Theor Appl Genet (1995) 91: 809–815

B. Arnholdt-Schmitt · S. Herterich · K.-H. Neumann

Physiological aspects of genome variability in tissue culture. I. Growth phase-dependent differential DNA methylation of the carrot genome (*Daucus carota* L.) during primary culture

Received: 5 April 1995 / Accepted: 21 April 1995

Abstract Investigations were performed on growth phase-dependent *Eco*RII site-specific DNA methylation of the carrot genome during primary culture to elucidate physiological aspects of genome DNA variability in tissue culture. While DNA methylation of the root cambium and the secondary phloem and petioles of carrot leaves were strikingly different, the methylation level of the secondary phloem seemed to be independent of cultivar origin, the age of the plants and the extent of secondary root growth. As was shown earlier a change in the differentiated state of the secondary phloem by tissue culture leads to changes in genome modification. Whereas de novo methylation was observed during the first 2 weeks of growth initiation, the results presented demonstrate genome de-methylation during the transition to stationary growth indicating differential genome methylation during different phases of culture. The presence of kinetin in the nutrient medium of the primary culture was found to be antagonistic to changes in genome modification in general. De novo methylation and subsequent de-methylation of the carrot genome are discussed as gross changes obviously essential to molecular genome differentiation during tissue culture.

Key words *Daucus carota* L. · Tissue culture · Differential DNA methylation · Cell-division growth · Kinetin

Introduction

Genomic DNA variation is a common phenomenon in cell or tissue culture systems. For commercial in vitro-production or breeding systems this kind of variability is a fundamental problem. Although known for at least two decades causal relationships are still obscure. However, in recent years increasing data have demonstrated that differentiation and de-differentiation, as well as growth by cell division, were accompanied by DNA amplification or loss of DNA sequences and/or DNA modification by methylation or de-methylation (Nagl 1987, 1989, 1990; Bassi 1990; Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993a; Jost and Saluz 1993). A relationship between physiology and genome variability is suggested, but systematic investigations are needed for further insights. In this paper, we focus on the originally stable pattern of tissue-specific DNA methylation of carrot secondary root phloem and changes in DNA modification after primary culture initiation in respect of the transition from linear to stationary growth. An accompanying paper (Arnholdt-Schmitt 1995) reports on the dependence of differential DNA replication on the growth phase, suggesting a relationship between quantitative genomic variation and cell determination.

In a majority of eukaryotic organisms genome activity, i.e. essentially transcription, replication and rearrangements (e.g. Cedar 1988; Holliday et al. 1990; Linn et al. 1990; Phillips et al. 1990; Riggs and Crispeels 1990; Meyer et al. 1992), and the structural organisation of chromatin (e.g. Ball et al. 1983; Klaas and Amasino 1989; Keshet et al. 1986; Selker 1990; Zlatanova 1990; Graessmann and Graessmann 1993), seems to be somehow related to DNA methylation. Nevertheless, in most cases it is still not known whether DNA methylation is the cause or the result of changes in genome activity (Jost and Saluz 1993). Specificity in DNA methylation may occur by specific sequence methylation or de-methylation. An additional suggestion is that DNA methylation by methyltransferase starts at structural- or sequence-specific crystallization points spreading non-specifically from such sites over all its methylation-sensitive adjacent regions (Adams and Burdon 1985; Adams 1990). In the latter case specificity would be the result of protection against the activity of the methyltransferase, for instance by DNA-protein binding (e.g. by transcription factors) or by topological protection. In this context elucidation of DNA modification events is also

Communicated by H. F. Linskens

B. Arnholdt-Schmitt (✉) · S. Herterich · K.-H. Neumann
Institut für Pflanzenernährung, Abteilung Gewebekultur, Justus-Liebig-Universität Giessen, Südanlage 6, D-35390 Giessen, Germany

important for the application of gene transfer methods, since the successful integration of active sequences is influenced by DNA methylation (Linn et al. 1990).

De novo methylation and de-methylation events are part of differential genomic changes. Tissue-specific DNA methylation of different sequences has been reported for animals as well as for plants (Shen and Maniatis 1980; Razin and Friedman 1981; Razin and Cedar 1984; Adams and Burdon 1985; Dhar et al. 1990; Edwards 1990; Jost et al. 1990; Riggs and Crispeels 1990; Behn-Krappa et al. 1991). The question arises whether the extent of DNA methylation at the total genome level contributes to the characterization of the complexity of the differentiation state of cells or tissues.

In previous papers we reported changes in the methylation pattern of the genomic DNA of carrot root explants during callus growth initiation (Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993a). In the present study we demonstrate tissue-specific methylation of the genomic DNA in carrot plants, as influenced by genotype, age, plant growth and primary cultures, at the transition from linear to stationary growth.

