseases [2–5]. Resveratrol is characterized by a high level of antioxidant activity, which exceeds the activity of vitamin E [6, 7]. In addition, there are data on the positive effect of resveratrol on the lifespan of organisms [8, 9]. Biologically active food supplements are currently being developed on the basis of this compound. Resveratrol has a high potential to be used in phytotherapy and pharmacology [5].

To date, no cheap and efficient approach to resveratrol production has been developed. Plants contain this compound in small quantities, and they require a long time for growth. Therefore, resveratrol extracted from plants is an expensive raw material for industrial BAC production. Cell cultures of grapes, provided that they are highly productive, could be an alternative source of resveratrol. However, the resveratrol content in cell cultures is usually known to be lower than that needed for the use of these cultures for industrial stilbene production [1, 10]; therefore, resveratrol biosynthesis must be induced using biotechnological approaches. Selection of the most productive cell lines, variation in the nutrient medium composition, application of precursors, and also exposure of cells to various elicitors are conventional methods for the induction of secondary metabolite synthesis in plant cell cultures. In addition to cell cultures, resveratrol is currently obtained by the addition of a resveratrol precursor to metabolically modified *Escherichia coli*, which expresses a plant stilbene synthase (STS) and 4-coumarat-CoA-ligase (4CL) [11].

The aim of the study is to investigate the effect of plant stilbene precursors on the production of resveratrol in grape cell cultures and to compare the effect with synthesis in genetically modified microorganisms.

MATERIALS AND METHODS

Plant material and cell cultures. Two model systems were used: a cell culture of *V. amurensis* V2 [12] with a low content of resveratrol (no more than 0.01% of the dry weight of cells) and a cell culture of *V. amurensis* transgenic at the *rolB* gene from *Agrobacterium rhizogenes* (VB2 [12]), characterized by an increased level of resveratrol biosynthesis (~0.5–1.5% of the dry weight). Callus culture V2 was obtained in 2002 from a young stem of an adult wild *V. amurensis* plant (*Vitaceae*), which was harvested in the south of Primorskii krai and was determined at the Department of Botany, Institute of Biology and Soil Science, Far Eastern Branch, Russian Academy of Sciences [12]. Transgenic culture VB2 was obtained as a result of a V2 suspension culture transformation with the *A. tumefaciens* GV3101/Pmp90RK strain, which carries a pPCv002-CaMVB vector construction [12]. The *rolB* gene is regulated by the 35S promoter of the 35S CaMV cauliflower mosaic virus [13].

An agar nutrient medium was modified according to Murasige and Skugu with the addition of 0.5 mg/L of 6-benzylaminopurine, 2 mg/L of α-naphthylacetic acid, 0.2 mg/L of thiamine, 0.5 mg/L of nicotinic acid, 0.5 mg/L of pyridoxine, 100 mg/L of mesoinozite, 100 mg/L of peptone, 25 g/l of sucrose, and 7 g/L of agar [14]. Nutrient medium (15 ml) was poured into each test tube with a 22 mm diameter and a height of 200 mm. The interval of subcultivation was 35–40 days in the dark at 24 ± 1°C.

Exposure to phenylalanine. Phenylalanine (Phe) (Pancreac, Spain) was diluted in 5% ethyl alcohol. Phe solutions were added to the nutrient media at concentrations of 0.1, 0.5, and 2 mM under aceptic conditions after autoclaving (the initial Phe solution was 20 mg/mL).

Nucleic acid extraction and obtainment of complementary DNA (cDNA). cDNA was obtained using 1.5 μg of total RNA using a kit for reverse transcription (Sileks M, Russia). For reverse transcription polymerase reaction (RT-PCR), 50 μL of reaction mixture was used which contained one-fold RT buffer; 0.25 mM each of four deoxynucleoside triphosphates; 0.2 μM primer, the sequence of which included 15 deoxythymidine triphosphates (the oligo-$(dT)_{15}$ primer); and 200 units of activity of a reverse transcriptase from the Moloni mouse leukemia virus. A reaction was carried out at 37°C for 1–2 h. Samples of the obtained products (0.5 μL) were then amplified by polymerase chain reaction (PCR).

Quantitative estimation of the *PAL* and *STS* gene expression. For the quantitative estimation of the *PAL* and *STS* gene expression, real-time PCR (RT-PCR) was used. RT-PCR for the genes was performed according to the recommendation described by Giuletti et al. [15]. Gene-specific pairs of primers and TaqMan samples were previously presented in [16]. cDNA was amplified using a PCR kit (Sintol, Russia), and an iQ5 thermocycler with a RT-PCR option was used (Bio-Rad Laboratories Inc., United States) with an optical system of program software (version 2.0). The RT-PCR conditions were previously described in detail in [17–19].

