Theor Appl Genet (1991) 82:283–288

THEORETICAL AND APPLIED GENETICS

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 concomittant 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). LoShiavo 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 cytokinine supplement to the IAA and inositol-containing medium used for the induction of rhizogenesis (Neumann 1972; Neumann et al. 1978; Schäfer et al. 1978; Dührssen 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 T_1 digestion and chloroform-isoamylalcohol 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 × 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 alowed 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-Spektralphotometer, 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

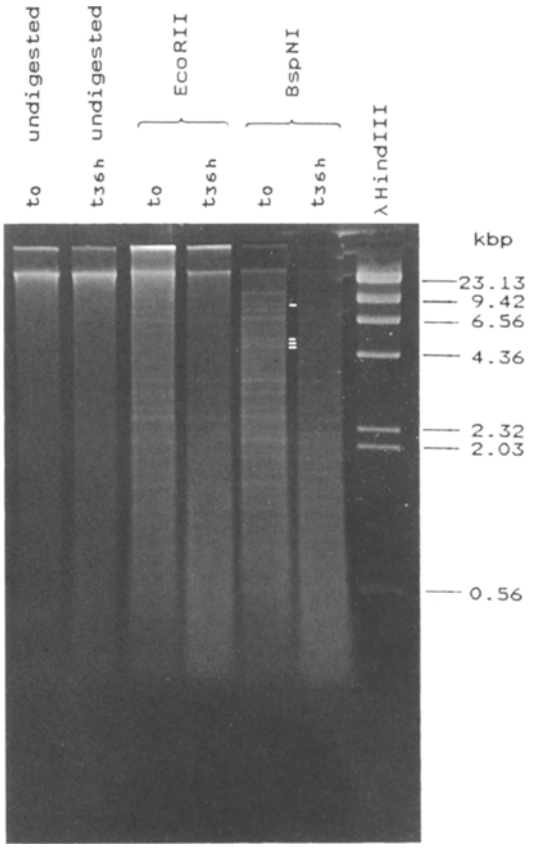

Fig. 1. Restriction pattern of DNA of phleom explants at t_0 and after 36 h of incubation in a hormone-free nutrient medium

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 t_0 (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-

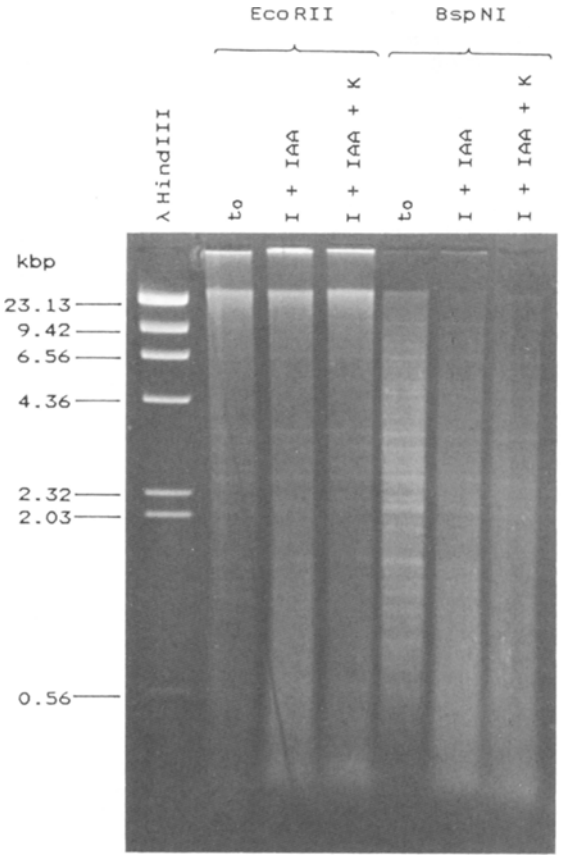

Fig. 2. Restriction pattern of DNA of phloem explants at t_0 and after 36 h of incubation in a nutrient medium supplemented with inositol (*I*) and 3-indole-acetic-acid (*IAA*) with or without kinetin (*K*)