Materials and methods

Plant material and tissue culture

The plant material used for this study originated from the cultivars Rote Riesen (red-orange, cylindrical form, late maturity), Rotin (red-orange, cylindrical form, middle-early maturity), Pariser Markt (red-orange, round form, early maturity) and Lobbericher (yellow, cylindrical form, late maturity). To perform culture experiments early in the year, fresh carrots of unknown origin imported from Italy were bought at the local market (experiments in Fig. 6). For tissue-culture experiments a primary culture system of the secondary phloem of the carrot roots was applied. Explants (2–4 mg) of this tissue can be easily taken by a trocar without the risk of contamination by other cell types. The explants were cultured in a liquid medium (Neumann 1966, 1968; see also Gartenbach-Scharrer et al. 1990) with permanent illumination (at about 4000 lux, osmium lumilux white) at 21 °C. Three-to-five explants were incubated in 15 ml of nutrient solution in T-tubes (Steward et al. 1952) and continuously rotated at 1 rpm. Ten tubes were cultured simultaneously for each variant. For DNA analysis a mixture of the callus material of each variant was taken and investigated in parallel experiments. The culture period and supplementation of the nutrient solution with m-inositol (50 ppm), 3-indole-acetic-acid (IAA; 2 ppm) and kinetin (0.1 ppm) was varied according to the experiment.

During culture, callus formation is induced indicating a change in the differentiation state of the phloem tissue, and defined growth stages can be reproducibly obtained within a rather limited space of time. After a lag-phase of 6–8 days exponential callus growth commences, mainly due to cell-division activity (r=0.813). In the presence of kinetin callus growth decelerates to stationary growth at about the 28th day of culture.

DNA isolation

Genomic DNA was extracted as described by Murray and Thompson (1980) and simplified by Power et al. (1986) (see Arnholdt-Schmitt et al. 1991). This was followed by digestion with ribonuclease A and T1 overnight and extraction with chloroform-isoamylalcohol. The quantitation of the DNA concentration was performed by the diphenylamine reaction using the method of Richards (1974) as described by Power et al. (1986). Isolated DNA was tested for high-molecular-weight preparation in comparison to λ-*Hin*dIII fragments in 1% agarose used as a length standard.

DNA restriction digests

Genomic DNA was digested completely by the restriction enzymes *Eco*RII (Gibco-BRL) and *Bsp*NI (Biozym diagnostic) using 3–4 U/μg of DNA for at least 4 h. The neo-schizomeric enzymes *Eco*RII and *Bsp*NI differ in that the former is not able to cleave the recognition site 5′-CC(AT)GG if the internal cytosine is methylated, whereas the latter cuts this sequence independently of methylation. The performance of *Eco*RII was studied thoroughly by Krüger et al. (1988a, b) and Pein et al. (1989). *Eco*RII activity was reported to lead to incomplete digestion if the recognition site was present only in low frequency in the investigated DNA. The enzyme obviously requires at least two bound sites for activation. Nevertheless, resistance of unmethylated DNA to *Eco*RII could be overcome by a second DNA sequence with further 5′-CC(AT)GG sites. In the genome of carrots the *Eco*RII recognition site occurs frequently (see *Bsp*NI restriction in Fig. 1) and so complete digestion of the genomic DNA of carrots can be assumed. Different methods of purification (additional CsCl-centrifugation or supplementation of PVP to the extract) and parallel restriction assays with the neo-schizomeric enzyme *Bst*NI, as well as the addition of λ-DNA as an internal standard to reference DNA preparations, did not indicate any contamination which was able to influence restriction enzyme activity even of contamination-sensitive enzymes like *Eco*RI (Arnholdt-Schmitt 1993b).

Agarose electrophoresis

Electrophoresis was performed with 2–3 μg of genomic DNA in 1% agarose (Bio-Rad Standard Low-M_r) for 15 h (50 V, submerged technique at 15°C). The gels were stained for 2 h in 2 μg/ml of ethidium bromide, and the results were documented photographically using a transilluminator with a wavelength of 302 nm.

Video-densitometric evaluation

Scanning of the restriction fragment pattern was carried out on the negatives by video-densitometry. In Figs. 1, 2 and 6 the scanning region started at the gel pocket (left side) whereas in Figs. 3, 4 and 5 the pockets were excluded. To correct for differences between the quantity of DNA of the different variants compared with what was actually loaded onto the gel, the integral of the sum of all fragments distributed as bands and the background in the gel of one variant was corrected to the total integral of the DNA fragments of the compared variant. Additionally, this procedure ensures that differences outside the evaluated region (e.g. in the region of the gel pocket) will be recognizable since deviations in a higher molecular region will be compensated by reverse deviations in lower molecular regions and vice versa.

Results

The level of DNA methylation in a genome can be evaluated by the extent of site-specific methylation in the recognition sequence of a methylation-sensitive restriction enzyme (Razin 1988). The recognition sequence 5′-CC(AT)GG occurs frequently in the carrot genome as could be confirmed by the marked extent of restriction by *Bsp*N I. Figure 1 shows the video-densitometric scans of the restriction profiles of the genomic DNA of the secondary

