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ORIGINAL PAPER

DNA methylation in different origin clonal offspring from a mature *Sequoiadendron giganteum* genotype

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Abstract A meristem-issued rejuvenated line was obtained in 1986 from a 100-year-old *Sequoiadendron giganteum* tree and has been since then micropropagated in tissue culture conditions maintaining its juvenile-like characteristics. By contrast, grafts and rooted microcuttings from the same genotype planted in outdoor conditions for several years exhibited mature foliage traits and the grafts started to produce cones, which are obvious indicators of physiological aging. These three different clonal lines were compared with regard to global DNA methylation assessed by HPLC. The in vitro rejuvenated line showed a much higher level of DNA methylation (23% as average value) than the two other outdoor origins from the same clone which displayed similar degrees of global methylation (average values of 13.4% for the grafts and 13.8% for the cuttings). Overall these DNA global methylation values obtained for the first time in *S. giganteum* are consistent with the level of methylation reported for many plants using the same HPLC protocols. The fact that shoots exhibiting a juvenile-like leaf morphology can be characterized by higher DNA methylation than mature-like ones is discussed in relation to physiological aging, referring to other studies on the same topic.

Keywords Aging · Coniferous species · DNA methylation · Maturational traits · Phase change

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Introduction

The monospecific genus *Sequoiadendron giganteum*, commonly known as giant sequoia, is very famous for the outstanding size and age of some of its oldest representatives in its native Sierra Nevada mountains (Hartesveldt et al. 1975). With individuals reaching some 3,500–4,000 years old, it is therefore a predestined species for studying several influences of aging in plants (Fortanier and Jonkers 1976). Besides, the potential of giant sequoia to be used for forest plantations has accounted for assessing its ability for true-to-type cloning, which was found, similarly to many tree species, to diminish drastically with increasing age and development (Monteuuis 1985). In addition to a significant decrease of organogenic capacities and of its ability for adventitious rooting more particularly (Monteuuis 1985; Monteuuis et al. 1987), aging was found to induce cyto- and histomorphological changes (Monteuuis 1989a, Monteuuis and Genestier 1989) as well as modification of certain metabolic pathways (Monteuuis and Gendraud 1987; Bon 1988a). Of noteworthy interest was the identification of a polypeptide associated with physiological aging in *S. giganteum* (Bon 1988b), giving more and more evidence of the epigenetic control of phase change phenomenon in trees (Monteuuis 1989b; Fraga et al. 2002b). DNA methylation also has been for a long time speculated to play a key role in the various maturational changes observed in plants during their ontogenetical development

(Schaffalitzky de Muckadell 1959; Fortanier and Jonkers 1976; Bonga 1982). The prevailing hypothesis was that during higher organism development, genomic DNA could become more methylated resulting in the modification or the switching on and off of gene expression responsible for the variation of the maturational traits noticed (Razin and Riggs 1980). Access to analytical methods for quantifying overall 5mC based on high performance liquid (HPLC) or even more sensitive capillary (HPCE) electrophoresis (Fraga et al. 2002a) has given the opportunity to study this topic more extensively, focusing more specifically on higher plants (Finnegan et al. 2000). In the heteroblastic tree species *Acacia mangium* Willd., Baurens et al. (2004) reported higher DNA methylation rates for the juvenile-like microshoots than for those of the mature type, irrespective of the source material age. In tissue-cultured chestnut, Hasbún et al. (2005) observed higher levels of methylated DNA in microshoots exhibiting juvenile characteristics than in the more mature-like ones originating from older parts of the same donor tree. The availability of different physiological age lines within a same giant sequoia clone (Monteuuis and Bon 1989) prompted us to pursue our multidisciplinary approach of phase change investigations on this species by looking at the DNA methylation aspects.

Materials and methods

Plant material

The experimental plant material consisted basically of three different clonal lines all deriving from a 100-year-old giant sequoia. Ramets were collected and grafted in 1983 (Monteuuis 1985) to give rise to the "G83" origin kept cultivated outdoors since then. These grafts were bearing cones when the samples were collected, and can therefore be considered as physiologically mature (Bonga 1982; Monteuuis and Bon 1989, 1990). A rejuvenated clonal line was obtained from in vitro meristem culture in 1986 from the same mature genotype (Bon and Monteuuis 1991; Monteuuis 1991). Part of the microshoots derived from this meristem-issued rejuvenated line were rooted, acclimatized then planted next to "G83" in 1990, and were named "C90". These "C90" plants reached an overall height of 4 m and exhibited mature foliage characteristics already but were not producing cones yet when the samples were collected. The rest of this meristem-issued rejuvenated line "R86" was kept cultivated in vitro on the elongation medium according to the procedure described by Monteuuis and Bon (1986) until the samples were collected.