Determination of the stilbene content in tissue samples of *V. ammurensis*. The quantitative and qualitative contents of stilbenes were determined by high-performance liquid chromatography (HPLC) at the Mountain Taiga Station, Far Eastern Branch, Russian Academy of Sciences, where samples of *V. amurensis* tissue, dried according to a previously described technique [20], were transferred. The quantity of resveratrol was determined by comparison with the resveratrol standard (Sigma-Aldrich, United States).

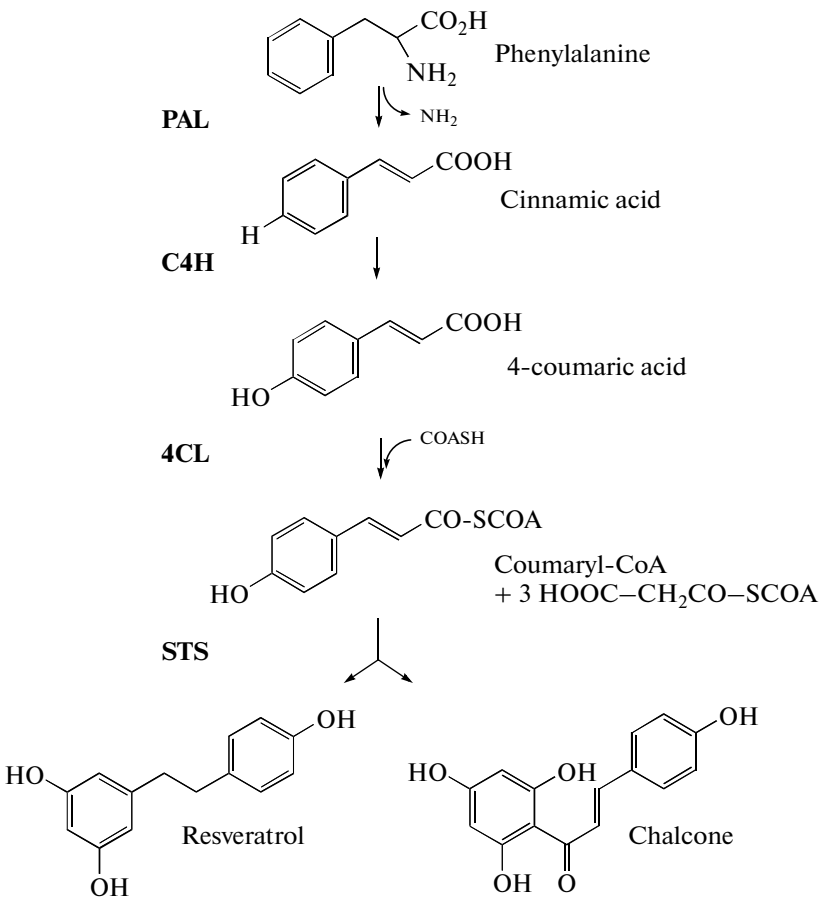
Statistics. The results were processed using the Statistica program (version 9.0). All the data were presented as a mean value ± standard error. The data obtained were verified using Student's paired criterion. A significance level of 0.05 was taken as the minimal value of the statistical difference for all experiments.

RESULTS AND DISCUSSION

Effect of Phe on the growth and biosynthesis of resveratrol in *V. amurensis* cell cultures. Resveratrol was synthesized through the phenylpropanoid route (Fig. 1); therefore, we assumed that an increase in resveratrol production by plant cells may be achieved through the stimulation of the phenylpropanoid pathway at various stages of biosynthesis. Phenylalanine–ammonia–lyase (PAL, EC 4.3.1.5) is the first enzyme, which catalyzes Phe deamination, thus converting it into cinnamic acid; therefore, the presence of Phe is an essential factor for the initiation of resveratrol biosynthesis.

We showed that Phe addition at a concentration of 0.5 and 2 mM into nutrient media inhibited the growth of row biomass in both V2 control culture and *rolB* transgenic VB2 cell culture (Table 1). The resveratrol content in the V2 control culture increased significantly (by a factor of 6.3–14.5) after Phe addition. At the same time, it should be noted that the maximal production of resveratrol by the V2 control culture (6.82 mg/L) was observed after the addition of 0.1 mM Phe. We also showed that the addition of 0.1 and 2 mM Phe causes an insignificant increase in the production of resveratrol in the *rolB* transgenic VB2 culture (17–20.7 mg/L, by a factor of 1.2–1.5, respectively).

