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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.

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Abstract Systematic investigations on the occurrence of differential DNA replication in carrot cultures, expressed at the total genome level, were performed. The genome of *Daucus carota* L. could be characterized by a pattern of repetitive *Bst*NI fragments that was independent of tissue specificity or cultivar differences. Characterization of the genomic DNA of the secondary phloem of carrot roots, in comparison to the DNA of the induced primary cultures at different growth phases, revealed dramatic differences in the copy number of the repetitive fragments. Highly proliferative tissue showed extensive reduction in the proportion of repetitive sequences in the genome in all of the 37 investigated variants. In contrast, during subsequent transition to stationary growth the repetitive fragments re-amplified. The results suggest that the quantitative genome organisation was involved in the regulation of the growth potential of cells. A hypothesis is discussed suggesting a determining influence of the observed differential DNA replication on cell-cycle rates and the cell program of proliferative tissue by structural and positioning effects on DNA loops. To study the causality of somaclonal variation, research on the relationship between physiological genome variability and the induction of heritable changes is recommended.

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

Introduction

Tissue-culture systems are known to be preferentially impaired by genetic variability (Larkin and Scowcroft 1981). Inhibition of somaclonal variation is of practical significance for the identical multiplication or preservation of germplasm. Somaclonal variation has been reported to be due to various kinds of alterations at the chromosomal or sequence level including quantitative and modificational changes (Peschke and Phillips 1992). In a companion paper Arnholdt-Schmitt et al. (1995) pointed to the physiological relevance of differential methylation in primary cultures of carrots in relation to cell-division growth. Cullis (1987) reported that environment-induced quantitative DNA variation, which was observed in the so-called "genotrophs" of flax, was comparable to the extent of DNA variation obtained in cultured cells and regenerated plants of flax. The induced rDNA variation in copy number occurred only in dividing cells of the stem meristems. The question arises whether the genome of proliferative cells – in vivo and in vitro – could be especially susceptible to quantitative genetic variation. Simultaneous "screening" of cell-division growth and quantitative genome variability is required but has not been systematically investigated until now (Peschke and Phillips 1992).

The plant genome is characterized by a high content of repetitive DNA sequences of mainly moderate copy numbers varying normally between 50% and 80% (Flavell et al. 1974; Nagl 1976; Dührssen and Neumann 1980; Bouchard 1982; Nagl et al. 1983; Rogowsky et al. 1991). Despite this great proportion of middle-repetitive sequences in the genome of plants their basic role in plant development or growth remains obscure. Most of this repetitive DNA is non-coding. Nevertheless, increasing data reveal a relationship between variation in the number of repetitive sequences and differentiation and de-differentiation processes (Natali et al. 1986; Altamura et al. 1987; for a review see Nagl 1987, 1989, 1990). Nagl (1979, 1992) has proposed that quantitative gross changes in repetitive DNA sequences should vary the growth parameters of cells. Highly proliferative cells differ from cells of quiescent tissues in metabolism and are also histologically distinguishable by the volume and structure of the corresponding nuclei (Cavalier-Smith 1978). Proliferative cells have a specific cell program that can obviously be established rapidly during growth induction and, as a gross change, it is assumed to be expressed at the DNA level.

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In a previous paper (Arnholdt-Schmitt et al. 1991) we reported on the amplification of some low molecular *Bsp*NI fragments during the first hours after inoculation of secondary phloem explants of the carrot root (*Daucus carota* L.). These amplifications were reversible and were not characteristic for proliferative callus cells. Additionally, if highly proliferative calli were induced to a significantly higher growth rate by a short subculture of 36 h, amplifications comparable to those observed during the first hours after inoculation did not appear (Arnholdt-Schmitt 1993a). Therefore, it was concluded that the early amplified sequences should be related exclusively to the transformation of non-dividing tissue to callus initiation. In carrot callus tissue with a high cell-division activity the copy number of most of the repetitive *Bst*NI or *Bsp*NI fragments was found to be dramatically reduced. This reduction in the proportion of repetitive DNA in the genome was even more pronounced if the calli were subcultured, resulting in an increased growth rate. A loss of repetitive sequences during tissue culture was also indicated in a former investigation involving reassociation kinetics (Dührssen 1983). Slot-blot hybridization experiments confirmed the reduced content of repetitive DNA in the genome of callus tissue growing under the influence of kinetin with high cell-division activity in comparison to calli growing in the absence of kinetin (Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993b). Also, in earlier experiments with analogous carrot callus systems, biochemical analysis had indicated that the DNA content per cell in rapidly growing tissues decreased (Steward et al. 1964; Neumann 1972; Schäfer 1976).

