Plant Cell Tiss Organ Cult (2012) 111:91–100
DOI 10.1007/s11240-012-0175-0

ORIGINAL PAPER

Effects of 5-azacytidine induced DNA demethylation on methyltransferase gene expression and resveratrol production in cell cultures *of Vitis amurensis*

A. P. Tyunin · K. V. Kiselev · Y. N. Zhuravlev

Received: 1 December 2011 / Accepted: 5 May 2012 / Published online: 17 May 2012

Abstract DNA becomes methylated in vivo through the action of a specific group of enzymes known as methyltransferases or methylases. Plants are known to possess the methyltransferases (Met), chromo methyltransferases (CMT), and domainrearranged methyltransferases (DRM) methylase families, which affect cytosine methylation within different contexts. DNA methylation has been proposed to play a role in secondary plant metabolism, but there is a lack of valid data connecting these two processes. In this study, we treated control and transformed with *rolB* gene from *Agrobacterium rhizogenes* cell cultures of *Vitis amurensis* with the demethylation agent 5-azacytidine (azaC). The purpose of the current investigation was to study effects of induced DNA demethylation on methyltransferase gene expression in connection to resveratrol production, a naturally occurring polyphenol that has a wide range of intriguing biological properties. Using semi-quantitative and real-time PCR, we showed that *rolB* gene transformation of *V. amurensis* cells decreased *Met* and *CMT* expression, but significantly increased *DRM* expression. AzaC treatment of the control and the *rolB*-transgenic calli significantly increased expression of all methylases (excluding *Met*). Following 3 months of azaC treatment, we detected significantly elevated levels of *rolB* gene expression in the transgenic calli. In current paper, we discuss how methylase expression may influence resveratrol biosynthesis and *rolB* transgene expression. Effects of azaC application are discussed.

Keywords 5-azacytidine · *RolB* · Secondary metabolism · Resveratrol · DNA methylation · Methyltransferases

Abbreviations

azaC	5-azacytidine
CMT	Chromo methyltransferase
DRM	Domainrearranged methyltransferase
STS	Stilbene synthase
DW	Dry weight

Introduction

Covalent DNA modification involves the addition of methyl groups to some nucleotides. This process results in the presence of two new methylated forms of nucleotides—5-methylcytosine and 6-methyladenine, in the polynucleotide DNA chain (Vanyushin 2005). While DNA methylation is found in the majority of organisms, the number of methylated cytosine bases is usually species- and tissue-specific (Martienssen and Richards 1995). Abnormal methylation patterns have negative effects on embryonic development and may sometimes even be lethal (Bitonti et al. 2002; Zluvova et al. 2001). In eukaryotes, DNA methylation primarily functions in defense reactions of the cells and maintaining genomic stability. Methylation also

Electronic supplementary material The online version of this article (doi:10.1007/s11240-012-0175-0) contains supplementary material, which is available to authorized users.

A. P. Tyunin (✉) · K. V. Kiselev · Y. N. Zhuravlev
Laboratory of Biotechnology, Institute of Biology and Soil Science, Far Eastern Branch of Russian Academy of Sciences, Vladivostok 690022, Russia
e-mail: Tyunin@biosoil.ru

A. P. Tyunin · K. V. Kiselev · Y. N. Zhuravlev
Department of Biochemistry and Biotechnology, Far Eastern Federal University, Vladivostok 690090, Russia

plays a major role in regulating gene expression during ontogenesis. In higher plants, cytosine methylation is common (Zluvova et al. 2001). Within eukaryotic DNA, most cytosine bases in invasive and transposable genome elements become hypermethylated. The methylation of cytosine nucleotides within promoter regions (e.g., CG islands) in most cases leads to transcriptional inactivation of the gene. It has been suggested that DNA methylation in promoter regions controls gene expression at different stages of ontogenesis (Finnegan and Kovac 2000). Additionally, DNA methylation is known to play an important role in the process of transcriptional gene silencing; this is induced by non-coding RNA transcripts via RNA-directed DNA methylation (RdDM) (Miki and Shimamoto 2008; Lang-Mladek et al. 2010). DNA methylation also contributes to transgene silencing during long-term cultivation of cell cultures, whereas induced DNA demethylation can restore transgene expression (Zeng et al. 2010). Finally, DNA methylation plays a large role in plants' abiotic and biotic stress response mechanisms (Alvarez et al. 2010; Baranek et al. 2010). Pathogenic infection by bacteria *Pseudomonas syringae* also leads to significant hypomethylation in host genomic loci (Ruiz-Ferrer and Voinnet 2009).

The process of methylation is performed by methylases. The main function of these enzymes is to transfer the methyl group from S-adenosyl-metheonine to cytosine residues in target DNA sequences. All methylases are monomeric proteins that contain variable N-termini and comparably conservative C-terminal catalytic domains. The N-terminus interacts with different proteins in multiple enzymatic complexes. The C-terminal catalytic domain contains six to ten highly conserved repeating motifs, which are responsible for the catalytic transfer of the methyl group from S-adenosyl-metheonine to DNA (Chinnusamy et al. 2009; Messeguer et al. 1991). The C-terminal catalytic domain also contains the variable target recognizing domain, which recognizes specific DNA sequences (Chinnusamy et al. 2009; Messeguer et al. 1991). Plants possess three methylases families: the methyltransferases (Met), chromo methyltransferases (CMT), and domainrearranged methyltransferases (DRM). Each methylases family affects cytosine methylation in unique way (Buryanov et al. 2005). Met family methylases are primarily active during the S-phase of cell replication. Met methylases are also responsible for maintaining methylation within symmetrical "CG" sites; in rare instances, these methylases can also affect cytosine methylation in a "CHG" context (Buryanov et al. 2005). The CMT methylase family is unique to plants. CMT methylases maintain cytosine methylation within symmetrical "CHG" sites, but also can affect de novo methylation within asymmetrical "CHH" sites. Their main function is to methylate centromeric, ribosomal, and satellite DNA within heterochromatin. The third family of plant methylases is the DRM family. The main function of DRM methylases is to block endogeneous transposons and transgene sequences (Law et al. 2010). These methylases utilize the si-RNA-induced RdDM mechanism and maintain CHH methylation through persistent de novo methylation (Law et al. 2010).

