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ORIGINAL ARTICLE

Involvement of DNA methylation in the regulation of *STS10* gene expression in *Vitis amurensis*

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Abstract DNA methylation is known to play an important role in various developmental processes and defense mechanisms in plants and other organisms. However, it is not known whether DNA methylation is implicated in the genetic regulation of plant secondary metabolism, including resveratrol biosynthesis. Resveratrol is a naturally occurring polyphenol that is present in grapes, peanuts, and other plant sources, and it exhibits a wide range of valuable biologically active properties. The transformation of the wild-growing grape *Vitis amurensis* with the oncogene *rolB* from *Agrobacterium rhizogenes* has been demonstrated to considerably increase resveratrol production. To investigate whether DNA methylation regulates resveratrol biosynthesis, we treated both *rolB* transgenic and empty vector control *V. amurensis* cell cultures with the DNA demethylation agent 5-azacytosine (azaC). The azaC treatment significantly increased stilbene synthase 10 gene (*VaSTS10*) expression and resveratrol content in the *V. amurensis* cell cultures. Using bisulfite sequencing, we examined the methylation status of *VaSTS10* in cell cultures under normal conditions and after azaC treatment. Both the promoter and 3′-end of the protein coding region of the *VaSTS10* gene were hypermethylated (54–67 %) in the control cell culture. The *rolB* transgenic cell culture had high levels of resveratrol and lower hypermethylation levels of the *VaSTS10* gene (20–47 %). The azaC treatment resulted in reduction in the DNA methylation levels in the promoter and coding regions of the *VaSTS10* gene in both cell cultures. These data suggest that the DNA methylation may be involved in the control of resveratrol biosynthesis via the regulation of *STS* genes expression.

Keywords 5-Azacytidine · Bisulfite sequencing · Epigenetics · Resveratrol · Stilbene synthase

Abbreviations

azaC	5-Azacytidine
DW	Dry weight
STS	Stilbene synthase

Introduction

DNA methylation plays an important role in the epigenetic control of gene expression in many eukaryotes (Martienssen and Richards 1995) and is very common in plant genomes (Finnegan et al. 1998). DNA methylation is often associated with gene silencing and is well-known to silence transposable elements (Dieguez et al. 1998; Okamoto and Hirochika 2001). Several reports have suggested that DNA methylation has a role in the regulation of plant development; however, evidence supporting this finding is sparse. Significant differences in cytosine methylation have been observed between different organs in species such as in tomatoes *Lycopersicon esculentum* (Messeguer et al. 1991), rice *Oryza sativa* (Xiong et al. 1999), and white cockles *Silene latifolia* (Zluvova et al. 2001). Moreover, cytosine

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methylation variations have been found between different developmental phases in pines *Pinus radiate* (Fraga et al. 2002) and peaches *Prunus persica* (Bitonti et al. 2002). Genome-wide demethylation causes abnormal development of *Arabidopsis thaliana* (Finnegan et al. 1998). Depending on the genes affected, a reduction in cytosine methylation can result in different effects on flowering time. Furthermore, treatment with vernalization or demethylation agents, such as 5-azacytidine (azaC), is known to promote flowering due to reduced levels of DNA methylation (Burn et al. 1993). While studies address the effects of DNA methylation on plant development, its role in plant secondary metabolism is currently unclear.

The stilbene synthase (STS, EC 2.3.1.95) genes are involved in resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) biosynthesis. Resveratrol is a plant-derived polyphenol compound that displays a wide range of intriguing biological properties. Resveratrol has been identified to have anti-inflammatory, antioxidant, and platelet anti-coagulant properties (Shankar et al. 2007). In addition, resveratrol is known to possess preventive properties against certain cancers and cardiovascular disease, and it has been documented to promote longevity (Aggarwal et al. 2004; Shankar et al. 2007). Stilbenes, including resveratrol, are synthesized via the phenylpropanoid pathway (Langcake and Pryce 1977). Stilbene synthase (STS) or resveratrol synthase condense three molecules of malonyl-CoA and one molecule of cumaryl-CoA to form resveratrol (Rupprich et al. 1980; Xu et al. 2010). STS exists as a multi-gene family in most stilbenoid-producing species, including grapes, peanuts, and pines (Fliegmann et al. 1992; Sparvoli et al. 1994; Fan et al. 2008; Kiselev 2011). Southern blot experiments suggested that the *Vitis vinifera* (*V. vinifera*) genome contains approximately 15–20 *STS* genes (Sparvoli et al. 1994). More recent analyses of the draft assembly of the *V. vinifera* genome confirmed the large size of these multi-gene families, with an estimated 13 *PAL* genes and 21–43 *STS* genes (Jaillon et al. 2007; Velasco et al. 2007). An analysis of *STS* gene expression in cultured *V. amurensis* cells transformed with the *rolB* gene from *Agrobacterium rhizogenes* (*A. rhizogenes*) was conducted previously. The results suggested that the *STS* family in *V. amurensis* consists of at least 10 *STS* genes that differ in both nucleotide sequence and expression patterns (Kiselev et al. 2009, 2010a; Kiselev and Dubrovina 2010).