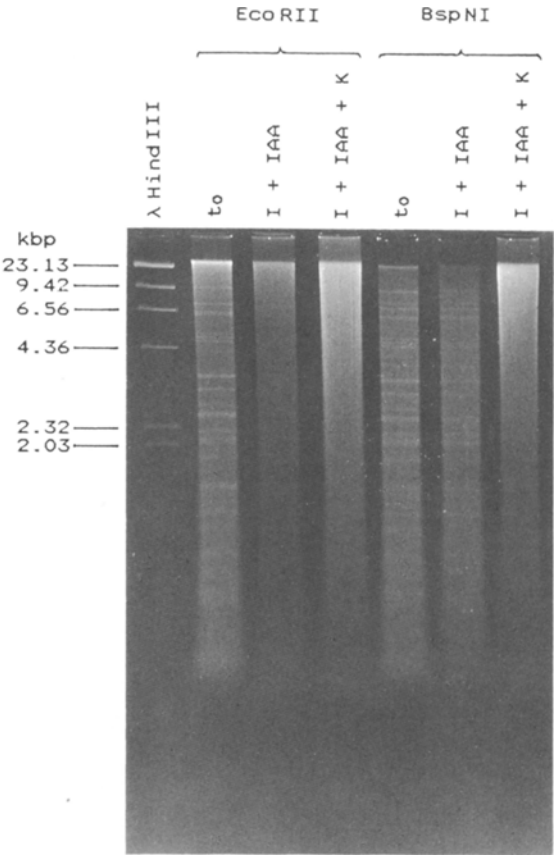

Fig. 3. Restriction pattern of DNA of phloem explants at t_0 and after 14 days of culture in a nutrient medium supplemented with inositol (*I*) and 3-indole-acetic-acid (*IAA*) with or without kinetin (*K*)

tured cells (36 h) the sequences adjacent to these repeated DNA fragments are now no longer methylated. There seem to be no differences in the methylation pattern of all cultures independent of the hormonal treatment. It has to be pointed out that at this stage of the culture period all treatments are in the same phase of the cell cycle due to a high natural synchrony during callus induction (Gartenbach-Scharrer et al. 1990).

After 2 weeks of culture in the IAA plus inositol treatment the explants reach a stationary growth phase, and the emergence of adventitious roots can be observed. However, cultures grown in a medium supplemented with kinetin as well as IAA and inositol still display high cell division activity and an apparent lack of organized structures at the same stage of the cultural cycle (Neumann 1968). At this stage the dominance of the low molecular weight fragments observed after 36 h of culture has disappeared in explants from both treatments (Fig. 3). Since without kinetin hardly any bands in the gel can be seen besides the undigested DNA at its top after digestion with EcoRII, in the cells cultured without kinetin most of the recognition sites adjacent to the repeated DNA sequences should be methylated. Apparently, in this treatment methylation now occurs in the recognition sites adjacent to the repeated DNA sequences which were unmethylated after 36 h of culture, to an even higher extent than in the original explants.

From the plus kinetin treatment at this culture stage mainly high molecular weight fragments were obtained to the same extent with both restriction enzymes. This indicates that at the recognition sites there is an absence of or only minor methylation in this treatment. Further, no bands of repeated DNA are visible following digestion with both enzymes. Obviously the methylation pattern and the amplification of DNA sequences are not stable in this system, and they change according to the hormonal supplement at t_0 as well as to the status of differentiation.

By use of the methylation-sensitive isoschizomers HpaII and MspI in the plus kinetin treatment it could be shown that less methylation also exists for the recognition site 5′-CCGG (Fig. 4). In this treatment also the repeated sequences cannot be observed after digestion with MspI. Consequently, here the recognition sites adja-

cent to these sequences should be specifically methylated at the outer cytosine (5′-cytosine). In the minus kinetin cultures no variation occurs in the appearance of the repetitive sequences after digestion with both enzymes. This suggests an additional response of the DNA organization.