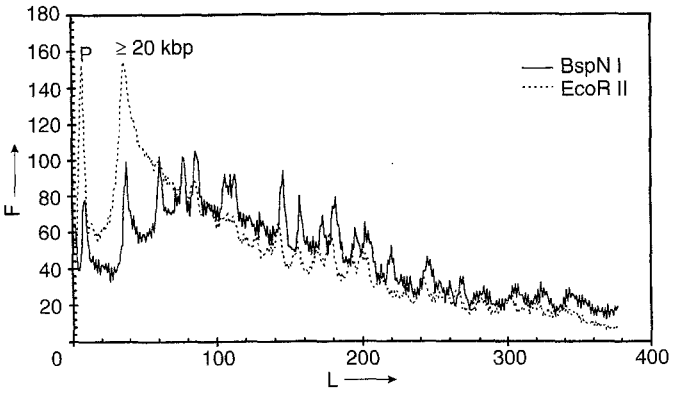

Fig. 1 Site-specific DNA methylation in the carrot genome. *Bsp*NI and *Eco*RII restriction profiles of the DNA of the secondary phloem of carrot roots. *F* relative fluorescence of the restriction fragments; *L* relative length of the electrophoretic run of the restriction fragments through the agarose gel given by the number of video-densitometric scan points. The scans include the gel pocket (*p*)

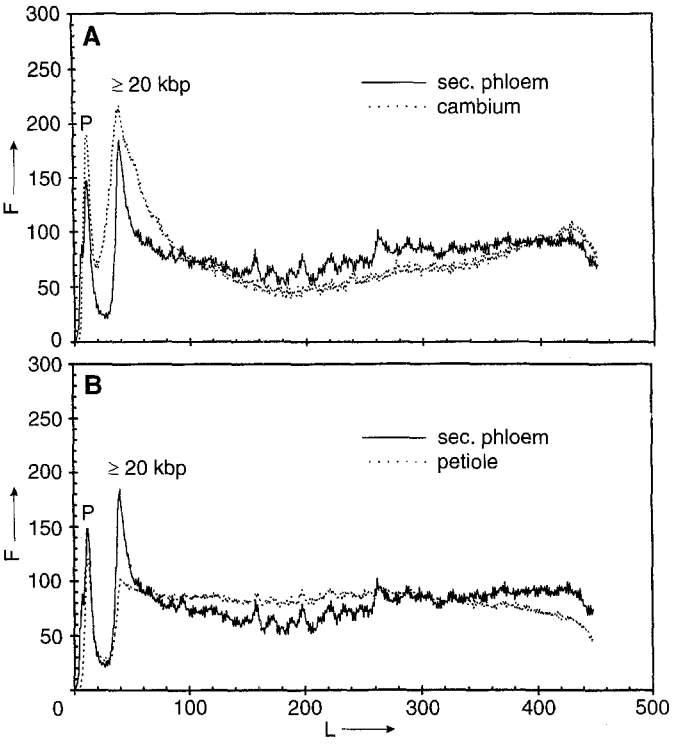

Fig. 2A, B Tissue-specific genome methylation. **A** Comparison of the *Eco*RII restriction profiles of the DNA of cambium and of the secondary phloem of mature carrot roots (cv Rotin). **B** Comparison of the *Eco*RII restriction profiles of the DNA of the secondary phloem of mature carrot roots and of petioles of about 7-week-old plants (cv Rotin). The scans include the gel pocket (*p*)

phloem of the carrot root after digestion by the neo-schizomeric enzymes *Bsp*NI and *Eco*RII. The increased amount of the higher molecular fragments of the methylation-sensitive *Eco*RII digest, in comparison to the digestion by the methylation-insensitive enzyme *Bsp*NI, reveals the degree of site-specific methylation in the investigated tissue. This modification seems to be distributed throughout the entire genome.

*Eco*RII profiles were used in our investigation to detect differences in the level of site-specific DNA methylation of the motif -CNG- of different variants (concerning *Eco*RII digestion see Materials and methods). In Fig. 2 the *Eco*RII fragment pattern of different tissues of the carrot plant were compared. The two graphs demonstrate tissue-specific methylation. The level of DNA methylation of the cambium of the carrot storage root was considerable higher than that of the secondary phloem of the root as was demonstrated by the greater amount of high-molecular *Eco*RII fragments (Fig. 2A), indicating a higher number of methylated recognition sequences in the DNA of the meristematic tissue which prevented cleavage by *Eco*RII. By contrast the DNA of the petiole tissue was notably less methylated than the DNA of both root tissues resulting in a smaller amount of high-molecular *Eco*RII fragments as was evident by comparison with the DNA of the secondary phloem of carrot roots (Fig. 2B).

Figure 3 shows that the genomic DNA of analogous tissues, even of morphologically highly distinctive cultivars (see Materials and methods), displays the same level of site-specific methylation. No substantial differences were obtained by comparison of the *Eco*RII profiles from three different cultivars. Additionally, Fig. 4 demonstrates that the *Eco*RII methylation pattern was obviously independent of the age of the plants investigated. Since the fresh weights of the roots were 56 g for the younger and 116 g for the older plant the level of site-specific DNA methylation was also obviously independent of the extent of secondary root growth. This was confirmed by a comparison of the DNA of the roots of cv Rote Riesen and cv Rotin (Fig. 3A). Secondary root growth until the selected harvest date had been strikingly different resulting in a 99 g fresh weight for the root of cv Rotin and a 253 g fresh weight for the root of cv Rote Riesen. Nevertheless, the pattern of *Eco*RII methylation of the DNA of the secondary root phloem for both carrot cultivars with cylindrical roots was almost identical. This observation also indicates that there was no relationship between the level of site-specific -CNG- methylation and the extent of secondary root growth.

