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Rapid changes in amplification and methylation pattern of genomic DNA in cultured carrot root explants (*Daucus carota* L.)

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Summary. Rapid genomic DNA variation due to methylation and copy number alteration was observed in carrot root explants 6 h after inoculation and during a 36-h period of exponential callus growth. De novo methylation and amplification of restricted *Bsp*NI fragments of low molecular weight occurred before cell cycle activation and should, therefore, be independent of progression through the S-phase of the cell cycle. Growth regulators seemed to influence the amplification pattern indirectly by regulating cell division activity. In exponentially growing callus tissue the copy number of most of the repetitive fragments was dramatically reduced. It is presumed that this reduction in the copy number of repetitive fragments is characteristic of 'rejuvenilization'. 3-Indole-acetic-acid (IAA) and inositol in the medium increased the degree of unspecific genomic DNA methylation in growing rhizogenic carrot callus tissue in the absence of kinetin, which inhibits root induction at that stage. A possible relation to the induction of rhizogenesis is considered. The observed reduction in number of repetitive restriction fragments and the increase in DNA methylation are gross changes covering the total genome. The results are discussed in relation to the controversy concerning the general biological significance of the methylation and amplification of DNA sequences.

Keywords: *Daucus carota* L. – Tissue culture – Growth regulator – Genomic DNA – Methylation – Differential replication – Rejuvenilization

Introduction

The occurrence of DNA methylation and DNA amplification in plants have some characteristics in common. Firstly, the extent of both phenomena is generally found to be much greater in the higher plants than in other organisms, although the proportion of repetitive DNA in different plant species can be highly variable – between 18% and 95% (Nagl 1976; Flavell 1982). Secondly, methylated DNA as well as repeated DNA sequences are distributed throughout the genome. The possibility of methylation occurring, however, is dependent on the presence of methylation-receptive C-G or C-X-G di- or trinucleotides (Gruenbaum et al. 1981). Thirdly, although both phenomena have been recognized for a long time and are described for various sequences in different organisms, no clear indication of an essential and definite biological function is available as yet. Variations in both methylation and amplification patterns have been observed to be especially great during in vitro culture.

Reported changes have been related to growth and differentiation processes and also to somaclonal variations (Arnholdt-Schmitt et al. 1991; Brown et al. 1991; Buiatti 1977; D'Amato 1984; Dührssen and Neumann 1980; Kikuchi et al. 1987; Lapitan et al., 1988; Larkin and Scowcroft 1981; Müller et al. 1990; Phillips et al. 1990; Schäfer et al. 1978). Because repeated DNA sequences may be highly methylated (Crowhurst and Gardner 1991; Deumling 1989; Ehrlich et al. 1982; Leclerc and Siegel 1987; Pages and Roizes 1982; Sturm and Taylor 1981) and because alterations in the degree of methylation have been observed in tissue culture-derived plants simultaneously with changes in amplification pattern it has been hypothesized that both events might be directly connected (Brown 1989).

Tissue culture systems that enable the induction of such alterations in DNA methylation and amplification as well as the carrying out of investigations during developmental processes can contribute to a better understanding of the appearance of both phenomena in general. It should be determined whether these events are correlated. In a previous paper we reported strong transient changes in the amplification and methylation pattern of genomic DNA in carrot root

explants after initiation of cell division activity as well as during callus growth with different cell division activity and at different states of differentiation as influenced by hormonal treatments (Arnholdt-Schmitt et al. 1991). Changes in the amplification pattern, however, were not necessarily linked to methylation. In the present paper we demonstrate rapid changes in the methylation and amplification pattern of genomic DNA 6 h after inoculation, i.e. before activation of the cell cycle, and during a 36-h period in the log-phase of callus growth, following a change in the hormonal composition of the nutrient medium to induce rhizogenesis.

Material and methods

Plant material and tissue culture

Explants (2–3 mg fresh weight) were obtained from the secondary phloem of mature carrot roots (*Daucus carota* L., cv 'Rote Riesen') and cultured in a liquid medium under continuous light at 21 °C as described earlier (Neumann 1966; Neumann 1968). For the investigations conducted during exponential callus growth 200 explants were cultured in 250 ml nutrient solution, whereas in experiments carried out shortly after inoculation about 4500 explants were incubated in 250 ml medium under what were otherwise identical conditions. Culture period and the degree to which the nutrient solution was supplemented with inositol (50 ppm), 3-indole-acetic-acid (IAA, 2 ppm) and kinetin (0.1 ppm) was varied according to the experiment.