In the present study systematic investigations on carrot primary cultures during a phase of high cell-division activity were performed to elucidate the relationship between growth rate and the loss of repetitive DNA sequences. The genomic DNA organisation of 37 different in vitro growth variants were compared in terms of the various genotypes (single plants and cultivars), the age of the plants used for tissue culture, and the temperature during culture. Additionally, the DNA of calli with high cell-division activity was compared to that of calli showing delayed growth at the 28th day of culture.

Materials and methods

Plant material and tissue culture

The plant material used for the investigations 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 Figs. 4 and 5). For tissue-culture experiments a primary culture system of the secondary phloem of the carrot roots was used. Explants (2–4 mg) of this tissue 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 and 28°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. The culture period and supplementation of the nutrient solution with kinetin (0.1 ppm), and additionally with m-inositol (50 ppm) and 3-indole-acetic-acid (IAA, 2 ppm), was varied according to the experiment.

During culture, callus formation is induced indicating a change of the differentiation state of the phloem tissue and defined growth stages can be obtained reproducibly within a rather limited space of time. After a lag-phase of 6–8 days exponential callus growth starts, due mainly 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 (Neumann et al. 1978)

For systematic investigations on the relationship between callus growth rates and quantitative changes in the genome at day 14 after inoculation, plants of the four carrot cultivars mentioned above were grown in the field (sowing date: 6. 5. 1991). Two plants of each cultivar were harvested at different dates (30. 8., 15. 10., 6. 11., 26. 11. and 19. 12. 91). The secondary phloem explants of the roots of the various plants were cultured at 21°C and 28°C in the presence of m-inositol, IAA and kinetin. Ten tubes containing approximately five explants were cultured simultaneously for each plant and temperature variation resulted in about 50 calli grown at 21°C or 28°C for each single plant genotype. For DNA analysis, variants were selected in terms of cultivar origin, plant age, temperature and callus growth, and a mixture of the callus material of each variant was taken and investigated in parallel experiments.

Cell number

The number of cells of the original explants and the calli were determined by the method of Neumann (1962, see also 1966). For maceration fresh tissue was frozen at –20°C in distilled water and incubated after thawing (about 1 ml/100 mg) in a mixture (1:1) of 0.1 N HCl and 10% v/v chromic acid for about 24 h.

DNA isolation

High-molecular-weight 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 enzyme *Bst*NI (Stratagene) or the isoschizomeric enzyme *Bsp*NI (Biozym diagnostic) using 3–4 U/µg DNA for at least 4 h. The endonuclease cleaves the recognition site 5′-CC(AT)GG independently of cytosine methylation. To assure that differences between the restriction analysis of the secondary phloem of the carrot roots and the cultured tissue had not been due to incomplete digestion by contaminations, DNA of the primary culture was purified and tested by different procedures including additional CsCl-density gradient centrifugation, supplementation of the extract with PVP and the addition of λ-DNA as an internal standard (data not shown, see Arnholdt-Schmitt 1993b).

Length determination of the repetitive restriction fragments was performed by the use of λ-*Hin*dIII fragments and the reciprocal method (local form) given by Southern (1979) and Elder and Southern (1983).

