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ORIGINAL PAPER

Influence of calcium influx induced by the calcium ionophore, A23187, on resveratrol content and the expression of *CDPK* and *STS* genes in the cell cultures of *Vitis amurensis*

K. V. Kiselev · O. A. Shumakova · A. Y. Manyakhin · A. N. Mazeika

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Abstract The present study examines the effect of calcium influx induced by the calcium ionophore (CI) on the biosynthesis of resveratrol and the expression of stilbene synthase (*STS*) and calcium-dependent protein kinase (*CDPK*) genes in cell cultures of *Vitis amurensis*, which have different levels of resveratrol production. The present study utilized the control cell culture V2 of *V. amurensis*, which contains no more than 0.02 % dry weight (DW) of resveratrol, in addition to *rolB* transgenic cell cultures VB1 and VB2, which have increased resveratrol contents (0.1–0.8 % DW). Treatment with the CI at a 1 μM concentration significantly increased *STS* gene expression (6 of 10 analyzed *STS* genes) and resveratrol production in the control V2 cell culture by fourfold; however, use of the CI at 10 μM significantly decreased resveratrol production by 2–4 fold in all cell cultures tested. In the control V2 grape cell culture, treatment with the CI increased expression of all of the *CDPK* genes except *VaCDPK1a* and *VaCDPK3a*. In the *rolB* transgenic VB2 grape cell culture treated with the CI, we detected alterations in expression of several *CDPK* genes, but these changes in gene expression were not significant. Our results indicated that treatment with 1 μM of the CI increased resveratrol content and production in control grape cells by selectively increasing the expression of *STS* genes. Conversely, the CI treatment did not significantly increase resveratrol content and production, or the expression of *CDPK* or *STS* genes in the *rolB* transgenic cells. Likely, untreated VB2 cells have increased concentrations of cytoplasmic calcium, and therefore, treatment with the CI did not significantly change *CDPK* expression. These results suggest that the *rolB* gene has an important role in the regulation of calcium-dependent transduction pathways in transformed cells.

Keywords Calcium ionophore · Callus culture · CDPK · Resveratrol · *STS* · *Vitis amurensis*

Abbreviations

CDPK	Calcium-dependent protein kinase
CI	Calcium ionophore
DW	Dry weight
STS	Stilbene synthase

Introduction

The polyphenol, *trans*-resveratrol (3,5,4′ trihydroxy-trans-stilbene), is one of the best-known plant secondary metabolites (Kiselev 2011). *Trans*-resveratrol is a beneficial molecule to human health, which explains its appeal for many research groups. *Trans*-resveratrol has been associated with the "French paradox" because its daily

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K. V. Kiselev (✉) · O. A. Shumakova
Laboratory of Biotechnology, Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, 690022 Vladivostok, Russia
e-mail: kiselev@biosoil.ru

K. V. Kiselev · O. A. Shumakova · A. N. Mazeika
Department of Biochemistry and Biotechnology, Far Eastern Federal University, 690090 Vladivostok, Russia

A. Y. Manyakhin
Mountain-Taiga Station, Far Eastern Branch of the Russian Academy of Sciences, Posyolok Gornotaezhnoe, Primorsky Krai, 692533 Ussuriisky Region, Russia

consumption in forms such as red wine (Jeandet et al. 1995; Stervbo et al. 2007) helps to prevent the development of cardiovascular diseases (Gronbaek et al. 2000). Resveratrol exerts its protective actions through the regulation of nitric oxide production (Giovannini et al. 2001), and it prevents thrombocyte aggregation by regulating the synthesis of eicosanoids (Olas et al. 2001). In addition to red wine, other foods may represent valuable sources of *trans*-resveratrol such as Itadori tea, peanuts, pistachios, peanut butter and chocolate (Burns et al. 2002; Tokusoglu et al. 2005; Counet et al. 2006; Hurst et al. 2008). The plant physiology of *trans*-resveratrol is interesting. *Trans*-resveratrol exhibits antifungal activity (Jeandet et al. 2002; Adrian and Jeandet 2006). For instance, in the leaves and berries, it acts as a phytoalexin that is produced in response to stresses such as wounding or pathogen attack (Langcake and Pryce 1976). In addition, some plants have been genetically modified to produce *trans*-resveratrol, including tobacco, tomato, and poplar, for the assessment of its potential role in health promotion and disease control (Giorcelli et al. 2004; Halls and Yu 2008; Delaunois et al. 2009).

Because *trans*-resveratrol has potential implications in human health and plant protection (Chong et al. 2009; Donnez et al. 2009; Fan et al. 2010; Kiselev 2011), it is important to understand how the resveratrol content is controlled in plants. It is known that calcium plays an important role in plant defense reactions, such as phytoalexin biosynthesis (Lecourieux et al. 2006; Ramani and Chelliah 2007). For example, it has been demonstrated that extracellular calcium availability, calcium influx and intracellular calcium mobilization are necessary for an increase in the yields of secondary metabolites (Preisig and Moreau 1994; Dmitriev et al. 1996; Ramani and Chelliah 2007). These reports demonstrate that elevated levels of cytoplasmic calcium play an important role in phytoalexin production. The inhibition of Ca^{2+} channels, calcium chelation or a block of intracellular calcium flux by means of special agents considerably reduced phytoalexin accumulation. However, contradictory reports have also appeared in the literature, indicating that calcium could negatively affect the production of some secondary metabolites (Cacho et al. 1995; Ning et al. 1998; Sanchez-Sampedro et al. 2005).

In our previous paper, we demonstrated that the high resveratrol content in the transgenic by *rolB* gene from *Agrobacterium rhizogenes* VB2 cell culture decreased after treatment with the calcium channel blockers $LaCl_3$, verapamil, and niflumic acid (Dubrovina et al. 2009). Additionally, expression of the calcium-dependent protein kinase (*CDPK*) genes significantly changed in the *rolB* transgenic cell cultures compared with the control cell cultures. CDPKs are implicated as the major primary sensors of Ca^{2+} flux in plants and play an essential role in plant defense responses, embryogenesis, germination, and seedling growth (Lecourieux et al. 2006; Kiselev et al. 2008; Jaworski et al. 2011). The mechanism by which the plant secondary metabolism is stimulated in *rol* transformed cell cultures is unknown (Dubrovina et al. 2009). A better understanding of this mechanism has fundamental and applied interests. At present, little is known about the properties and functions of the RolB protein. RolB causes significant morphological and biochemical changes in transgenic plant cells (Altamura et al. 1994). An important recent finding is that RolB has nuclear localization and can interact with protein 14-3-3 and modulate its activity (Moriuchi et al. 2004). In the current study, we investigated the influence of calcium influx induced by the calcium ionophore (CI), A23187, on the resveratrol content and the expression of *STS* and *CDPK* genes in control and *rolB* transgenic cell cultures of *V. amurensis*.