The cell cultures of a variety of plant species contain low resveratrol levels [up to 0.03 % of dry weight (DW)], and treatments with UV irradiation, elicitors, precursors, or other agents result in an increase in resveratrol production (Ku et al. 2005; Tassoni et al. 2005; Kiselev et al. 2007; Ferri et al. 2011). We previously showed that transformation of cultured *V. amurensis* cells with the *rolB* gene from *A. rhizogenes* greatly enhanced resveratrol production in transformed calli (Kiselev et al. 2007). Southern blot analysis revealed that DNA from VB2 cells contained at least two copies of the *rolB* gene (Kiselev et al. 2011a). Semi-quantitative RT-PCR and real-time PCR analyses determined that the *rolB* gene was expressed in VB2 cells (Kiselev et al. 2007, 2009). In these transgenic cultures, resveratrol production increased more than 100-fold (up to 3.15 % of DW) compared with the controls or the resveratrol levels that were observed in other studies performed in plant cell cultures (Ku et al. 2005; Tassoni et al. 2005; Kiselev et al. 2007). However, after long-term cultivation, the *rolB* transgenic culture of *V. amurensis* loses its ability to produce high levels of resveratrol (Dubrovina and Kiselev 2011). The transgenic cells produce significantly less resveratrol (0.1–0.8 % of DW) after 2.5 years of cultivation. Nonetheless, these content levels are still significantly higher compared with the control cell culture. Cytosine DNA methylation is known to influence the expression of secondary metabolism genes and transgenes. We hypothesize that the reduced levels of resveratrol production that are observed in the long-term cultures of transgenic *V. amurensis* cells are caused by cytosine DNA methylation. Therefore, we used both control and *rolB* transgenic *V. amurensis* cells as a model system to investigate the effect of DNA methylation on resveratrol metabolism.

Earlier, we determined that azaC led to an increase in resveratrol production in *V. amurensis* cell cultures VV and VB2 (Kiselev et al. 2011a; Tyunin et al. 2012). We also found statistically increased expression levels only of the *VaSTS10* gene in the both analyzed cell cultures of *V. amurensis*. The increase in *VaSTS10* expression was the highest among those for other analyzed *STS* genes; therefore, we proposed that the cytosine methylation of the *VaSTS10* gene, or its regulatory regions, suppresses resveratrol biosynthesis. Therefore, the aim of this study was to analyze the methylation pattern of the *VaSTS10* gene under both normal conditions and after azaC treatment. Because bisulfite sequencing is widely used to detect cytosine methylation within a DNA sequence at one-base resolution, we chose this method to identify the methylation status of the *VaSTS10* gene in *V. amurensis* cell cultures. The data demonstrate a negative correlation between the cytosine methylation levels of *VaSTS10* and the level of resveratrol production in the cell cultures. According to the bisulfite sequencing data, both the promoter and protein coding regions of the *VaSTS10* gene were hypermethylated under normal conditions (especially in the control cell culture VV) when both the level of *VaSTS10* gene expression and resveratrol production were low. These results indicate that cytosine DNA methylation plays an important role in the regulation of resveratrol biosynthesis via the modulation of *VaSTS10* gene expression.

Materials and methods

Vitis amurensis cell culture

As described previously, the V2 callus culture of wild-growing grape, *Vitis amurensis* Rupr. (Vitaceae), was established in 2002 (Kiselev et al. 2007). The VV culture was established via the co-cultivation of the V2 cell suspension with *A. tumefaciens* GV3101/pMP90RK strains containing the pPCV002 plasmid vector carrying a kanamycin resistance gene (*nptII*) (Kiselev et al. 2007). The *rolB* transgenic *V. amurensis* callus culture (designated VB2) was obtained in 2004 via the transformation of the V2 cell suspension with *A. tumefaciens* strain GV3101 containing the binary plasmid vector pPCV002-CaMVB/pMP90RK, as described previously (Kiselev et al. 2007). In the pPCV002-CaMVB vector, the *rolB* gene is under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Spena et al. 1987).

The VV and VB2 callus cultures were cultivated in 35-day subculture intervals in the dark at 24–25 °C in 15 ml of $W_{B/A}$ solid medium (Kiselev et al. 2009). Reagents for cell culture were purchased from Sigma (St Louis, MO, USA) and Serva (Heidelberg, Germany). The samples were harvested from the 35-day cultures during their linear growth phase and when the highest resveratrol content was observed. These samples were weighed and used for quantitative real-time PCR. The calli were dried using hot air (50 °C for 2 h), and their levels of resveratrol were measured (Dubrovina et al. 2010).

5-Azacytidine treatments

The reagents for tissue culture were obtained from Sigma or ICN (New York, USA). Sterile aqueous solutions of azaC were added to autoclaved media in the 200 μM, because it was shown that this concentration significantly increased resveratrol content and *VaSTS10* expression in the grape cells (Kiselev et al. 2011a).