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 tech-

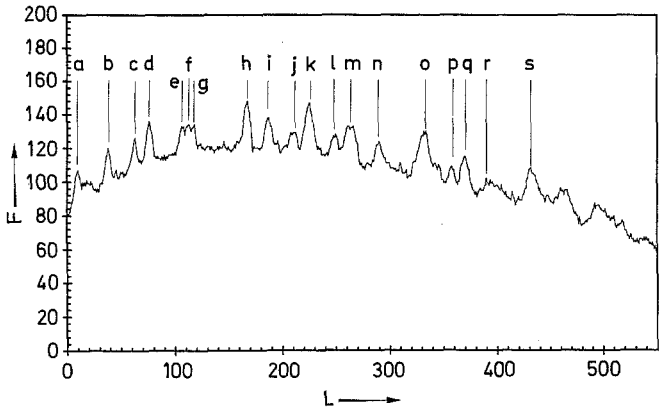

Fig. 1 The length of the sequences of the reproducible *Bst*NI fragment pattern occurring in the carrot genome. *F* relative fluorescence of the restriction fragments; *L* relative length of the electrophoretic run of the restriction fragments through the agarose gel given by the number of video-densitometric scan points. *a* ≥20 kbp, *b* 11–15 kbp, *c* 8.2 kbp, *d* 7.1 kbp, *e* 5.2 kbp, *f* 5.0 kbp, *g* 4.8 kbp, *h* 3.5 kbp, *i* 3.1 kbp, *j* 2.8 kbp, *k* 2.6 kbp, *l* 2.3 kbp, *m* 2.1 kbp, *n* 1.9 kbp, *o* 1.5 kbp, *p* 1.3 kbp, *q* 1.2 kbp, *r* 1.0 kbp, *s* 0.9 kbp

nique at 15°C). The gels were stained for 2 h in 2 µg/ml 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. To level out 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, which might possibly not have been scanned by mistake, will be recognized since deviations in a higher molecular region will be compensated for by reverse deviations in lower molecular regions and vice versa.

Results

*Bst*NI digestion of the carrot genome leads to an unusual and distinctive pattern of restriction fragments (Arnholdt-Schmitt et al. 1991; Arnholdt-Schmitt 1993a). Figure 1 shows the video-densitometric scan of the *Bst*NI restriction pattern defining at least 19 bands indicating characteristic repetitive DNA fragments of the carrot genome. Dührssen et al. (1980) estimated the content of repetitive DNA in the carrot genome by reassociation kinetics. These authors obtained about 92% reassociation including 46% repetitive DNA which belonged primarily to the moderately repeated DNA fraction displaying on average 390 copies (Dührssen 1983). Thirty-one single carrot plant genotypes originating from five different genetic backgrounds gave the restriction pattern shown in Fig. 1. An identical *Bst*NI or *Bsp*NI pattern of repetitive fragments was obtained for the different carrot cultivars (cv Rote Riesen, cv Rotin, cv Pariser Markt, cv Lobbericher and an unkown variety from the local market) and different tissues (petioles as well as the cambium and secondary phloem of carrot roots) investigated. The genome of only one single plant (cv Lobbericher) differed slightly by one additional and one missing band in the fragment pattern (data not shown).

Explants of the quiescent tissue of the secondary carrot root phloem from 26 single plant genotypes of five genetic origins and different plant ages were induced by primary culture to proliferate at high cell-division activity at 21°C and/or 28°C (a total of 37 in vitro growth variants) (see also Materials and methods). The growth potential of the different variants determined at day 14 after inoculation varied strongly. The average fresh weight obtained by the various variants displayed a minimum value of 14.2 mg/callus and a maximum of 161.3 mg/callus. Despite these great discrepancies in growth potential all of the 37 investigated in vitro growth variants displayed a similar dramatic reduction in the amount of repetitive fragments during high cell-division activity as could be seen by the reduced intensity of the repetitive *Bst*NI bands in comparison to the repetitive fragment pattern at t_0 (see e.g. in Figs. 2 and 3). Figure 2 demonstrates the restriction pattern of the genomic DNA of two in vitro variants which originated from two single plants of different ages of the same cultivar in comparison to the restriction pattern at t_0. Callus growth of the root explants, which is mainly due to cell-division activity ($r=0.813$), was significantly different ($P<0.001$) due to the two original plants. The explants of plant "J", which was harvested in December (see Materials and methods), achieved only 25.3 mg and 275.8×10^3 cells per callus after 2 weeks of culture in the presence of kinetin, whereas those of plant "A", which was harvested and inoculated late in august, achieved a six-fold higher fresh weight of 152.0 mg with 1763.2×10^3 cells per callus. Nevertheless, there were no striking dissimilarities in the restriction patterns concerning the intensity of the repetitive fragment bands between the primary cultures at t_{14d} (Fig. 2B, C). By contrast, although the difference between the average callus fresh weight of plant "J" and the original explants was also six-fold, the band intensity of the genome at t_0 (Fig. 2A) differed significantly in comparison to the genomes of the primary cultures, indicating a higher amount of repeated DNA fragments in the quiescent tissue. This result shows that the actual growth rate of the primary cultured secondary phloem of the carrot roots until day 14 obviously did not affect the overall degree of reduction in the number of repetitive fragments. The evaluation of 37 growth variants indicated that the elimination of the repetitive DNA fragments was found to be independent of the genotype (variety and single plants), as well as of the age, of the plants used for callus growth induction.