Materials and methods

Vitis amurensis cell cultures

The V2 callus culture of the wild-growing grape *Vitis amurensis* Rupr. (Vitaceae) was established in 2002, as described previously (Kiselev et al. 2007). The *rolB* transgenic *V. amurensis* callus cultures (designated VB1 and VB2) were obtained in 2004 by transformation of the V2 cell suspension with the *Agrobacterium tumefaciens* strain GV3101 containing the binary plasmid vector pPCV002-CaMVB/pMP90RK, as described (Kiselev et al. 2007). In the pPCV002-CaMVB construction, the *rolB* gene was under the control of the cauliflower mosaic virus 35S promoter. High levels of *rolB* expression in the transformed callus culture of *V. amurensis* resulted in more than a 100-fold increase in resveratrol production, up to 3.15 % dry weight (DW), compared to that of the control. Unfortunately, the high level of the resveratrol content in the *rolB* transgenic callus culture of *V. amurensis* was not stable during long-term cultivation. The high resveratrol content decreased to 0.2–1 % DW after 3 years of cultivation; however, it is still significantly higher than the control cell culture V2. In the current study, we repeated the experiments using calcium blockers in *rolB* transgenic cell cultures. As a control, we used the non-transgenic cell culture V2. The influence of the calcium blockers on resveratrol content in the V2 cell culture is identical to the influence on resveratrol content in the cell culture VV, described in Dubrovina et al. (2009). Therefore, we used the control V2 and the *rolB* transgenic cell culture VB2 of *V. amurensis* as the model system for the investigation of the influence of the CI on resveratrol metabolism.

The V2 and VB2 callus cultures were cultivated with 35-day subculture intervals in the dark at 24–25 °C in test tubes with 15 ml of $W_{B/A}$ media (Kiselev et al. 2009a) with 0.7 % agar. Inoculum biomass was 0.21 g (14 g/l). The reagents for the cell culture medium were purchased from Sigma Chemical Co (MO, USA) and Serva Feinbiochemica & Co (Heidelberg, Germany). Samples were harvested from 35-day cultures (linear growth phase and the highest resveratrol content), weighed and used for quantitative real-time PCR. The calli were dried under hot air flow (50 °C for 3 h) and used for resveratrol determination.

Total RNA isolation and quantitative real-time PCR

Total RNA isolation was performed using the CTAB-based extraction protocol developed by Bekesiova et al. (1999). Briefly, 0.3 g of fresh cells were homogenized in 1.3 ml of buffer containing 2 % mercaptoethanol (AppliChem, Germany); 100 mM Tris (Biomedicals, France), pH 8.0; 2 M sodium chloride (NaCl, Sigma, USA); 25 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA, Biomedicals, Germany), pH 8.0; 2 % N-Cetyl-N,N,N-trimethylammonium bromide (CTAB, Panreac, Spain); and 2 % polyvinylpyrrolidon K30 (PVP, AppliChem, Germany). The mixture was incubated for 5 min at 65 °C in a water bath. After the above incubation, 500 µl of chloroform was added. The samples were then centrifuged at full speed (≥10,000*g*) for 15 min (5415R, Eppendorf, Germany). Approximately 1 ml of the water phase was mixed with 200 µl of 10 M lithium chloride (LiCl, Panreac, Spain) and incubated at +4 °C overnight. After the above incubation, the samples were centrifuged at full speed for 20 min. The pellets were dried at +37 °C for 40 min and then dissolved in 100 µl of distilled water. Next, 300 µl ethanol was added, and the samples were then incubated at −20 °C overnight. After the above incubation, the samples were centrifuged at full speed for 20 min (Kiselev et al. 2009a, 2011a). The pellets were dried at +37 °C for 20 min and finally dissolved in 150 µl of distilled water.

Complementary DNAs were synthesized, as described previously (Dubrovina et al. 2009; Kiselev et al. 2009b). Previously, by sequencing of cloned RT-PCR products at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEBRAS using ABI 3130 Genetic Analyzers (Applied Biosystems, Foster City, USA), as described (Kiselev et al. 2011b), we obtained cDNA sequences of 10 *CDPK* and 10 *STS* genes of *V. amurensis*. The gene-specific primer pairs and TaqMan-probes for the *rolB*, *CDPK1a*, *1d*, *1e*, *1L*, 2a, *3a*, *STS1-STS10*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and *Actin1* genes of *V. amurensis* are presented in Kiselev et al. (2009a, 2010a) and in the Electronic supplementary material Table 1.

For TaqMan real-time PCR, cDNA was amplified, as described (Kiselev et al. 2010b), 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 (15 ng) cDNA sample, and 0.25 µM of each primer and probe (real-time PCR Kit, Syntol, Russia). The amplification conditions consisted of one 2 min cycle at 95 °C followed by 50 cycles of 10 s at 95 °C and 25 s at 62 °C. The TaqMan PCR assays were performed in an iCycler thermocycler supplied with iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Inc., USA), and the data were analyzed with the iQ5 Optical system software v.2.0 according to the manufacturer's instructions (Dubrovina et al. 2009). 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). *V. amurensis* actin and *GAPDH* genes (GenBank ac. no. AY907207; GU585870) were used as endogenous controls to normalize variance in the quality and the amount of cDNA used in each real-time RT-PCR experiment. These genes were selected in previous studies as the most relevant reference genes for real-time PCR of *Vitis* genes (Reid et al. 2006). 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 the *V. amurensis* actin and *GAPDH* genes was labeled with a FAM reporter dye at the 5′-end and a RTQ-1 quencher dye at the 3′-end, and the TaqMan probe for *CDPK* and *STS* genes was labeled with a 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 with *V. amurensis* actin as the endogenous control and five independent experiments with *GAPDH* as the endogenous control (Shumakova et al. 2011).

Frequency analysis of RT-PCR products (FAPP)

The PCR products of the *CDPK* genes obtained with degenerate primers (Dubrovina et al. 2009) were subcloned into a pTZ57R/T plasmid using the InsT/Aclone PCR Product Cloning Kit (FERMENTAS, Vilnius, Lithuania). The clones were amplified with M13 primers and sequenced. The amino acid sequences of the CDPK fragments of *V. amurensis* were deduced from the nucleotide sequences with the Gene Runner 3.05 program and compared with the known CDPK sequences of other organisms using the BioEdit 7.0.8 and BLAST software programs. Overall, 289, 325, and 306 *CDPK* clones of the V2, VB1, and VB2 cultures, respectively, were sequenced. All sequences were numbered in the order of sequencing. These results and data on the total *CDPK* expression, assayed with the degenerate primers, were used to estimate the expression level (in relative units) for each of the

CDPK genes. The relative expression was estimated to = the total *CDPK* expression normalized to the expression of the actin gene × the % of the clones of each *CDPK* transcript variant/100 (Kiselev and Dubrovina 2010).