HPLC analyses

Transgenic lines harboring the pPCV002 and pPCV002-CaMVB constructs were analyzed for the presence of stilbenes by HPLC analyses as described previously (Kiselev et al. 2011a).

Bisulfite sequencing

The DNA methylation status of the *VaSTS10* 5′-upstream protein coding region (GeneBank accession number JQ780328) was investigated using the bisulfite sequencing method. The *VaSTS10* gene was divided into several PCR products (Fig. 1). Genomic DNA was isolated as described previously (Kiselev et al. 2011b). 50 mg of *V. amurensis* cells dried in a thermostat (at +37 °C) was homogenized with the addition of 50 mg Al_2O_3. The obtained powder was mixed with 800 μl of buffer containing 0.2 % mercaptoethanol, 100 mM Tris, pH 7.5–8.0, 0.7 M NaCl, 40 mM EDTA, pH 7.5–8.0, and 1 % hexadecyltrimethylammoniumbromide (CTAB). The mixture was incubated at 60 °C for 1.5 h under stirring. Then, 300 μl of chloroform were added, the probe was gently mixed for 5 min, and centrifuged for 5 min at 13,200*g* at 4 °C (5415R, Eppendorf, Hamburg, Germany). 400 μl of water phase was mixed with 1 ml of ethanol and incubated at −20 °C overnight. Then, probes were centrifuged for 7 min at 13,200*g* at 4 °C. Pellets were dried at +37 °C and dissolved in 150 μl of distilled water.

A total of 1.5 μg of *V. amurensis* genomic DNA was subjected to bisulfite modification using the Zymo Research EZ DNA Methylation-Gold Kit (Irvine, CA, USA), as per the manufacturer's instructions. The DNA was converted using the following conditions: 95 °C for 4 min and 47 °C for 1.5 h. The cloned PCR products from the 5′-upstream region and parts of the coding region were used as positive control for the bisulfite chemical reactions. The level of C to T transitions in the converted PCR products was greater than 95 %.

The following primers, designed according to the Zymo Research recommendations for the EZ DNA Methylation-Gold Kit, were used to amplify the corresponding sequences from the promoter regions of the *VaSTS10* gene −508 to −122 of the 5′-upstream region: PS2, 5′-ATG GTY AAA TTA TAG YAT TYG GGG, and PA2, 5′-TTT CAC CAA CCC TCA TCC ACT T. Although we designed two pairs of primers for the 5′-upstream region of the *VaSTS10* gene, we obtained PCR products of the 5′-upstream region of the

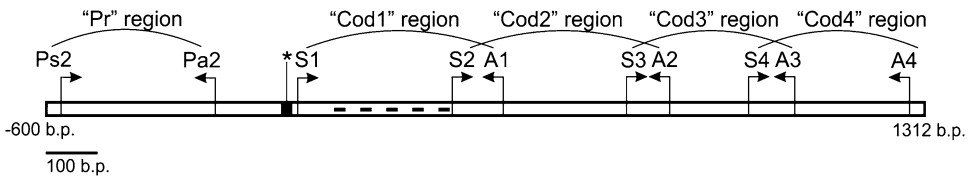

Fig. 1 Schematic diagram of the *VaSTS10* promoter and protein coding regions. PS2, PA2, S1, S2, S3, S4, A1, A2, A3, and A4 primers. For further explanation, see "Materials and methods". The *asterisk* indicates the start codon, and the dashes indicate the intron position

VaSTS10 gene only using the PS2 and PA2 primers. For example, using PS1 and PA1 primers, which were designed to amplify from −261 of the 5′-upstream region to +50 of the coding region of the *VaSTS10* gene, we did not detect PCR products of the 5′-upstream region of the *VaSTS10* gene, only some other parts of Vitis DNA. Thus, the corresponding sequences from the protein coding region of the *VaSTS10* gene were amplified using the following primers: the first area of the coding region (Cod1), S1: 5′-TGT GGA GGA AAT TAG AAA TGY T and A1: 5′-CCC ACT CTT TAA RAR CCT TCA AT. The second area of the coding region (Cod2), S2: 5′-AAA YAT TGG TGY TTA TAT GGY T and A2: 5′-RCT CCT RCA TTA TTC TCA RCA A. The third area of the coding region (Cod3), S3: 5′-TYA GTY AGA AGA GTA ATG TTG TA and A3: 5′-TCA AAC ATT TCT CTA TAT TTT C. The fourth area of the coding region (Cod4), S4: 5′-AAG GTG YTA TTG YAG GYA AYT T and A4: 5′-TCC AAA RCC AAA CAA RAC RCC C.