The polyphenol *trans*-resveratrol (3,5,4′ trihydroxy-trans-stilbene) is a secondary plant metabolite produced by the *Vitaceae* species. Resveratrol is involved in conferring pathogenic resistance in grapes and is known to possess unique pharmacological properties (Aggarwal et al. 2004; Bavaresco et al. 2009; Kiselev 2011). The metabolite is 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. STS is a multi-gene family in most stilbenoid producing species including grapes, peanuts, and pine (Kiselev 2011).

The role cytosine DNA methylation plays in plant secondary metabolism is unclear. There are no data describing the conditional methylation status of *STS* genes and their promoter regions. However, indirect evidence does suggest a link between DNA methylation and resveratrol biosynthesis. Previous studies have shown that *rolB* gene transformation of *A. rhizogenes* can increase resveratrol production (Kiselev et al. 2007). The mechanism of inducing secondary plant metabolite production by *rol* transformation is still unknown (Veena and Taylor 2007). Nevertheless, elevated *rolB* expression in *V. amurensis* VB2 resulted in more than a 100-fold increase in resveratrol production. Increased *rolB* expression brought resveratrol production up to 3.15 % dry weight (DW) compared to the level in control cells (Kiselev et al. 2007). However, the *rolB*-transgenic culture VB2 of *V. amurensis* lost its ability to produce high levels of resveratrol in long-term culture; after 2.5 years of cultivation, resveratrol content decreased to a range of 0.1–0.8 % DW. Our previous experiments showed that treatment with 200 μM of 5-azacytidine (azaC), which is known to block DNA methylation, resulted in enhancement of resveratrol production. Resveratrol expression increased in 1.9 and 2-fold in *V. amurensis* control VV and *rolB*-transgenic VB2 calli, respectively (Kiselev et al. 2011). Therefore, the purpose of the present study is to investigate whether methyltransferase-mediated DNA methylation is linked with resveratrol biosynthesis in *V. amurensis* cell cultures. We proposed that the methyltransferases expression pattern and methylation status of the *STS* genes could provide us an answer. In this study, we analyzed the expression of DNA methyltransferases in control and *rolB*-transgenic *V. amurensis* cell cultures. Methyltransferease expression levels were compared relative to resveratrol content and the total level of DNA methylation, before and after azaC treatment.

Materials and methods

Vitis amurensis cell cultures

As described previously (Kiselev et al. 2007), the V2 callus culture of wild-growing grape *Vitis amurensis* Rupr. (*Vitaceae*) was established in 2002. The VV culture was established in 2004 by co-cultivation of the V2 cell suspension with *Agrobacterium tumefaciens* GV3101/pMP90RK strains containing the pPCV002 (empty vector) plasmid vector carrying a kanamycin resistance gene *nptII*. The *rolB*-transgenic *V. amurensis* callus culture (designated VB2) was obtained in 2004 by transformation of the V2 cell suspension with *A. tumefaciens* strains GV3101/pMP90RK containing the binary plasmid vector pPCV002–CaMVB as described previously (Kiselev et al. 2007). In pPCV002–CaMVB, the *rolB* gene is under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Spena et al. 1987). The plasmid also contained the *nptII* gene that was placed under the control of the nopaline synthase promoter. Gene-specific PCR analysis on DNA that was isolated from VB2 cells confirmed that they contained the *rolB* and *nptII* gene sequences (Kiselev et al. 2007). Semi-quantitative reverse transcriptase RT-PCR and real-time PCR analyses showed that the *rolB* gene expressed in the VB2 cells (Kiselev et al. 2007, 2009a).

VV and VB2 callus cultures were cultivated at 35-day subculture intervals in the dark at 24–25 °C in test tubes with 15 ml nutrient medium $W_{B/A}$ medium (Kiselev et al. 2009a). Inoculum biomass was 0.2 g per tube or 13.3 g/l (each callus was weighed using an electronic balance). Reagents for cell culture were purchased from Sigma Chemical (St.Louis, MO, USA) and Serva Feinbiochemica (Heidelberg, Germany). Samples were harvested from 35-day-old cultures (linear phase of growth and the highest resveratrol content), weighed, and used for analyses. To confirm the demethylation effect of azaC and to avoid the effects of its photolability, we passed the VB2 calli to a fresh culture medium containing 50 and 200 μM of azaC every 3 days. The calli were dried under hot air flow (50 °C for 2 h) and used to determine the levels of resveratrol (Kiselev et al. 2009a).

AzaC treatments

All reagents for tissue culture, as previously described (Kiselev et al. 2011), were obtained from Sigma or ICN Pharmaceuticals (Costa Mesa, CA, USA). Sterile aqueous solutions of azaC were added to autoclaved medium at the designated concentrations (50 and 200 μM).

Total RNA isolation and semi-quantitative RT-PCR

Total RNA isolation was performed using a CTAB (hexadecyltrimethylammonium bromide)-based extraction protocol developed by Bekesiova (1999). Complementary DNAs were synthesized using 1–3 μg of RNA by the RNA PCR Kit (Silex M, Moscow, Russia). The reactions were performed in 50-μl aliquots of the reaction mixture, which contained RT buffer, 0.2 mM each of the 4 dNTPs, 0.2 μM of oligo-$(dT)_{15}$ primer, 200 U of M-MLV-polymerase at 37 °C for 1–2 h. The 0.2–5 μl samples of re-verse transcription products were then amplified by polymerase chain reaction.

To amplify sequences corresponding to *VaMet* genes, the degenerate primers were designed according to GenBank amino acid sequences of Met from different plant species (GenBank accession no. NM_124293, NM_001111716, GU011683). Primers to *VaCMT* were designed according to GenBank amino acid sequences of CMT (NM_106722, NM_118020, NM_105645). The same approach used for *VaDRM* expression gave no results, so the most conserved regions of *DRM* nucleotide sequences derived from the related species *Vitis vinifera* were used as templates for primer design (XM_002264190, XP_002273972). Degenerate primers corresponding to *VaMet*, *VaCMT*, and *VaDRM* genes are presented in the supplementary file Table S1.

The semi-quantitative RT-PCR reactions were carried out in conditions described previously (Kiselev et al. 2009a). In the semiquantitative RT-PCR reactions, PCR products were collected after 25, 30, 35, 40, 45, 50 and the linearity of the PCR was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) by calculating cycle/concentration curves. The linearity of the PCR was determined for the *VaMet*, *VaCMT*, and *VaDRM* genes between 40 and 45 cycles. Therefore, we used 40 cycles for amplification *VaMet*, *VaCMT*, *VaDRM* genes.