For each category of plant material, the samples collected at the same period consisted of 1-cm long shoot apical parts, which had just entered the resting phase for "G83" and "C90" sources, while "R86" was actively growing in vitro. These samples were stored after collection in a freezer at −80°C a few days before the extraction procedure started.

Genomic DNA extraction

Plant material DNA extraction was performed using a mixed alkyl trimethyl ammonium bromide (MATAB) procedure with the additional information: 250 mg of plant sample were ground in liquid nitrogen then immediately incubated in 2 ml of pre-warmed extraction buffer (100 mM Tris–HCl pH 8, 20 mM EDTA, 1.4 M NaCl, 2% w/v MATAB, 1% w/v PEG6000 (poly ethylene glycol) and 0.5% w/v sodium sulfite, 20% w/v Igepal CA630, 20% w/v lithium dodecyl sulfate, 20% w/v sodium deoxycholate) at 74°C for 20 min. After purification with 2 ml of CIAA (chloroform/isoamylalcohol 24:1, v:v), DNA extracts were precipitated with 1.6 ml of isopropanol then resuspended in 1 ml of buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 0.7 M NaCl) before purification on anion exchange columns QIAGEN TIP 20 following the manufacturer's instructions (QIAGEN, Valencia, CA, USA).

HPLC analyses

The method employed for DNA enzymatic hydrolysis was adapted from Jaligot et al. (2000). DNA samples (20 µg each) were added to 10 ml of a 0.5 U/µl solution of nuclease P1 (Sigma N8630) and 35 µl of a 0.017 U/µl solution of alkaline phosphatase (Sigma P4252). The reaction volume was adjusted to 200 µl with the digestion buffer (30 mM $NaCH_3$, 0.1 mM $ZnCl_2$, pH 5.3). Hydrolysis of DNA to nucleosides was performed in triplicate at 37°C for 3 h. The reaction was stopped by the addition of 490 µl of absolute ethanol; then the samples were centrifuged at 11,000*g* for 15 min. The supernatant was transferred to a new tube, vacuum-dried and nucleosides were resuspended in 1 ml of sterile water. The extracts were then filtered (0.2 µm) prior to HPLC analysis. An isocratic elution method was followed using a modified version of the buffer described by Gehrke et al. (1984): 50 mM KH_2PO_4, 8% (v/V) methanol, pH 3.5 (instead of 4.4), on a Supelcosil LC-18S reverse-phase column (SUPELCO Inc., Bellefonte, Pa., 25 cm × 4.6 mm; particle diameter: 5 µm), with a flow rate of 0.8 ml/min and a run time of 30 min. The improved conditions used allowed highly efficient separation of the peaks corresponding to dC and uracil (compounds identified by their respective retention time and UV spectra). The effluent was monitored at the wavelength of 285 nm with a photodiode array detector (Beckmann Germany). The percentage of 5mdC

was calculated using the formula: (5mdC)/[(dC) + (5mdC)] were (5mdC) and (dC) are the respective concentrations of the two forms of dC deduced from the calibration curves for external standards of known concentrations, monitored simultaneously with the samples.

Statistical analyses

A total of 10–12 independent analytical HPLC measurements were performed for each origin. The data were statistically treated using an analysis of variance computer program (GLM procedures, SAS Institute, Inc. 2000), and the Student–Newman Keuls mean comparison test (Sokal and Rohlf 1995). A probability level of $P \leq 0.05$ was considered significant for all the statistical analyses. Results are expressed as means.

Results and discussion

The rejuvenated line "R86" (Bon and Monteuuis 1991; Monteuuis 1991) obtained from one apical meristem excised from a 100-year-old *S. giganteum* tree and set in culture in 1986 has been successfully subcultivated since then on the "M20" elongation medium (Monteuuis and Bon 1986) displaying during all these years the same organogenic (data not shown) and morphological features as the juvenile clone used as control (Monteuuis 1991). This demonstrates the possibility of physiologically rejuvenating (Bon and Monteuuis 1991) and maintaining this physiological rejuvenation in vitro using suitable tissue culture protocols, a mature genotype of this coniferous species. Actually, the "R86" plant material exhibited awl-shaped leaves (Hartesveldt et al. 1975) with much longer free parts than those of the mature type from "G83" and "C90" outdoor origins of the same genotype (Fig. 1). This trait characterizes the juvenile phase in giant sequoia (Hartesveldt et al. 1975) and can be considered as an indicator of adventitious rooting ability and thus of physiological aging in this species (Monteuuis 1985). As stated by Schaffalitzky de Muckadell (1959) long ago, modifications of leaf morphology reflects changes in physiological aging occurring within the shoot apical meristem, from which derived during the ontogenetical process all the different parts of the aerial body of a tree.