Also, we analyzed the methylation level of the 5′-upstream region and the 3′ end of the coding region of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *actin* grape genes using primers 5′-TTT TTY AAT YAA GTT GAY TGT YTA, 5′-AAA CTA RAA CAC TTA CCC ATR ATC TA (for the 5′-upstream region of *GAPDH*); 5′-TTT TTG TGA TGG AAG GTG TTT ATG T, 5′-TCA CAT RTT CTT TAT RTA ATR TCC CA (for the 3′ end of the coding region of *GAPDH*); 5′-TTG AGT AAG GGA TTT GTT TGG AGG T, 5′-TTR CTR CTT RAC AAA TAT TCA CCT T (for the 5′-upstream region of *actin*); 5′-ATG AAG TGT GAT GTT GAT ATY AGG AA, 5′-ACC ARA TTC ATC ATA TTC ACC CTT (for the 3′ end of the coding region of *actin*).

Amplification reactions were performed in volumes of 25 µl containing 10 mM Tris–HCl (pH 8.5), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % gelatin, 0.1 mM Triton X-100, 0.25 mM of each dNTP, 0.25 µM of primer, and 1 unit of Taq polymerases ("Silex M", Russia) (Kiselev et al. 2010a). Analysis was performed in an iCycler thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) programmed for an initial denaturation step of 2 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 20 s at 52 °C, 40 s at 72 °C, and a last cycle of 72 °C for 20 min, using the fastest available transitions between each temperature.

The PCR products were subcloned into the pTZ57R/T plasmid using the InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania) and sequenced using a Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Biosystems, Forster City, CA, USA), following the manufacturer's protocol and recommendations at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEBRAS (Dubrovina et al. 2009; Shumakova et al. 2011) with an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Two independent amplifications each for the *VaSTS10* promoter and protein coding regions from DNA of VV and VB2 cell cultures were carried out. Two groups of clones for each region were separately collected from the two PCR reactions and sequenced. Thus, the total number of analyzed clones (not less than 14 clones) for each region is the result of two collections of clones obtained from two PCR products. After ethanol purification, the DNA sequences were determined using an ABI 3130 Genetic Analyzer (Perkin-Elmer Biosystems). The BLAST search program was used for sequence analysis. Multiple sequence alignments were performed using the ClustalX program (Altschul et al. 1990). Coding regions of *STS* genes are highly conserved, but we compared sequences of the clones with the sequence of *VaSTS10* gene (GeneBank JQ780328), which DNA was sequenced earlier. If the obtained clone sequences differed more than by 1 % of the nucleotides from the *VaSTS10* gene (analyzed only A, T, and G nucleotides) we did not use this sequence in our work. We used only highly identical to *VaSTS10* gene sequences (electronic supplementary material Fig. S1). The quantity of the sequences, different from the *VaSTS10* gene, was usually no more than 5–10 %.

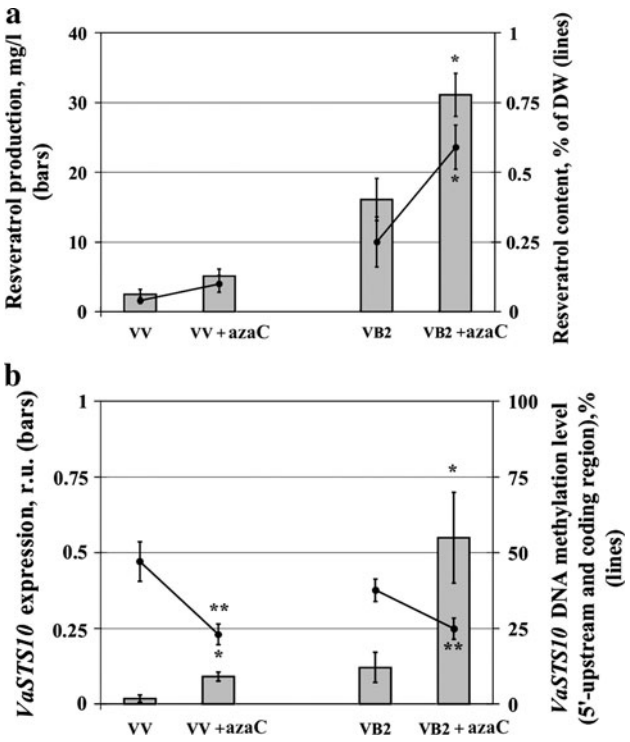

Fig. 2 Resveratrol production and resveratrol content (**a**). *VaSTS10* expression and *VaSTS10* DNA methylation levels in the 5′-upstream and coding regions (**b**) in azaC-treated *V. amurensis* VV and VB2 callus cultures. The data are presented as the mean ($n = 14$) ± SE obtained from two independent experiments. *$P < 0.05$; **$P < 0.01$ versus the values measured from the untreated VV or VB2 cultures

