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Heterochromatin and repetitive DNA frequency variation in regenerated plants of *Helianthus annuus* L.

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Abstract Plant regeneration from cotyledons of seeds of a single progeny of a pure line of *Helianthus annuus* was studied in respect of the nuclear DNA contents of control and regenerated plants. Control plants were divided into two groups: those developed from seeds at the periphery of the inflorescence (showing a high basic 4C DNA content) and those from seeds developed in the middle of the inflorescence (showing a low basic 4C DNA content). It was observed that plants from peripheral seeds have a higher morphogenetic potential than those from central seeds. Cytophotometric analyses indicated that plants regenerated from cotyledons of both peripheral and central seeds show the same basic 4C DNA amount, which is higher that that observed in vivo in peripheral seeds. Molecular analysis by slot blotting and hybridization with different DNA families showed that the difference in nuclear DNA content between plants from peripheral and central seeds in vivo are mainly related to differences in the frequency of highly repeated, "slow" medium repeated (MR2), and ribosomal DNA families; by contrast, the increase in DNA amount in regenerated plants is mainly due to "fast" medium repeated sequences (MR1). Moreover, the frequency of kinetically isolated "unique" sequences was higher in peripheral seeds than in central ones and still higher in regenerated plants. Optical-density measurements of interphase nuclei showed an increase of heterochromatin in regenerated plants, suggesting that, whatever DNA is amplified in these plants, it remains condensed and probably inactive.

Key words *Helianthus annuus* · Heterochromatin · Nuclear DNA content · Plant regeneration · Repetitive DNA

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Introduction

From the first reports on the plasticity of the flax genome (giving rise to so-called "genotrophs" Evans et al. 1966), wide intraspecific variation in nuclear DNA content has been described in numerous other species (Price 1988; Arumuganathan and Earle 1991; Cavallini and Natali 1991).

Within *Helianthus annuus*, genome-size variation has been reported by several authors (Nagl and Capesius 1976; Olszewska and Osiecka 1983; Cavallini et al. 1986; Michaelson et al. 1991): DNA amounts, as determined by Feulgen cytophotometry of prophase nuclei of root and shoot apical meristems, differ widely (up to 58%) among cultivars and even within one and the same progeny of plants from a homozygous line (up to 25%) (Cavallini et al. 1986, 1989).

These variations are not due to changes in chromosome number or to alterations in chromosome structure; rather, they seem to be related to the differential replication of particular DNA families (mostly repetitive, Natali et al. 1993); this takes place during embryogenesis and occurs to a different extent according to the position of the seed on the capitulum: seeds developed at the periphery of the head have a greater genome size than seeds developed in its central part (Cavallini et al. 1989).

Intraspecific nuclear DNA content variations may affect plants by altering nucleotypic parameters, such as mitotic and meiotic cycle time, nuclear volume, and cell volume (Bennett 1972). In multicellular organisms, these parameters may cumulatively determine modifications in life-cycle duration and/or plant mass (Bennett 1987;

Cavallini et al. 1993a; Biradar et al. 1994). In the sunflower (Natali et al. 1993), cell proliferation is affected by DNA content: mitotic cycle time is longer in large genome-sized seedlings; moreover, genome size was positively correlated with the surface area of leaf epidermal cells. Possibly by additive effects, sunflower plants with larger DNA amounts flower later than plants with a low DNA amount (Natali et al. 1993). It was suggested that the variations of nuclear DNA content and organization observed in the sunflower may play a role in determining developmental variability in plant populations, and so buffer the effects of altered environmental conditions (Natali et al. 1993).

Nuclear DNA content variations have been often related to environmental stimuli and can be produced by stress conditions occurring during plant development (Walbot and Cullis 1985; Cullis 1990; Schneeberger and Cullis 1991). For this reason, a study of nuclear DNA content, at both cytophotometric and molecular levels, of sunflower plants regenerated through in vitro culture seemed to us of some interest. It is known, in fact, that in vitro culture may determine cryptic DNA alterations, such as the amplification, under-replication or under-representation of certain DNA sequences or families (Nagl 1990; Cecchini et al. 1992); these alterations in turn might produce some of the so-called somaclonal variants (Larkin and Scowcroft 1981; Brown and Lörz 1986; Karp 1991). In the present paper we report the results of analyses on sunflower plants with a different genome size and on their regenerants.

Materials and methods

Plant material

A single progeny of the sunflower "A" line, selfed for 10 years, was used in our experiments. Analyses were performed on single plantlets developed from seeds taken at the periphery or in the middle of the head (hereafter called PC and CC plantlets, respectively).