The *VaMet* primers were used for amplification of a 446 bp *VaMet* transcript, Ta 51 °C, elongation time 27 s. The *VaCMT* primers were used for amplification of a 245 bp *VaCMT* transcript, Ta 50 °C, elongation time 15 s. The *VaDRM* primers were used for amplification of a 325 bp *VaDRM* transcript, Ta 51.5 °C, elongation time 19 s. The data of *VaMet*, *VaCMT*, and *VaDRM* expression are presented as relative fluorescent units normalized to expression of the corresponding *V. amurensis* actin 1 gene (Kiselev et al. 2007). The gene expression of *VaMet*, *VaCMT*, and *VaDRM* data were derived from 3 independent experiments.

Sequence analysis

RT-PCR products were sequenced as described (Kiselev et al. 2006) at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEBRAS using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequencing of each gene was performed at least 3 times. The BLAST search program was used for sequence

analysis. Multiple sequence alignments were performed using the BioEdit 7.0.8 program found at http://www.mbio.ncsu.edu/BioEdit/bioedit.html. All the sequencing data presented as electronic supplementary material.

Screening of cDNA (VaMet, VaCMT, VaDRM) clones

For control VV calli, 50 μM azaC + VV, 200 μM azaC + VV; control VB2 calli 50 μM, azaC + VB2, and 200 μM azaC + VB2), cDNA from 28, 14, 40, 28, and 18 clones, respectively, were sequenced for *VaMet*; 32, 17, 45, 25, 24, and 40 clones, respectively, were sequenced for *VaCMT*; and 30, 37, 31, 18, 28, and 29 clones, respectively, were sequenced for *VaDRM*. These results and the data on total *VaMet, VaCMT*, and *VaDRM* expression assayed obtained using the degenerate primers were used to estimate the expression level (in relative units) for each of the *VaMet, VaCMT*, and *VaDRM* genes. The relative expression was estimated as the total *VaMet*, *VaCMT*, or *VaDRM* expression normalized to expression of the actin gene, multiplied by the percent clones of each *VaMet*, *VaCMT*, and *VaDRM* transcript variant and divided by 100 (Kiselev et al. 2009b; Dubrovina et al. 2009; Kiselev and Tchernoded 2009; Kiselev and Dubrovina 2010). All the sequencing data presented as electronic supplementary material.

Quantitative real-time PCR analysis

For TaqMan real-time PCR, cDNAs were amplified (Kiselev et al. 2010; Shumakova et al. 2011) in 20-μl reaction mixtures containing 1 × TaqMan Buffer B, 2.5 mM $MgCl_2$, 250 μM dNTP, 1 U Taq DNA polymerase, 0.5 μl (15 ng) cDNA, and 0.25 μM of each primer and probe (Real-time PCR Kit, Syntol, Russia). PCR amplification consisted of one cycle of 2 min at 95 °C, followed by 50 cycles of 10 s at 95 °C and 25 s at 62 °C. The TaqMan PCR assays were performed in an iCycler thermocycler that was equipped with the iQ5 Multicolor Real-Time PCR detection system (Bio Rad, Hercules, CA, USA), and data were analyzed using the iQ5 Optical System software v.2.0 according to the manufacturer's instructions. The normalized expression was calculated using the $2^{-\Delta\Delta CT}$ method, and transcript levels were determined relative to the most highly expressed sample (Kiselev et al. 2010). The *VaActin1* gene (GenBank AY680701) was used as an endogenous control to normalize the variance in the quality and amount of cDNA used in each real-time PCR experiment. A no-template control was included in each assay, and no-cycle threshold (Ct) values were consistently obtained after 50 cycles of PCR. The data shown are the averages of 3 PCR analyses. To amplify sequences corresponding to *VaMet1a*, *VaMet2a*, *VaMet3a* genes, the specific primers were designed according to GenBank nucleotide sequences of *Met* derived from related species *V. vinifera* (XM_002267164, XM_002267248, XM_002268202). Specific primers for *VaCMT1a* and *VaCMT2a* were designed according to GenBank nucleotide sequences of *CMT* from *V. vinifera* (XM_002275896, XM_002283319). To amplify sequences corresponding to *VaDRM1a*, *VaDRM2a* nucleotide sequences of *DRM* derived from the *V. vinifera* were used as templates for primer design (XM_002264190, XP_002273972). To amplify sequences corresponding to *rolB* transgene we used as a template for primers design region from 10,509 to 11,285 bp within T-DNA sequence of *A. rhizogenes* (K03313). All primers and TaqMan probes corresponding to certain genes are presented in the supplementary file Table S1.

High-performance liquid chromatography

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

Methylation-sensitive DNA fragmentation assay

In our study, we used the BstHH I enzyme (50 u/μL, SibEnzyme, Novosibirsk, Russia) to confirm effect of azaC treatment on DNA samples from tested cell cultures. This restriction enzyme is sensitive to cytosine methylation within its restriction site (GCG*C); thus, restriction proceed successfully only if cytosine methylation within the restriction site was absent. The DNA samples from treated with 200 μM of azaC and untreated calli of VV and VB2 cell cultures were treated with BstHHI restrictase for 50 min at 50 °C and immediately separated by electrophoresis on a 1.3 % agarose gel.