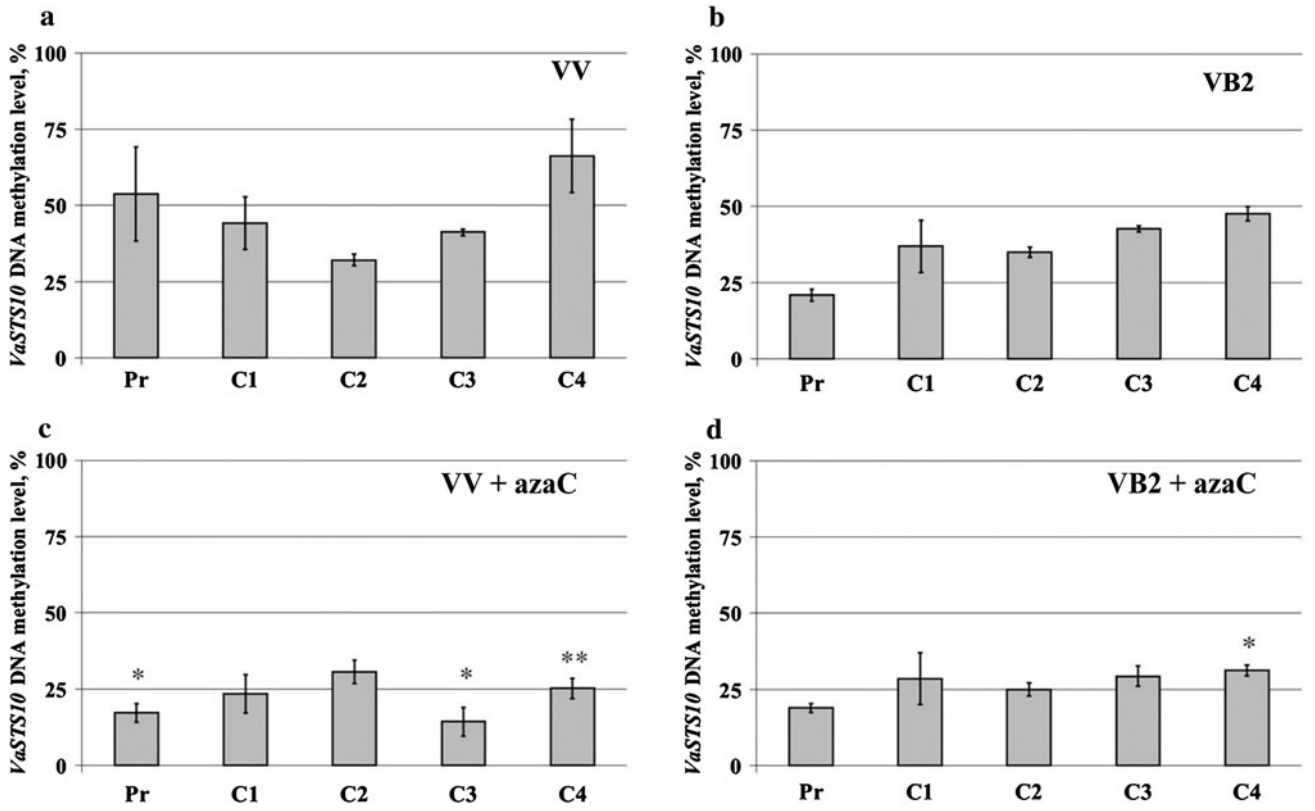

Fig. 3 Analysis of the total DNA methylation status of the different regions of the *VaSTS10* gene under normal conditions in VV (**a**) and VB2 (**b**) cell cultures and after azaC treatment (**c**, **d**). Pr: methylation levels in the 5′-upstream region of *VaSTS10*; C1: Cod1 region methylation levels; C2: Cod2 region methylation levels; C3: Cod3 region methylation levels; C4: Cod4 region methylation levels. The data are presented as the mean ($n = 14$) ± SE obtained from two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus the values measured from the untreated VV or VB2 cultures

Statistical analysis

Statistical analysis was performed using Statistica 10, and statistical significance was determined using the paired Student's *t* test. The data are presented as mean ± standard error (SE). We required that $P < 0.05$ for statistical significance.

Results

VaSTS10 gene expression and total methylation of *VaSTS10* in *V. amurensis* callus cultures

We compared our results from this study with previously reported data (Kiselev et al. 2011a) by detecting resveratrol content, resveratrol production, and *VaSTS10* gene expression in the VV and VB2 callus cultures. Our results were similar to those described earlier by Kiselev et al. 2011a (Fig. 2a). We demonstrated that both resveratrol content and production in VV and VB2 cells were increased twofold upon the addition of azaC. Increasing resveratrol content in VB2 cells and in cells treated with azaC correlated with increased *VaSTS10* gene expression (Fig. 2b).

We also estimated the total DNA methylation level of *VaSTS10* in the VV and VB2 callus cultures. Interestingly, the total level of *VaSTS10* gene methylation in the VB2 callus culture was 1.2-fold lower compared with the methylation in the VV cell culture; however, this difference was not significant (Fig. 2b). The addition of azaC significantly decreased the total methylation level of the *VaSTS10* gene in both the VV and VB2 cell cultures (Fig. 2b). Furthermore, we determined the methylation levels of both the 5′-upstream and protein coding regions of *VaSTS10*.