Figure 3 demonstrates that the loss of repetitive DNA sequences in the genome of rapidly dividing cells was insensitive to temperature. This was true independently of the interaction of the temperature of 21°C or 28°C with the different plant genotypes in respect of the cell-division rates during callus proliferation.

At the delayed growth phase of the primary cultures at day 28 the reverse process was observed in the genome compared to day 14. Figure 4 illustrates that the same re-

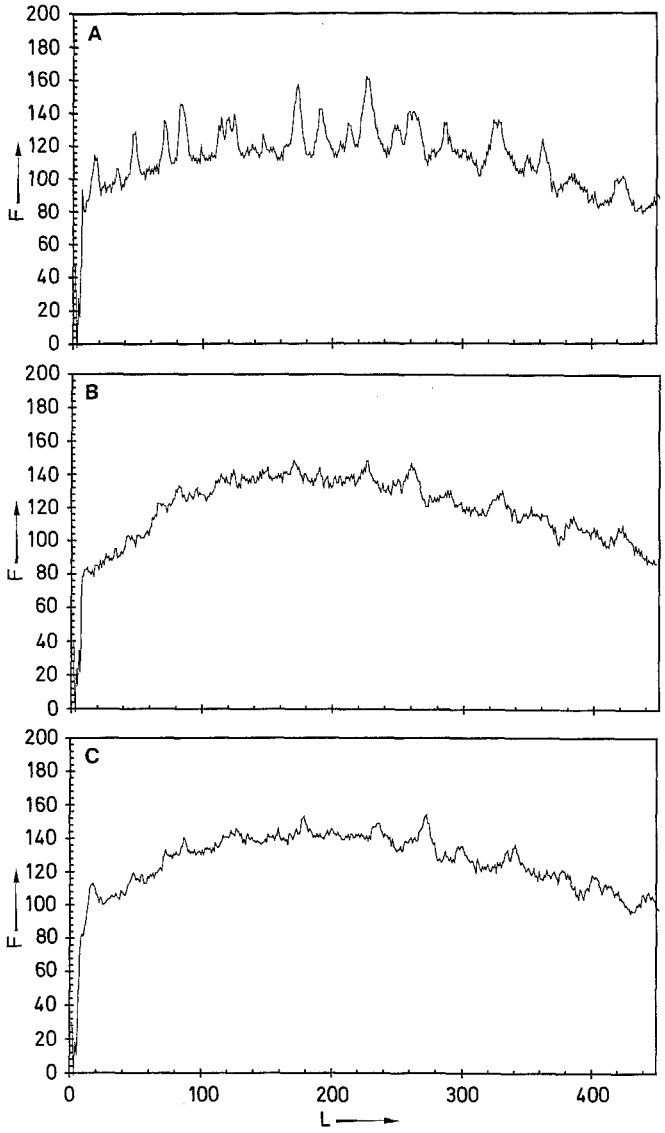

Fig. 2A–C Reduction in the amount of repetitive fragments in the carrot genome during primary culture in relation to callus growth rate. **A** *Bst*NI fragment pattern of the DNA of the secondary root phloem of plant "J" (cv Rote Riesen) at t_0. **B** *Bst*NI fragment pattern of the DNA of cultured secondary root phloem explants of plant "J" (cv Rote Riesen) at t_{14d} (grown at 21°C). **C** *Bst*NI fragment pattern of the DNA of cultured secondary root phloem explants of plant "A" (cv Rote Riesen) at t_{14d} (grown at 21°C). For fresh weights and cell numbers per explants/calli see text