Statistical analysis

Statistical analysis employed the Statistica 9.0 program. The results were represented 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

The effect of azaC treatment in grape cell cultures

We chose 5-azacytidine as a demethylation agent based on its structural similarity to cytosine. AzaC can be incorporated into replicating DNA but cannot be methylated; this is due to the lack of a free valence in the fifth position of the pyramidine ring. Non-specific DNA demethylation is a well-documented result of azaC treatment of the analyzed

Table 1 Biomass accumulation and resveratrol production in grape callus cultures that are treated with 50 and 200 μM of azaC

Callus line	Fresh biomass (g/L)	Dry biomass (g/L)	Resveratrol content (% dry wt.)	Resveratrol production (mg/L)
VVk	175.1 ± 32.1	6.4 ± 0.6	0.04 ± 0.02	2.5 ± 1.4
VV-50	102.9 ± 19.3*	6.1 ± 0.5	0.06 ± 0.02	3.7 ± 1.5
VV-200	81.7 ± 11.7**	5.3 ± 0.4	0.09 ± 0.04	4.8 ± 2.4
VB2 k	115.2 ± 17.4	6.9 ± 0.5	0.22 ± 0.05	15.2 ± 3.1
VB2-50	93.6 ± 10.2	6.4 ± 0.7	0.28 ± 0.08	18.0 ± 7.2
VB2-200	89.7 ± 9.5	6.2 ± 0.5	0.47 ± 0.08*	29.2 ± 5.5*

The data are presented as mean ± SEM obtained from 3 independent experiments, *$P < 0.05$; **$P < 0.01$ versus values of the untreated VV or VB2 cultures

cells (Weber et al. 1990; Xiong et al. 1999; Tokuji et al. 2011). Previous studies (Kiselev et al. 2011) demonstrated that the addition of 50 and 200 μM of azaC to VV and VB2 grape cultures resulted in a 1.3–2-fold decrease in fresh biomass accumulation. Furthermore, the addition of 200 μM of azaC increased in a 1.4–2-fold resveratrol content and production in VV and VB2 calli compare with untreated cells (Kiselev et al. 2011). In this research control VV and *rolB*-transgenic VB2 grape cell cultures were treated with two concentrations of azaC during 35 days. The addition of 50 and 200 μM of demethylation agent to cell culture medium decreased calli growth (Table 1). Addition of 200 μM of azaC to VV and VB2 cultures caused a 1.9–2-fold increase in resveratrol production in comparison to levels in untreated calli (Table 1). Both cultures were still growing and accumulating fresh biomass (Table 1). Thus, cells remained alive during the 35 days of azaC treatment. The expression of main genes that control resveratrol biosynthesis (such as *STS*) were affected by this demethylation agent: the VV culture showed an increase of *VaSTS10* gene expression; the VB2 culture—of *VaSTS5*, *VaSTS6*, and *VaSTS10* gene expression (Kiselev et al. 2011).

Due to photolability of azaC, we decided to confirm that azaC retains its action during the whole period of treatment. Therefore, the experiment was repeated using 3 days-long subcultivation on freshly prepared azaC-containing medium during 35 days. The obtained data indicate that azaC did not undergo degradation during the experiments. In general all results on methylases expression were proved by data obtained using freshly prepared medium.

Methylation-sensitive BstHH I restriction digestion was used to confirm DNA demethylation in grape cell cultures treated with azaC. This restriction enzyme digests the unmethylated DNA at the target site (GCG*C). DNA isolated from calli treated with 200 μM of azaC was digested with

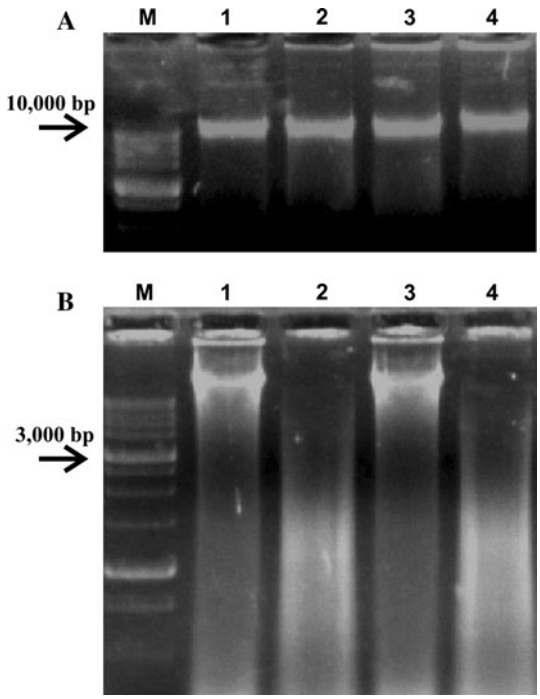

Fig. 1 Electrophoregram of total DNA samples before (**a**) and after **b** BstHH I treatment. 1: Total DNA from the VV culture; 2: Total DNA from the VV culture treated with 200 μM of azaC; 3: Total DNA from the VB2 culture; 4: Total DNA from the VB2 culture treated with 200 μM of azaC; M: synthetic molecular weight marker

BstHH1. The digested DNA was then compared to samples from the control group to confirm the demethylation effect of azaC (Fig. 1). Based on this assay, it could be concluded that the azaC treatment caused DNA demethylation in the test cultures.

RolB expression

The expression levels of the *rolB* gene were measured using quantitative real-time PCR. According to previously published data, the level of *rolB* expression in azaC-treated VB2 cells does not significantly increased in comparison to untreated VB2 calli (Kiselev et al. 2011). Our results show a significant enhancement of *rolB* gene expression only in VB2 cultures subcultivated for 3 months in medium with azaC (Fig. 2).

Treatment of VV and VB2 cell cultures with azaC also led to a 2-fold increase in resveratrol production in both used cell cultures (Table 1). The resveratrol content increased by similar degrees in *rolB*-transgenic and in nontransgenic cells. Therefore, the increase of resveratrol production by azaC is not correlated with the increase in *rolB* gene expression and *rolB* gene expression increased significantly only after 3 months of azaC treatment (Fig. 2). According to last report from Fan et al. (2011) cytosine methylation significantly affects transgene expression but not caused its

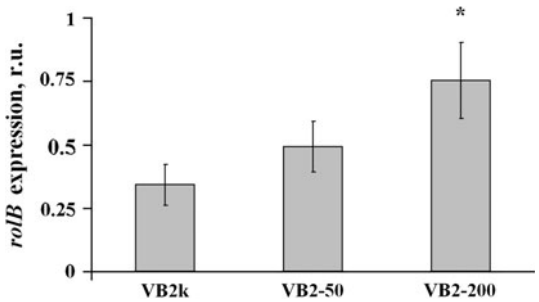

Fig. 2 *RolB* gene expression levels in *V. amurensis* VB2 cell cultures (treated with azaC for 3 months) as detected by real-time PCR. VB2 cell lines VB2-K/VB2-50/VB2-200 were differentially treated with either 50 or 200 μM azaC, respectively. Data are presented as the mean ± SEM and are obtained from 3 independent experiments. * $P < 0.05$; r.u.: relative units

silencing in transgenic orange. We suppose that the expression of *rolB* sequence was decreased by cytosine DNA methylation during long-term cultivation, but not silenced as its expression was still detectable.