As has been previously shown, inoculation and rapid cell-division activity during callus initiation of secondary phloem explants of carrot roots is accompanied by an increase in genomic DNA methylation (Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993a). The extent of this increase was variable and dependent on kinetin, additional to m-inositol and IAA, in the nutrient solution. In the presence of kinetin de novo methylation seemed to be suppressed, or at least delayed, at inoculation as well as during the phase of rapid callus growth mainly by cell-division activity (Arnholdt-Schmitt 1993a). Nevertheless, the callus growth rate by cell-division was not related to the level of DNA methylation as is shown in Fig. 5 which demonstrates the *Eco*RII profiles of two selected tissue-culture growth variants originating from different roots. This was also true when differences in callus fresh weight at the 14th

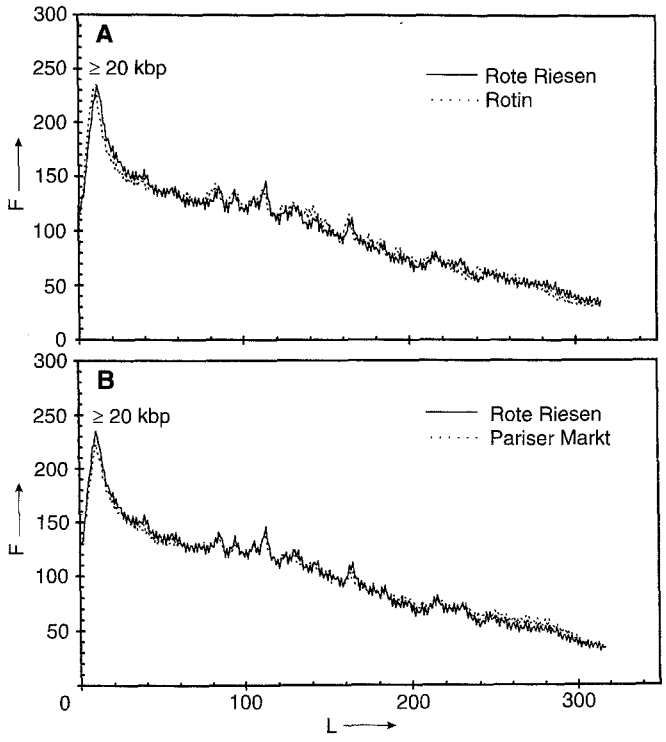

Fig. 3A, B Genome methylation of different genotypes. **A** Comparison of the *Eco*RII restriction profiles of the DNA of the secondary phloem of carrot roots of the cultivars Rote Riesen and Rotin. **B** Comparison of the *Eco*RII restriction profiles of the DNA of the secondary phloem of carrot roots of the cultivars Rote Riesen and Pariser Markt. The scans do not include the gel pocket

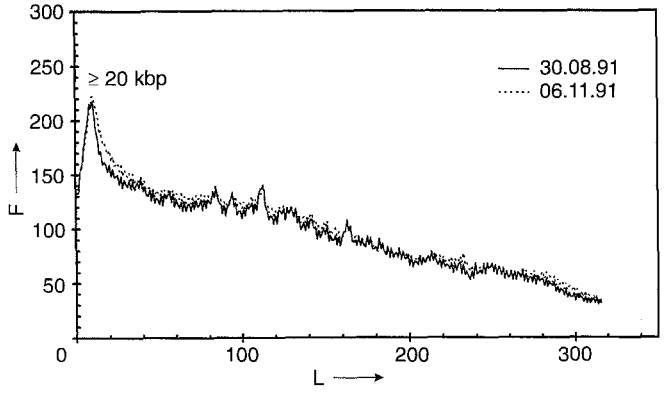

Fig. 4 Genome methylation and plant age. *Eco*RII restriction profiles of the DNA of the secondary phloem of carrot roots (cv Pariser Markt) which were harvested at different times (sowing date: 6. 5. 1991). The scans do not include the gel pocket

day of culture were due to the higher temperature of 28°C during callus growth (data not shown).

In Fig. 6 *Eco*RII profiles of the DNA of callus tissues of the rapid cell-division phase at the 14th day were compared to *Eco*RII fragment patterns at the 28th day of culture, i.e. during transition to stationary growth. As can be seen from all three graphs, during transition to the stationary growth phase of cultured secondary phloem explants

