Variable methylation and differential replication of genomic DNA in cultured carrot root explants during growth induction as influenced by hormonal treatments

B. Arnholdt-Schmitt, B. Holzapfel, A. Schillinger, and K.-H. Neumann
Institut für Pflanzenernährung, Abteilung Gewebekultur, Justus-Liebig-Universität Giessen, Südanlage 6, W-6300 Giessen, FRG

Received January 4, 1991; Accepted January 23, 1991
Communicated by H.F. Linskens

Summary. The methylation and amplification pattern of genomic DNA of carrot root explants (Daucus carota L.) undergoes transitory changes during the cultural cycle. A high degree of variation was observed as early as 36 h after the incubation of fresh explants in the nutrient medium and, depending on the hormonal treatment significant modifications occurred during 14 days of culture. Proliferative tissue conditioned by kinetin showed an extensive reduction in DNA methylation. Changes in the DNA amplification pattern were not necessarily linked to methylation.

Key words: Daucus carota L. – Tissue culture – Hormones – DNA methylation – Differential replication – Transitory genomic variation

Introduction

A rather general phenomenon in cell culture systems is the occurrence of genomic variations that apparently can also be transmitted to regenerated plants (Buiatti 1977; Larkin and Scowcroft 1981; Meins 1983; D’Amato 1984; Karp 1989). During recent years evidence has accumulated that relates these somaclonal variations to changes in the methylation pattern of the DNA of the cultured cells (for review see Phillips et al. 1990).

Some investigators have reported a preferential accumulation of methylated cytosine in repeated DNA sequences (Deumling 1981; Sturm and Taylor 1981; Ehrlich et al. 1982; Pages and Roizes 1982, for review see Adams and Burdon 1985; Leclerc and Siegel 1987), and Brown et al. (1987) observed a concomitant variation in the amplification and methylation pattern of the DNA of tissue culture-derived maize plants (see also Brown 1989). Apparently the hyper/hypomethylation pattern of the DNA induced in cell culture systems can be stabilized and then transmitted to plants regenerated from these cultures (Brown and Lörz 1986; Lörz 1990). LoShiaio et al. (1989), however, were able to show some relation between methylation and the course of somatic embryogenesis in carrot cell cultures. Further, the extent of methylation in cell cultures was increased by high auxin concentrations in the medium; this state was reversible after transfer to lower auxin concentrations. Therefore, it seems that two types of methylation changes can be induced, one genetically fixed and the other of a more transitory nature and possibly related to development. If the latter is a more general phenomenon, then such methylation processes should also occur during callus induction and organogenesis. In this paper we report our investigations on the methylation pattern of carrot root explants during both the initiation of cell division in this original “quiescent” tissue, and the initiation of adventitious roots. In rhizogenic cultures the DNA concentration per cell was increased concurrently with some higher DNA turnover and qualitative variations in satellite DNA as compared to highly proliferative undifferentiated cultures. The high cell division activity of the latter system requires a cytokinin supplement to the IAA and inositol-containing medium used for the induction of rhizogenesis (Neumann 1972; Neumann et al. 1978; Schäfer et al. 1978; Dürrsen and Neumann 1980).

Here we report investigations on the correlation between differential DNA replication and DNA methylation. To determine gross changes in the organization and modificational processes superimposed on the variations in individual genes we used genomic DNA for the investigations.

Materials and methods

Tissue culture

Freshly cut explants (2–3 mg fresh weight) of the secondary phloem of the tap root of Daucus carota L. (cv “Rote Riesen”) were aseptically cultured in a liquid medium as described earlier (Neumann 1966; Neumann 1968). Culture period and supplementation of the nutrient solution with inositol (50 ppm), 3-indole-acetic-acid (IAA, 2 ppm) and kinetin (0.1 ppm) was varied according to the experiment.
DNA isolation, restriction digests and electrophoresis

