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Methylated DNA changes associated with the initiation and maintenance of *Vitis vinifera in vitro* shoot and callus cultures: A possible mechanism for age-related changes

by

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S u m m a r y : Tissue culture technologies are an important aspect of the genetic modification of grapevine (*Vitis vinifera*). The molecular basis of this phenomenon is not well understood, however, the extent of DNA methylation is recognised as a factor in the control of gene expression. This study explores the possibility that DNA methylation may have a role in grapevine culture responses. DNA methylation profiles were constructed for *Vitis vinifera*, cv. Sultanina, during plantlet micropropagation, callus induction and proliferation.

Methylation of genomic DNA and ribosomal RNA genes (rDNA) was found in glasshouse-grown plants, micropropagated plantlets and callus cultures. An analysis of rDNA showed that glasshouse-grown plants had 74.6 % of the recognition sequences for *Hpa* II methylated at the internal cytosine position CmCGG, whereas 7.7 % of the recognition sequences appeared to be methylated at the external cytosine (mCmCGG). The rDNA profiles of micropropagated subcultures S_0 (initial subcultures) and S_4 (fourth subcultures), representing one year of *in vitro* growth, showed that the percentage of recognition sequences containing a methylated external cytosine increased from 7.7 % in glasshouse-grown plants to 64.5 % for S_0 and 72.5 % for the S_4 subculture. The implications of these findings for the *in vitro* manipulation of grapevine used in genetic modifications are discussed.

K e y w o r d s : biotechnology, molecular biology, tissue culture, grapevine.

Introduction

It is important to establish reproducible tissue culture procedures for the regeneration of plants and for transformation techniques. The regeneration of *Vitis vinifera* protoplasts is problematic (MULLINS 1990); this species demonstrates morphogenetic recalcitrance, a characteristic of many woody plants (THORPE and HARRY 1990). Moreover, the tissue culture of woody species is notorious for the production of deleterious polyphenols and tannins (COLLINS and SYMONS 1992; HARDING and ROUBELAKIS-ANGELAKIS 1994), lipid peroxidation products (BENSON and ROUBELAKIS-ANGELAKIS 1992) and its oxidative stress status may lead to tissue culture recalcitrance (BENSON and ROUBELAKIS-ANGELAKIS 1994). In addition, contaminating microorganisms can affect phytosanitary status, and vitrification and lack of totipotency are problematic in many woody plant tissue culture systems (THORPE and HARRY 1990). In the case of *Vitis*, nodal cuttings can be introduced readily into tissue culture and these micropropagated shoot cultures have produced virus-free stocks of grapevine (ALLEWELDT and POSSINGHAM 1988; MULLINS 1990). Micropropagated shoot cultures are a convenient supply of pathogen/pest-free material for tissue culture and protoplast studies. However, tissue culture recalcitrance is still a problem which may be solved by testing a wide range of genotypes, manipulating tissue culture medium components and the physical growth environment, assessing explant responses and rejuvenation (BONGA 1987; VASIL 1987; THORPE and HARRY 1990).

Tissue culture responses resulting in recalcitrance can be related to the physiological state of the explant, transitions from juvenile to mature growth and the developmental changes associated with the ageing process (MEIER-DINKEL and KLEINSCHMIT 1990; PIERIK 1990). As maturation progresses, growth rates often decline and the ability to propagate material vegetatively diminishes, whilst the establishment of mature plant tissue in culture requires the reverse process (rejuvenation) to operate (DURZAN 1990). Many mechanisms have been associated with the plant maturation process and DNA methylation has been suggested to be a possible factor (BONGA 1987). To date, there has been no direct evidence to support this assumption in grapevine. Analysis of DNA methylation may offer an approach to study developmental competence *in vitro*. Several DNA methylation studies have been applied to assess the underlying biochemical and molecular processes for cereal recalcitrance (BROWN *et al.* 1989). Furthermore, the cell ageing phenomenon has been correlated with methylated DNA sequences (MORRISH and VASIL 1989; PALMGREN *et al.* 1991).