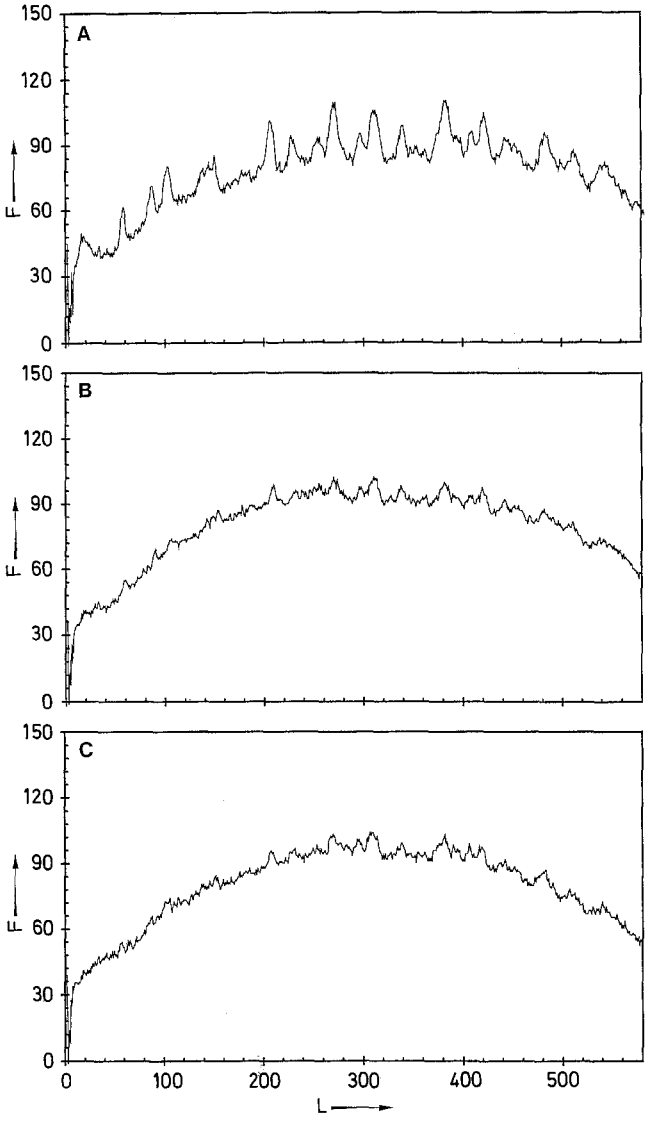

Fig. 3A–C Reduction in the amount of repetitive fragments in the carrot genome (cv Rote Riesen, plant "F") during primary culture at 21°C and 28°C. **A** *Bst*NI fragment pattern of the DNA of the secondary root phloem at t_0. **B** *Bst*NI fragment pattern of the DNA of secondary root phloem explants cultured 14 days at 21°C (51.1 mg/callus). **C** *Bst*NI fragment pattern of the DNA of secondary root phloem explants cultured 14 days at 28°C (79.3 mg/callus)

petitive fragments, which were reduced in their number at high cell-division activity at day 14, re-amplified at the transition to stationary growth at day 28. Parallel experiments with another two single plants (altogether nine evaluated in vitro variants) confirmed the reproducibility of amplifications at this stage of the primary culture. Again this quantitative change in genome organisation was not dependent on the absolute growth rate during callus culture. Even a subculture at day 14, which resulted in a strong increase in the fresh weight of callus material at day 28, did not influence the amplification events. Additionally, re-amplification at day 28 occurred also in the absence of kinetin during subculture (data not shown). As can be seen in Fig. 5, two fragments of 4.0 kbp and 3.7 kbp had been amplified at the transition to stationary growth to a greater extent than they had been found in the original tissue of the plants. These additionally amplified fragments are also evident in Fig. 4 (L: 185, 196), when compared with the restriction fragment patterns typically obtained at t_0 (see e.g. in Figs. 1, 2, 3 and 5).