The DNA methylation data resulting from the HPLC profiles obtained (Fig. 2) for *S. giganteum* samples analyzed showed also a big difference according to the different origins of the same clone. The juvenile-like "R86" in vitro samples are characterized by a much higher proportion of 5mC than the two other "G83" and "C90" more mature outdoor origins ($P < 0.0001$), which showed similar degrees of DNA methylation (average values of 19.1 vs. 13.8% for C90 and 13.4% for G83, respectively). Minimal variation was observed among DNA data within each sample of the three different plant material origins, attesting the good reproducibility of the independent measurements and the reliability of the results (Fig. 3).

These call for several comments. First of all, the values found are consistent overall with the level of DNA methylation reported in the literature for many plants, mostly Angiosperms, utilizing the same HPLC methodology (Diaz-Sala et al. 1995; Finnegan 1996; Fraga et al. 2002a). DNA methylation has been less investigated in Gymnosperms, with particular mention for Greenwood et al. (1989) with DNA methylation levels averaging 20% in larch, and Fraga et al. (2002b) who found unexpectedly high 5mC methylation values in *Pinus radiata*. This can be due to the nature of tissues used for the analyses (Fraga et al. 2002a, b), with the impossibility with the HPLC technology to restrict the investigations to the meristematic

Fig. 1 Morphological features of the apical shoot samples corresponding to the three clonal lines from the same 100-year-old *Sequoiadendron giganteum* genotype investigated. The in vitro "R86" rejuvenated line (*left*) exhibited awl-shaped leaves with much longer free parts than those from the "C90" (*middle*) and "G83" (*right*) outdoor origins of the mature type. *Bar scale* 0.5 cm long

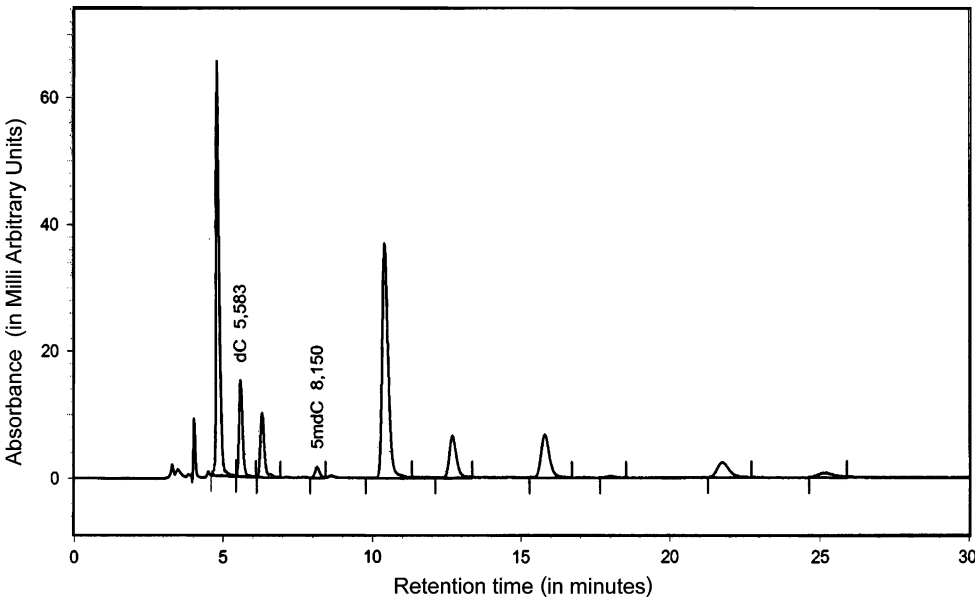

Fig. 2 HPLC representative chromatogram of *Sequoiadendron giganteum* DNA hydrolyzed nucleosides (detection vawelength: 285 nm, column used: Supelcosil LC-18S reverse-phase, elution buffer: 50 mM KH_2PO_4, 8% (v/V) methanol, pH 3.5). *dC* deoxycytidine, *5mdC* 5-methyl-deoxycytidine