Ribosomal RNA genes (rDNA) have been shown to be methylated in plants (VON KALM *et al.* 1986; BLUNDY *et al.* 1987; ELLIS *et al.* 1989; COLLINS and SYMONS 1992; HARDING 1994) and nucleolus organiser region (NOR) activity has been correlated with DNA methylation (FLAVELL *et al.* 1983; WATSON *et al.* 1987; FLAVELL *et al.* 1988). Altered patterns of rDNA expression may be one factor which contributes towards recalcitrance and the underlying mechanism for alteration is methylation of rDNA (HARDING

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1995). In the present paper, the methylation of rDNA of *Vitis* has been studied in relation to manipulations associated with the establishment of grapevine tissue cultures and dedifferentiated growth (callogenesis). The methylation status of rDNA has been examined using methylation-sensitive and insensitive (isoschizomers) restriction enzymes.

Materials and methods

Plant material and tissue cultures: Glasshouse-grown plants, *in vitro* plantlets and callus cultures of *V. vinifera* L. cv. Sultanina (Thompson Seedless) were used in the present studies. Plantlets were established and callus cultures induced and maintained on the Roubelakis medium (ROUBELAKIS-ANGELAKIS and ZIVANOVITCH 1991) as described by ROUBELAKIS-ANGELAKIS and KATSIRDAKIS (1990).

Preparation of nuclear DNA from glasshouse plants: Healthy green leaves (5 g) were selected from several plants, and sequentially washed in tap water, distilled water, 20 mM potassium chloride, and finally in 5 mM EDTA. Leaves were homogenised in 50–100 ml of cold (4 °C) buffer (0.1 M sodium citrate, 0.35 M glucose, 5 mM EDTA, 0.1 M diethyl dithiocarbamic acid, 2 % (w/v) polyvinylpyrrolidone (PVP, mol.wt. 40,000), and 1.0 % (w/v) bovine serum albumin (BSA) adjusted to pH 6.0) in a Waring blender at high speed for 60 s. The homogenate was filtered through several layers of cheese cloth and centrifuged at 1000 *g* for 10 min at 4 °C to pellet the nuclei. The pellet was resuspended in 5–10 ml of lysis solution (1 g of caesium chloride dissolved in 1 ml of 2 % (w/v) sodium lauryl sarcosine) and incubated at 4 °C for 10 min. After centrifugation at 10,000 *g* for 20 min 500 µl of ethidium bromide (10 mg/ml) was added to the cleared lysate, and the density was adjusted to 1.58–1.60 g/ml with solid caesium chloride. DNA was separated by ultra-centrifugation (Beckman) in a vertical rotor (Vti 65) at 40,000 rpm (ca. 150,000 *g*) for 12–16 h, removed, and the ethidium bromide extracted with isopropanol. The DNA was dialysed against TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA) as described by SAMBROOK *et al.* (1989).

Preparation of total DNA from *in vitro* plantlets and callus cultures: Samples of healthy green, expanded leaves or proliferating callus (1–5 g) were ground to a fine powder with liquid nitrogen and extracted in 5 ml of buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM sodium chloride, 2 % (w/v) PVP (mol.wt. 40,000) and 50 mM beta-mercaptoethanol), and 2 ml of 10 % (w/v) SDS and incubated for 1 min at 65 °C. After incubation, 2.5 ml of 5 M potassium acetate were added and the mixture incubated at 4 °C for 20 min. The precipitate was centrifuged at 12,000 rpm for 10 min. The supernatant was removed and extracted with 10 ml of phenol/chloroform and centrifuged for 10 min at 10,000 rpm, followed by a repeated extraction with 10 ml chloroform/isoamyl alcohol and centrifugation. DNA was precipitated by removing the supernatant and adding an equal volume of cold (-20 °C) isopropanol. The DNA was centrifuged at 10,000 rpm for 10 min and the pellet was washed with 70 % (w/v) ethanol and subsequently with 50 % (w/v) ethanol, dried and resuspended in 200–300 µl of TE buffer (see HARDING and ROUBELAKIS-ANGELAKIS 1994).