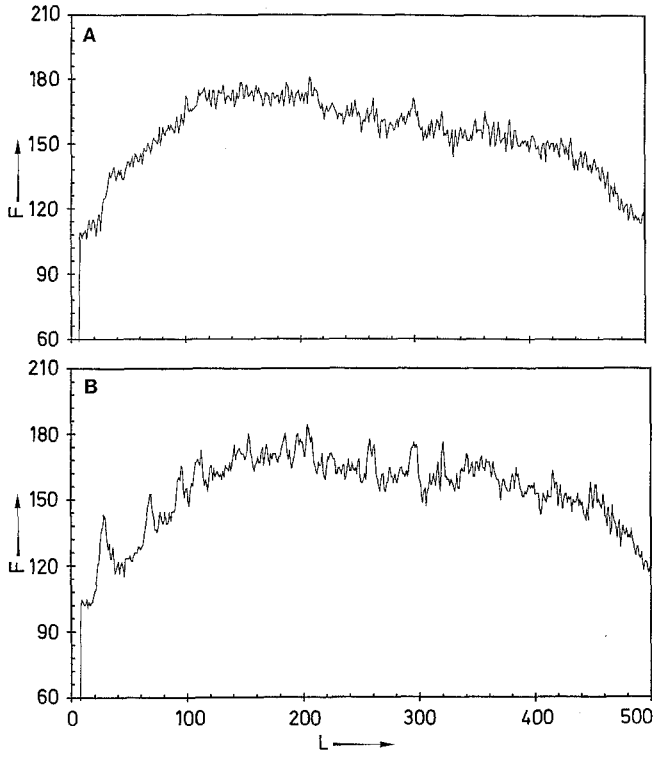

Fig. 4A, B Amplification of repetitive fragments at the transition to stationary growth. **A** *Bst*NI fragment pattern of the DNA of secondary root phloem explants cultured in the presence of m-inositol, IAA and kinetin at day 14. **B** *Bst*NI fragment pattern of the DNA of secondary root phloem explants cultured in the presence of m-inositol, IAA and kinetin at day 28 (*a–m*: see in Fig. 1)

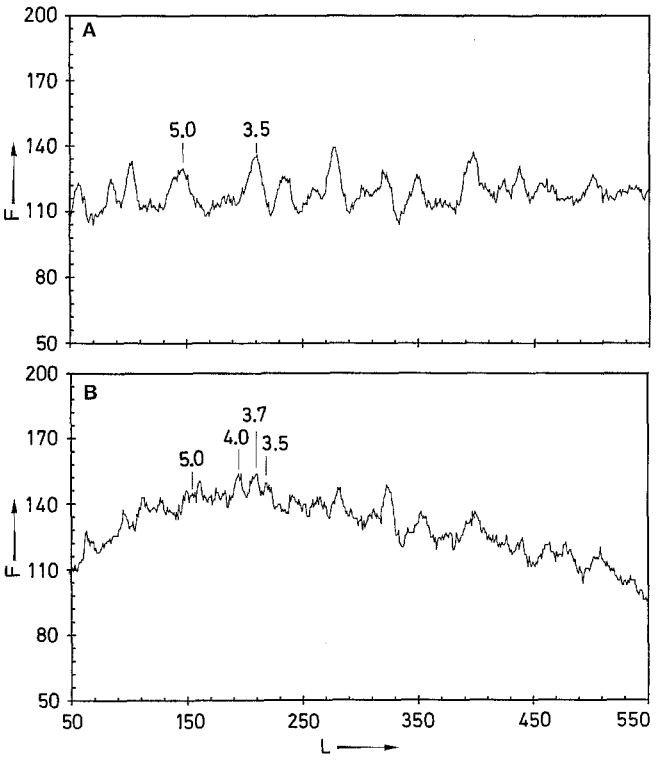

Fig. 5A, B Newly amplified sequences at the transition to stationary growth. **A** *Bst*NI fragment pattern of the DNA of the secondary root phloem at t_0. **B** *Bst*NI fragment pattern of the DNA of cultured secondary root phloem explants subcultured at day 14 until day 28 in the absence of kinetin; fragment length is given in kbp