CI treatments

The CI was obtained from Sigma (Calcium ionophore: A23187, St. Louis, USA). Sterile solutions of the CI (as ethanol solutions) were added to the autoclaved media aseptically in the desired concentrations (1 and 10 μM).

High-performance liquid chromatography

V. amurensis cell cultures were analyzed for the presence of stilbenes by HPLC analysis as described (Dubrovina et al. 2010).

Statistical analysis

Statistical analysis employed the Statistica 9.1 program. The data are presented as mean ± standard error (SEM) and were tested by paired Student's *t* test. The 0.05 level was selected as the point of minimal statistical significance in all analyses.

Results and discussion

Effects of the CI, A23187, on growth and resveratrol accumulation in callus cultures of *V. amurensis*

Figure 1a demonstrates that treatment with the CI at a concentration of 10 μM dramatically decreased the growth of the V2, VB1, and VB2 cell cultures of *V. amurensis* by 3–11 fold, but the calli were still growing and accumulating fresh biomass (from inoculum of 14 to 29–35 g/l). This finding indicates that the cells remained alive during the 35 days of CI treatment. At a concentration of 1 μM, the CI also significantly decreased the growth of the VB1 and VB2 cell cultures of *V. amurensis* by 1.4–2.9 fold. While 1 μM of the CI also decreased the growth of the V2 cell culture by 1.2 fold, this decrease was not significant.

Use of the CI at a concentration of 1 μM significantly increased the content and production of resveratrol by 3–4 fold in the V2 cell culture (Fig. 1b, c). The highest resveratrol content and production was detected in the VB2 cell culture that was not treated with the CI (0.46 % DW or 17.8 mg/l, respectively). Cultivation in the presence of the CI significantly decreased the resveratrol content and production in both the VB1 and VB2 cell cultures by 1.2–3 fold (Fig. 1b, c). Furthermore, increasing the calcium concentration in the nutrient media also decreased the resveratrol content and production in the VB1 and VB2 cell cultures, although this decrease was not significant (Fig. 1b, c).

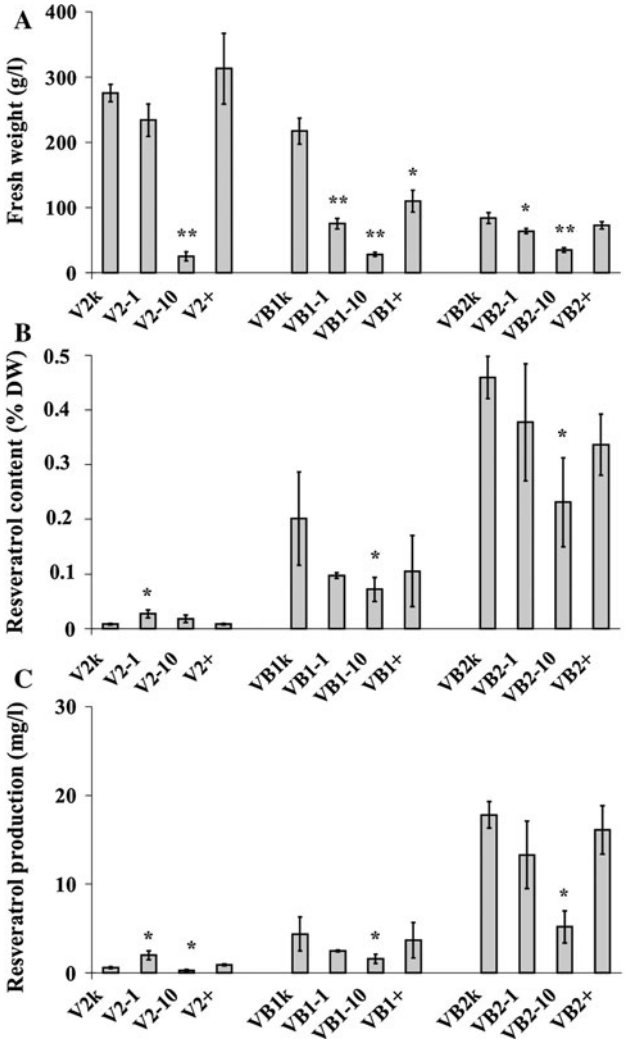

Fig. 1 Biomass accumulation (**a**), resveratrol content (**b**), and resveratrol production (**c**) in V2 VB1, and VB2 callus cultures of *V. amurensis* treated with the CI. V2k, V2 cell culture growing on standard media; V2-1, on media treated with 1 μM CI; V2-10, on media with 10 μM CI; V2+, on media with a threefold higher calcium concentration; VB1k, VB1 cell culture growing on standard media; VB1-1, on media with 1 μM CI; VB1-10, on media with 10 μM CI; VB1+, on media with a threefold higher calcium concentration. VB2k, VB2 cell culture growing on standard media; VB2-1, on media with 1 μM CI; VB2-10, on media with 10 μM CI; VB2+, on media with a threefold higher calcium concentration. These data are presented as the mean ± SEM and were obtained from three independent experiments; $*P < 0.05$, $**P < 0.01$ versus values of the V2, VB1 or VB2 cultures that were not treated with the CI

Expression of the *rolB* and *STS* genes

The expression levels of the *rolB* and *VaSTS* genes were estimated by quantitative real-time PCR (Fig. 2). The level

of *rolB* gene expression in the CI-treated cells of *V. amurensis* was not considerably altered when compared to the untreated cells (Fig. 2). Real-time PCR analysis has shown that after a 5-year culture period, the VB2 cells have higher levels of *rolB* gene expression than the VB1 cells.

Stilbenes, including resveratrol, are synthesized via the phenylpropanoid pathway (Langcake and Pryce 1977). Stilbene synthase or resveratrol synthase (STS, EC 2.3.1.95) condenses three molecules of malonyl-CoA and one molecule of cumaryl-CoA to form resveratrol (Rupprich et al. 1980). The *STS* genes exist as a multigene family in most stilbenoid-producing species, including grape, peanut, and pine (Fliegmann et al. 1992; Sparvoli et al. 1994; Kiselev et al. 2009a). Analyses of the first drafts of the *V. vinifera* genome sequence confirmed that it contained a large number of all of the multigene families, with an estimated 21–43 *STS* genes (Jaillon et al. 2007; Velasco et al. 2007). It has been suggested that the STS family in *V. amurensis* consists of at least 10 *STS* genes, which differ in nucleotide sequence and expression patterns (Kiselev et al. 2009a, 2010a; Kiselev and Dubrovina 2010).