DNA isolation

High-molecular-weight genomic DNA was extracted as described by Murray and Thompson (1980) and simplified by Power et al. (1986); this was followed by digestion with the ribonuclease A and T_1 overnight and extraction with chloroform-isoamylalcohol. For the data shown in Fig. 3 video-densitometric evaluation after electrophoresis and desoxyribonuclease digestion were used to control the result of the DNA isolation procedure and to assure that no RNA remained in the extract. The quantitative estimation of DNA concentration was performed by the diphenylamine reaction using the method of Richards (1974) as described by Power et al. (1986).

DNA restriction digests

Genomic DNA was digested by the restriction enzymes *Bsp*NI (Biozym diagnostic), *Eco*RII, *Hpa*II and *Msp*I (Gibco-BRL) using 3–4 U/μg DNA for at least 4 h. The isoschizomeric enzymes *Eco*RII and *Bsp*NI differ in that *Eco*RII is not able to cleave the recognition site 5′-CC(AT)GG if the internal cytosine is methylated, whereas *Bsp*NI cleaves this sequence independently of methylation. The *Bsp*NI restriction fragment pattern is therefore on additional aid by which to demonstrate differences in copy number between variants. *Hpa*II and *Msp*I recognize the cutting sequence 5′-CCGG. Both are blocked by methylation, but while *Hpa*II is not able to cleave if the inner 3′-cytosine is modified, digestion by *Msp*I is prevented by methylation of the outer 5′-cytosine.

Agarose electrophoresis

Electrophoresis was performed with 2 μg of genomic DNA in 1% agarose (Bio-Rad Standard Low-M_r) for 15 h (50 V, submerged technique). Lambda *Hin*dIII DNA fragments (1 μg) were used as size markers. The gels were stained for 2 h in 2 μg/ml ethidium bromide, and the results were documented photographically (Kodak Tri-X Pan, 400 ASA) using a trans-illuminator with a wavelength of 302 nm.

Densitometric evaluation

Scanning of the restriction fragment pattern was carried out on the negatives by video-densitometry. To level differences between the quantity of DNA of the different variants for comparison that was actually loaded onto the gel, the measured integral of the sum of all fragments distributed as bands and background in the gel of the various variants was corrected to the total integral of the DNA fragments of the control at t_0.

Results

Figure 1 shows the restriction pattern of genomic DNA isolated from phloem explants that were cultured in a nutrient solution supplemented with inositol, IAA and kinetin for 14 days and then transferred to the kinetin-free medium for 36 h. It can be seen that at t_0 genomic DNA of phloem tissue shows some methylation at the recognition site 5′-CC(AT)GG. Since most of the bands became more distinct after digestion with the methylation-insensitive enzyme *Bsp*NI than with the *Eco*RII digest, methylation seems to be distributed throughout the genome. Additionally, the disappearance or the reduction in intensity of some bands (*Eco*RII fragments marked with white lines) after restriction with *Bsp*NI indicates that at least some of the repeated DNA sequences of these bands also contained methylated recognition sites. Obviously, only some of the fragments with repetitive DNA of a given size are methylated at the sites adjacent to the recognition sequence. This means that a sample of fragments can consist of mixtures of molecules with either methylated or unmethylated sites. Also, the proportion of repeated DNA sequences of a given fragment size with methylated sites differs between various bands. For instance, the three fragments of 5.14 kbp, 4.95 kbp and 4.77 kbp (*Bsp*NI fragments marked with white lines) correspond in that they have a strikingly higher proportion of repetitive fragments with methylated adjacent sites than repetitive fragments of other molecular sizes.

After 14 days of culture in a nutrient medium supplemented with kinetin, IAA and inositol the explants grew as morphologically undifferentiated callus that showed very active cell division. The transfer of such cultures to a kinetin-free medium at t_{14d} induces rhizogenesis that becomes perceptible approximately 14 days later. Cultures which are kept in the original medium continue to grow as undifferentiated callus for a longer period until rhizogenesis can also be observed (Neumann 1968).