Purification of DNA derived from *in vitro* cultures: Quiagen columns were used to purify the DNA from grapevine with the following modifications: The column (pack 100) was equilibrated with 1 ml of buffer 1 (750 mM sodium chloride, 50 mM Mops, pH 7.0 and 15 % (v/v) ethanol). DNA samples (0.5–1 ml) were adjusted to the same composition as buffer 1, and the column was aspirated with the DNA sample. The column was washed 6 times with 0.5 ml of fresh buffer 2 (1000 mM sodium chloride, 50 mM Mops, pH 7.0 and 15 % (v/v) ethanol). DNA was eluted from the column by washing with two 250 µl volumes of buffer 3 (1500 mM sodium chloride, 50 mM Mops, pH 8.0 and 15 % (v/v) ethanol). The efficiency of DNA recovery was improved by further washing the column with two 250 µl volumes of buffer 4 (2000 mM sodium chloride, 50 mM Mops, pH 8.0 and 15 % ethanol). The eluents from buffers 3 and 4 were collected in a 1.8 ml Eppendorf tube. DNA was precipitated with 2 volumes of cold ethanol (-20 °C), and centrifuged at 10,000 rpm (Eppendorf) for 10 min. The pellets were dried, resuspended in 10 µl of TE buffer and pooled.

Restriction enzyme digestion, gel electrophoresis and Southern blotting: Digestion of DNA (2 to 5 µg) was performed with 5 units of each restriction enzyme for 2 to 4 h at conditions described for the enzyme in the appropriate buffer (BRL) or 37 °C in potassium glutamate buffer (MCCLELLAND *et al.* 1988). Digested DNA (2 µg/track) was fractionated in a 1.2 % agarose gel with Tris-borate-EDTA buffer for 2 h at 5 V/cm. After electrophoresis, the gel was depurinated, denatured, neutralised and blotted onto neutral nylon membrane filters (Tropilon, Tropix) as described by SAMBROOK *et al.* (1989).

Hybridisation conditions: Membranes were incubated at 65 °C for 4 h in pre-hybridisation solution (0.5 % (w/v) PVP-360, 2.0 % (w/v) SDS, 0.2 % (w/v) heparin, 1 mM EDTA, 1 M sodium chloride and 50 mM Tris-HCl pH 7.5). Plasmid pTa 71 (GERLACH and BEDBROOK 1979) containing the ribosomal genes derived from wheat was biotin labelled and heat denatured. Hybridisation was performed for 16–20 h at 65 °C under the same conditions as pre-hybridisation; after hybridisation filters were washed once in the fresh pre-hybridisation solution, then in 4 x SSC, 0.1 % (w/v) SDS for 5 min at 65 °C, and 2 x SSC at 20 °C for 2 min.

Plasmid biotin labelling procedure: Plasmid DNA was nick-translated (NT) as follows: DNA (1–2 µg) was incubated with 2.0 µl of x10 NT buffer (0.5 M Tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 µg/ml BSA), and 2 µl of biotin-7-dATP (0.4 mM stock, BRL), dCTP, dGTP and dTTP each from a 1 mM stock. Pancreatic DNase I (0.1 mg/ml) was diluted 1/1000 (in 10 mM Tris-HCl pH 7.5 buffer) and 4 µl added to the reaction mixture with 1 µl (5 units) of DNA polymerase I (Gibco BRL) in a total volume of 20 µl prior to incubation at 15 °C for 1 h. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA. Separation of unincorporated biotin-7-dATP from the biotin labelled DNA was achieved by adding 20 µl of a 1 % (w/v) dextran blue to the reaction mixture, and passing the mixture over a Sephadex G50 column (5 ml). Biotin labelled DNA was co-eluted with the dextran blue dye.

Detection of chemiluminescent DNA signals: Hybridised membranes were washed in blocking buffer for 30 min. Avidin-alkaline phosphatase conjugate (1 mg, Sigma) was diluted (1:15,000) in conjugate buffer and the membrane incubated for 30 min. Approximately 20 ml of diluted avidin conjugate was used for 10 cm^2 of membrane. The membrane was rinsed in washing solution, then washed in assay buffer prior to incubation with the dioxetane substrate AMPPD for 5 min. The membrane was removed and sealed in a clear plastic bag. Chemiluminescent detection was performed in cassettes containing X-ray film (Fuji HR-G) without screens at room temperature (see HARDING 1992).