Cotyledons from seeds at the periphery or in the middle of the head were cultured in vitro according to Pugliesi et al. (1991). Mature achenes were sterilized for 2 min in 70% ethanol, then for 20 min in 3% commercial sodium hypochlorite plus 0.01% triton X-100 and finally washed in sterile distilled water. Forty-eight hours after germination in Petri dishes (moistened with distilled water and kept at 25 °C under a 16-h photoperiod), cotyledons were excised and cultured on solid Murashige and Skoog (1962) medium (MS) supplemented with 0.4 mg/l of indolacetic acid plus 4 mg/l of kinetin, pH 5.7. Plates were incubated at 25 °C under a 16-h photoperiod (light intensity 35 $\mu E\,m^{-2}\,s^{-1}$). After about 3 weeks, regenerated adventitious buds were excised, cultured on MS without growth regulators until they attained a length of 10–25 mm, and then subcultured on 1/3 strength MS, with a reduced sucrose concentration (1%) and without hormones, to induce rooting. After rooting, plantlets were transferred into pots with a sterilized mixture of vermiculite, peat and mud, covered with plastic bags and placed under a light intensity of $250\,\mu E\,m^{-2}\,s^{-1}$. After 15–20 days, the plastic bags were removed and plants were allowed to develop up to the physiological maturation of seeds.

Single regenerated plants were analyzed, keeping separated those regenerated from cotyledons of seeds developed at the periphery of the capitulum and those from seeds developed in the middle of the capitulum (hereafter called PR and CR plantlets).

Cytophotometric analyses

Leaflets collected from in vivo or regenerated seedlings, fixed in ethanol-acetic acid 3:1 (v/v), were treated with a 5% aqueous solution of pectinase (Sigma) for 20 min at 37 °C, squashed in a drop of 45% acetic acid and, after removing the cover slip, air dried. Squashes were then hydrolyzed in 1 - N HCl for 8 min at 60 °C, Feulgen stained (in 0.5% fuschsin) at room temperature for 1 h and washed three times in SO_2 water prior to dehydration and mounting. Preparations made to collect data which were to be directly compared were processed simultaneously. Feulgen/DNA absorptions in individual cell nuclei were measured at 550 nm with a Barr and Stroud GN5-type integrating microdensitometer.

Using a Leitz MPV3 integrating microdensitometer, the Feulgen/DNA absorption of chromatin fractions with differing condensation were determined on the same slides by measurements of the same 4C (G_2) interphase nucleus, after selecting different threshold of optical density in the instrument (Cavallini and Natali 1989). The instrument read all parts of the nucleus where optical density is greater than the preselected limit: at low thresholds all the nucleus (euchromatin plus heterochromatin) is measured, while only heterochromatin is read at high thresholds. Absolute values of the first derivative curve of the absorption at different thresholds allow one to discriminate among differently condensed chromatin fractions.

DNA extraction and fractionation

DNA was purified according to the method devised by Doyle and Doyle (1989) with modifications. Leaves from individual seedlings were ground in a mortar in CTAB isolation buffer [1.5% (w/v) CTAB (Sigma), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0] at 60 °C. Samples were incubated at 60 °C for 30 min with occasional gentle swirling and then extracted once with chloroform/isoamyl alcohol (24:1, v/v). After centrifugation (5000 rpm) at room temperature, nucleic acids were precipitated from the aqueous phase by adding 2/3 vol of cold isopropanol and then spooled with a glass hook, washed in 76% ethanol, 10 mM ammonium acetate for 1–2 h, allowed to dry briefly and resuspended in water.

For further purification, solid CsCl and ethidium bromide were added to the nucleic acid solution up to final concentrations of 0.8 mg/ml and 200 µg/ml, respectively. The solution was centrifuged at 44000 rpm in a Beckman L5-65 ultracentrifuge using the 65 Ti rotor, and the DNA band visualized under long-wave UV illumination was collected. Ethidium bromide was than removed by gentle inversion of the solution with n-butanol and dialyzed against water at 4 °C for 3 h. Finally, DNA was ethanol-precipitated and resuspended in the appropriate buffer.

For fractionating DNA at different Cot values, it was solubilized in 0.12 M Na-phosphate buffer, pH 7.0, and sheared by sonication in an MSE sonicator at medium energy output for 5 × 5 s with 10 s intervals at 4 °C. The DNA was then denatured for 10 min at 103 °C and allowed to reassociate according to Britten et al. (1974) up to the Cot values reported in Natali et al. (1993): $Cot < 0.22$ for highly repeated sequences (HR), $0.22 < Cot < 2.1$ for "fast" medium repeated sequences (MR1), $2.1 < Cot < 100$ for "slow" medium repeated sequences (MR2), and $Cot > 100$ for "unique" sequences. DNA was fractionated by elution through a hydroxylapatite column equilibrated in the same buffer as above: single-strand DNA was eluted with the same buffer and reassociated sequences were recovered by elution with 0.5 M phosphate buffer. By successive renaturations and elutions HR, MR1, MR2 and unique DNA families were isolated.

DNA blotting

For slot DNA blots, 1-µg DNA samples were denatured by heating at 37 °C for 10 min in 0.5 M NaOH and then neutralized by the addition of an equal volume of 2 M ammonium acetate. Scalar dilutions of DNA from 0.5 to 0.0625 µg were loaded onto nylon filters (Hybond-N; Amersham) using a commercial slot-blotting apparatus (Minifold II; Schleicher and Schuell). The DNA fractions obtained as described

above were used as probes. An *Eco*-RI $18s + 25s$ rDNA repeat of *Phaseolus coccineus*, cloned into lambda EMBL-4 arms and subcloned into lambda pUC13 vectors (Maggini et al. 1992), was also used. DNA probes were labelled with digoxygenin-11-dUTP by a DIG-DNA labelling kit (Boehringer).