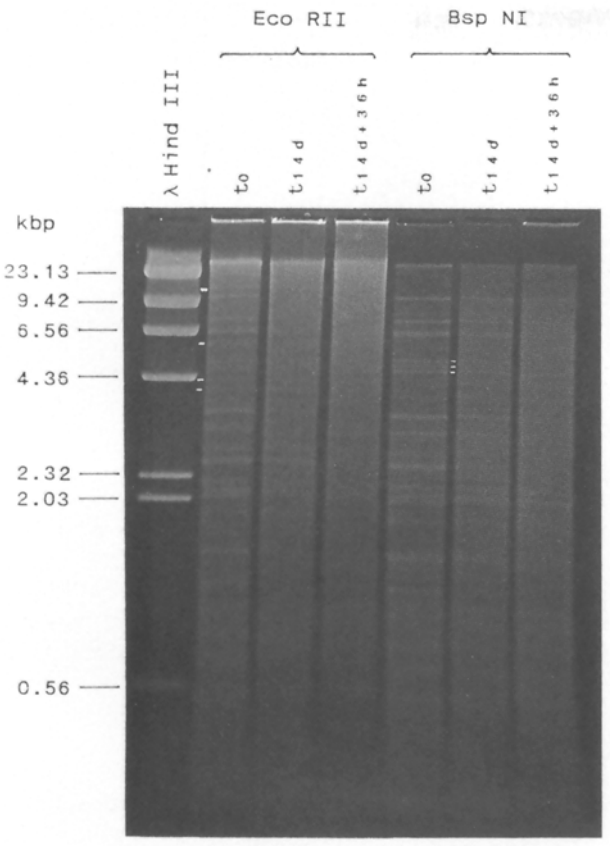

Fig. 1. Restriction pattern of genomic DNA of phloem explants. The explants were cultured in a nutrient solution supplemented with inositol, 3-indole-acetic-acid (IAA) and kinetin (K) for 14 days and then transferred to the kinetin-free medium for 36 h. Fresh weight per callus increased during the 36 h culture period from 39.1 mg to 56.1 mg (5.8 mg to 7.7 mg dry weight)

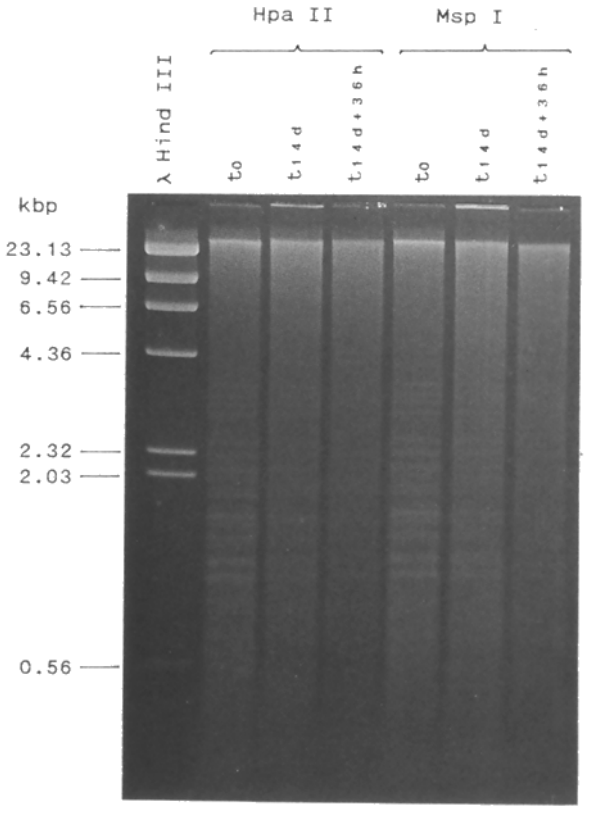

Fig. 2. Restriction pattern of genomic DNA of phloem explants. The explants were cultured in a nutrient solution supplemented with inositol, 3-indole-acetic-acid (IAA) and kinetin (K) for 14 days and then transferred to the kinetin-free medium for 36 h. Fresh weight per callus increased during the 36-h culture period from 39.1 mg to 56.1 mg (5.8 mg to 7.7 mg dry weight)