Assay for methylated DNA sequences: Restriction enzyme analysis was performed as described by SAMBROOK *et al.* (1989). The methylation status of DNA sequences was examined by comparing the activities of an isoschizomer pair of restriction enzymes. The methylation-sensitive restriction enzyme *Hpa* II, and -insensitive *Msp* I recognise the same target sequence CCGG, however *Hpa* II activity is inhibited by methylation of the internal cytosine (CmCGG), whereas *Msp* I activity is inhibited by methylation of the external cytosine in the target sequence (mCCGG or mCmCGG). Similarly, the isoschizomers *Eco* RII and *Bst* NI recognise the same target sequence CC(A/T)GG. *Eco* RII activity is inhibited when the internal cytosine (CmCAGG) is methylated, however *Bst* NI cleaves several methylated sequences (CmCAGG, mCCAGG and mCmCAGG), but is inhibited if both external and internal cytosines are hemi-methylated (hmChmCGG, MCCLELLAND and NELSON 1988). *Hae* III restriction enzyme activity is inhibited by methylation of the internal cytosine in the recognition sequence (GGmCC).

Results

Caesium chloride purified nuclear DNA isolated from glasshouse-grown *V. vinifera* plants was digested with several restriction enzymes. Fig. 1 a shows an ethidium bromide stained agarose gel containing digested DNA samples; the terminal tracks represent lambda DNA digested with *Eco* RI/*Hin*d III as molecular weight markers. The sin-