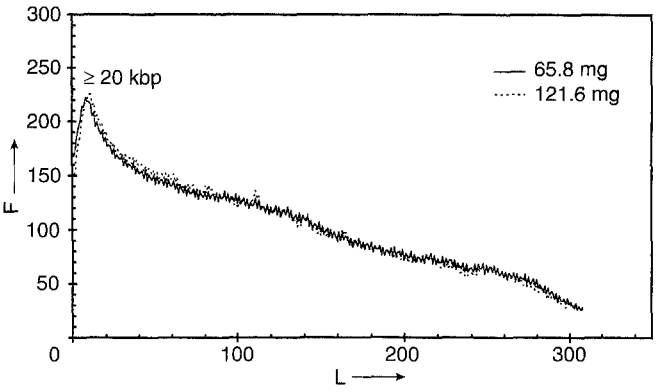

Fig. 5 Genome methylation and callus growth rates. *Eco*RII restriction profiles of the DNA of cultured secondary phloem explants of the roots of two carrot plants 2 weeks after inoculation (t_{14d}) in a medium supplemented with m-inositol, IAA and kinetin (cv Lobbericher). Fresh weights are given in mg per callus. The scans do not include the gel pocket

de-methylation at the *Eco*RII recognition site occurred predominantly in the genome leading to a slightly smaller degree of high-molecular fragments by *Eco*RII digests. This result was achieved in 8 out of 9 experiments (three single plants and three different culture conditions for each plant as displayed in Fig. 6; replications are not shown). The rate of de-methylation was independent of a subculture at the 14th day after inoculation (Fig. 6B), which resulted in an increase in fresh weight per callus of about 34% at the 28th day of the culture period (data in the legend to Fig. 6). Surprisingly, a subculture at the same time to a nutrient solution depleted of kinetin increased the degree of de-methylation as can be seen by the smaller amount of higher molecular fragments if kinetin is not present in the medium (Fig. 6C). This result indicates that kinetin seems to impair changes in the methylation state of the genome in general, since the rate of de novo methylation during the initiation of primary culture of the secondary phloem explants of the carrot roots was, as was shown earlier (see above), also reduced by supplemented kinetin.

Discussion

The investigations presented in this paper show that different tissues of carrot plants can be distinguished by different levels of *Eco*RII site-specific DNA methylation. Additionally, *Eco*RII restriction analysis of the DNA of the secondary phloem of carrot roots from three cultivars displayed almost identical restriction profiles. The level of methylation was independent of the age or the extent of the secondary root growth of the investigated plants. These results indicate that the pattern of methylation covering the total genome seems to be related primarily to the differentiated state of a specific tissue. Genome modification is obviously superimposed on differences in the genetic background of different cultivars.

In this context it has to be remembered that coding gene sequences have been estimated to make up at best only

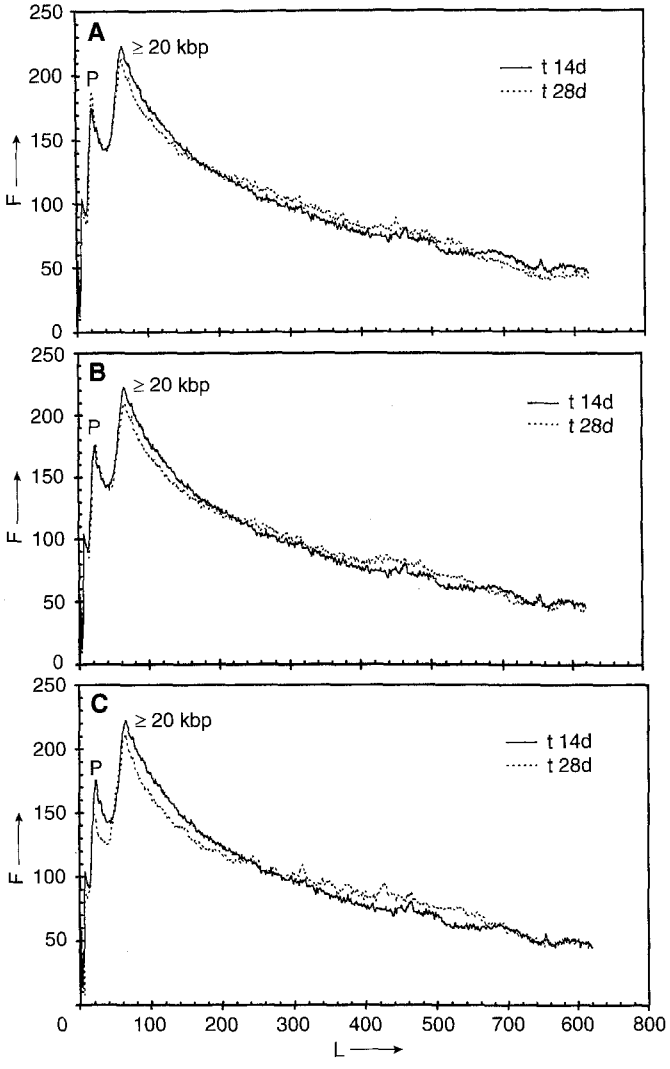

Fig. 6A–C Genome de-methylation during the transition to stationary callus growth. **A** *Eco*RII restriction profiles of the DNA of secondary phloem explants, which were cultured during 14 and 28 days in the presence of m-inositol, IAA and kinetin. **B** *Eco*RII restriction profile of the DNA of secondary phloem explants, which were cultured during 14 days in the presence of m-inositol, IAA and kinetin, in comparison to the profile of the DNA of explants which were subcultured at the 14th day in fresh medium until day 28. **C** *Eco*RII restriction profile of the DNA of secondary phloem explants, which were cultured during 14 days in the presence of m-inositol, IAA and kinetin, in comparison to the profile of the DNA of explants which were subcultured at the 14th day in fresh medium depleted of kinetin until day 28. Fresh weights per callus were 82 mg (t_{14d}), 375 mg (t_{28d}; in **A**), 423 mg (t_{28d}, subculture at t_{14d} without kinetin; in **C**) and 502 mg (t_{28d}, subculture at t_{14d} with kinetin; in **B**). The scans include the gel pocket (*p*)