Figure 1 shows that before the transfer of the callus at t_{14d} the DNA shows the same fragment pattern as at t_0 (*Bsp*NI digest at t_0 and t_{14d}) and that there are also no clear differences in the degree of overall methylation if *Eco*RII digests at t_0 and t_{14d} are compared. The extent of repetitive fragments, however, is considerably reduced at t_{14d} (*Bsp*NI). To assure that the enzyme activity was not inhibited in this variant by any unknown substances lambda DNA was added to the extraction buffer as an internal standard; it was subsequently found to be completely restricted by both *Bsp*NI and by *Eco*RI, the latter being known to be especially sensitive to contaminations (figure not shown). This reduction of fragments with repeated DNA sequences continued rapidly during a 36-h cultivation of this callus material after transfer into the kinetin-free medium ($t_{14d+36h}$ *Bsp*NI) and was of such an extent that eventually bands were barely visible against the background of randomly distributed fragments. Remarkably, at least two fragments seemed to be more stable than the others: the so-called 'relic' DNA fragment with the highest molecular weight (Bedbrook et al. 1980) and a fragment of about 2.1 kbp.

Although a 2-week culture of the callus in the kinetin-supplemented medium elicited no visible change in DNA methylation compared to t_0, transfer into a kinetin-free but IAA- and inositol-supplemented medium and further culture for 36 h led to de novo methylation resulting in a higher degree of general methylation of the genomic DNA. This can be seen by the higher proportion of uncleaved DNA after *Eco*RII digestion (Fig. 1, $t_{14d+36h}$).

Results obtained with a second pair of isoschizomeric restriction enzymes, i.e., *Hpa*II and *Msp*I, seem to confirm the general tendency as demonstrated by the *Eco*RII/*Bsp*NI set (Fig. 2). Differences in band intensity on the gel in this system, however, are not a clear indication of either a variation in copy number or the degree of methylation because a control enzyme that cleaves the same recognition site independently of cytosine methylation is lacking. Still, the use of these enzymes showed that no clear differences seem to exist