Despite the fact that the degree of resveratrol content increase was higher in the V2 culture, the highest production of resveratrol among the analyzed samples was exactly achieved by the VB2 transgenic culture (20.7 mg/L); that is 3.1 times higher than by the V2 culture after Phe addition. This is attributed to the fact that resveratrol production by the VB2 transgenic culture without the addition of plant stilbene precursors



Scheme of resveratrol and chalcone biosynthesis. PAL is phenylalanine–ammonia–lyase, C4H is cinnamate-4-hydroxylase, 4CL is 4-coumarat-CoA-ligase , STS is a stilbene synthase, and CHS is a chalcone synthase.

was initially higher by 17.5 times than that in the control V2 culture.

The final enzyme in the biosynthesis of resveratrol and its derivatives in the phenylpropanoid route is the stilbene synthase (STS, EC 2.3.1.95), which condenses three molecules of malonyl-CoA with one coumaryl-CoA molecule; the final product of this reaction is resveratrol (Fig. 1). Coumaryl-CoA is formed as a result of thioester bond formation between the carboxyl group of CA and coenzyme A by the cinnamate-4-hydrozylase (C4H) enzyme. Therefore, we assumed that the CA addition should activate the last stages of resveratrol biosynthesis, thus enhancing the production of resveratrol by *V. amurensis* cell cultures. The main results of the effect of CA exposure on the production of cell biomass and resveratrol by grape cell cultures were previously obtained in [16].

The expression of the *PAL* and *STS* genes was then analyzed in V2 and VB2 cell cultures exposed to Phe, the protein products of which participate in the biosynthesis of resveratrol.

Phe effect on *rolB, PAL*, and *STS* gene expression. The level of *rolB*, *PAL*, and *STS* gene expression was estimated by RT-PCR in grape cell cultures after the addition of Phe. The expression of the *rolB* gene by a transgenic culture changed insignificantly after Phe addition (Table 2) [16]; therefore, we are inclined to attribute the observed changes in the VB2 culture after the application of a precursor to the effect of the introduced compounds on the expression of resveratrol biosynthesis genes.

We showed that, after Phe application to the control V2 *V. amurensis* culture, the expression of all *PAL* genes tended to increase, but the reliability of the expression increase depended on the quantity of the added Phe and *PAL* gene (Table 2). Thus, we observed a significant 2.5-fold increase in the expression of the *PAL2* gene. The expression of the *PAL3* gene increased significantly (by a factor of 7.8) after the addition of 0.5 mM Phe into nutrient media of the V2 culture (Table 2).

The expression of nine out of ten *STS* genes also tends to increase after Phe addition into the nutrient media of the V2 control culture that depended on the quantity of Phe added and the *STS* gene. A concentration of 0.1 M Phe increased significantly the expression of the *STS* and *STS2* genes (by a factor of 3.3–5, respectively) (Table 3). The addition of 0.5 mM Phe

Table 1. Effect of Phe on the growth and biosynthetic production of V2 and VB2 *V. amurensis* cell cultures

Production	Phe, mM							
	V2				VB2			
	0	0.1	0.5	2	0	0.1	0.5	2
Fresh biomass, g/L	208.9 ± 21.1	204.3 ± 26.0	119.2 ± 4.9**	110.7 ± 5.4**	75.9 ± 16.4	65.4 ± 17.3	49.0 ± 4.4	38.5 ± 4.0*
Dry biomass, g/L	8.3 ± 0.9	5.2 ± 0.6	8.1 ± 0.9	6.7 ± 0.7	5.5	5.7	2.8	6.7
Resveratrol, % of the dry weight	0.009 ± 0.001	0.131 ± 0.071*	0.071 ± 0.03*	0.057 ± 0.024*	0.254 ± 0.069	0.298 ± 0.071	0.339 ± 0.063	0.309 ± 0.132
Total production of resveratrol, mg/L	0.8 ± 0.2	6.8 ± 1.5*	5.8 ± 1.4*	3.8 ± 1.2	14.0 ± 2.2	17.0 ± 3.5	9.5 ± 1.9	20.7 ± 5.3

Note: to Tables 1–3: V2k and VB2k is the expression of *rolB* and *PAL* in V2 and VB2 cell cultures without Phe application; V2-0.1 and VB2-0.1 are expression in V2 and VB2 cell cultures after the addition of 0.1 mM Phe; V2-0.5 and VB2-0.5 are expression in V2 and VB2 cell cultures after the addition of 0.5 mM Phe; V2-2 and VB2-2 are expression in V2 and VB2 cell cultures after the addition of 2 mM Phe; * is $p < 0.05$; ** is $p < 0.01$; a comparision was made with the *rolB* and *PAL* gene expression in V2 and VB2 cell cultures without Phe application.