about 1% of plant genomes (Nagl et al. 1983). Palmgren et al. (1991) reported highly distinctive levels of DNA methylation in different cell lines and cell types in unorganized carrot suspension cultures. These authors assumed that their results, and those in the literature concerning variations in the level of DNA methylation of organs or tissues, may be primarily due to changes in the relative distribution of the different cell types in a given cell population. For the present investigation it was obviously advantageous not to use extracts of the whole carrot root but rather DNA extracts from the relative homogeneous tissue of the secondary root phloem (see Neumann 1969), which was already differentiated and in a so-called "quiescent" state. Thus, differentiation at a fully developed stage seems to be characterized by a stable pattern of overall DNA methylation, at least in the carrot genome. Also for the human genome, Behn-Krappa et al. (1991) reported indistinguishable patterns of cell type-specific DNA methylation over a wide range of DNA sequences in different individuals even those with different genetic origins (East-Asian or Caucasian).

A change in the differentiated state of the secondary phloem by tissue culture conditions was followed by changes in the methylation pattern. Reports on differential DNA methylation by cell or tissue culture normally compare the results obtained by "in vitro" to others obtained "in vivo" (Brown et al. 1989; Palmgren et al. 1991; Galaud et al. 1993), or else distinguish between callus and cell-culture systems (Quemada et al. 1987; Brown et al. 1989; Bezdek et al. 1991; Palmgren et al. 1991) or between embryogenic and non-embryogenic callus cultures (Morrish and Vasil 1989). An essential aspect of the previous and present investigations was the use of a primary culture system with well defined growth phases (see Materials and methods). Previous studies (Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993a) have shown that de novo methylation of the genomic DNA occurred characteristically during the inoculation and primary culture of carrot explants of the secondary phloem of the roots. On the other hand at the transition to stationary growth de-methylation primarily occurred in the genome. As was evident from earlier investigations the growth phase- or kinetin-dependent de-methylation coincides with a higher content of protein per unit DNA or per cell (Neumann 1968, 1972). This suggests that the observed de-methylation at the total genome points to an increased transcription rate indicating the initiation of a new molecular program in cells of the callus tissue. As will be shown in an accompanying paper (Arnholdt-Schmitt 1995; see also Arnholdt-Schmitt 1993b), differential amplification occurs as a gross change in the genomic DNA of the callus tissue at the same time, i.e. at the 28th day of culture. Although differential replication and DNA methylation should be basically independent events (see Arnholdt-Schmitt 1993a) it can not be excluded that amplification contributed to the de-methylation of the genome. Razin and Cedar (1984) have pointed to a possible relationship between rapid replication events and de-methylation if amplification were to lead to limiting levels of methyltransferase followed by a decrease in maintenance methylation.

The de novo DNA methylation and de-methylation which accompanied changes in the differentiation state of the carrot tissues in primary culture could be impaired by exogenous cytokinin activity. Obviously kinetin was antagonistic to changes in genome modification in general. This observation is in agreement with the known physio-

logical effect of cytokinins in vivo concerning the senescence-delaying potential of these hormones. Evidently, cytokinin activity can be effective in genome modification only if the cultured material is dynamically changing with respect to molecular differentiation. Therefore, it is not surprising that LoSchiavo et al. (1989) did not find any change in DNA methylation by the application of cytokinins to a cell suspension, which apparently had already established a stable DNA modification pattern expressed by 16% methylated cytosines.

The increase in methylation of the genome during the first 2 weeks of primary culture was obviously not directly linked to the induction or initiation of growth (Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993), but seems to be related to the de-stabilization of the differentiation state of the original tissue of the plant. It is suggested that de novo methylation as a gross change in the genome indicates a de-differentiation of the tissue presumably implying a blockage of former cell programs (see also LoSchiavo et al. 1989). The transition of the carrot tissues to stationary growth was accompanied by de-methylation. And at this phase too the change in DNA modification was independent of the cell-division rate. The results of Palmgren et al. (1991) also indicate that the DNA of freshly cultivated segments of carrot hypocotyl was more strongly methylated than the original tissue. By contrast, the DNA of the established cell suspension showed less methylation in comparison to freshly inoculated tissue. These results suggest that de-differentiation at the genome level by non-specific de novo methylation is followed by de-methylation, which seems to be related to specific molecular "re-programming" of the tissue.

This hypothesis is in good agreement with the experiments of Kafri et al. (1992) and the assumptions of Razin and Cedar (1993) with respect to differential changes in DNA methylation during mouse gametogenesis and embryogenesis. It has to be stressed that in carrot plants the meristematic root tissue, i.e. the cambium, also displayed a high level of genomic DNA methylation while the DNA of the secondary root phloem, originating from the meristem by cell division and subsequent differentiation, was less methylated. During somatic embryogenesis in carrot suspensions with a stabilized auxin-dependent level of DNA methylation at 16% LoSchiavo et al. (1989) reported an initial decrease of DNA methylation followed by an increase in methylation during further development. Specific de novo methylation related to development was also assumed by Monk et al. (1987) in their investigations on mouse embryos.