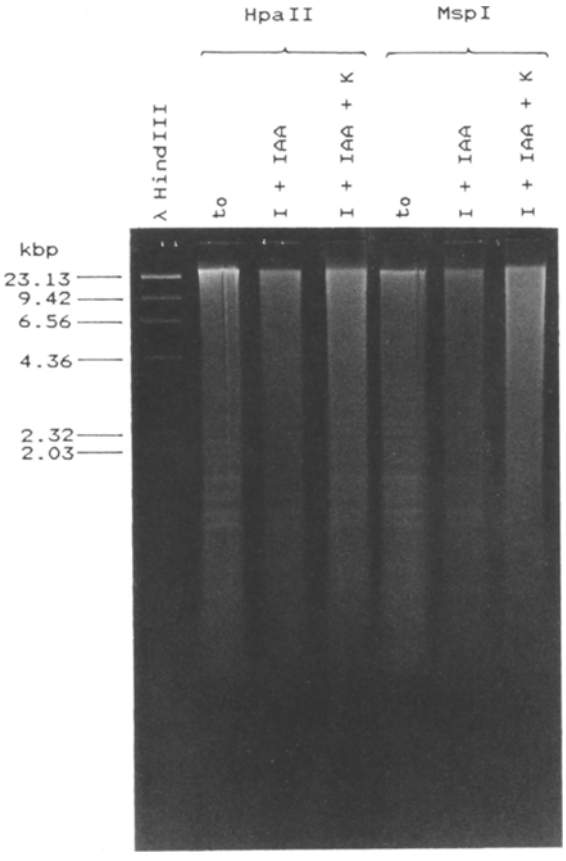

Fig. 4. Restriction pattern of DNA of phloem explants at t_0 and after 14 days of culture in a nutrient medium supplemented with inositol (*I*) and 3-indole-acetic-acid (*IAA*) with or without kinetin (*K*)

Evidence for differential replication was also obtained by using hybridization assays of undigested total DNA with genomic HaeIII fragment probes (Table 1). In the minus kinetin treatment a considerable higher rate of amplification of some DNA sequences is obvious, especially in comparison to the plus kinetin sample but also to some extent to the original phloem explants. Some variation in amplification magnitude, however, is similar in cultured explants independent of the hormonal supplement as compared to the orignal tissue at t_0 (>8.4 kbp; 3.1–4.8 kbp; 1.5–2.1 kbp), and therefore should be induced by the conditions of culture. In the plus kinetin sample some fragments with a molecular size between 0.5 and 1.1 kbp indicate specific underreplication. The frequency of these fragments in the minus kinetin sample and in the original explants is comparable.

Discussion

In the literature some publications are available that report a close correlation between repeated DNA sequences and its methylation (see introduction). Nevertheless, until now it has not been clear whether the methylation of repeated DNA sequences is changed on existing DNA or whether there is a differential replication of DNA sequences with a specific methylation pattern. Although a number of theories on the function of methylated DNA have been published, none of these is able to cover all of the results described. In many cases a correlation between differentiation states and methylation pattern seems to exist and frequently an inactivation of DNA sequences is associated with methylation, but not in all cases (for reviews see Doerfler 1983; Bird 1984; Razin et al. 1984; Adams and Burdon 1985; Cedar 1988; Selker 1990).