Table 2. Data on the expression of *rolB* and *PAL* genes in V2 and VB2 cultures after Phe application obtained by RT PCR

Gene	Phe							
	V2k	V2-0.1	V2-0.5	V2-2	VB2k	VB2-0.1	VB2-0.5	VB2-2
rolB	0	0	0	0	0.55 ± 0.21	0.56 ± 0.18	0.55 ± 0.22	0.63 ± 0.23
PAL1	0.11 ± 0.04	0.10 ± 0.03	0.08 ± 0.03	0.26 ± 0.10	0.53 ± 0.20	0.43 ± 0.21	0.36 ± 0.11	0.36 ± 0.10
PAL2	0.31 ± 0.09	0.68 ± 0.19	0.58 ± 0.13	0.82 ± 0.10*	0.42 ± 0.11	0.31 ± 0.08	0.37 ± 0.06	0.36 ± 0.14
PAL3	0.10 ± 0.07	0.09 ± 0.05	0.77 ± 0.08**	0.51 ± 0.21	0.28 ± 0.17	0.24 ± 0.11	0.24 ± 0.14	0.25 ± 0.13
PAL4	0.09 ± 0.04	0.17 ± 0.11	0.13 ± 0.05	0.21 ± 0.14	0.64 ± 0.20	0.24 ± 0.14	0.22 ± 0.15	0.23 ± 0.13
PAL5	0.11 ± 0.06	0.12 ± 0.05	0.11 ± 0.05	0.33 ± 0.09	0.81 ± 0.19	0.36 ± 0.17	0.35 ± 0.16	0.29 ± 0.20

increased significantly the expression of the *STS1, STS2, STS3, STS4, STS6, STS8*, and *STS10* genes (by a factor of 2.4–92.2). A concentration of 2 mM also increased significantly the expression of the *STS4, STS6, STS8*, and *STS10* genes.

The expression of the *PAL* genes tends to decrease after Phe addition in VB2 transgenic cultures (Table 2). The expression of the *STS* genes in a VB2 cell culture after Phe addition was found to be within the limits of experimental error, except the *STS7* and *STS5* genes, the expression of which increased significantly after the addition of 0.5 mM Phe (by a factor of 3.9–4.1, respectively) (Table 3).

It is possible that the increase in the production of resveratrol in the control culture after Phe addition was due to the direct activation of the phenylpropanoid route. An indication of this is the significant activation of the expression of several genes from the *PAL* family (*PAL2* and *PAL3*). A product of *PAL* gene expression is an enzyme, which indirectly deaminates Phe into cinnamic acid (Fig. 1), thus increasing the quantity of the substrate for a further cascade of reactions of the phenylpropanoid route that finally leads to an increase in the resveratrol quantity. In a transgenic VB2 culture, the phenylpropanoid route is normally in an active state, because a VB2 culture differs by an increased resveratrol content (Table 1) [12, 14] and *PAL* and *STS* gene expression (Tables 2, 3) [14]; therefore, Phe addition leads to an insignificant increase in the production of resveratrol relative to the increase in the V2 control culture.

The main results on the effects of CA exposure on the expression of *PAL* and *STS* genes were previously obtained and presented in [16]. The expression of the *PAL* genes remained unchanged after the addition of CA into the nutrient media of the V2 and VB2 *V. amurensis* cell cultures or tended to decrease with increasing CA concentration, and the expression of two out of ten *STS* genes increased significantly [16]. Therefore, CA addition does not lead to the activation of the phenylpropanoid route accompanied by an increase in the expression of *PAL* genes as was observed after Phe addition because the expression of four out of five *PAL* genes was inhibited significantly [16]. We assume that

Table 3. Data on the *STS* gene expression in V2 and VB2 cell cultures after Phe addition obtained by RT PCR