In the control V2 calli treated with the CI, the expression of *VaSTS1-VaSTS6* was significantly increased by 3–6 fold compared to the untreated cells, while the expression of *VaSTS7-VaSTS10* was not significantly changed (Fig. 2). The greatest increase in expression of the described *STS* genes was detected in the V2 cell culture treated with 1 μM of the CI (Fig. 2). Cultivation of the VB1 and VB2 cell cultures in the presence of the CI or in the nutrient media with 3 × the typical calcium chloride concentration did not significantly increase *STS* expression (Fig. 2).

Expression of the *CDPK* genes

CDPK expression estimated by FAPP

CDPK amplicons of the expected size were obtained by performing PCR on the cDNA isolated from the V2, VB1, and VB2 cultures using degenerate primers (Fig. 3). In the V2, VB1, and VB2 cells treated with the CI, *CDPK* expression was higher than in the untreated control; however, the difference was not significant (Fig. 3). In the VB1 and VB2 cells, the total *CDPK* expression was higher than in the control, as was described previously (Dubrovina et al. 2009); however, the difference was not significant (Fig. 3). The *CDPK* amplicons were cloned and sequenced. Using the BioEdit 7.0.8 program, the *V. amurensis CDPK* sequences were subdivided into three subfamilies (*VaCDPK1*, *VaCDPK2* and *VaCDPK3*) corresponding to 3 of the 4 *CDPK* subfamilies found in *Agrobacterium thaliana* (Cheng et al. 2002). The *VaCPK1a*, *VaCPK1b*, *VaCPK1c*, *VaCPK1d*, *VaCPK1e* and *VaCPK1L* transcripts had the highest degree of similarity to subfamily II of the *A. thaliana CDPK* genes (Dubrovina et al. 2009). The sequenced fragment of the *VaCPK2a* transcript was most similar to subfamily III, while the *VaCPK3a* and *VaCPK3b* transcripts were most similar to subfamily I of the *A. thaliana CDPK* genes (Dubrovina et al. 2009). The sequences of *V. amurensis CDPK* kinase domains, which were restricted by degenerate primers, were translated into their respective amino acid sequences. *CDPK* transcripts whose amino acid sequences differed by more than one residue were placed into separate genes, as described previously (Dubrovina et al. 2009).

Table 1 was compiled based on the number of various *V. amurensis CDPK* clones. *CDPK* expression was estimated based on the number of clones of each transcript (Table 1) and the total *CDPK* expression (Fig. 3). The *VaCPK1a*, *VaCPK1d*, *VaCPK1L1*, *VaCPK1L2*, *VaCPK2a* and *VaCPK3a* transcripts were expressed in the V2 cell culture (Table 1). The CI treatment resulted in an increase in expression in 4 of the 6 *CDPK* genes expressed in the untreated V2 cells, while the expression of *VaCPK1a* and *VaCPK3a* decreased by 1.2–1.3 fold.

In cDNA samples isolated from the VB1 callus culture compared with the V2 cell culture, 5 new *CDPK* transcripts (*VaCDPK1b*, *VaCDPK1c*, *VaCDPK1e*, *VaCDPK3aS* and *VaCDPK3b*) were detected, with the *VaCDPK1a*, *VaCDPK1d*, *VaCDPK1L1*, *VaCDPK1L2*, *VaCDPK2a* and *VaCDPK3a* transcripts found in the control calli (Table 1). Four new *CDPK* transcripts (*VaCDPK1e*, *VaCDPK1aS*, *VaCDPK3b* and *VaCDPK3aS*) were found in cDNA samples isolated from the VB2 callus culture compare with the V2 cell culture.

In the V2 cell cultures treated with 1 μM of the CI, we detected increasing levels of *CDPK* expression of five and decreasing levels of one out of the six expressed *CDPK* genes, while five out of eleven and five out of ten expressed *CDPK* genes increased their expression level in the *rolB* transgenic VB1 and VB2 cell cultures, respectively (Table 1). Furthermore, in the *rolB* transgenic cell cultures treated with 1 μM of the CI, we detected decreases in the expression of *VaCDPK1c*, *VaCDPK1d*, *VaCDPK1L1*, *VaCDPK1L2*, *VaCDPK2a*, *VaCDPK3a* and *VaCDPK3aS* (for VB1 cell culture). In the V2, VB1, and VB2 cell cultures treated with 10 μM of the CI two out of six, four out of eleven, and four out of ten *CDPK* genes decreased their expression level, respectively (Table 1).

In cDNA from the *rolB* transgenic cultures, we found short *CDPK* transcripts whose Ser/Thr kinase domains lacked either 48, 63 or 114 bp without a frameshift (*VaCPK1aS* and *VaCPK3aS*). Previously, we described such modifications of the Ser/Thr kinase domains of the grape *CDPKs* in Dubrovina et al. 2009. Currently, we describe the same transcripts. The *VaCPK1aS* and