Discussion

The results presented show that the induction of the quiescent secondary root phloem of carrots to primary callus growth was accompanied by non-random epigenetic changes expressed at the total genome level. In 100% of the investigated in vitro growth variants the identified repetitive *Bst*NI or *Bsp*NI restriction fragments were found to be strongly reduced in their copy number at the phase of high cell-division activity. At a later stage, during transition to stationary growth, this loss of repetitive DNA fragments tended to be reversed. The same repetitive fragments were now amplified at this stage and, additionally, two repeated fragments seemed to be amplified to an even higher degree than they had been in the original tissue. The sites of differential replication leading to genome variability seem to be genetically determined. The reproducibility of these results indicate a physiological significance of the observed differential replications related to the growth phases of the primary culture system.

The extent of reduction in copy number does not seem to be related to the growth rates of the calli. Therefore, the loss of sequences could not have been the result of cell-division cycles, and passive mechanisms such as under replication (Bassi et al. 1984; Nagl 1990) or unequal recombination (Harley et al. 1982) could be excluded. Rather the results indicate active elimination of the repetitive sequences. As was shown in a previous paper (Arnholdt-Schmitt 1993a) a subculture of only 36 h during the phase of high cell-division activity was enough to initiate an additional significant loss of repetitive fragments. Disintegration of repetitive sequences, which resulted in extrachromosomal DNA fractions, and a subsequent loss during mitosis, has been reported in the literature (Nuti Ronchi et al. 1973; Mellerowicz et al. 1992). On the other hand a degradation by deoxyribonuclease activity is also possible. Matousek et al. (1987) found a rapid and reversible increase of deoxyribonuclease activity during exponential growth in callus cultures of *Nicotiana tabacum*. Jenns and Bryant (1978) investigated the activity of a deoxyribonuclease which was bound to chromatin. The authors reported a dramatic increase in the activity of the nuclease directly before DNA replication starts. In non-replicating cells deoxyribonuclease seems to be inhibited by an enzyme-inhibitor-complex (see also Szopa and Wagner 1980; Rudnicki et al. 1988). The investigations by Rudnicki et al. (1988) demonstrated a reduced degree of the inhibitor-complex in cells displaying high cell-division activity. In the present study the elimination of repetitive sequences seems to occur non-specifically in the sense that most of the repetitive fragments that could be recognized as dis-

tinct *Bst*NI or *Bsp*NI fragments by total genome analysis seemed to be involved. Non-specific activity, which leads to gross epigenetic changes at the genome level, is also assumed for methyltransferase (Adams 1990). In both cases, specificity might be achieved by local inhibition of enzyme activity by binding factors or by structural disposition. Previous experiments also indicated differences in the stability of the repetitive fragments (Arnholdt-Schmitt 1993a).

What significance and what implications can be attributed to the elimination of such a quantity of repeated DNA? Do these observations either confirm or disprove the thesis of "junk DNA" (Doolittle and Sapienza 1980; Orgel and Crick 1980) in eukaryotic genomes? The investigations presented included several primary cultures with strongly different potentials for callus growth. Since the extent of the loss of repetitive fragments was not correlated with cell-division rates, the deduced active elimination of repeated sequences could not have been the rate-determining step for cell-division growth, but rather might have been rate limiting. This suggestion is supported by the fact that high cell-division activity obviously could not be achieved without the removal of a substantial portion of the repetitive sequences from the genome, and secondly by the fact that further induction to increased growth was again accompanied by a further elimination of repetitive sequences (Arnholdt-Schmitt 1993a). Additionally, transition to stationary growth was accompanied, reversibly, by the amplification of repetitive sequences. It is assumed that the elimination of repeated DNA fractions during primary culture was responsible for the cells of the originally quiescent tissue being able to divide with a high cell-division activity. This determination was obviously part of the growth induction procedure.