It is evident that in future we will have to define the terms de-differentiation and differentiation more precisely at the molecular level. This should include differential modification by methylation of the total genome. As will be shown in a further paper this should also consider differential replication as a gross event (see also Arnholdt-Schmitt et al. 1991 and Arnholdt-Schmitt 1993a,b). Since de-differentiation seems to be a prerequisite for the expression of totipotency in tissue culture by somatic embryogenesis (e.g. Brettschneider et al. 1993) a genomic marker for de-differentiation might be helpful for studying the induction of somatic embryogenesis in recalcitrant species.

DNA methylation is known to contribute to somaclonal variation (Phillips et al. 1990). Therefore, further research needs to be done to elucidate the relationship between physiological DNA variability in tissue culture and the transmission of stable changes in genome modification in the case of plants regenerated from tissue culture.

References

Adams RLP (1990) DNA methylation. Biochem J 265:309–320

Adams RLP, Burdon RH (1985) Molecular biology of DNA methylation. Springer, New York Berlin Heidelberg Tokyo

Arnholdt-Schmitt B (1993a) Rapid changes in amplification and methylation pattern of genomic DNA in cultured carrot root explants (*Daucus carota* L.). Theor Appl Genet 85:793–800

Arnholdt-Schmitt B (1993b) Hormonsensitivität und genomische DNS-Variabilität in ihrem Bezug zur Ertragsbildung bei *Daucus carota* L. ssp. *sativus*. Ein Beitrag zur Zellbiologie pflanzlichen Wachstums. Habilitationsschrift, Giessen

Arnholdt-Schmitt B (1995) Physiological aspects of genome variability in tissue culture. II. Growth phase-dependent quantitative variability of repetitive *Bst*NI fragments of primary cultures of *Daucus carota* L. Theor Appl Genet 91:816–823

Arnholdt-Schmitt B, Holzapfel B, Schillinger A, Neumann KH (1991) Variable methylation and differential replication of genomic DNA in cultured carrot root explants during growth induction as influenced by hormonal treatments. Theor Appl Genet 82:283–288

Ball DJ, Gross DS, Garrard WT (1983) 5-Methylcytosine is localized in nucleosomes that contain histone H1. Proc Natl Acad Sci USA 80:5490–5494

Bassi P (1990) Quantitative variations of nuclear DNA during plant development: a critical analysis. Biol Rev 65:185–225

Behn-Krappa A, Hölker I, Sandaradura De Silva U, Doerfler W (1991) Patterns of DNA methylation are indistinguishable in different individuals over a wide range of human DNA sequences. Genomics 11:1–7

Bezdek M, Koukalova B, Brzobohaty B, Vyskot B (1991) 5-Azacytidine-induced hypomethylation of tobacco HRS60 tandem DNA repeats in tissue culture. Planta 184:487–490

Brettschneider R, Lörz H, Stirn S (1993) In vitro Kultur und Gentransfer bei Getreide. BioEngineering 9:31–36

Brown PTH, Yoneyama K, Lörz H (1989) An investigation into the role of 5-Azacytidine in tissue culture. Theor Appl Genet 78: 321–328

Cedar H (1988) DNA methylation and gene activity. Cell 53:3–4

Dhar MS, Pethe VV, Gupta VS, Ranjekar PK (1990) Predominance and tissue specificity of adenine methylation in rice. Theor Appl Genet 80:402–408

Edwards YH (1990) CpG islands in genes showing tissue-specific expression. In:Holliday et al. (1990) 207–215

Galaud JP, Gaspar T, Boyer N (1993) Inhibition of internode growth due to mechanical stress in *Bryonia dioica*: relationship between changes in DNA methylation and ethylene metabolism. Physiol Plant 87:25–30

Gartenbach-Scharrer U, Habib S, Neumann KH (1990) Sequential synthesis of some proteins in cultured carrot explant (*Daucus carota*) cells during callus induction. Plant Cell, Tissue Organ Cult 22:27–35

Graessmann M, Graessmann A. (1993) DNA methylation, chromatin structure and the regulation of the gene. In: Jost and Saluz (1993) 404–424

Holliday R, Monk M, Pugh JE (eds) (1990) DNA methylation and gene regulation. Phil Trans R Soc Lond B 326:177–338

Jost JP, Saluz HP (eds) (1993) DNA methylation: molecular biology and biological significance. Birkhäuser, Basel Boston Berlin

Jost JP, Saluz HP, McEwan I, Feavers IM, Hughes M, Reiber S, Liang HM, Vaccaro M (1990) Tissue-specific expression of the avian vitellagenin gene is correlated with DNA hypomethylation and in vivo specific protein-DNA interactions. In: Holliday et al. (1990) 231–240

Kafri T, Ariel M, Brandeis M, Shemer R, Urven L, McCarrey J, Cedar H, Razin A (1992) Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev 6:705–714