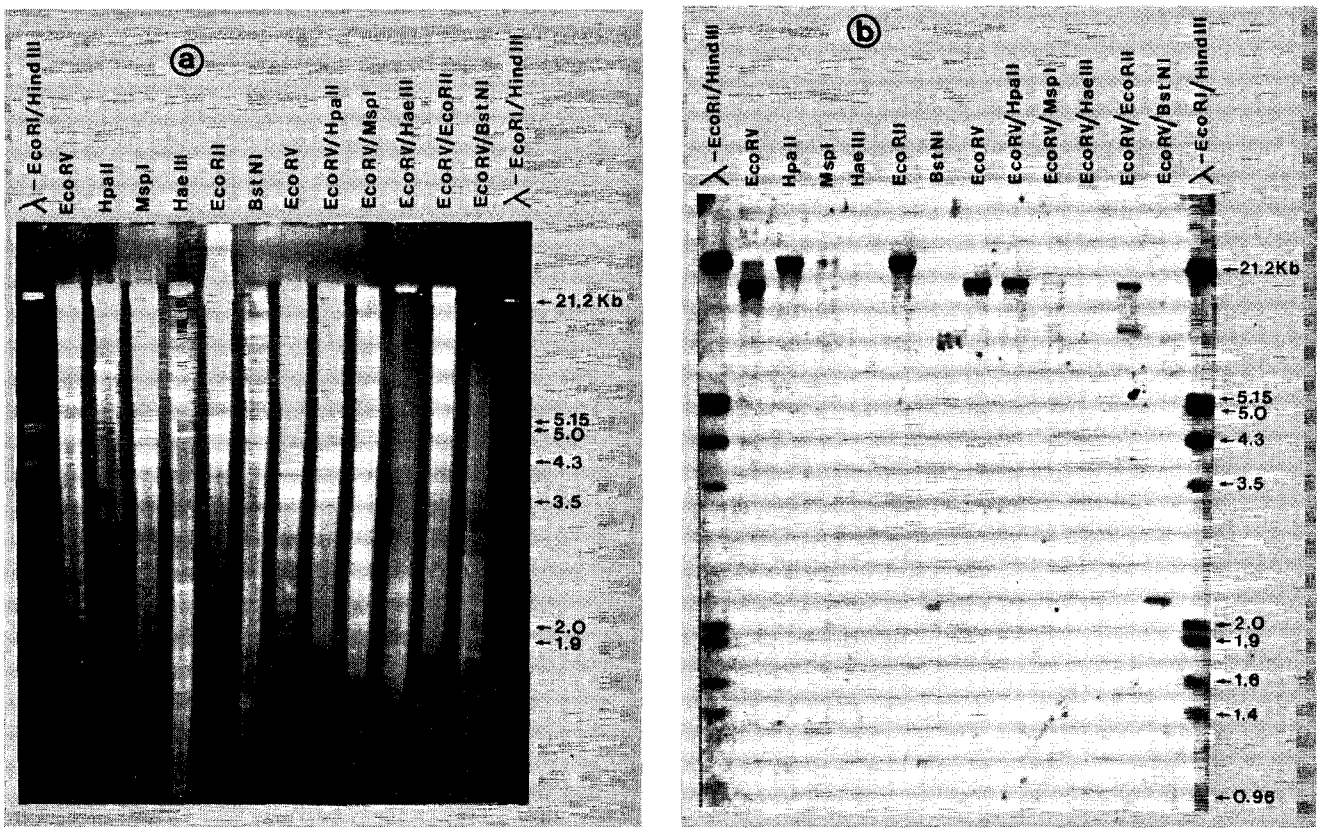

Fig. 1: An ethidium bromide stained agarose gel (**a**) containing digested nuclear DNA derived from grapevine digested with several restriction enzymes and (**b**) an image derived from a Southern blot of the same agarose gel by the chemiluminescent detection of rDNA sequences.

gle *Hpa* II restriction digestion showed genomic DNA to consist of relatively undigested fragments of high molecular weight in the range 5.0 to 21 kb. Whereas genomic DNA digested by *Msp* I produced numerous fragments in a lower molecular weight range (< 5 kb). This digestion pattern was further shown with *Eco* RII, where genomic DNA was essentially undigested, while *Bst* NI completely cut the genomic DNA. Genomic DNA digested with *Hae* III produced a range of relatively low molecular weight fragments. The digestion properties of restriction enzymes for grapevine genomic DNA were repeated in the double restriction enzyme digestions with *Eco* RV. This agarose gel was Southern blotted and hybridised to a biotin labelled rDNA probe, and homologous rDNA sequences on the membrane filter were detected by chemiluminescence (Fig. 1 b). The rDNA sequences in the *Eco* RV sample migrate as a single fragment, whereas the *Hpa* II and *Eco* RII samples are essentially undigested higher molecular weight fragments. In comparison, the methylation-insensitive isoschizomers *Msp* I/*Bst* NI completely digested the rDNA. These differences in rDNA were further shown in the double digestions with *Eco* RV.

Total DNA was extracted from plantlets derived from several micropropagation subcultures; DNA was isolated from the plantlet first introduced from the glasshouse into culture 0 (S_0) and subsequent subcultures (1,2,3 and 4 corresponding to S_1, S_2, S_3 and S_4 see Fig. 2). The time for glasshouse nodal cuttings to become established in culture and the interval between two subculture cycles was 2-3 months. The time between the S_0 and S_4 subcultures was approximately 1 year. There was a progressive decrease in the amount of total DNA extracted from successive subcultures (up to S_3) compared to the initial plantlet (unpublished results).

Quiagen purified total DNA extracted from plantlets was digested with several restriction enzymes. Fig. 3 a shows an ethidium bromide stained agarose gel containing digested DNA derived from micropropagated subcultures S_0 and S_4; DNA was digested with *Eco* RV (track 1), *Eco* RV/*Hpa* II (track 2) and *Eco* RV/*Msp* I (track 3). Tracks a and b represent Lambda DNA digested with *Eco* RI/*Hin*d III and *Hin*d III as molecular weight markers. Identical DNA was loaded in each track (1,2 and 3) for each subcultures S_0 and S_4. Approximately 50 % less DNA was loaded in S_4 sample compared to S_0 and this accounts for the difference in the intensity of ethidium bromide staining. There were no obvious differences in the digested (tracks 1, 2 and 3) genomic DNA samples. (The distortion of the migrating DNA in the agarose gel is due to the use of potassium glutamate digestion buffer; this electrophoresis artefact characteristically occurs at molecular weights below 2.0 kb, and does not effect the resolution of high molecular fragments). This agarose gel was Southern blotted, hybridised and homologous rDNA sequences were detected by chemiluminescence as described for glasshouse plants. Fig. 3 b shows the rDNA sequences of plantlets derived from micropropagated subcultures S_0 and S_4. The arrows indicate the rDNA repeat unit produced from an *Eco* RV digestion (track 1) and the remaining intact rDNA fragments after *Hpa* II (track 2) and *Msp* I (track 3) double restriction enzyme digestions with *Eco* RV.