Methylases expression

For investigation of total methylase gene expression quantitative analysis of total *VaMet*, *VaCMT*, and *VaDRM* gene expression standardized to actin gene expression was used (Fig. 3). Chosen approach showed that *VaMet* expression in control VV culture increased proportionally to the concentration of demethylation agent in the medium. Addition of 200 μM of azaC to the cell culture medium of VV correlated with more than a 2-fold increase in *VaMet* gene expression as compared with control calli group. *VaMet* expression in *rolB*-transgenic culture was also altered by the addition of 50 μM azaC, whereas the addition of 200 μM azaC resulted in a dramatically decrease in *VaMet* expression as compared to the control calli group. Total *VaCMT* expression (Fig. 3) increased proportionally to the concentration of demethylation agent in VV and VB2 treated cultures. Chromo methylase expression increased more than 2.5-fold in both studied cultures with the addition of 200 μM of azaC to the medium, as compared to levels in the control calli group. A significant change in total *VaDRM* expression (Fig. 3) was only detected in VV cell cultures that were treated with 200 μM of azaC.

Our study demonstrates that azaC-induced DNA demethylation leads to the expression enhancement of almost all detectable methylases. The level of expression enhancement is not uniform, however, even among transcripts from the same family. It is possible that the increase in DNA methyltransferase expression in *V. amurensis* cell cultures serves to defend genome stability in conditions of total DNA demethylation. Furthermore, the total level of *VaDRM* gene expression was considerably higher in the

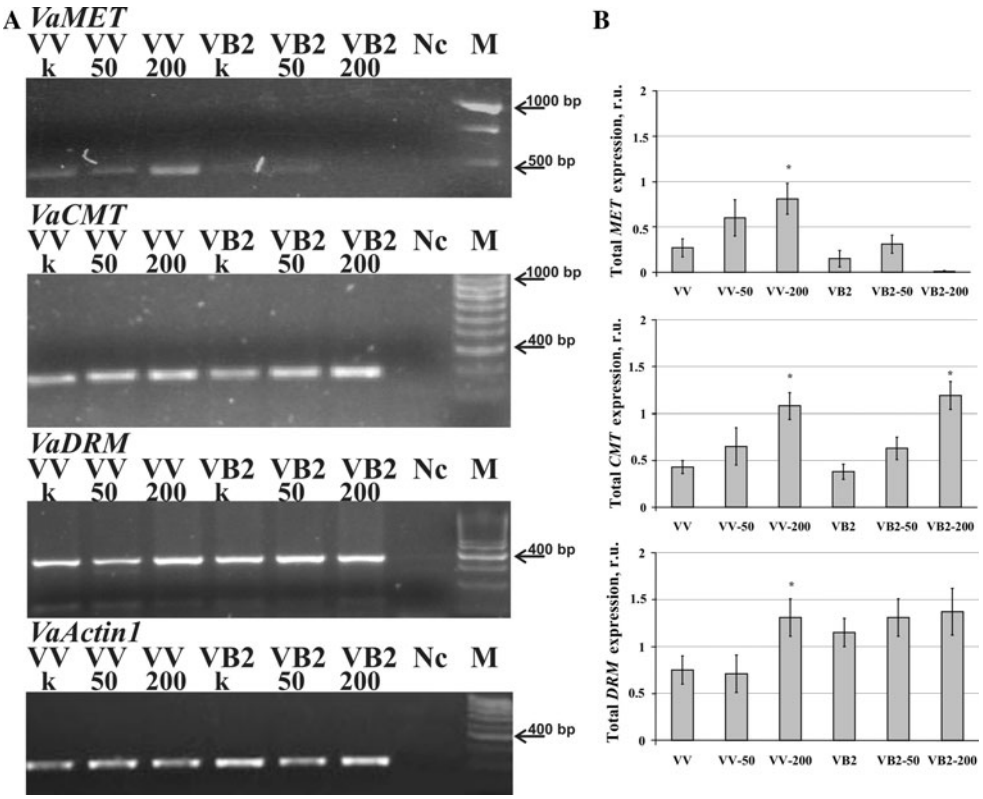

Fig. 3 Total gene expression of *VaMet*, *VaCMT*, and *VaDRM* in VV and VB2 cell cultures of *V. amurensis*, which were treated with 50 and 200 μM of azaC. **a** Electrophoregram of RT-PCR products of *VaMet*, *VaCMT*, *VaDRM*, and *VaActin1*. *Lines* 1, 2, and 3: VV culture samples obtained from the control group and treated with 50 and 200 μM of azaC; *Lines* 4, 5, 6: VB2 culture samples obtained from control group and treated with 50 and 200 μM of azaC. Nc: negative control (PCR mixture without the cDNA); M: synthetic marker. **b** Quantitative analysis of total *VaMet*, *VaCMT*, and *VaDRM* gene expression standardized to actin gene expression. Data are presented as the mean ± SEM obtained from 3 independent experiments. * $P < 0.05$; r.u.: relative units

rolB-transgenic cultures than it was in the VV cell culture under normal conditions. This can also be compared to the lower total levels of *VaCMT* and *VaMet* gene expression.

We used frequency analysis of RT-PCR products technique (Kiselev and Dubrovina 2010) to measure expression levels of individual methylases transcripts within mentioned methlyases families. By following this method five *VaMet* family methylase transcripts were detected in studied grape cell cultures: *VaMet1a*, *VaMet2a*, *VaMet3a*, *VaMet4a*, and *VaMet5a* each contained a different nucleotide sequence (Electronic supplementary material Table S2 and Fig. S1). Addition of azaC significantly increased levels of expression transcripts demonstrated high levels of expression in normal conditions. Thus, *VaMet1a*, *VaMet2a*, and *VaMet3a* transcripts increased in their expression after addition of the demethylation agent. The expression of the *VaMet1a* transcript increased by 2-fold in the VV culture that was treated with 200 μM of azaC (Fig. 4a). *VaMet2a* and *VaMet3a* transcripts were also detected in the VV cell culture under conditions of induced total DNA demethylation.