In our investigations on carrot callus cultures a clear variation in methylation and differential replication was

Table 1. Hybridization rates of HaeIII fragments with the genomic DNA of carrot root phloem explants at t_0 or callus tissues cultured for 14 days in the presence of 50 ppm inositol and 2 ppm IAA with or without 0.1 ppm kinetin (±kin)

HaeIII fragments kbp	Phloem t_0 (pl.u.)	−Kin (pl.u.)	+Kin (pl.u.)	−Kin:phloem (%)	+Kin:phloem (%)	−Kin:+kin (%)
>8.4	90	63	63	70	70	100
4.8–8.4	52	78	54	150	104	144
3.1–4.8	21	42	48	200	229	88
2.1–3.1	19	23	15	121	79	153
1.5–2.1	16	11	7	69	44	157
1.1–1.5	36	44	35	122	97	126
0.7–1.1	41	39	31	95	76	126
0.5–0.7	40	40	29	100	73	138
0.3–0.5	22	37	28	168	127	132

pl.u., Planimetric units

evident. As early as 36 h after the initiation of the experiment, changes in copy number and in methylation of the DNA of the original explants could be observed. After 2 weeks of culture, however, again a different methylation and amplification pattern exists in the DNA of explants undergoing the same treatments. This is a clear indication that these two characteristics of DNA, i.e., methylation and differential replication, are liable to transitory changes in this tissue culture system. Such variations seem to occur in addition to the stable and therefore genetically transmittable alterations in the methylation pattern described by Brown and Lörz (1986) and Brown (1989). It remains to be seen whether transitory variations under certain conditions can be stabilized.

The variation in amplification and methylation pattern can be influenced by the hormones added to the nutrient medium at t_0. This is particularly obvious for kinetin at day 14 (compare Fig. 3 and Table 1). In both sets of experiments with the minus kinetin treatment repeated DNA sequences are more abundant than in the plus kinetin cultures. The use of methylation-sensitive enzymes shows that in this treatment the amplified DNA fragments are also highly methylated in their adjacent recognition sites. A similar coincidence of amplification and methylation can be also seen by comparison of the low molecular weight fragments for the cultures supplemented with IAA, inositol and plus or minus kinetin after 36 h of incubation. However, a comparison of the DNA of original explants with those cultured in hormone-free medium for 36 h indicates (Fig. 1, e.g., fragments marked with white lines) that the recognition sites of not all repeated DNA fragments are methylated, nevertheless, they are reduced to a similar extent in the genome after 36 h. From this it can be derived that changes in the amplification pattern are not necessarily linked to methylation.

Further, in the plus kinetin treatment differences in the position of 5-methylcytosine in the recognition site from that in the minus kinetin treatment could also be shown, and therefore a de novo methylation of pre-existing DNA has to be considered.

In explaining the increase in the low molecular weight fragments at the expense of larger fragments in Figs. 1 and 2 we should not ignore that this also could result from the integration of transposable elements containing the recognition site of the restriction enzyme applied, though insertion events of this magnitude would be at the very least astonishing given our present knowledge. A loss of such elements could then be responsible for the dominance of higher molecular weight fragments after 2 weeks of culture.

It has been considered that demethylation drugs could be helpful in stimulating differential gene activity in tissue cultures, for instance with respect to somatic embryogenesis (Brown et al. 1989). The results presented in this paper indicate that genomic DNA methylation can be significantly reduced in a purely normal process during tissue culture apparently dependent on the hormones present in the medium. Gross demethylation of the genome, however does not necessarily mean activation of genes related to differentiation since in our system the reduction in methylation is correlated with a high proliferation rate of the cells without any morphological differentiation. Thus, if alterations in sequence hypomethylation should play a role in the induction of differential processes they should be specific.

In summary, three ways of transitory changes in the amplification and methylation pattern during callus development were observed in our investigations, i.e., the amplification of DNA sequences with methylated 5′-CC(AT)GG sequences; amplification of sequences with the same recognition sites but without methylation; de novo methylation of the 5′-cytosine in the recognition site (5′-CCGG). All three possibilities of transitory changes could contribute to strong variation in the organization and modification of total DNA during the culture cycle. Considering the great quantitative extent of variation at any given stage of development of the culture system with respect to changes in methylation as well as amplification pattern mainly others than gene sequences should be involved.

Acknowledgement. Thanks are due to Dr. M. Henze at the Institut für Genetik of the Justus-Liebig-Universität Giessen for help with densitometric evaluations.