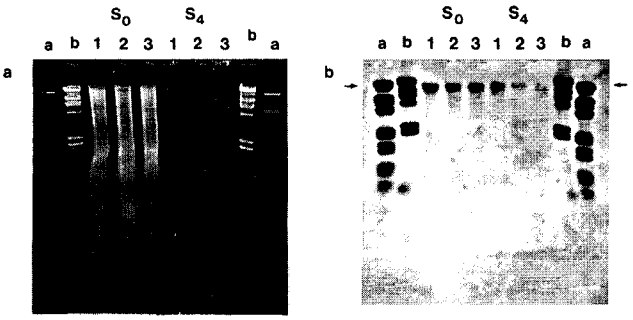

Fig. 3: An ethidium bromide stained agarose gel (**a**) containing DNA derived from micropropagated subcultures S_0 and S_4 digested with *Eco* RV (track 1), *Eco* RV/*Hpa* II (track 2) and *Eco* RV/*Msp* I (track 3). — The agarose gel was Southern blotted and rDNA sequences were detected by chemiluminescence (**b**).

Total DNA was extracted from callus cultures induced from plantlets derived from the micropropagated S_0 subculture. DNA was extracted from the S_0 plantlets and callus at several times in the culture cycle. There was a 10-fold decrease in the amount of total DNA extracted from the S_0 plantlets and low values for successive callus cultures (unpublished results). Purified total DNA isolated from callus cultures was digested with several restriction enzymes. Fig. 4 a shows an ethidium bromide stained agarose gel

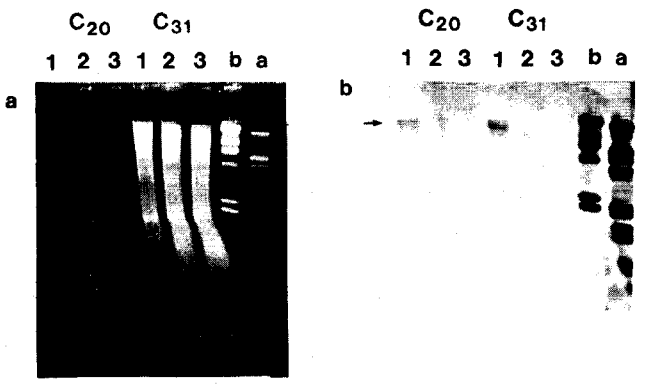

Fig. 4: An ethidium bromide stained agarose gel (**a**) containing DNA derived from 20 (C_{20}) and 31 (C_{31}) weeks old callus cultures digested with *Eco* RV (track 1), *Eco* RV/*Hpa* II (track 2) and *Eco* RV/*Msp* I (track 3).
The agarose gel was Southern blotted and rDNA sequences were detected by chemiluminescence (**b**).

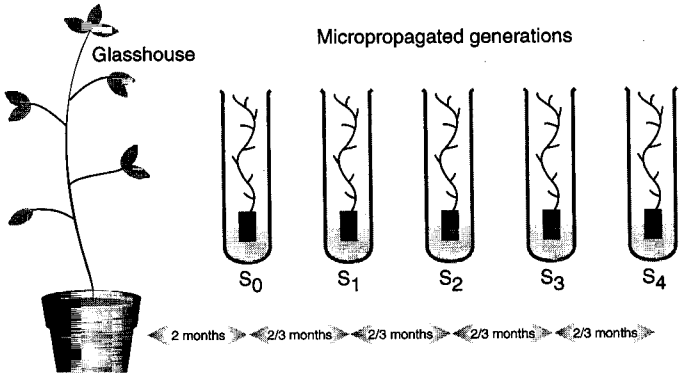

Fig. 2: A diagram illustrating the micropropagation process.

containing digested DNA derived from 20 (C_{20}) and 31 (C_{31}) weeks old callus cultures; DNA was digested with *Eco* RV (track 1), *Eco* RV/*Hpa* II (track 2) and *Eco* RV/*Msp* I (track 3). Lambda DNA was digested with *Hind* III (b) and *Eco* RI/*Hind* III (a) as molecular weight markers. The faint intensity of staining for the C_{20} sample is due to approximately 75 % less DNA being loaded on the gel compared to the C_{31} sample. The agarose gel was Southern blotted, hybridised and rDNA sequences were detected as described above. Fig. 4 b shows the rDNA of 20 and 31 weeks old callus cultures, the arrow indicates the intact rDNA repeat fragments produced from an *Eco* RV digestion (track 1), whereas these rDNA fragments are absent in tracks 2 and 3 indicating the lack of methylated sequences in these DNA samples.