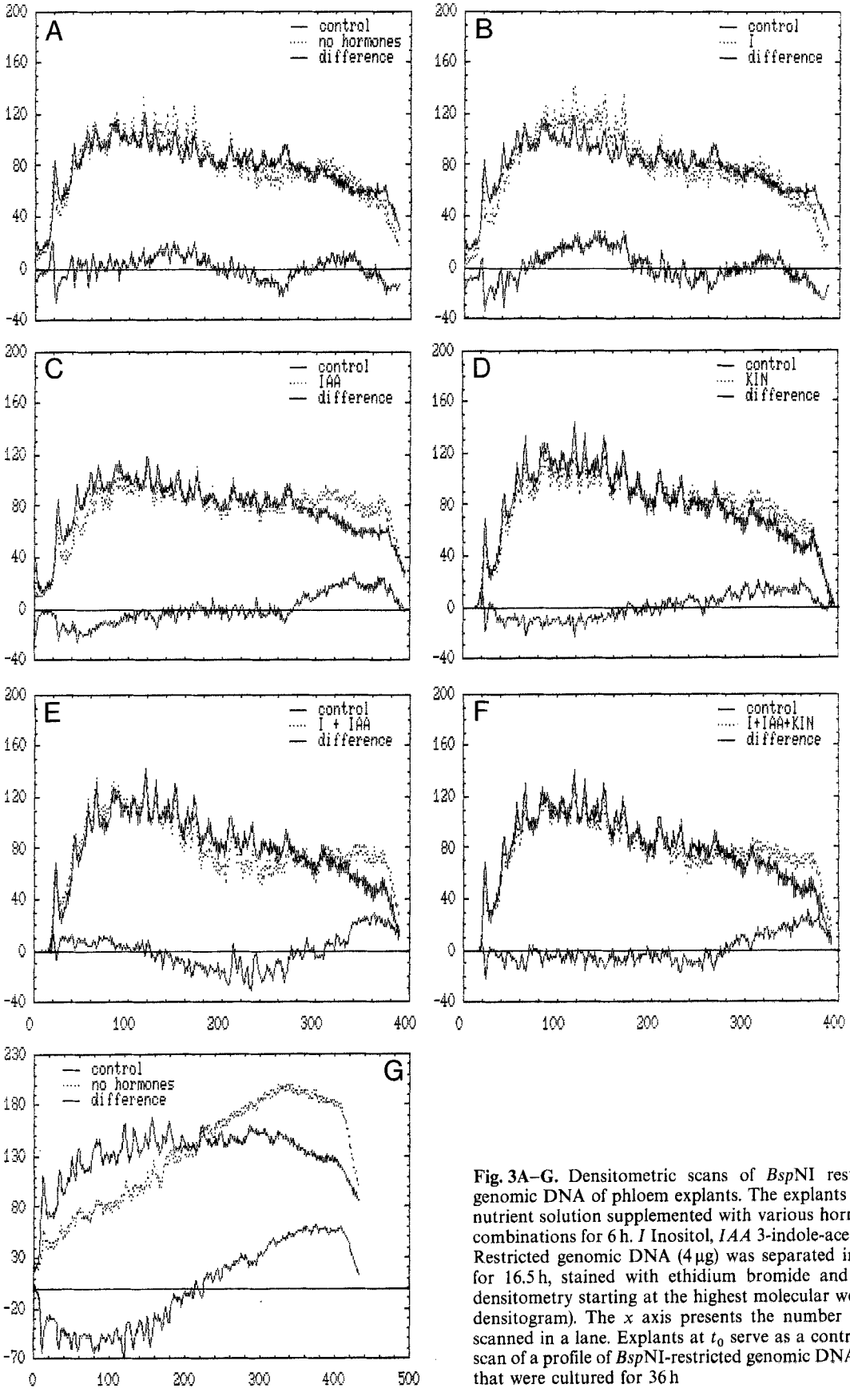

Fig. 3A–G. Densitometric scans of *Bsp*NI restriction patterns of genomic DNA of phloem explants. The explants were incubated in a nutrient solution supplemented with various hormones and hormone combinations for 6 h. *I* Inositol, *IAA* 3-indole-acetic-acid, *Kin* kinetin. Restricted genomic DNA (4 µg) was separated in 1% agarose (50 V) for 16.5 h, stained with ethidium bromide and scanned by video-densitometry starting at the highest molecular weight (*left side* of the densitogram). The *x* axis presents the number of points that were scanned in a lane. Explants at t_0 serve as a control. **G** Densitometric scan of a profile of *Bsp*NI-restricted genomic DNA of phloem explants that were cultured for 36 h

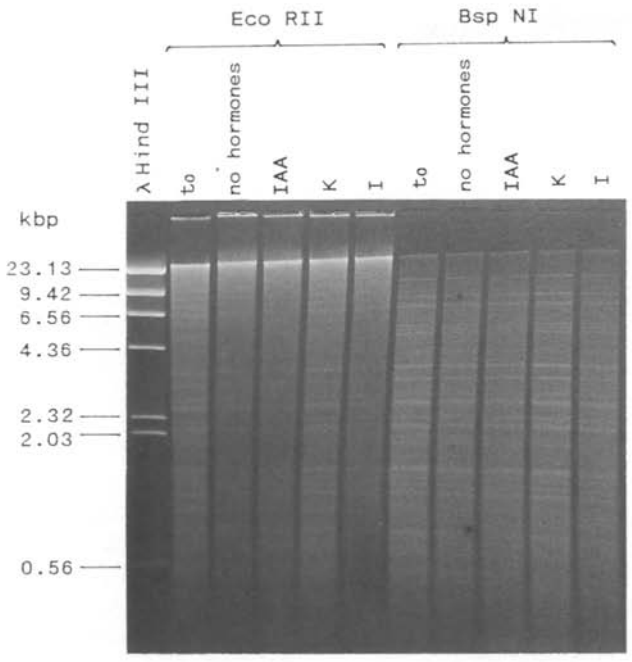

Fig. 4. Restriction pattern of genomic DNA of phloem explants at t_0 and 6 h after inoculation in a nutrient solution without phytohormones or supplemented with 3-indole-acetic-acid (*IAA*), kinetin (*K*) or inositol (*I*)