Gross elimination of repetitive sequences from the genome should lead to a lower DNA content per nucleus and should imply striking changes in genome organisation. Early biochemical experiments with an analogous carrot callus system had already indicated a reduction in the DNA content of about 50% in callus cells with a high cell-division activity (Steward et al. 1964) but with stable diploid chromosome numbers (Mitra et al. 1960). Recent progress in structural DNA research has revealed that the eukaryotic chromatin is organized in topologically independent loops obviously representing replicon units (for reviews see Bonifer et al. 1991; Van Driel et al. 1991; Nagl 1992). The ends of the loops are attached to the nuclear framework at the so-called matrix attachment regions (MARs) or scaffold attachment regions (SARs), which are suggested to be identical to the origins of replication (Opstelten et al. 1989). Additionally, DNA loops seem to be strongly involved in the structural regulation of gene expression (Stief et al. 1989; Bonifer et al. 1990; Hall et al. 1991; Ludérus et al. 1992; Allen et al. 1993). Elimination of a large portion of middle-repetitive sequences, which are usually distributed throughout the genome (e.g. Kuhrová et al. 1991; Rogowsky et al. 1991; Liu et al. 1992; Solano et al. 1992; Leeton and Smyth 1993), must necessarily result in the shortening of the DNA loops of the genome with consequent effects on the position of remaining sequences. There are, in fact, some investigations in the literature pointing to changes in genome organisation in actively dividing tissues. Fujimura and Komamine (1982) studied the initiation of somatic embryogenesis in carrot cell suspensions and reported that the average replicon size was reduced to about one third at the preglobular stage. Also, Jacqmard and Houssa (1988) studied the replicon size in the apical meristems of white mustard during the morphogenetic transition from the vegetative to the flower-forming condition. At this stage, showing a dramatic increase in the proportion of replicating cells, the replicon size rapidly decreased to 50%. Houssa et al. (1990) achieved the same effect on replicon size by stimulating cell-division activity in the meristematic apical meristem of white mustard by cytokinin treatment. These authors did not analyse the underlying mechanism but suggested an activation of additional replicon origins. Considering the present results from the carrot callus system the elimination of repetitive sequences could also be responsible for a shortening of replicon sizes.

In addition to the replication fork rate and the synchrony in replicon activation, the size of replicons is critical to the duration of the S-phase of the cell cycle, influencing the number of replication origins per unit length of DNA (Francis et al. 1985; Van't Hof 1985; Waterborg and Shall 1985). Since repetitive sequences were found to be involved in the condensation of chromatin, and so obviously contribute to early and late replication (Blumenthal et al. 1974; Flavell 1980; Waterborg and Shall 1985; Olszewska et al. 1990), it would be of interest to further clarify, whether the observed elimination of repetitive sequences during high cell-division activity resulted in the structural activation of replication origins, leading to a higher degree of synchrony in DNA replication during the S-phase. Houssa et al. (1990) reported a synchronization of the activation of replicon origins in white mustard meristems in response to cytokinin treatment. An increase of a dispersed fraction of chromatin in relation to the more condensed fraction was reported by Havelange and Jeanny (1984) in evoked meristems of white mustard.

In conclusion, our results demonstrate a non-random, physiological, quantitative variability of DNA sequences in the carrot genome during the cell-division growth of primary cultures. It became obvious, that differential replication, together with changes of the DNA methylation pattern (Arnholdt-Schmitt et al. 1995), is part of the normal cell physiology of dividing cells in tissue culture. Nevertheless, it is assumed that this phase of genome instability during culture may preferentially provide an opportunity for stress-induced heritable changes in genome organisation. Further studies will be necessary to elucidate the interference of physiological genome variability and stress inductors or conditions in tissue culture.

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