Genomic DNA was extracted as described by Murray and Thompson (1980) and simplified following the protocol of Power et al. (1986); this was followed by ribonuclease A and T₁ digestion and chloroform-isoamyl alcohol extraction. DNA quantitation was carried out with diphenylamine using the method of Richards (1974) as described by Power et al. (1986). Genomic DNA was digested with different restriction enzymes using 3–4 U/µg DNA for at least 4 h. The enzymes employed were HaeIII (Boehringer), EcoRII, HpaII, MspI (Gibco-BRL) and BspNI (BIOzym diagnostic). EcoRII is a methylation-sensitive isoschizomeric enzyme of BstNI. EcoRII cannot cleave if the internal cytosine of the recognition sequence 5'-CC(AT)GG is methylated, while BstNI cleaves in this situation. HpaII and MspI cleave at the cutting sequence 5'-CCGG; HpaII activity is blocked by methylation of the internal 3'-cytosine, whereas MspI cannot cleave if the 5'-cytosine is methylated. Gel electrophoresis was performed in 1% agarose for 15 h with lambda HindIII DNA fragments or defined lambda HaeIII DNA fragments as size markers. The gels were stained with ethidium bromide.

Fractionation of genomic DNA and labelling of HaeIII fragments

Ten micrograms of DNA was digested with HaeIII, and the fragments were separated in 1% low-melting point agarose (Gibco-BRL). The gel with the separated fragments was divided into nine sections identical in gel size. In a parallel run the DNA quantity in each piece of gel was determined after phenol extraction. In each of the nine fractions 80 ng DNA were labelled with biotin-16dUTP (Boehringer) for 20 h at 23 °C in the presence of low-melting point agarose using a kit for "Random primed DNA labeling" (Boehringer). The labelled fragments were separated from the nucleotides on a 1-ml Sephadex-G75 column and detected in a slot blot with the BlueGene Detection System (Gibco-BRL).

Slot blot hybridization assays

Aliquots of 1 ng genomic DNA were slot blotted on nitrocellulose and hybridized in 6 x SSC and 0.7% skim milk powder at 70 °C for 1 h with each of the nine HaeIII fragment fractions (see above). Washing conditions allowed homology of 80%–100% (Schneider and Müller 1988). The hybridization rate was detected for 1 h with the BlueGene Detection System (Gibco-BRL) and scanned by measurement of remission (Chromatogramm-Spektrophotometer, Zeiss), and then evaluated planimetrically.

Results

In Fig. 1 the restriction fragment pattern of the original root phloem explants are compared to that of explants cultured for 36 h in a hormone-free nutrient medium. At this stage the first round of cell division is completed in this system (Gartenbach-Scharrer et al. 1990). In the original explants there is already methylation of some of the recognition sites of the restriction enzymes employed (EcoRII, BspNI). However, only some of the recognition sites adjacent to repeated DNA fragments are methylated, whereas others having the same fragment size are not (e.g., see fragments marked with white lines). This could be due either to the occurrence of two different types of the same DNA with respect to methylation in a given cell or to variations in the same DNA sequence in different cells of a given callus.

In explants cultured in the hormone-free medium basically the same fragment pattern as that observed in the original explants occur, however, an increase in the number of low molecular weight fragments in the lower half of the gel at the expense of higher molecular weight fragments can be seen. This indicates variation in the amplification of DNA sequences with the dominance of DNA with a high number of recognition sites. Again, only some of the recognition sites were methylated. Almost the same restriction fragment pattern can be observed if IAA in combination with inositol with or without kinetin is supplemented to the nutrient medium (Fig. 2). However, in the presence of kinetin the low molecular weight fragment fraction seems to be less dominant than in those cultured with IAA and inositol only. The degree of methylation of DNA of the cultured explants seems to be comparable to that of the DNA of the original explants at t₀ (see digestion of EcoRII). Following BspNI digestion however, no difference in the intensity of the bands as observed for the DNA of the original explants between the restriction pattern of the isoschizomeric enzymes is perceptible, indicating that in cul-