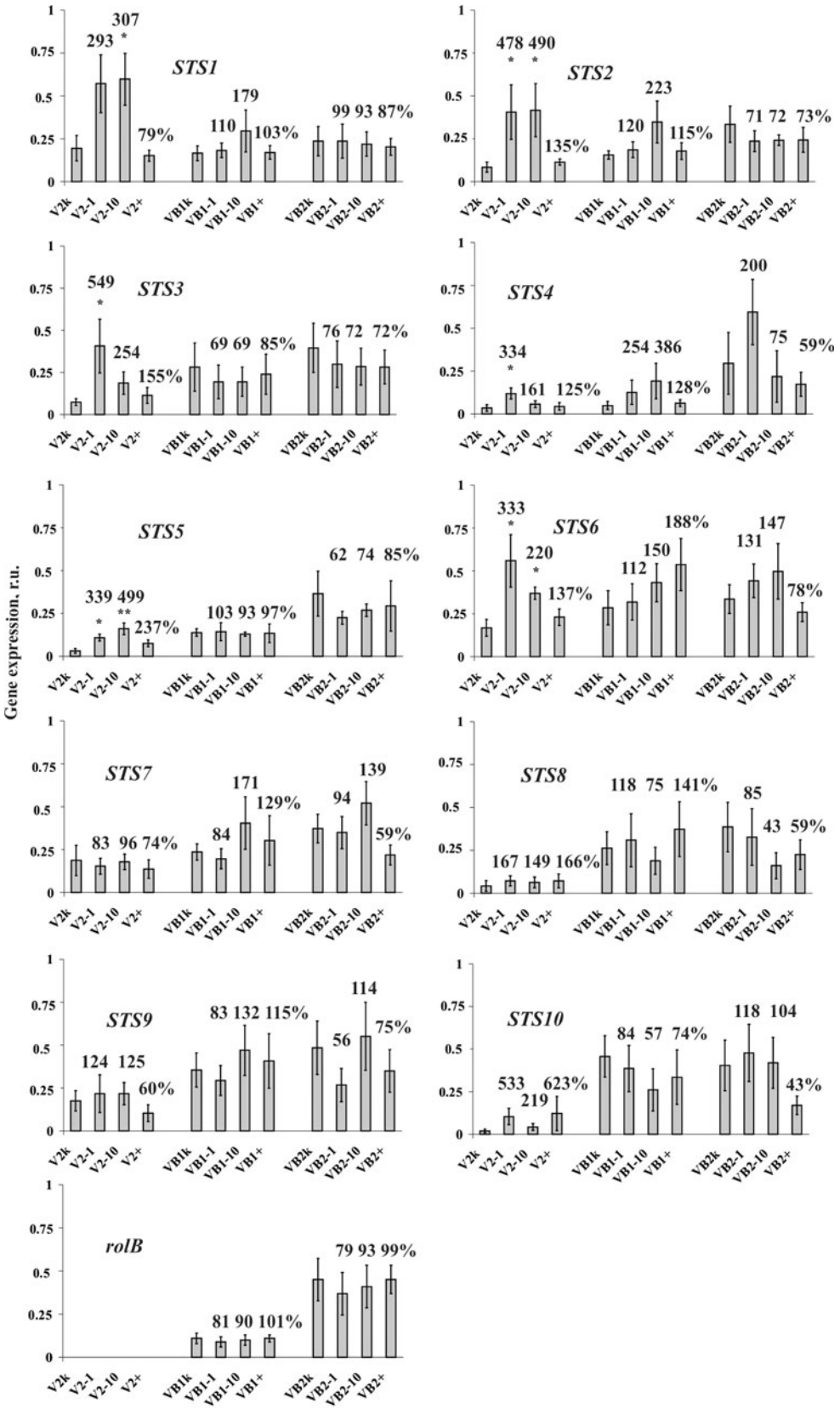

Fig. 2 *rolB* and *VaSTS* expression levels detected by real-time PCR in *V. amurensis* cell cultures V2, VB1, and VB2 treated with the CI. Presented numerals in the figure are percentages from V2, VB1 or VB2 without the CI treatment; *r.u.* relative units. The data are presented as the mean ± SEM and were obtained from three independent experiments; $*P < 0.05$, $**P < 0.01$ versus values of the V2, VB1 or VB2 culture without CI

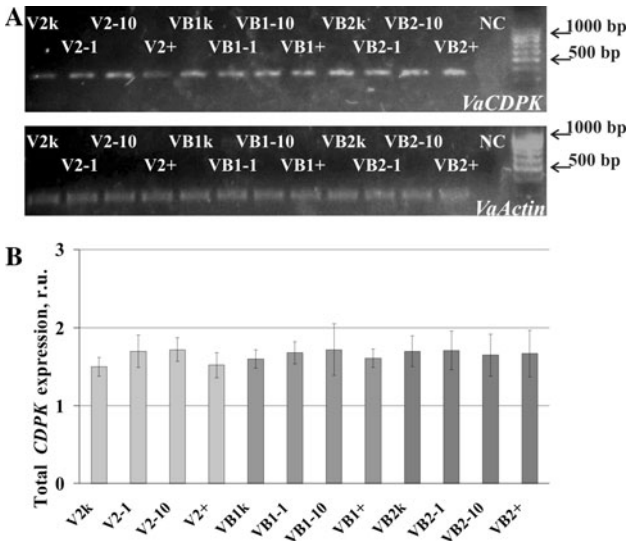

Fig. 3 Expression of *VaCDPKs* in the V2, VB1, and VB2 grape cell cultures. **a** Electrophoretic separation of *V. amurensis* RT-PCR products of *VaCDPK* and *VaActin1*. Total RNA was isolated from 35-day calli (linear phase of growth and the highest resveratrol content). V2k, V2 cell culture growing on standard medium; V2-1, on medium with 1 μM CI; V2-10, on medium with 10 μM CA; V2+, on medium with 3-times increased calcium concentration; VB1k, VB1 cell culture growing on standard medium; VB1-1, on medium with 1 μM CI; VB1-10, on medium with 10 μM CI; VB1+, on medium with 3-times increased calcium concentration. VB2k, VB2 cell culture growing on standard medium; VB2-1, on medium with 1 μM CI; VB2-10, on medium with 10 μM CI; VB2+, on medium with 3-times increased calcium concentration. **b** Quantification of the *VaCDPK* transcripts by microchip technology (Kiselev et al. 2009a). The designation r.u. indicates relative fluorescence units. The data obtained from three independent experiments were averaged and presented as the mean ± SEM

VaCPK3aS transcripts were identical to the *VaCPK1a* and *VaCPK3a* transcripts, with the only difference being the absence of 48–114 bp (Dubrovina et al. 2009). Interestingly, the missing sequences did not comprise a single exon. Similar, short *CDPK* transcripts were found in other plants. For example, the *PgCDPK1as* and *PgCDPK2ds* transcripts lacking 15 and 51 bp, respectively, were found in cDNA from the *rolC* and *rolB* transgenic *Panax ginseng* cell cultures (Kiselev et al. 2008, 2009b, 2010c). We excluded any anomalies resulting from ligation, cloning or amplification of the *VaCPK1aS* and *VaCPK3aS* transcripts (Dubrovina et al. 2009). We noticed that the kinase domains of some plant and animal kinases are modified similarly to those in the *VaCPK1aS* and *VaCPK3aS* transcripts (Xiong and Yang 2003; Park et al. 2006; Kurihara et al. 2007). The CI treatment did not lead to an appearance of the "short" *CDPK* transcripts in the V2 cell cultures, whereas the quantity of short *CDPK* transcripts increased by 2–4 fold in the VB1 and VB2 cell cultures.

In addition to the short *CDPK* transcripts, we found long *CDPK* transcripts in the cDNA of the control V2 and the *rolB* transgenic *V. amurensis* cell cultures. These transcripts (*VaCDPK1L1* and *VaCDPK1L2*) contained a 35 bp insertion in the catalytic subdomain VII (Dubrovina et al. 2009). The sequences of these transcripts were most similar to the *VaCDPK1a* transcript. Treatment with the CI increased expression of the long *CDPK* transcripts in the V2 cell cultures by 2–9 fold but did not increase the *VaCDPK1L* expression in the VB1 and VB2 cell cultures.