We detected four CMT family methylases transcripts with different nucleotide sequences in studied grape cell cultures: *VaCMT1a*, *VaCMT2a*, *VaCMT3a* and *VaCMT4a* (Fig. 5; Electronic supplementary material Table S3 and Fig. S2). Treatment with azaC led to significant enhancement in gene expression of the transcripts that were already well-expressed in normal conditions. These transcripts include *VaCMT1a* and *VaCMT2a* in both cell cultures (Fig. 5a). The increase in gene expression was proportional to the concentration of demethylation agent in the medium. Thus, adding 200 μM of azaC increased *VaCMT2a* expression 4-fold in control VV culture. This is compared to the 3.4-fold increase in *VaCMT1a* expression in VB2 culture, after adding the same concentration of azaC. It is necessary to note that azaC treatment led to the cessation of *VaCMT3a* and *VaCMT4a* expression in VV and in VB2 culture (Fig. 5a). As with the *VaMet* family transcripts, it is possible that we could not measure the expression of these transcripts in the context of the significant expression level increases that were observed in other transcripts.

We detected two transcripts of *VaDRM* family different with their nucleotide sequences: *VaDRM1a* and *VaDRM2a* (Fig. 6a; Electronic supplementary material Table S4 and Fig. S3). Frequency analysis of RT-PCR products revealed that azaC treatment did not affect the expression of *VaDRM1a* in both cell cultures. *VaDRM2a* expression significantly increased in the VV culture only after the culture was treated with 200 μM of azaC. The *rolB*-transgenic culture was not found to show significant changes in *VaDRM2a* expression.

Real-time PCR analysis was used to verify expression results on individual methylases transcripts as a more sensitive method. Real-time PCR data revealed that *VaMet1a*, *VaMet2a*, and *VaMet3a* gene expression increased in the VV culture after the addition of azaC. We also detected the presence of these *VaMet* transcripts in the VB2 culture treated with 200 μM of azaC (Fig. 4b), but the levels of the transcripts were lower than they were in the untreated VB2. Real-time PCR analysis confirmed previous *VaMet* gene expression results, which were obtained from VB2 calli that were passed to a fresh azaC medium every 3 days (Fig. 4c). The *VaCMT* expression data from the VV and VB2 cultures were confirmed using real-time PCR (Fig. 5b). Real-time PCR analysis indicated a significant increase in *VaCMT1a*

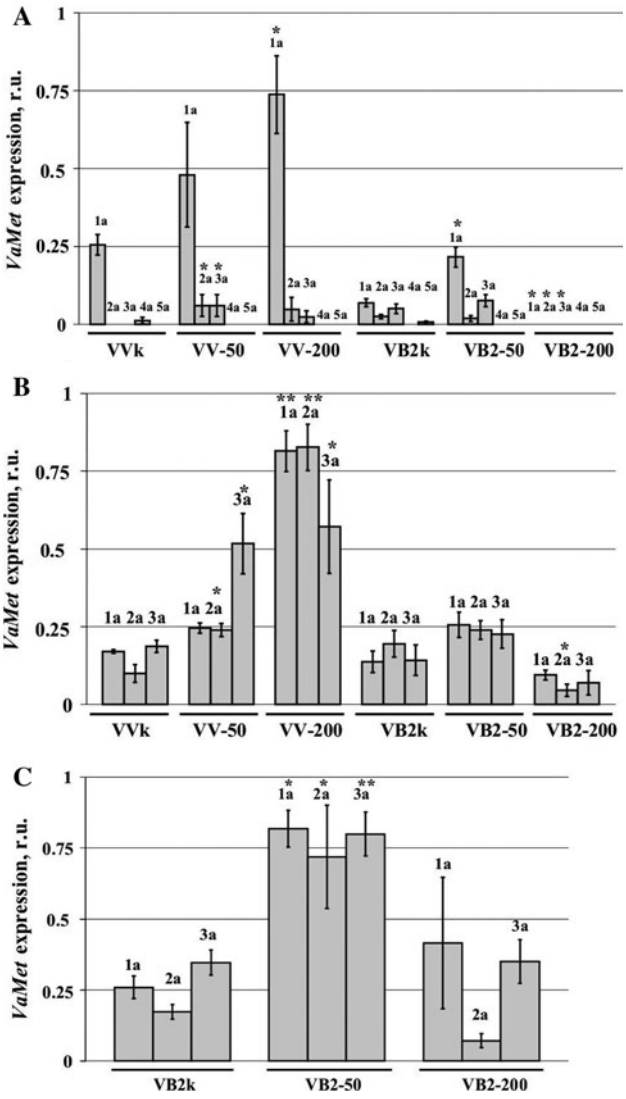

Fig. 4 Expression of certain *VaMet* genes in VV and VB2 *V. amurensis*. Expression values are derived from RT-PCR with degenerate primers (**a**), real-time PCR (**b**), and real-time PCR of VB2 calli in azaC medium that was changed every 3 days (**c**). Data are presented as the mean ± SEM obtained from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; r.u.: relative units