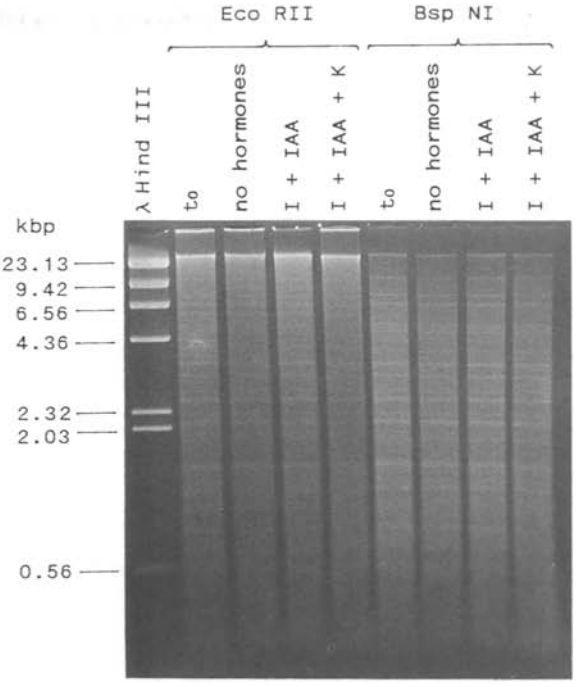

Fig. 5. Restriction pattern of genomic DNA of phloem explants at t_0 and 6 h after inoculation in a nutrient solution without phytohormones or supplemented with inositol (*I*) and 3-indole-acetic-acid (*IAA*) with or without kinetin (*K*)

in the degree of methylation of the inner and outer cytosine in the recognition sequences under all treatments.

The strong and rapid variations in copy number of restriction fragments and the overall degree of methylation during callus growth raised the question of whether changes in the methylation or amplification pattern need a passage through the S-phase of the cell cycle. As shown earlier by Gartenbach-Scharrer et al. (1990), activation of the cell cyle in freshly isolated explants of carrot root requires more than 12 h culture in a nutrient medium. At explantation the majority of the cells are in the G_1-phase. To test the essential requirement of a passage through the S-phase, the restriction fragment pattern after 6 h of culture following explantation was investigated. In addition, studies on the influences of some growth regulators were included.

As was shown in a previous paper (Arnholdt-Schmitt et al. 1991) during the first 36 h of the culture of phloem explants in a nutrient solution there was an increase in *Bsp*NI fragments of various low molecular weights distributed over the lower half of the gel that was independently of the presence of hormones in the medium. A densitometric scan is given as an example in Fig. 3G that demonstrates a *Bsp*NI profile of genomic DNA of phloem explants that were incubated for 36 h in a nutrient solution lacking hormones. For comparison, in Fig. 3A–F, it can be seen that a 6-h incubation of phloem explants was sufficient to initiate the process of amplification of sequences with a higher proportion of 5′-CC(AT)GG sites if IAA and kinetin alone or in combination and additionally supplemented with inositol were present in the nutrient solution (Fig. 3C–F). In the absence of any hormone (Fig. 3A) there was no increase in low-molecular weight *Bsp*NI fragments after a 6-h incubation of phloem explants although amplification could be observed 36 h later (Fig. 3G and Arnholdt-Schmitt et al. 1991). The addition of only inositol to the nutrient medium also did not enhance the proportion of smaller fragments in the genome during 6 h of culture (Fig. 3B).

Figures 4 and 5 show the restriction pattern that originated from digesting genomic DNA of the different variants with *Eco*RII and *Bsp*NI to demonstrate differences in the site-specific methylation intensity of the 5′-CC(AT)GG sequence. The extent of methylation in comparison to that at t_0 seemed to be stable during the first 6 h of culture in the presence of kinetin alone (Fig. 4, *Eco*RII) and inositol together with IAA (Fig. 5, *Eco*RII). The degree of methylation clearly became stronger, however, than that at t_0 in the presence of inositol or IAA alone (Fig. 4) or of inositol simultaneously with IAA and kinetin (Fig. 5) or even in the absence of any phytohormone in the nutrient

medium (Fig. 4, 5). This could be seen by the less efficient cleavage of *Eco*RII although the restriction patterns originating from *Bsp*NI digests were comparable.