Keshet I, Lieman-Hurwitz J, Cedar H (1986) DNA methylation affects the formation of active chromatin. Cell 44:535–543

Klaas M, Amasino RM (1989) DNA methylation is reduced in DNaseI-sensitive regions of plant chromatin. Plant Physiol 91: 451–454

Krüger DH, Barcak GJ, Reuter M, Smith HO (1988a) *Eco*RII can be activated to cleave refractory DNA recognition sites. Nucleic Acids Res 16:3997–4008

Krüger DH, Schroeder C, Reuter M, Bickle TA, Bogdarina IG, Buryanov YI (1988b) Use of bacterial virus T7 as a tool for the study of DNA methylation. Gene 74:85–87

Linn F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize A 1 gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol Gen Genet 222:329–336

LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variation as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–331

Meyer P, Linn F, Heidmann I, Meyer H, Niedenhof I, Saedler H (1992) Endogenous and environmental factors influence 35*s* promotor methylation of maize A1 gene construct in transgenic petunia and its colour phenotype. Mol Gen Genet 231:345–352

Monk M, Boubelik M, Lehnert S (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 99:371–382

Morrish FM, Vasil IK (1989) DNA methylation and embryogenic competence in leaves and callus of napiergrass (*Pennisetum purpureum* Schum.). Plant Physiol 90:37–40

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Nagl W (1987) Replication. Progr Bot 49:181–191

Nagl W (1989) Replication. Prog Bot 51:173–180

Nagl W (1990) Gene amplification and related events. Biotechnol Agric For 11:153–201

Nagl W, Jeanjour M, Kling H, Kühner S, Michels I, Müller T, Stein B (1983) Genome and chromatin organisation in higher plants. Biol Zentralbl 102:129–148

Neumann KH (1966) Wurzelbildung und Nucleinsäuregehalt bei Phloem-Gewebekulturen der Karottenwurzel auf synthetischem Nährmedium verschiedener Hormonkombinationen. Phytohormones et l'Organogenèse 38:95–102

Neumann KH (1968) Über Beziehungen zwischen hormonal gesteuerter Zellteilungsgeschwindigkeit und Differenzierung. Ein Beitrag zur Physiologie der pflanzlichen Ertragsbildung. Habilitationsschrift, Giessen

Neumann KH (1969) Mikroskopische Untersuchungen zum Wachstumsverlauf von Explantaten verschiedener Gewebepartien der Karottenwurzel in Gewebekultur. Mikroskopie 25:261–271

Neumann KH (1972) Untersuchungen über den Einfluß des Kinetins und des Eisens auf den Nukleinsäure- und Protein-stoffwechsel von Karottengewebekulturen. Z Pflanzenernaehr Bodenkd 131: 211–220

Palmgren G, Mattsson O, Okkels T (1991) Specific levels of DNA methylation in various tissues, cell lines, and cell types of *Daucus carota*. Plant Physiol 95:174–178

Pein CD, Reuter M, Cech D, Krüger DH (1989) Oligonucleotide duplexes containing CC(A/T)GG stimulate cleavage of refractory DNA by restriction endonuclease *Eco*RII. FEBS Lett 245: 141–144

Phillips RL, Kaeppler SM, Peschke VM (1990) Do we understand somaclonal variation? In: Nijkamp HJJ, Van der Plas LHW, Van Aartrijk, J (eds) Progress in plant cellular and molecular biology. Proc VIIth Intl Congr Plant Tissue Cult 9:131–141

Power JB, Davey MR, Freeman JP, Mulligan BJ, Cocking EC (1986) Fusion and transformation of plant protoplasts. Methods Enzymol 118:578–595

Quemada H, Roth EJ, Lark KG (1987) Changes in methylation of tissue-cultured soybean cells detected by digestion with the restriction enzymes *Hpa*II and *Msp*I. Plant Cell Rep 6:63–66

Razin A (1988) Assays for studying DNA methylation. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant Mol Biol Manual B3: Kluwer Academic Publishers, Dordrecht, pp 1–28

Razin A, Cedar H (1993) DNA methylation and embryogenesis. In: Jost und Saluz (1993) 343–357

Razin A, Cedar H (1984) DNA methylation in eukaryotic cells. Int Rev Cytol 92:159–185

Razin A, Friedman J (1981) DNA methylation and its possible biological roles. Prog Nucleic Acid Res 25:33–52

Richards GM (1974) Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal Biochem 57:369–376

Riggs CD, Chrispeels MJ (1990) The expression of phytohemagglutinin genes in *Phaseolus vulgaris* is associated with organ-specific DNA methylation patterns. Plant Mol Biol 14:629–632

Selker EU (1990) DNA methylation and chromatin structure: a view from below. Trends Biochem Sci 15:103–107

Shen CKJ, Maniatis T (1980) Tissue-specific DNA methylation in a cluster of rabbit b-like globin genes. Proc Natl Acad Sci USA 77:6634–6638

Steward FC, Caplin SM, Millar FK (1952) Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. Ann Bot 16:57–77

Zlatanova J (1990) Histone H1 and the regulation of transcription of eukaryotic genes. Trends Biochem Sci 15:273–276