By computer assisted image analysis the density per unit area was measured for the rDNA repeat fragments represented in Figs. 1 b and 3 b for the restriction enzymes *Eco* RV, *Eco* RV/*Hpa* II and *Eco* RV/*Msp* I. The values for the residual rDNA fragments after double digestion were expressed as percentage of the total (*Eco* RV) and indicates the degree of sequence specific methylation within the rDNA. The Table shows methylated rDNA changes associated with the initiation and maintenance of grapevine *in vitro* shoot cultures. Plants grown in the glasshouse had 74.6 % of the recognition sequences for *Hpa* II methylated at the internal cytosine position CmCGG, whereas 7.7 % of the recognition sequences appear to be methylated at the external cytosine, presumably as a double methylated sequence (mCmCGG). In comparison, the rDNA in plantlets derived from micropropagated subcultures S_0 and S_4 showed 72.9 % and 73.9 % of the *Hpa* II recognition sequences have the internal cytosine methylated. However, the percentage of recognition sequences containing a methylated external cytosine increased from 7.7 % in glasshouse plants to 64.5 % for S_0 and to 72.5 % for the S_4 subculture.

Table

Methylated rDNA changes associated with the initiation and maintenance of *Vitis vinifera in vitro* shoot cultures

Plant material	Restriction enzymes	Density per unit area	Percentage methylation	Methylated sequence
GH	*Eco* RV	7502	0.0	-
GH	*Eco* RV/*Hpa*II	5598	74.6	CmCpGG
GH	*Eco* RV/*Msp*I	576	7.7	mCmCpGG
Initial S_0	*Eco* RV	838	0.0	-
Initial S_0	*Eco* RV/*Hpa*II	611	72.9	CmCpGG
Initial S_0	*Eco* RV/*Msp*I	541	64.5	mCmCpGG
Final S_4	*Eco* RV	608	0.0	-
Final S_4	*Eco* RV/*Hpa*II	448	73.7	CmCpGG
Final S_4	*Eco* RV/*Msp*I	440	72.5	mCmCpGG

GH = glasshouse

Discussion

The objective of the present study is to explore the possibility that the methylation status of *Vitis vinifera* changes as a result of *in vitro* manipulations. This may have consequences for understanding and controlling the problems of recalcitrance often associated with the *in vitro* culture manipulations of *Vitis*. This investigation aims to evaluate the methylation status of the ribosomal RNA genes in the *Vitis* genome. Ribosomal RNA genes (rDNA) are central to cell function and development. The coding regions within the rDNA transcription (repeat) unit comprise the 25S, 5.8S and 18S genes (HARDING 1991) and are organised as a tandem array located in the nucleolus organiser region (NOR). Any impairment of rDNA expression, for example *via* methylation, reduces the pool of available RNA for the maintenance of cytoplasmic ribosomes. Ribosome turnover processes would further reduce the level of protein synthesis and have direct consequences for cellular growth and development. Therefore, hypothetically this may have an influence on developmental processes and the maintenance of totipotency resulting in recalcitrance.

Single restriction enzyme digestions with *Hpa* II/*Msp* I showed genomic DNA to be methylated at the recognition sequence CCGG; extensive methylation at the recognition sequence CC(A/T)GG was further shown comparing the *Eco* RII/*Bst* NI digestions (Fig. 1 a). These results were confirmed in the double restriction enzyme digestions with *Eco* RV. These data indicate that grapevine genomic DNA has a predominance for methylation at the internal cytosine position in these recognition sequences consistent with previous findings (GRUENBAUM *et al.* 1981; THOMAS *et al.* 1993). However, the range of DNA fragments produced with *Hae* III showed the internal cytosine of this recognition sequence (GGCC) was not methylated. *Eco* RV has a unique restriction enzyme site within the transcriptional unit of the tandem array to produce a repeat length of approximately 15 kb; the rDNA sequences were shown in the *Eco* RV samples as a single fragment (Fig. 1 b). The extent of methylated recognition sites within the rDNA for *Hpa* II and *Eco* RII were shown in the remaining intact fragments as compared to their methylation-insensitive isoschizomers *Msp* I/*Bst* NI. The differences in the rDNA fragment intensities in the double restriction enzyme digestions with *Eco* RV were the basis for examining methylation changes.