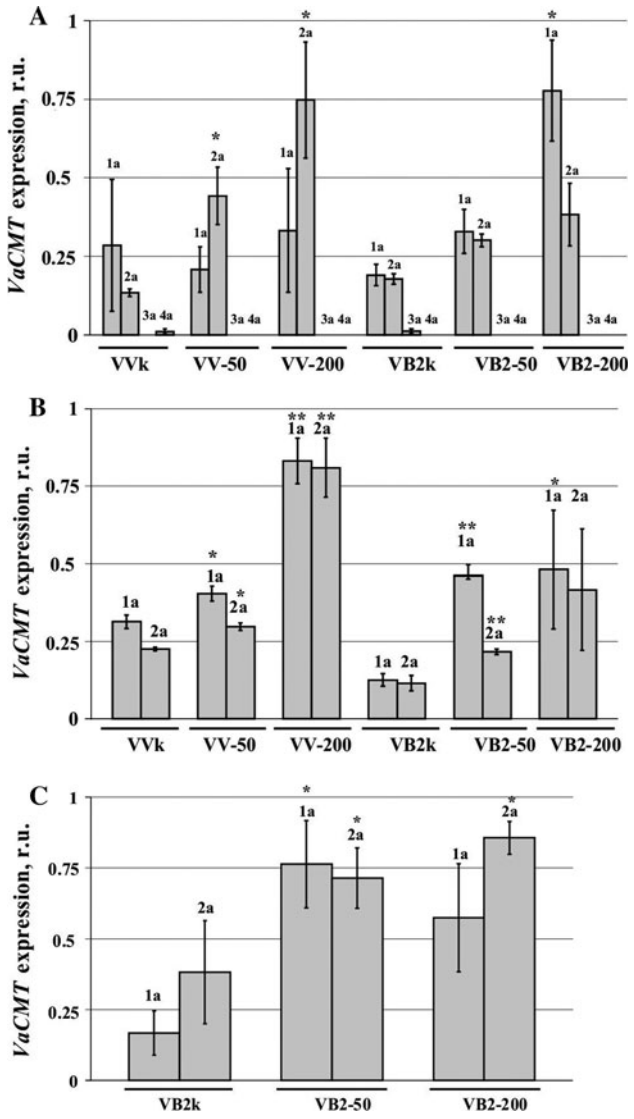

Fig. 5 Expression of certain *VaCMT* genes in VV and VB2 *V. amurensis*. Expression values are derived from RT-PCR with degenerate primers (**a**), real-time PCR (**b**), and real-time PCR of VB2 calli in azaC medium that was changed every 3 days (**c**). Data are presented as the mean ± SEM obtained from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; r.u.: relative units

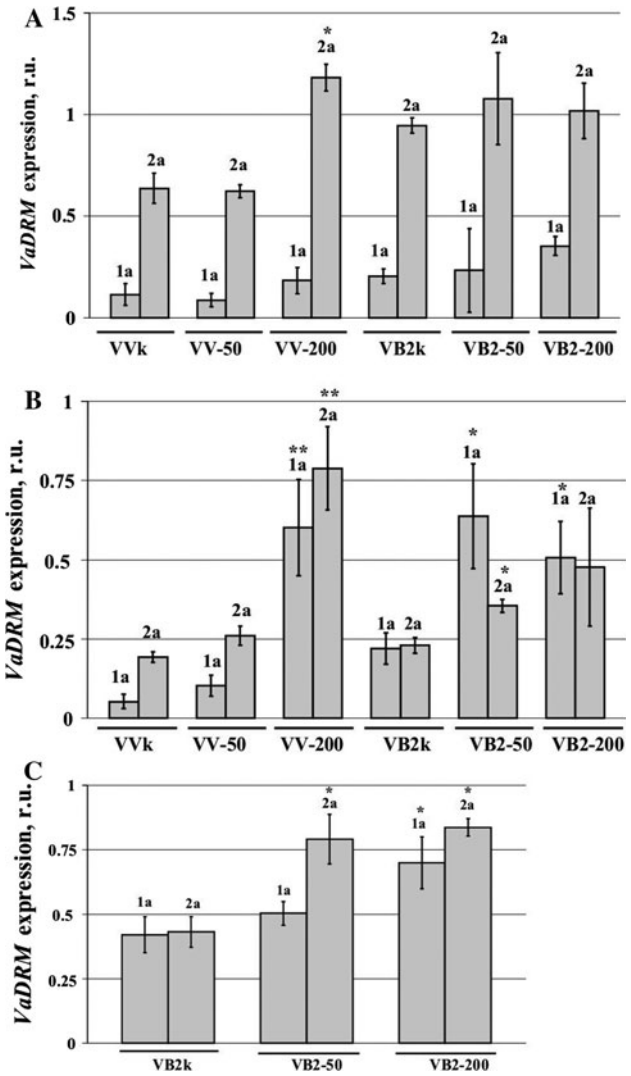

Fig. 6 Expression of certain *VaDRM* genes in VV and VB2 *V. amurensis*. Expression values are derived from RT-PCR with degenerate primers (**a**), real-time PCR (**b**), and real-time PCR of VB2 calli in azaC medium that was changed every 3 days (**c**). Data are presented as the mean ± SEM obtained from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; r.u.: relative units

and *VaCMT2a* gene expression under demethylating conditions in the VV and VB2 cultures (Fig. 5b). Analysis also confirmed the semi-quantitative RT-PCR *VaCMT* gene expression data; these results were obtained from VB2 calli passed to fresh medium containing azaC every 3 days (Fig. 5c). *VaDRM2a* gene expression increased in a dose-dependent manner after azaC was added to VB2 cell culture (Fig. 6b). Real-time PCR analysis also revealed that the expression of *VaDRM1a* and *VaDRM2a* increased by 12-fold and 4-fold respectively in the VV cell cultures that were treated with 200 μM of azaC. Real-time PCR analysis of *VaDRM* gene expression confirmed our semi-quantitative RT-PCR results; these data were obtained from VB2 calli cultivated in azaC medium, which was changed every 3 days (Fig. 6c). In VV and VB2 cell cultures, *VaDRM1a* expression was significantly higher in untreated *rolB*-transgenic cultures than it was in the untreated VV cultures (Fig. 6b).

Conclusion

The investigation of DNA methyltransferases expression under azaC treatment is scientifically and agriculturally significant. Interestingly, expression of individual methylases was differentially affected by azaC even within the

methylase subfamilies. This finding suggests that different methylases play different roles in cellular response to chemical DNA demethylation. Furthermore, we suggest that detected individual methylases transcripts are quite differ in their functional ability to methylate DNA, but this proposal awaits further investigation.

AzaC is used in agriculture to increase protein content in wheat and maize seeds (Vanyushin et al. 1990). Treatment with this demethylation agent leads to a great perturbation within whole plant epigenome and may result in increased DNA damage or double-stranded breaks (Tokuji et al. 2011; Hudson et al. 2011). In this case our report is the first to describe how plant methylases expression patterns are affected by azaC on example of resveratrol-producing *V. amurensis* cell cultures. AzaC treatment leads to reactivation of transposable elements and silenced genes (Hudson et al. 2011). Recent findings showed that azaC can also reduce expression of a chloroplast genome with abnormal phenotype of *Arabidopsis* seedlings (Hudson et al. 2011). According to our data azaC significantly induced methylases expression that can lead to random hypermethylation in normally unmethylated loci and result in its silencing. Taken together, these facts doubt using azaC in agriculture.