References

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

Bird AP (1984) DNA methylation – how important in gene control? Nature 307:503–504

Brown PTH (1989) DNA methylation in plants and its role in tissue culture. Genome 31:717–729

Brown PTH, Lörz H (1986) Molecular changes and possible origins of somaclonal variation. In: Semal J (ed) Somaclonal variation and crop improvement. Martinus Nijhoff, Boston, pp 148–159

Brown PTH, Göbel E, Lörz H (1987) Methylation and gene copy changes in maize somaclonal variation. Abstr XIVInt Bot Congr Berlin, p 153

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

Buiatti M (1977) DNA amplification and tissue cultures. In: Reinert J, Bajaj YPS (eds) Applied and fundamental aspects of plant cell, tissue and organ culture. Berlin Heidelberg New York; pp 358–374

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

D'Amato F (1984) Cytogenetics of plant cell and tissue cultures and their regenerates. CRC 3:73–112

Deumling B (1981) Sequence arrangement of a highly methylated satellite DNA of a plant, *Scilla*: A tandemly repeated inverted repeat. Proc Natl Acad Sci USA 78,1:338–342

Doerfler W (1983) DNA methylation and gene activity. Annu Rev Biochem 52:93–124

Dührssen E, Neumann KH (1980) Characterization of satellite-DNA of *Daucus carota* L. Z Pflanzenphysiol 100:447–454

Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C (1982) Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. Nucleic Acids Res 10:2709–2721

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

Karp A (1989) Can genetic instability be controlled in plant tissue cultures? JAPTC Newsl 58:2–11

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Leclerc RF, Siegel A (1987) Characterization of repetitive elements in several *Cucurbita* species. Plant Mol Biol 8:497–507

Lörz H (1990) Zell- und Gewebekultur, somatische Embryogenese and in vitro-Regeneration von Getreide. Botanikertagung Regensburg 30.9.–5.10., p. 45

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 variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–331

Meins F (1983) Heritable variation in plant cell culture. Annu Rev Plant Physiol 34:327–346

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

Neumann KH (1966) Wurzelbildung und Nukleinsäuregehalt bei Phloem-Gewebekulturen der Karottenwurzel auf synthetischen Nährmedien verschiedener Hormonkombinationen. Les Congr Colloques de l'Universite de Liege 38:96–102

Neumann KH (1968) Über Beziehungen zwischen hormonal gesteuerter Zellteilungsgeschwindigkeit und Differenzierung, ein Beitrag zur Physiologie der pflanzlichen Ertragsbildung. Habilitation Justus-Liebig-Universität Giessen

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

Neumann KH, Pertzsch Ch, Moos M, Krömmelbein G (1978) Untersuchungen zur Charakterisierung des Ernährungssystems von Karottengewebekulturen: Photosynthese, Kohlenstoff- und Stickstoffernährung, Mineralstoffverbrauch. Z Pflanzenernaehr Bodenk 141:299–311

Pages M, Roizes G (1982) Tissue specificity and organization of CpG methylation in calf satellite DNA I. Nucleic Acids Res 10:565–576

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 of VIIth Int Congr on Plant Tissue 9:131–141

Power JB, Davey MR, Freeman JP, Mulligan BJ, Cocking EC (1986) Fusion and transformation of plant protoplasts. In: Methods in enzymology, vol. 118. Academic Press, New York, pp 578–595

Razin A, Cedar H, Riggs AD (1984) DNA methylation, biochemistry, and biological significance. Springer, Berlin Heidelberg New York

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

Schäfer A, Blaschke JR, Neumann KH (1978) On DNA metabolism of carrot tissue cultures. Planta 139:97–101

Schneider J, Müller L (1988) DNA-DNA-Hybridisierung mit biotinylierten DNA-Sonden. Forum Mikrobiologie 6:254–262

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

Sturm KS, Taylor JH (1981) Distribution of 5-methylcytosine in the DNA of somatic and germline cells from bovine tissues. Nucleic Acids Res 9:4537–4546