The establishment of explants from glasshouse grown plants in culture was accompanied by several initial and temporal molecular changes. During micropropagation (Fig. 2), the average yield of DNA derived from *in vitro* grown plantlets (subcultures S_1, S_2, S_3 and S_4) dramatically decreased with time compared to the initial culture (S_0). This change was observed in callus, where the amount of DNA decreased 10-fold compared to the initial *in vitro* plantlet that was used to induce callus cultures (unpublished results). An increase in the degree of methylated rDNA sequences was detected in the initiation and maintenance of *in vitro* shoot cultures (Table), whereas the rDNA was demethylated in established callus cultures (Fig. 4).

These results indicate there are dynamic processes operating on the genome during the establishment of *in vitro* cultures.

The findings in this study may have consequences for grapevine biotechnology. Tissue culture manipulations are one central feature for several *Vitis* genetic manipulations (transformation and somatic hybridization) and micropropagated shoot cultures are a convenient source for the preparation of protoplasts (THEODOROPOULOS and ROUBELAKIS-ANGELAKIS 1990). It is often assumed that the starting plant material is in the correct 'physiological state' for protoplast isolation and tissue culture and the only indicators of this condition are plantlet vigour and morphology. However, as grapevine material is introduced and maintained in culture the rDNA repeats become increasingly methylated. It is unclear, why this should occur but DNA methylation has been correlated with NOR inactivation (FLAVELL *et al.* 1983; WATSON *et al.* 1987; FLAVELL *et al.* 1988) and this may be a stress-induced mechanism (HARDING 1994). A limited pool of ribosomal RNA coupled with further ribosome turnover would constrain the ability to produce efficient levels of protein synthesis in a new phase of plant growth. Furthermore, the rDNA methylation patterns would be passed on to tissue cultures, for example protoplasts, and during the conditions of high metabolic demand associated with protoplast growth and development, the ability of protoplasts to produce *de novo* proteins would be impaired. This 'physiological state' is an important factor likely to contribute to grapevine protoplast recalcitrance further supported by the observation that demethylation of the rDNA is associated with active cell division and growth of grapevine callus cultures.

The rDNA of developing pea cotyledons were found to be hypomethylated compared to leaf tissue (WATERHOUSE *et al.* 1986) and studies with young pea seedlings showed that as development progressed the level of rDNA methylation decreased (WATSON *et al.* 1987). It is generally regarded that demethylation is associated with gene expression, and methylation is related to gene inactivation (HOLLIDAY 1989). The application of anti-methylating agents (LOSCHIAVO *et al.* 1989) to recalcitrant tissue cultures for understanding the basis for gene regulation has limited use (BROWN *et al.* 1990), DNA methylation may play a specific role in development, however the use of chemicals which demethylate large regions of the genome may be inappropriate tools to reveal these mechanisms. Importantly, the present study has revealed that the developmental transitions associated with *in vitro* manipulations are accompanied by changes in methylation status.

The tissue culture of many mature hardwoods and conifers is problematic and cellular changes accompany transitions from juvenile to mature tissues (BONGA 1987). Several age-related processes have been described and alterations in DNA methylation have been suggested as a factor associated with ageing (SLAGBOOM and VIJG 1989). Therefore, as plants progress through the various stages of development, specific regions of the genome may become increasingly methylated. In the case of perennial woody species, the methylation 'imprint' on the DNA may persist for several years. The evidence presented here for grapevine suggests that this is possible, especially as 73–75 % of the recognition sequences for *Hpa* II in the rDNA are methylated (Table), and this methylated state (CmCGG) is maintained during one year of culture. Moreover, the degree of rDNA methylation at the sequence that inhibits *Msp* I activity (mCmCGG) increased during the same culture period. The establishment of woody species in tissue culture and subsequent regeneration relies on the success of rejuvenation (BONGA 1987). The methylation 'imprint' may be erased during meiosis, which enables the genetic information to be reprogrammed at the onset of rejuvenation: DNA methylation would be a mechanism to account for epigenetic changes. The initiation of grapevine callus cultures and demethylation of the rDNA may indicate the molecular basis for rejuvenation in woody perennials. Although DNA methylation is a controversial issue, there is amounting evidence to suggest that it plays a role in the control of gene expression. The results in this paper support the idea as rDNA methylation is a factor in grapevine culture responses.

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