Gene	Phe							
	V2k	V2-0.1	V2-0.5	V2-2	VB2k	VB2-0.1	VB2-0.5	VB2-2
STS1	0.14 ± 0.04	0.41 ± 0.12	0.67 ± 0.19*	0.40 ± 0.12	0.11 ± 0.02	0.10 ± 0.03	0.20 ± 0.06	0.15 ± 0.04
STS2	0.07 ± 0.02	0.34 ± 0.09*	0.79 ± 0.17**	0.37 ± 0.13	0.12 ± 0.04	0.13 ± 0.05	0.23 ± 0.09	0.24 ± 0.09
STS3	0.05 ± 0.02	0.39 ± 0.21	0.44 ± 0.09**	0.37 ± 0.20	0.19 ± 0.04	0.18 ± 0.04	0.38 ± 0.14	0.32 ± 0.10
STS4	0.01 ± 0.01	0.14 ± 0.02**	0.41 ± 0.10**	0.44 ± 0.25*	0.15 ± 0.08	0.16 ± 0.10	0.48 ± 0.25	0.15 ± 0.06
STS5	0.12 ± 0.07	0.21 ± 0.04	0.23 ± 0.09	0.20 ± 0.6	0.16 ± 0.03	0.17 ± 0.04	0.67 ± 0.19*	0.41 ± 0.09*
STS6	0.14 ± 0.04	0.37 ± 0.21	0.36 ± 0.14*	0.61 ± 0.19**	0.35 ± 0.09	0.28 ± 0.10	0.44 ± 0.13	0.43 ± 0.18
STS7	0.14 ± 0.08	0.11 ± 0.05	0.12 ± 0.06	0.16 ± 0.05	0.19 ± 0.06	0.14 ± 0.04	0.73 ± 0.17*	0.22 ± 0.08
STS8	0.02 ± 0.01	0.23 ± 0.15	0.19 ± 0.05*	0.45 ± 0.14*	0.09 ± 0.02	0.20 ± 0.12	0.36 ± 0.16	0.22 ± 0.11
STS9	0.03 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	0.31 ± 0.19	0.13 ± 0.04	0.29 ± 0.14	0.28 ± 0.09	0.35 ± 0.14
STS10	0.01 ± 0.01	0.22 ± 0.16	0.31 ± 0.19	0.22 ± 0.09*	0.17 ± 0.07	0.15 ± 0.06	0.33 ± 0.18	0.22 ± 0.07

CA application in nutrient media increased the resveratrol content via a selective action on the expression of certain *STS* genes, i.e., through the activation of the last stage in resveratrol biosynthesis (of only certain reactions of the phenylpropanoid route).

Comparison of the efficiency of stilbene conversion into resveratrol in cells of plants and microorganisms. After the addition of 0.1 mM Phe to the control V2 *V. amurensis* grape cell culture, resveratrol production increased by 8.5 times (Table 1), and after the addition of 0.1 mM CA the production of resveratrol increased by 16 times [16]. These results indicate that the activation of the last stages of the phenylpropanoid route has a stronger effect on the biosynthesis of resveratrol in cell cultures. Thus, we showed that the CA efficiency in the resveratrol biosynthesis increase is almost two times higher than that associated with the addition of Phe.

Results have been obtained recently which are of interest for the biotechnological production of resveratrol: they showed that, after CA application, in metabolically modified *E. coli*, which carries transfected *4CL* and *STS* genes from plants in its plasmid, the yield of resveratrol comprised about 100 mg/L [11]. We decided to compare the level of Phe and CA conversion into resveratrol in *V. amurensis* cell cultures and in metabolically modified *E. coli*. As a result, after the introduction of 2 mM Phe or 0.5 mM CA [16] into a *V. amurensis* cell culture, the highest level of resveratrol production by grape cells was found to be 0.09 (20.7 mg/L) and 0.16 mM (36.8 mg/L). It should be noted that, compared to microorganisms, in the experiment, the concentration of resveratrol was 0.06 (14 mg/L) (Table 1) and 0.11 mM (25.8 mg/L) [16] in the norm in the *rolB*-transgenic cell culture, i.e., resveratrol production after Phe application increased by 0.03 mM (6 mg/L); after CA addition, by 0.05 mM (11 mg/L). Thus, the efficiency of precursor conversion into resveratrol by grape cells comprised no more than 10%. Metabolically modified *E. coli* transforms 1 mM CA into 0.44 mM resveratrol [11]; therefore, the efficiency of CA conversion into resveratrol in bacteria comprises 44%. Hence, *V. amurensis* grape cells convert precursors into resveratrol four times less effectively than cells of metabolically modified *E. coli*. The efficiency of resveratrol conversion from the applied precursors in *V. amurensis* cell cultures is less, possibly due to the fact that plant cells carry enzymes for both its biosynthesis and degradation to low-molecular-weight compounds or oligomerization into high-molecular-weight derivatives [21, 22].

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