Discussion

The investigations presented here show that changes in the degree of genomic DNA methylation and variations in the copy number of DNA sequences occur to a great extent during the tissue culture of carrot root explants. Apparently, de novo methylation and DNA amplification can take place without it being necessary for the cell to pass through the S-phase of the cell cycle. The possibility of an increase in the methylation of fragments with repetitive DNA does not seem to be dependent on a simultaneous increase in the number of copies of repeated DNA sequences (Figs. 1, 4 and 5). Additionally, the reduction in the number of repetitive fragments during callus growth is not correlated to the extent of genomic DNA methylation (Fig. 1). Therefore, and in agreement with earlier investigations (Arnholdt-Schmitt et al. 1991) it can be concluded that, in general, differential DNA replication and DNA methylation are potentially independent events.

Evidently, some repetitive fragments are linked. For instance, it can be shown for some low-molecular-weight fragments that as a population they are simultaneously amplified shortly after inoculation (Fig. 3). Another example of linkage is the higher proportion of repetitive fragments methylated at recognition sites simultaneously occurring to the same extent at the three fragments of 5.14 kbp, 4.95 kbp and 4.77 kbp (*Bsp*NI digest at t_0 marked in Fig. 1) in comparison to the degree of methylation shown by other repetitive fragments. Obviously, these sequences are under joint control, and it is possible to presume that the linkage should have its structural basis in the chromosomes.

Since an increase in low-molecular-weight restriction fragments could only be observed during the earlier stages of the cultural cycle and was apparently independent of the hormonal treatment, this extra DNA synthesis seems to be specific for the initiation of tissue cultures. Nevertheless, the formation of these fragments may be delayed as for instance, was shown when the hormones were absent from the medium (Fig. 3A, G). This observation is in good agreement with results obtained in earlier investigations from others on the appearance of newly synthesized GC-rich satellite DNA during the first hours after inoculation and its disappearance afterwards (Buiatti 1977).

The induction of originally quiescent phloem root tissue to a state of morphologically undifferentiated growth with a high activity of cell division occurs simultaneously with a dramatic reduction in the level of the repetitive fragments. A comparison of data published earlier (Arnholdt-Schmitt et al. 1991) with those of the present investigation indicates that the recognition sites of the restriction enzymes used might be lost simultaneously to a varying degree in different sets of experiments, thereby altering digestion with the restriction enzyme. Since the cells of the cultures investigated during the exponential growth phase can be regarded as 'rejuvenilized', this decrease in fragments with repeated DNA sequences could be characteristic of juvenilization. This presumption, however, should be verified with other tissue of the same plant and also with other species.

In this context it is of interest that Deumling and Clermont (1989) reported extensive chromatin diminution, especially of highly repetitive DNA sequences, in cultured cells of *Scilla siberica* to be a necessary prerequisite to induce regeneration of these monocotyledons. Also, we found that some repetitive genomic DNA fragments become reduced during the induction of somatic embryogenesis in young carrot petiole tissue (not published).

Since no specific hormone regulation could be found either during the first hours of culture (Fig. 3) or upon the transfer of exponentially growing calli from the kinetin-containing medium to a medium supplemented only with IAA and inositol (Fig. 1), the influence of growth regulators on differential replication seems to be more indirect.

With respect to methylation, however, it now seems to be obvious that in growing cultures the application of auxin together with inositol leads to de novo methylation if kinetin is not present (Fig. 1, see also Arnholdt-Schmitt et al. 1991). This is in agreement with a report by LoShiavo et al. (1989) on the reversible influence of various auxins in carrot cell cultures even though they used increasing levels of auxins in the presence of 0.25 ppm 6-benzyl-adenine. Additionally, since without kinetin rhizogenesis is induced as a new differentiational pathway in our system, the present results seem to support the hypothesis of these authors that an increase in methylation brought about by auxin might be necessary for the ability to change differentiational programs. LoShiavo et al. (1989) did not find any effect of cytokinine on methylation, but comparing the data from the present investigation with that of an earlier paper (Arnholdt-Schmitt et al. 1991) in which DNA methylation was reduced in kinetin-treated cultures it seems as though the influence of a cytokinine on the degree of methylation could be dependent on the reduction in number of methylation-sensitive recognition sites. Additionally, the results presented in Figs. 1 and 4 indicate that de novo methylation might be suppressed or at least delayed

by kinetin. The increase in DNA methylation in cells newly stimulated to initiate cell cycle activity, however, did not occur specifically in response to the exogenous hormones tested as far as can be detected with the methods used in our investigations.