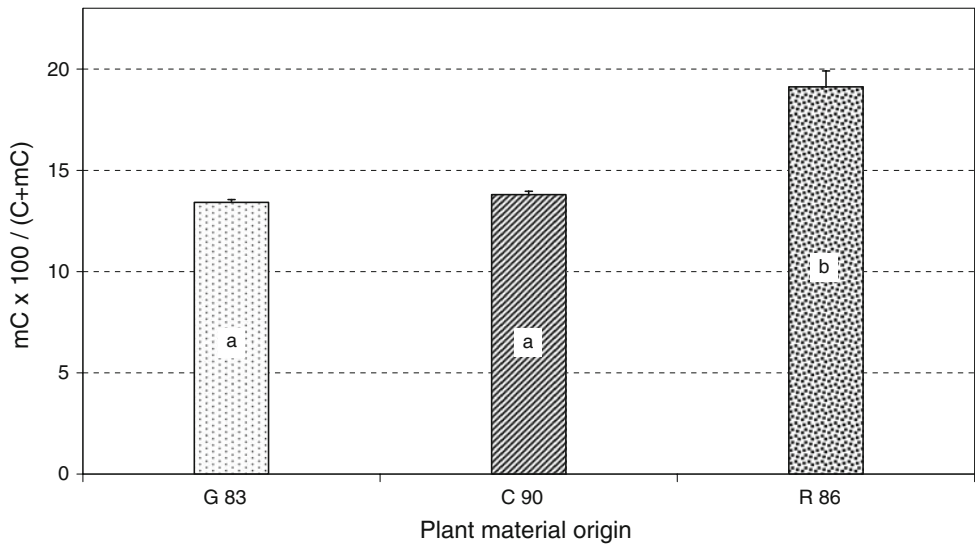

Fig. 3 Percentage of DNA methylation in the three 100-year-old giant sequoia-derived clonal lines investigated i.e. the grafts field planted in 1983 ("G83"), the rejuvenated and rooted microcuttings field planted in 1990 ("C90") and the rejuvenated line maintained in vitro ("R86"). *Bars* represent standard errors corresponding to 10–12 independent measurements for each plant material category, and *letters* distinguish average values which are significantly different at the 5% level. See text for more information

tissues only, contrary to what Fraga et al. (2002b) did on *P. radiata* thanks to the HPCE method utilized. In addition to the type of tissues investigated, and more specifically the respective proportion of meristematic versus differentiated ones, the particularities of HPCE protocols may account also for the higher DNA methylation values observed than usually reported for HPLC (Fraga and Esteller 2002; Fraga et al. 2002a; Valledor et al. 2007).

The advantages of studying the molecular aspects of phase change phenomenon within a same genotype for avoiding any genotypic interferences is obvious and was already argued (Baurens et al. 2004). The fact that the in vitro morphologically and physiologically rejuvenated line (Bon and Monteuuis 1991; Monteuuis 1991) showed that noticeably higher DNA methylation levels than the outdoor mature grafts of the same clone is not consistent with the prevailing views assuming that DNA methylation increases with maturity (Bonga 1982). However, apart from the previously mentioned study from Fraga et al. (2002b) on *P. radiata*, only a very limited number of concrete results have supported so far this statement, particularly in trees. Greenwood et al. (1989) did not observe any significant difference of DNA methylation between juvenile and mature scions in larch. In tissue-cultured *Acacia mangium* microshoots, Baurens et al. (2004) concluded the absence of significant difference in global DNA methylation between the juvenile and mature sources of plant material, which could be distinguished, however, by more qualitative age-related DNA methylation markers detected by MSAP.

In in vitro cultivated chestnut microshoots, Hasbún et al. (2005) found higher levels of methylated DNA in the juvenile-like microshoots than in the more mature-like ones originating from older parts of the same donor tree, similarly to what we observed in *Acacia mangium* (Baurens et al. 2004) and here on giant sequoia. In this later case, the comparison between in vitro and ex-vitro lines of the same clone is innovative regarding the possible influence of specific in vitro conditions on DNA global methylation (Diaz-Sala et al. 1995; Hasbún et al. 2005; Valledor et al. 2007). Moreover, it can be assumed according to Hasbún et al. (2005) and Valledor et al. (2007), the difference of DNA methylation between the "R86" in vitro line and the outdoor materials would have been even more salient if these latter were actively growing, as resting shoots are reported to be higher methylated (Hasbún et al. 2005; Valledor et al. 2007). The fact that "C90" origin displayed similar DNA methylation level as the grafts exposed to the same outdoor conditions and exhibiting the same mature foliage characteristics, contrary to "R86", is consistent with previous studies on the same genotype, comparing propagation by grafting and cuttings, in relation to the time needed to become morphologically mature and to lose gradually its ability for adventitious rooting (Monteuuis 1985). The relationship observed here again between phase change-related foliar dimorphism (Schaffalitzky de Muckadell 1959) and global DNA methylation measurement strengthens the view that leaf morphology must be considered as an indicator of physiological aging, regardless of the chronological age of the donor plant (Fortanier and Jonkers 1976; Monteuuis 1989a, b).

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