CDPK expression estimated by real-time PCR

To confirm and verify the above data, *CDPK* expression levels were estimated by real-time PCR (Fig. 4). The results were consistent with the above data (Table 1, Fig. 2), suggesting that *VaCDPK1d*, *VaCDPK1e*, *VaCDPK1L*, and *VaCDPK2a* expression increased significantly in the CI-treated V2 cell culture, while the expression levels of *VaCDPK1a* and *VaCDPK3a* were significantly decreased compared to the untreated V2 cells (Table 1). The expression of 6 analyzed *CDPK* genes did not change significantly in the CI-treated VB1 and VB2 cell cultures or the V2, VB1, and VB2 cell cultures growing in the presence of an increased concentration of calcium in the nutrient media, except *VaCDPK1e* and *VaCDPK2a* expression in the V2 cell culture (Table 1).

Conclusion

Previous experiments using calcium channel blockers indicated that inhibition of calcium influx from the extracellular space and/or efflux from intracellular calcium pools into the cytoplasm blocked resveratrol synthesis in the *rolB* transgenic *V. amurensis* calli (Dubrovina et al. 2009). These experiments suggested that *rolB*-mediated activation of resveratrol production in *V. amurensis* cell cultures is associated with Ca^{2+} flux into the cytoplasm. In this study, we examined the influence of CI, a calcium ionophore, on the levels of resveratrol content and *CDPK* and *STS* gene expression in three cell cultures of *V. amurensis*. Treatment with CI at a concentration of 1 μM significantly increased the production of resveratrol in the control V2 cell culture; however, the CI at a concentration of 10 μM significantly decreased the resveratrol production in the V2, VB1, and VB2 cell cultures. The decrease in resveratrol production was a result of cell growth suppression. In the control cell culture, CI treatment increased the expression of all of the expressed *CDPK* genes, with the exception of *VaCDPK1a* and *VaCDPK3a* expression, and six of the ten analyzed *STS* genes. In the *rolB* transgenic cell cultures treated with the CI, we detected both increases and decreases in the expression levels of different *CDPK* and *STS* genes. However, these alterations in gene

Table 1 Expression profiles of the *CDPK* genes from the *V. amurensis* cell cultures and quantity of *CDPK* clones (in parentheses) isolated from RT-PCR probes

CDPK transcripts	Expression of *CDPK*, relative units (r.u.)[a]											
	V2k[b]	V2-1[c]	V2-10[d]	V2+[e]	VB1k[f]	VB1-1[g]	VB1-10[h]	VB1+[i]	VB2k[j]	VB2-1[k]	VB2-10 [l]	VB2+[m]
VaCDPK1a	0.539 (52)	0.577 (19)	0.465 (15)	0.471 (11)	0.499 (22)	0.665 (29)	0.543 (22)	0.529 (21)	0.378 (39)	0.619 (18)	0.565 (14)	0.561 (21)
VaCDPK1b	0 (0)	0 (0)	0 (0)	0 (0)	0.011 (1)	0.017 (1)	0.038 (2)	0.012 (1)	0 (0)	0 (0)	0 (0)	0 (0)
VaCDPK1c	0 (0)	0 (0)	0 (0)	0 (0)	0.021 (2)	0.017 (1)	0.019 (1)	0.023 (2)	0 (0)	0 (0)	0 (0)	0 (0)
VaCDPK1d	0.014 (2)	0.161 (4)	0.225 (6)	0.075 (2)	0.094 (6)	0.050 (2)	0.197 (7)	0.136 (8)	0.253 (19)	0.122 (3)	0.204 (3)	0.054 (2)
VaCDPK1e	0 (0)	0 (0)	0 (0)	0 (0)	0.011 (1)	0.017 (1)	0.038 (2)	0.012 (1)	0.012 (2)	0.056 (2)	0.039 (2)	0.039 (1)
VaCDPK1aS	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.012 (2)	0.028 (1)	0.039 (2)	0.039 (1)
VaCDPK1L1	0.013 (1)	0.023 (1)	0.114 (2)	0.036 (1)	0.032 (3)	0.017 (1)	0.038 (2)	0.041 (4)	0.160 (4)	0.122 (3)	0.204 (3)	0.054 (2)
VaCDPK1L2	0.013 (1)	0.023 (1)	0.086 (1)	0.051 (1)	0.022 (2)	0.017 (1)	0.019 (1)	0.026 (2)	0.148 (2)	0.122 (3)	0.039 (2)	0.054 (2)
VaCDPK2a	0.044 (6)	0.046 (2)	0.114 (2)	0.051 (1)	0.011 (1)	0.017 (1)	0.038 (2)	0.012 (1)	0.198 (10)	0.122 (3)	0.039 (2)	0.039 (1)
VaCDPK3a	0.869 (103)	0.860 (19)	0.701 (15)	0.857 (21)	0.878 (55)	0.872 (39)	0.752 (29)	0.795 (41)	0.520 (62)	0.402 (11)	0.499 (18)	0.833 (35)
VaCDPK3b	0 (0)	0 (0)	0 (0)	0 (0)	0.011 (1)	0.017 (1)	0.038 (2)	0.012 (1)	0.007 (1)	0.017 (1)	0.019 (1)	0.058 (3)
VaCDPK3aS	0 (0)	0 (0)	0 (0)	0 (0)	0.011 (1)	0 (0)	0 (0)	0.012 (1)	0.012 (2)	0.028 (1)	0.019 (1)	0.019 (1)

[a] Relative units calculated as total *CDPK* expression × percentage of clones/100 (Kiselev and Dubrovina 2010)

[b] Total *CDPK* expression was 1.49 r.u., see Fig. 2

[c] Total expression was 1.70 r.u.

[d] Total expression was 1.72 r.u.

[e] Total expression was 1.52 r.u.

[f] Total expression was 1.60 r.u.

[g] Total expression was 1.68 r.u.

[h] Total expression was 1.72 r.u.

[i] Total expression was 1.61 r.u.

[j] Total expression was 1.70 r.u.

[k] Total expression was 1.71 r.u.

[l] Total expression was 1.65 r.u.

[m] Total expression was 1.67 r.u.