The reduction in repetitive restriction fragments and the increase in DNA methylation described in both the present paper and in earlier investigations (Arnholdt-Schmitt et al. 1991) mainly demonstrate unspecific gross changes in DNA organization and modification that should be superimposed on such variations in individual genes. This phenomenon might be related to difficulties in finding definite functions for both methylation and the amplification of DNA sequences. The apparent absence or at least the limited degree of methylated bases in some organisms and strong variations among organisms in nuclear DNA content mainly due to different amplification rates without a similar quantitative change in gene sequences ['C-value-paradoxon' (Nagl 1976)] even provoke doubts on the general essentially of DNA methylation and amplification events. Nevertheless, if variations in methylation and amplification pattern do occur, changes in the structural DNA organization will be considerable, and therefore they should be of importance. This can already be shown in some studies, mainly on animals but also on plants, for the expression of a number of genes related to the methylation of distinct sequences (reviewed in John and Amasino 1989; Linn et al. 1990) as well as for gene amplification (Donn et al. 1984; Shah et al. 1986; Shyr and Widholm 1990).

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References

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

Bedbrook JR, Jones J, O'Dell M, Thompson RD, Flavell RB (1980) A molecular description of telomeric heterochromatin in *Secale* species. Cell 19:545–560

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

Brown PTH, Göbel E, Lörz H (1991) RFLP analysis of *Zea mays* callus cultures and their regenerated plants. Theor Appl Genet 81:227–232

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. Springer, Berlin Heidelberg New York, pp 359–374

Crowhurst RN, Gardner RC (1991) A genome-specific repeat sequence from kiwifruit (*Actinidia deliciosa* var 'deliciosa'). Theor Appl Genet 81:71–78

D'Amato F (1984) Cytogenetics of plant cell and tissue cultures and their regenerates. Crit Rev Plant Sci 3:73–109

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 78:338–342

Deumling B, Clermont L (1989) Changes in DNA content and chromosomal size during cell culture and plant regeneration of *Scilla siberica*: selective chromatin diminution in response to environmental conditions. Chromosoma 97:439–448

Donn G, Tischer E, Smith JA, Goodman HM (1984) Herbicide resistand alfalfa cells: an example of gene amplification in plants. J Mol Appl Genet 2:621–635

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

Flavell RB (1982) Sequence amplification, deletion, and rearrangement: major sources of speciation during species divergense. In: Dover GA, Flavell RB (eds) Genome evolution. Academic Press, London New York, pp 301–323

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 Org Cult 22:27–35

Gruenbaum Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860–862

John MC, Amasino RM (1989) Extensive changes in DNA methylation patterns accompany activation of a silent T-DNA ipt gene in *Agrobacterium tumefaciens*-transformed plant cells. Mol Cell Biol 9:4298–4303

Kikuchi S, Takaiwa F, Oono K (1987) Variable copy number DNA sequences in rice. Mol Gen Genet 210:373–380

Lapitan NLV, Sears RG, Gill BS (1988) Amplification of repeated DNA sequences in wheat × rye hybrids regenerated from tissue culture. Theor Appl Genet 75:381–388

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

Linn F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize A1 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 variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–331

Müller E, Brown PTH, Hartke S, Lörz H (1990) DNA variation in tissue-culture-derived rice plants. Theor Appl Genet 80:673–679

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

Nagl W (1976) Zellkern und Zellzyklen. Ulmer, Stuttgart

Neumann KH (1966) Wurzelbildung und Nukleinsäuregehalt bei Phloem-Gewebekulturen der Karottenwurzel auf synthetischen Nährmedien verschiedener Hormonkombinationen. Les Congress et 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

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: Nijkam HJJ, Van der Plas LHW, Van Aartrijk J (eds) Proc VIIth Int Congr Plant Tissue Cell Cult, vol 9. Amsterdam, pp 131–141

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

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

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hirokana CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG, Fraley RT (1986) Engineering herbicide tolerance in transgenic plants. Science 233:478–481

Shyr YY, Widholm JM (1990) Glyphosate resistance and gene amplification in selected *Daucus carota* suspension cultures. In: Nijkam HJJ, Van der Plas LHW, Van Aartrijk Y (eds) Proc VIIth Int Congr Plant Tissue Cell Cutt, vol 9. Amsterdam, pp 148–152

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