VaSTS10 methylation in the 5′-upstream and protein coding regions in *V. amurensis* callus cultures

Under normal conditions, we established that in the 5′-upstream region in *VaSTS10* in the VV cell line there is a large number of methylated cytosines (i.e., approximately 60 % of all cytosines) (Fig. 3a). The level of cytosine methylation in the 5′-upstream region of *VaSTS10* in the

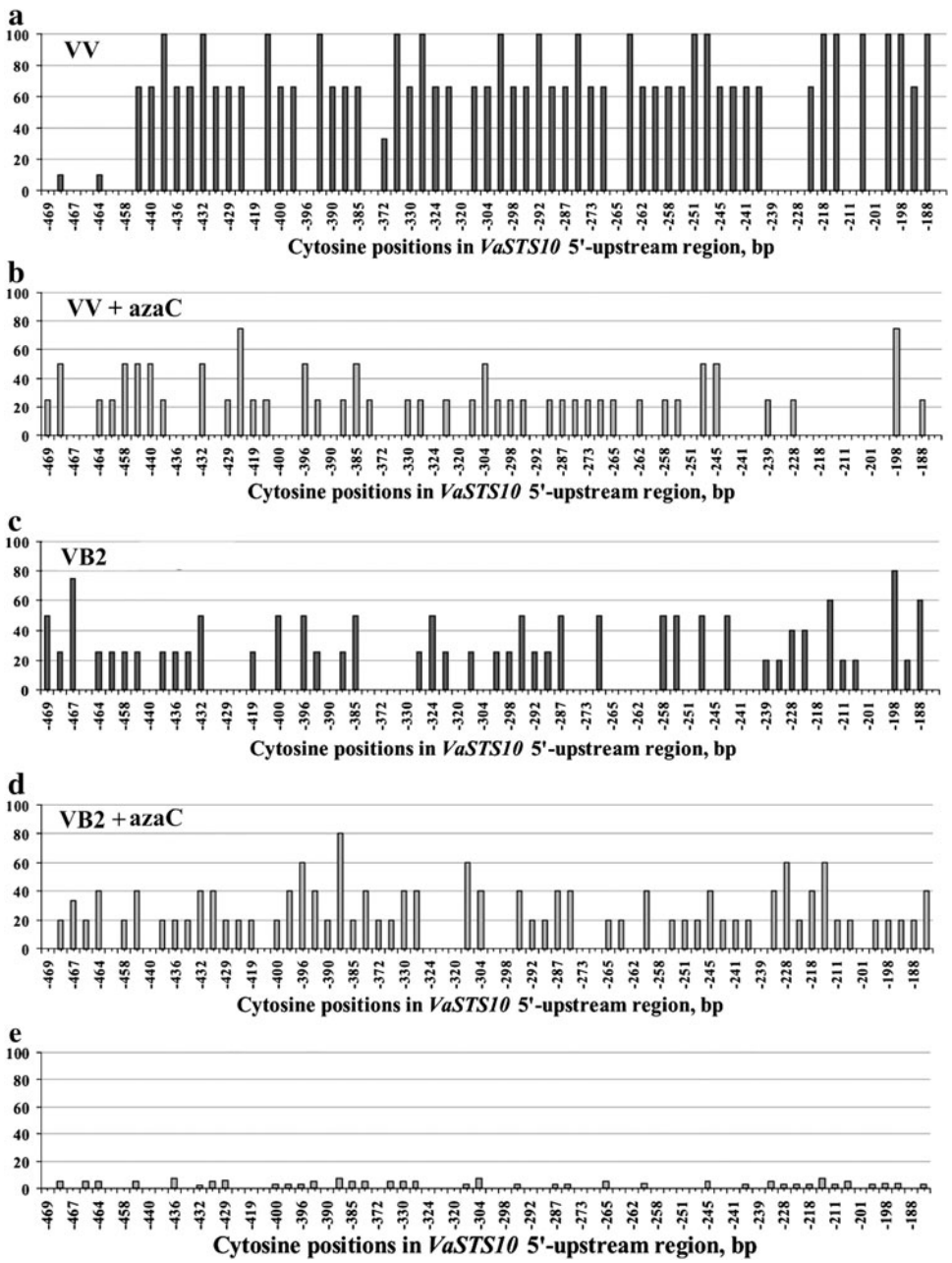

Fig. 4 Analysis of DNA methylation status in the *VaSTS10* 5′-upstream region in both VV and VB2 *V. amurensis* callus cultures treated with azaC (**a**–**d**) and in converted PCR products (**e**). The horizontal axis represents all cytosines present in the *VaSTS10* 5′-upstream region (*the arrow* indicates the portion of the *VaSTS10* 5′-upstream region analyzed, which was from −508 to −122). The *vertical axis* represents the percentage of methylated cytosines

VB2 cells was significantly lower (approximately in two- to threefold) than in the VV cell culture (Fig. 3b). The lowest methylation level among the different parts of the protein coding regions of the *VaSTS10* gene was detected in the second coding region (32–35 % of all cytosines), and the highest methylation level was observed in the fourth coding region (48–66 %) (Fig. 3a, b). Incubation with azaC significantly decreased methylation in the 5′-upstream region, as well as in coding regions 1, 3, and 4. The methylation levels in the second coding region decreased after the azaC addition; however, this reduction was not significant (Fig. 3c, d).