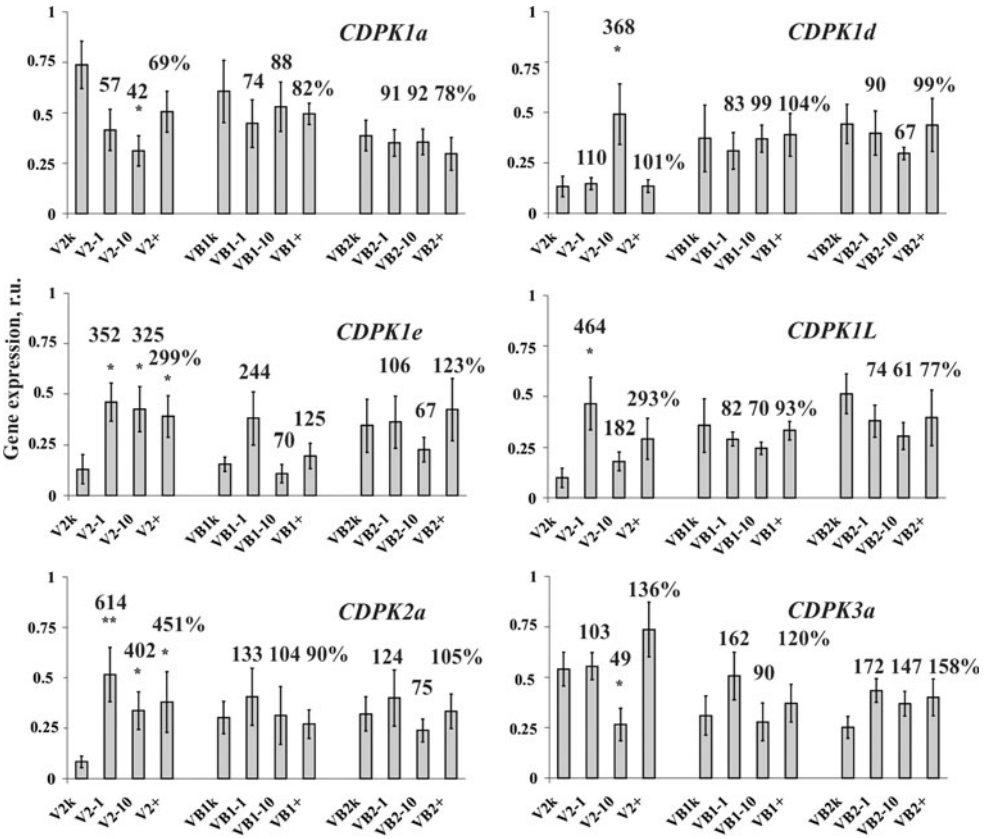

Fig. 4 *CDPK* expression levels detected by real-time PCR in the *V. amurensis* cell cultures, V2, VB1, and VB2, treated with the CI. Presented numerals in the figure are percentages from V2, VB1 or VB2 without CI; *r.u.* relative units. The data are presented as mean ± SEM obtained from three independent experiments, $*P < 0.05$, $**P < 0.01$ versus values of the V2, VB1, or VB2 culture without CI

expression were not significant.Thus, CI at concentration of 1 μM increased the content and production of resveratrol and the expression of a large number of *CDPK* and *STS* genes in the control grape cells, while it did not increase these parameters in the *rolB* transgenic cells. The results suggest that untreated *rolB* transgenic grape cells exhibit increased elevations in cytoplasmic calcium levels, and, therefore, the treatment with the CI did not significantly change *CDPK* expression in the *rolB* transgenic calli. We propose that activation of resveratrol biosynthesis by the *rolB* gene is closely connected with changes in the normal functioning of calcium signaling pathway.

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References

Adrian M, Jeandet P (2006) *Trans*-resveratrol as an antifungal agent. In: Aggarwal BB, Shishodia S (eds) Resveratrol in health and disease. CRC Press, Boca Raton, pp 475–497

Altamura MM, Capitani F, Gazza L, Capone I, Costantino P (1994) The plant oncogene *rolB* stimulates the formation of flowers and root meristemoids in tobacco thin cell layers. New Phytol 126: 283–293

Bekesiova I, Nap JP, Mlynarova L (1999) Isolation of high quality DNA and RNA from leaves of the carnivorous plant *Drosera rotundifolia*. Plant Mol Biol Rep 17:269–277

Burns J, Yokota T, Ashihara H, Lean MEJ, Crozier A (2002) Plant foods and herbal sources of resveratrol. J Agric Food Chem 50:3337–3340

Cacho M, Moran M, Tarrago JF, Corchete P (1995) Calcium restriction induces cardenolide accumulation in cell-suspension cultures of *Digitalis-thapsi* L. Plant Cell Rep 14:786–789

Cheng SH, Willmann MR, Chen HC, Sheen J (2002) Calcium signaling through protein kinases. The arabidopsis calcium-dependent protein kinase gene family. Plant Physiol 129:469–485

Chong JL, Poutaraud A, Hugueney P (2009) Metabolism and roles of stilbenes in plants. Plant Sci 177:143–155

Counet C, Callemien D, Collin S (2006) Chocolate and cocoa: new sources of trans-resveratrol and trans-piceid. Food Chem 98:649–657

Delaunois B, Cordelier S, Conreux A, Clement C, Jeandet P (2009) Molecular engineering of resveratrol in plants. Plant Biotechnol J 7:2–12

Dmitriev A, Djatsok J, Grodzinsky D (1996) The role of Ca^{2+} in elicitation of phytoalexin synthesis in cell culture of onion (*Allium cepa* L.). Plant Cell Rep 15:945–948

Donnez D, Jeandet P, Clement C, Courot E (2009) Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. Trends Biotechnol 27:706–713

Dubrovina AS, Kiselev KV, Veselova MV, Isaeva GA, Fedoreyev SA, Zhuravlev YN (2009) Enhanced resveratrol accumulation in *rolB* transgenic cultures of *Vitis amurensis* correlates with

unusual changes in *CDPK* gene expression. J Plant Physiol 166:1194–1206

Dubrovina AS, Manyakhin AY, Zhuravlev YN, Kiselev KV (2010) Resveratrol content and expression of phenylalanine ammonia-lyase and stilbene synthase genes in *rolC* transgenic cell cultures of *Vitis amurensis*. Appl Microbiol Biotechnol 88:727–736

Fan EG, Zhang K, Zhu MZ, Wang QA (2010) Obtaining resveratrol: from chemical synthesis to biotechnological production. Mini-Rev Organ Chem 7:272–281

Fliegmann J, Schroder G, Schanz S, Britsch L, Schroder J (1992) Molecular analysis of chalcone and dihydropinosylvin synthase from Scots pine (*Pinus sylvestris*), and differential regulation of these and related enzyme activities in stressed plants. Plant Mol Biol 18:489–503

Giorcelli A, Sparvoli F, Mattivi F, Tava A, Balestrazzi A, Vrhovsek U, Calligari P, Bollini R, Confalonieri M (2004) Expression of the stilbene synthase (*StSy*) gene from grapevine in transgenic white poplar results in high accumulation of the antioxidant resveratrol glucosides. Transgenic Res 13:203–214

Giovannini L, Migliori M, Longoni BM, Das DK, Bertelli AAE, Panichi V, Filippi C, Bertelli A (2001) Resveratrol, a polyphenol found in wine, reduces ischemia reperfusion injury in rat kidneys. J Cardiovasc Pharmacol 37:262–270

Gronbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G, Sorensen TIA (2000) Type of alcohol consumed and mortality from all causes, coronary heart disease and cancer. Ann Int Med 133:411–419

Halls C, Yu O (2008) Potential for metabolic engineering of resveratrol biosynthesis. Trends Biotechnol 26:77–81