Bacterially infected tissues show a reduction in DNA methylation. Infection may activate the expression of disease resistance genes that conduct surveillance against pathogens (Alvarez et al. 2010). In our last report, we demonstrated that azaC treatment caused an increase in *STS* expression (Kiselev et al. 2011). This may indicate the expression activation of disease resistance genes. As a result, plant DNA methylation and resveratrol production may be interconnected processes. Unfortunately, in present work we could not find correlation of certain methylases gene expression with levels of resveratrol production, therefore, further investigation of possible roles of DNA methylases in plant secondary metabolism is necessary.

Acknowledgments The authors express their thanks to Alexandra S. Dubrovina for helpful comments on the manuscript. This work was supported by grant of the Russian Foundation for Basic Research (10-04-00189-a), by grants of the Far East Division of the Russian Academy of Sciences.

References

Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y (2004) Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 24: 2783–2840

Alvarez ME, Nota F, Cambiagno DA (2010) Epigenetic control of plant immunity. Mol Plant Pathol 11:563–576

Baranek M, Krizan B, Ondrusikova E, Pidra M (2010) DNA-methylation changes in grapevine somaclones following in vitro culture and thermotherapy. Plant Cell, Tissue Organ Cult 101:11–22

Bavaresco L, Fregoni C, Van Zeller de Macedo Basto Gonçalves MI, Vezzulli S (2009) Physiology and molecular biology of grapevine stilbenes: an update. In: Roubelakis-Angelakis KA (ed) Grapevine molecular physiology and biotechnology, 2nd edn. Springer, London, pp 341–364

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

Bitonti MB, Cozza R, Chiappetta A, Giannino D, Castiglione MR, Dewitte W, Mariotti D, Van Onckelen H, Innocenti AM (2002) Distinct nuclear organization, DNA methylation pattern and cytokinin distribution mark juvenile, juvenile-like and adult vegetative apical meristems in peach (*Prunus persica* (L.) Batsch). J Exp Bot 53:1047–1054

Buryanov YI, Shevchuk TV (2005) DNA methyltransferases and structural–functional specificity of eucarioyic DNA modification. Biochem-Moscow 70:730–742

Chinnusamy V, Zhu JK (2009) RNA-directed DNA methylation and demethylation in plants. Sci China, Ser C Life Sci 52:331–343

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 J, Liu X, Xu SX, Xu Q, Guo WW (2011) T-DNA direct repeat and 35S promoter methylation affect transgene expression but do not cause silencing in transgenic sweet orange. Plant Cell, Tissue Organ Cult 107:225–232

Finnegan EJ, Kovac KA (2000) Plant DNA methyltransferases. Plant Mol Biol 43:189–201

Hudson K, Luo S, Hagemann N, Preuss D (2011) Changes in global gene expression in response to chemical and genetic perturbation of chromatin structure. PLoS ONE 6:e20587

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 analyzing gene expression based on frequency analysis of RT-PCR products obtained with degenerate primers. Acta Physiol Plant 32: 495–502

Kiselev KV, Tchernoded GK (2009) Somatic embryogenesis in the *Panax ginseng* cell culture induced by the *rolC* oncogene is associated with increased expression of *WUS* and *SERK* genes. Russ J Genet 45:511–518

Kiselev KV, Kusaykin MI, Dubrovina AS, Bezverbny DA, Zvyagintseva TN, Bulgakov VP (2006) The *rolC* gene induces expression of a pathogenesis-related beta-1,3-glucanase in transformed Ginseng cells. Phytochemistry 67:2225–2231

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, 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) *CDPK* gene expression in somatic embryos of *Panax ginseng* expressing *rolC*. Plant Cell Tiss Organ Cult 99:141–149

Kiselev KV, Turlenko AV, Zhuravlev YN (2010) 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, Tyunin AP, Manyakhin AY, Zhuravlev YN (2011) Resveratrol content and expression patterns of stilbene synthase genes in *Vitis amurensis* cells treated with 5-Azacytidine. Plant Cell, Tissue Organ Cult 105:65–72

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

Lang-Mladek C, Popova O, Kiok K, Berlinger M, Rakic B, Aufsatz W, Jonak C, Hauser MT, Luschnig C (2010) Transgenerational inheritance and resetting of stress-induced loss of epigenetic gene silencing in *Arabidopsis*. Mol Plant 3:594–602

Law J, Jacobsen E (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11:204–220

Martienssen RA, Richards EJ (1995) DNA methylation in eukaryotes. Curr Opin Genet Dev 5:234–242

Messeguer R, Ganal MW, Steffens JC, Tanksley SD (1991) Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. Plant Mol Biol 16:753–770

Miki D, Shimamoto K (2008) De novo DNA methylation induced by siRNA targeted to endogenous transcribed sequences is gene-specific and OsMet1-independent in rice. Plant J 56:539–549

Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. Annu Rev Plant Biol 60:485–510

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

Spena A, Schmulling T, Koncz C, Schell JS (1987) Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants. EMBO J 6:3891–3899

Tokuji Y, Takano S, Tonomura M, Tanaka S, Igari K, Watanabe T (2011) Influence of 5 '-azacytidine on promoting recovery of cell competence for shoot organogenesis in Arabidopsis. Plant Cell, Tissue Organ Cult 106:289–297

Vanyushin BF (2005) Enzymatic DNA methylation is an epigenetic control for genetic functions of the cell. Biochem-Moscow 70:598–611

Vanyushin BF, Sevostyanova SS, Kirnos MD, Saidova NS (1990) DNA methylation inhibitor 5-azacytidine induced increase in protein content in wheat. Izvestiya: Biol 1:75–83

Veena V, Taylor CG (2007) *Agrobacterium rhizogenes*: recent developments and promising applications. In Vitro Cell Dev Biol Plant 43:383–403

Weber H, Ziechmann C, Graessmann A (1990) In vitro DNA Methylation inhibits gene expression in transgenic tobacco. EMBO J 9:4409–4415

Xiong LZ, Xu CG, Saghai Maroof MA, Zhang Q (1999) Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. Mol Gen Genet 261:439–446

Zeng F, Qian J, Luo W, Zhan Y, Xin Y, Yang C (2010) Stability of transgenes in long-term micropropagation of plants of transgenic birch (*Betula platyphylla*). Biotechnol Lett 32:151–156

Zluvova J, Janousek B, Vyskot B (2001) Immunohistochemical study of DNA methylation dynamics during plant development. J Exp Bot 52:2265–2273