We analyzed the methylation status of the individual cytosines in the *VaSTS10* gene sequence. We determined that a majority of the cytosines are methylated (from 60 to 100 %) in the 5′-upstream region of *VaSTS10* in the VV cell culture (Fig. 4a). The majority of the *VaSTS10* methylated cytosines in the VV cell culture were located in the central and 3′-end of the 5′-upstream region. In the DNA of the VB2- and azaC-treated grape cells, the quantity of methylated cytosines was reduced compared with the VV cell culture under normal conditions, and the majority of cytosines were methylated approximately 20–50 % (Fig. 4b–d).

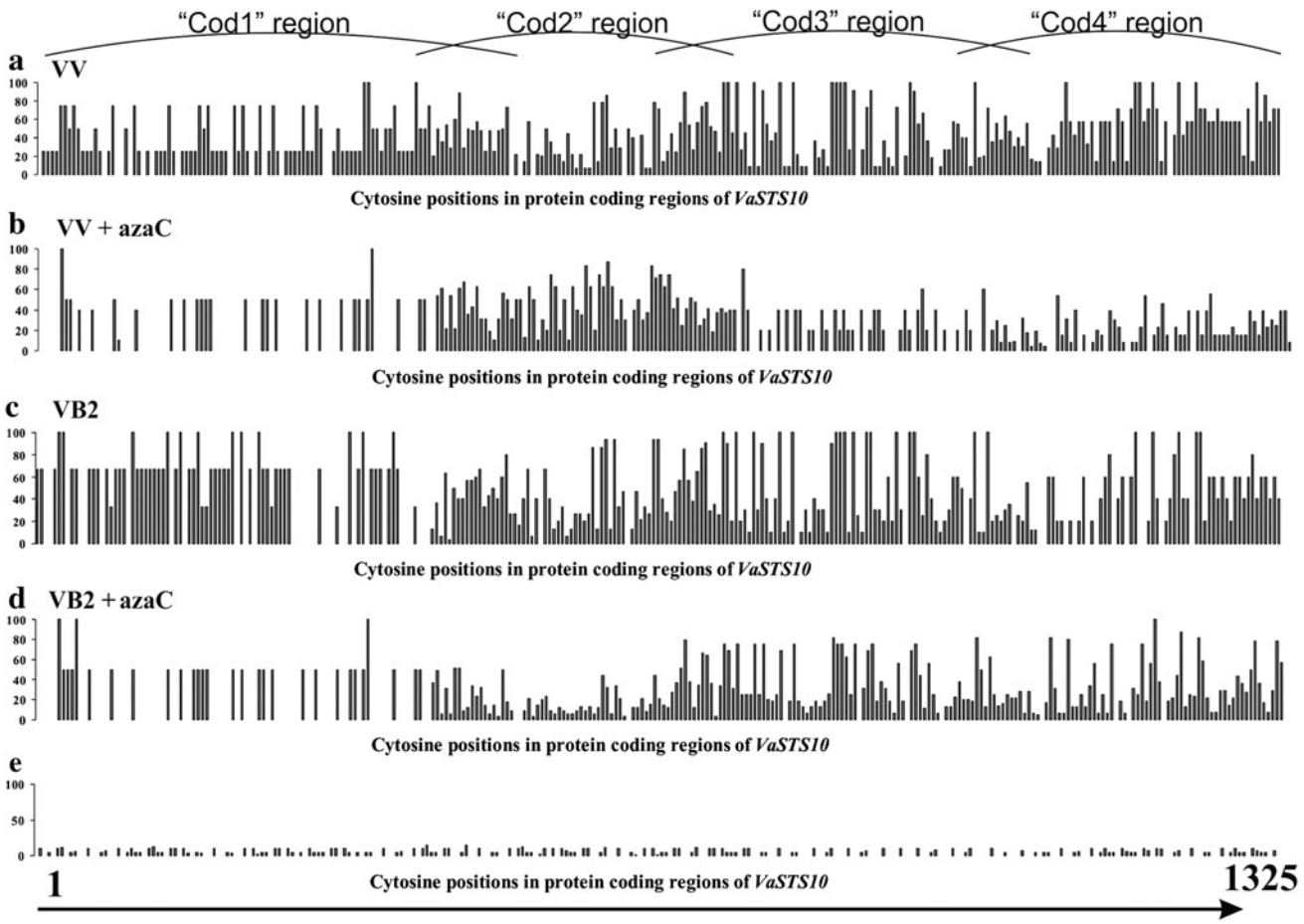

Fig. 5 Analysis of DNA methylation in the *VaSTS10* protein coding region in VV and VB2 *V. amurensis* callus cultures treated with azaC (**a**–**d**) and in converted PCR products (**e**). The horizontal axis represents all cytosines present in the *VaSTS10* protein coding region, and the vertical axis represents the percentage of methylated cytosines

Table 1 Methylation status in the *VaSTS10* 5′-upstream and protein-coding regions under normal conditions in *V. amurensis* callus cultures VV, VB2 and after azaC treatment