Hurst WJ, Glinski JA, Miller KB, Apgar J, Davey MH, Stuart DA (2008) Survey of the trans-resveratrol and trans-piceid content of cocoa-containing and chocolate products. J Agric Food Chem 56:8374–8378

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Adam-Blondon AF, Weissenbach J, Quetier F, Wincker P (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463

Jaworski K, Szmidt-Jaworska A, Kopcewicz J (2011) Two calcium dependent protein kinases are differently regulated by light and have different activity patterns during seedling growth in *Pharbitis nil*. Plant Growth Regul 65:369–379

Jeandet P, Bessis R, Sbaghi M, Meunier P, Trollat P (1995) Resveratrol content of wines of different ages: relationship with fungal disease pressure in the vineyard. Am J Enol Vitic 46:1–4

Jeandet P, Douillt-Breuil AC, Bessis R, Debord S, Sbaghi M, Adrian M (2002) Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. J Agric Food Chem 50:2731–2741

Kiselev KV (2011) Perspectives for production and application of resveratrol. Appl Microbiol Biotechnol 90:417–425

Kiselev KV, Dubrovina AS (2010) A new method for analysing gene expression based on frequency analysis of RT-PCR products obtained with degenerate primers. Acta Physiol Plant 32: 495–502

Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN (2007) The *rolB* gene–induced overproduction of resveratrol in *Vitis amurensis* transformed cells. J Biotechnol 128:681–692

Kiselev KV, Gorpenchenko TY, Tchernoded GK, Dubrovina AS, Grishchenko OV, Bulgakov VP, Zhuravlev YN (2008) Calcium-dependent mechanism of somatic embryogenesis in *Panax ginseng* cell cultures expressing the *rolC* oncogene. Mol Biol 42:243–252

Kiselev KV, Dubrovina AS, Bulgakov VP (2009a) Phenylalanine ammonia-lyase and stilbene synthase gene expression in *rolB* transgenic cell cultures of *Vitis amurensis*. Appl Microbiol Biotechnol 82:647–655

Kiselev KV, Turlenko AV, Zhuravlev YN (2009b) *PgWUS* expression during somatic embryo development in a *Panax ginseng* 2c3 cell culture expressing the *rolC* oncogene. Plant Growth Regul 56:237–243

Kiselev KV, Dubrovina AS, Isaeva GA, Zhuravlev YN (2010a) The effect of salicylic acid on phenylalanine ammonia-lyase and stilbene synthase gene expression in *Vitis amurensis* cell culture. Russ J Plant Physiol 57:415–421

Kiselev KV, Grishchenko OV, Zhuravlev YN (2010b) *CDPK* gene expression in salt tolerant *rolB* and *rolC* transformed cell cultures of *Panax ginseng*. Biol Plant 54:621–630

Kiselev KV, Turlenko AV, Zhuravlev YN (2010c) Structure and expression profiling of a novel calcium–dependent protein kinase gene *PgCDPK1a* in roots, leaves, and cell cultures of *Panax ginseng*. Plant Cell Tiss Organ Cult 103:197–204

Kiselev KV, Shumakova OA, Tchernoded GK (2011a) Mutation of *Panax ginseng* genes during long-term cultivation of ginseng cell cultures. J Plant Physiol 168:1280–1285

Kiselev KV, Tyunin AP, Manyakhin AY, Zhuravlev YN (2011b) Resveratrol content and expression patterns of stilbene synthase genes in *Vitis amurensis* cells treated with 5-azacytidine. Plant Cell Tiss Organ Cult 105:65–72

Kurihara D, Kawabe A, Matsunaga S, Nakagawa K, Fujimoto S, Uchiyama S, Fukui K (2007) Characterization of a splicing variant of plant Aurora kinase. Plant Cell Physiol 48:369–374

Langcake P, Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Physiol Plant Pathol 9:77–86

Langcake P, Pryce RJ (1977) A new class of phytoalexins from grapevines. Experientia 33:151–152

Lecourieux D, Ranjeva R, Pugin A (2006) Calcium in plant defence-signalling pathways. New Phytol 171:249–269

Moriuchi H, Okamoto C, Nishihama R, Yamashita I, Machida Y, Tanaka N (2004) Nuclear localization and interaction of RolB with plant 14-3-3 proteins correlates with induction of adventitious roots by the oncogene *rolB*. Plant J 38:260–275

Ning W, Wang JX, Liu YM, Li N, Cao RQ (1998) The effects of Ca^{2+} during the elicitation of shikonin derivatives in *Onosma paniculatum* cells. In vitro Cell Dev Biol-Plant 34:261–265

Olas B, Wachowicz B, Szewczuk J, Saluk-Juszczak J, Kaca W (2001) The effect of resveratrol on the platelet secretory process induced by endotoxin and thrombin. Microbios 105:7–13

Park SH, Choi J, Kang JI, Choi SY, Hwang SB, Kim JP, Ahn BY (2006) Attenuated expression of interferon-induced protein kinase PKR in a simian cell devoid of type I interferons. Mol Cells 21:21–28

Preisig CL, Moreau RA (1994) Effects of potential signal-transduction antagonists on phytoalexin accumulation in tobacco. Phytochemistry 36:857–863

Ramani S, Chelliah J (2007) UV-B-induced signaling events leading to enhanced-production of catharanthine in *Catharanthus roseus* cell suspension cultures. BMC Plant Biol 7:61

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 6:27

Rupprich N, Hildebrand H, Kindl H (1980) Substrate specificity in vivo and in vitro in the formation of stilbenes—biosynthesis of rhaponticin. Arch Biochem Biophys 200:72–78

Sanchez-Sampedro MA, Fernandez-Tarrago J, Corchete P (2005) Enhanced silymarin accumulation is related to calcium deprivation in cell suspension cultures of Silybum marianum (L.) Gaertn. J Plant Physiol 162:1177–1182

Shumakova OA, Manyakhin AY, Kiselev KV (2011) Resveratrol content and expression of phenylalanine ammonia-lyase and stilbene synthase genes in cell cultures of *Vitis amurensis* treated with coumaric acid. Appl Biochem Biotechnol 165:1427–1436

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol Biol 24:743–755

Stervbo U, Vang O, Bonnesen C (2007) A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine. Food Chem 101:449–457

Tokusoglu O, Unal MK, Yemis F (2005) Determination of the phytoalexin resveratrol (3,5,4′-trihydroxystilbene) in peanuts and pistachios by high-performance liquid chromatographic diode array (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS). J Agric Food Chem 53:5003–5009

Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A, Pruss D, Pindo M, Fitzgerald LM, Vezzulli S, Reid J et al (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One 2:e1326

Xiong LZ, Yang YN (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15: 745–759