	CG	CHG	CHH
VV	48.8 ± 4.6	61.7 ± 4.3	47.0 ± 1.8
VB2	40.0 ± 4.8	49.1 ± 3.7	26.1 ± 1.5
VV + azaC	33.1 ± 3.9*	24.2 ± 3.1**	22.1 ± 1.6**
VB2 + azaC	36.0 ± 4.3	37.3 ± 3.8*	21.0 ± 1.1

The data are presented as the mean ($n = 14$) ± SE obtained from two independent experiments, * $P < 0.05$; ** $P < 0.01$ versus the values measured from the untreated VV or VB2 cultures

Next, we analyzed the methylation status of the individual cytosines in the protein coding region of *VaSTS10*, including a comparison between the DNA from the control VV cell culture and the DNA from the VB2 cell culture. The main difference in the DNA was the hypermethylation of the 3′-end of the protein coding region in the DNA from the VV cell culture (Fig. 5a). The azaC treatment reduced the DNA methylation levels in the *VaSTS10* protein coding region under both cell cultures conditions (Fig. 5b, c).

Finally, we analyzed the methylation status in the *VaSTS10* 5′-upstream and protein coding regions in the DNA from both the VV and VB2 cell cultures (Table 1). In general, the methylation pattern in the VV and VB2 cell cultures was the same, with the highest methylation levels detected at the CG and CHG positions. The only significant difference observed between the two cell culture conditions was that the level CHH methylation of was twofold lower in the DNA of VB2 cell culture versus the DNA from the VV cell culture (Table 1). The azaC treatment reduced *VaSTS10* DNA methylation levels in the CG, CHG, and CHH positions in both cell cultures (Table 1).

Table 2 Analysis of the total DNA methylation status of the 5′-upstream region and 3′ end of coding regions of the *actin* and *GAPDH* genes under normal conditions in VV and VB2 cell cultures

	5′-Upstream region of *GAPDH*	5′-Upstream region of *actin*	3′ End of coding region of *GAPDH*	3′ End of coding region of *actin*
VV cell culture	10.9 ± 1.9	12.1 ± 1.3	12.2 ± 2.6	17.3 ± 2.1
VB2 cell culture	10.8 ± 2.1	12.2 ± 1.2	11.5 ± 2.6	16.4 ± 3.1

The data are presented as the mean ($n = 14$) ± SE obtained from two independent experiments; all presented data between VV and VB2 cultures were not significantly different

GAPDH and *actin* methylation in the 3′ end of the protein coding regions in *V. amurensis* callus cultures

We have analyzed the methylation levels at the 5′-upstream region and 3′ end of coding regions of the *actin* and *GAPDH* genes (Table 2). The level of methylation for *GAPDH* gene was 10.9–12.2 % (VV cell culture) and 10.8–11.5 % (VB2 cell culture). The level of methylation for *actin* gene was 12.1–17.3 % (VV cell culture) and 12.2–16.4 % (VB2 cell culture). Thus, the methylation level of the house-keeping genes did not significantly change in grape cells after transformation with the *rolB* oncogene.

Discussion

In this report, we investigated the levels of cytosine DNA methylation on a gene involved in plant secondary metabolism. We chose a member of the stilbene synthase multigene family because this family is involved in the biosynthesis of *trans*-resveratrol, which is one of the most well-understood plant secondary metabolites (Kiselev 2011). From the stilbene synthase multigene family, we investigated the *VaSTS10* gene because the expression of this gene is significantly increased after treatment with the demethylation agent azaC (Kiselev et al. 2011a).

Both the *VaSTS10* promoter and 3′-end of the protein coding region were hypermethylated (59–70 %) in the control cell culture VV. Moreover, the control cell culture produced low levels of resveratrol compared with the levels measured (26–51 %) in the *rolB* transgenic cell culture VB2, which produced high levels of resveratrol. The azaC treatment significantly reduced (1.4- to 2.1-fold) the levels of DNA methylation in the *VaSTS10* promoter and coding regions in both cell cultures. The stilbene synthase family in *V. vinifera* and *V. amurensis* consists of numerous members (Sparvoli et al. 1994; Kiselev et al. 2011a), and we showed the methylation pattern of only one member. However, our results indicate that DNA methylation may be involved in the control of resveratrol biosynthesis via the regulation of *STS* gene expression. In the future, it will be interesting to investigate the methylation patterns of another *STS* gene, especially those highly expressed under normal conditions, such as *VaSTS1* or *VaSTS2*, which are upregulated by salicylic acid (Kiselev et al. 2010b).

Another interesting finding was that the function of *rolB* correlates with the DNA methylation of the genes involved in resveratrol biosynthesis, though DNA methylation of house-keeping genes did not change significantly. The *rol* genes act via transcriptional activation of defense genes; however, the mechanism of this activation is unclear (Veena and Taylor 2007). The present data suggest that the *rolB* oncogene can induce alterations in the DNA methylation patterns in plants, and this information may be helpful for understanding the function of the *rol* genes.

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