Hybridization was performed according to Sambrook et al. (1989). Filters were washed sequentially in 2 × and 0.3 × SSC containing 0.05% SDS at 65 °C for the rDNA probe and at 60 °C for the HR, MR1 and MR2 probes, and in 2 × and 1 × SSC containing 0.05% SDS at 62 °C for the unique-sequence probe. Hybridization was detected by a DIG-DNA detection kit (Boehringer). Filters were then scanned in a Vernon PH1-type densitometer, and the tracings were used for quantitative determinations. As a control, all filters were rehybridized with *Pst*I maize alcohol-dehydrogenase cDNA cloned into pBR322 vectors (Dennis et al. 1984) and labelled with a ^{32}P-dCTP Random Primer kit (Amersham). Filters were then autoradiographed and scanned as above.

Statistical treatment

All experiments were performed on six plants of each group (PC, CC, PR, and CR) and were repeated three times. Optical-density measurements were performed on 20 interphase nuclei of six plantlets for each group.

Each mean value was normalized (CC = 100, in every experiment) to facilitate comparisons. Standard analysis of variance and the Scheffé test (1959) were applied.

Results

The frequency of plant regeneration from sunflower cotyledons is reported in Table 1. It is evident that cotyledons excised from seeds developed at the periphery of the capitulum showed a higher regeneration capability than those developed in the middle of the inflorescence.

Cytophotometric measurements of prophase nuclei from the marginal meristem of young leaflets of both in vivo and regenerated plants are reported in Table 2. A 10.63% significant difference in basic 4C DNA content was observed between plantets from peripheral and central seeds in vivo, which confirms previous results (Natali et al. 1993). A further increase in genome size is observed in regenerated plants compared to the controls (Table 2); moreover, it is worth noting that the basic 4C DNA content is the same in PR and CR plantlets, regardless of the position in the inflorescence of the seeds from which the cotyledon was explanted.

Molecular analyses were then performed to study the DNA families involved in the variations. The sunflower genome was divided into four fractions according to the DNA reassociation kinetics studies of Natali et al. (1993): HR, MR1, MR2 and unique sequence families. Moreover an *Eco*RI $18s + 25s$ rDNA repeat was used as a probe. In Fig. 1 the relative frequency of each fraction is reported in the four groups of plants and Table 3 gives an analysis of variance of the densitometric data. It is evident that, in vivo, HR, MR2, unique and rDNA sequences are represented more in PC than in CC genomes, while MR1 sequences are equally represented. Concerning HR and MR2 sequences, in regenerated plantlets a quantitative difference is maintained between PR and CR at the same extent as in vivo, i.e. between PC and CC plantlets. Ribosomal DNA sequences show slight increases of their frequences in both PR and CR plantlets, compared to PC and CC plantlets, respectively. MR1 and unique sequences show an increase of their frequency in regenerated plantlets: this increase is larger in CR than in PR. It is conceivable that the increases of MR1 and unique sequences are responsible for the higher increase of 4C basic DNA content in CR than in PR plantlets.

Finally, chromatin condensation of interphase 4C (G_2) nuclei was studied in the four groups of plants. Interphase nuclei were Feulgen-stained, measured at different thresholds of optical density, and the frequency of differently condensed chromatin fractions was calculated by first derivative curves of absorption: values at 1–4 thresholds refer to euchromatin content, while values at 5–9 thresholds indicate the content of differently condensed heterochromatin. Mean optical density profiles of the four groups of plants are reported in Fig. 2: significant differences are observed between regenerated and control plants concerning heterochomatin frequency; the same descending order, CR > PR > PC > CC is found for heterochromatin frequency and for basic DNA content (Table 2).

Table 1 Plant regeneration from cotyledons of the pure line of *H. annuus* used in these experiments. Cotyledons were excised from seeds which were distinguished on the basis of their position on the inflorescence (P = peripheral seeds, C = central seeds)

Seeds	No. of explants	No. of regenerating explants	% ± SE
P	235	113	48.09 ± 2.10
C	182	71	39.01 ± 2.22

Table 2 Feulgen absorption of early prophases (= 4C DNA; arbitrary units; mean ± SE) in six plants of *H. annuus* belonging to four groups (CC, PC, CR, PR, see text) and an analysis of variance. Values were normalized (CC = 100) to facilitate comparisons; means followed by the same letter are not significantly different at the 1% level with Scheffe's ranking test (*** $P \leqslant 0.001$, ** $P \leqslant 0.01$; * $P \leqslant 0.05$)

CC	PC	CR	PR
100.00 ± 0.53 A	110.63 ± 0.53 B	115.00 ± 0.22 C	114.57 ± 0.27 C

Source of variation	Degrees of freedom	SS	MS	F
Among groups	3	2633.05	877.68	737.55***
Among plants	5	12.16	2.43	2.04
Error	63	74.80	1.19	
Total	71	2720.01	38.31	

Discussion

Cytophotometric and molecular experiments confirm the existence of large quantitative DNA variations in

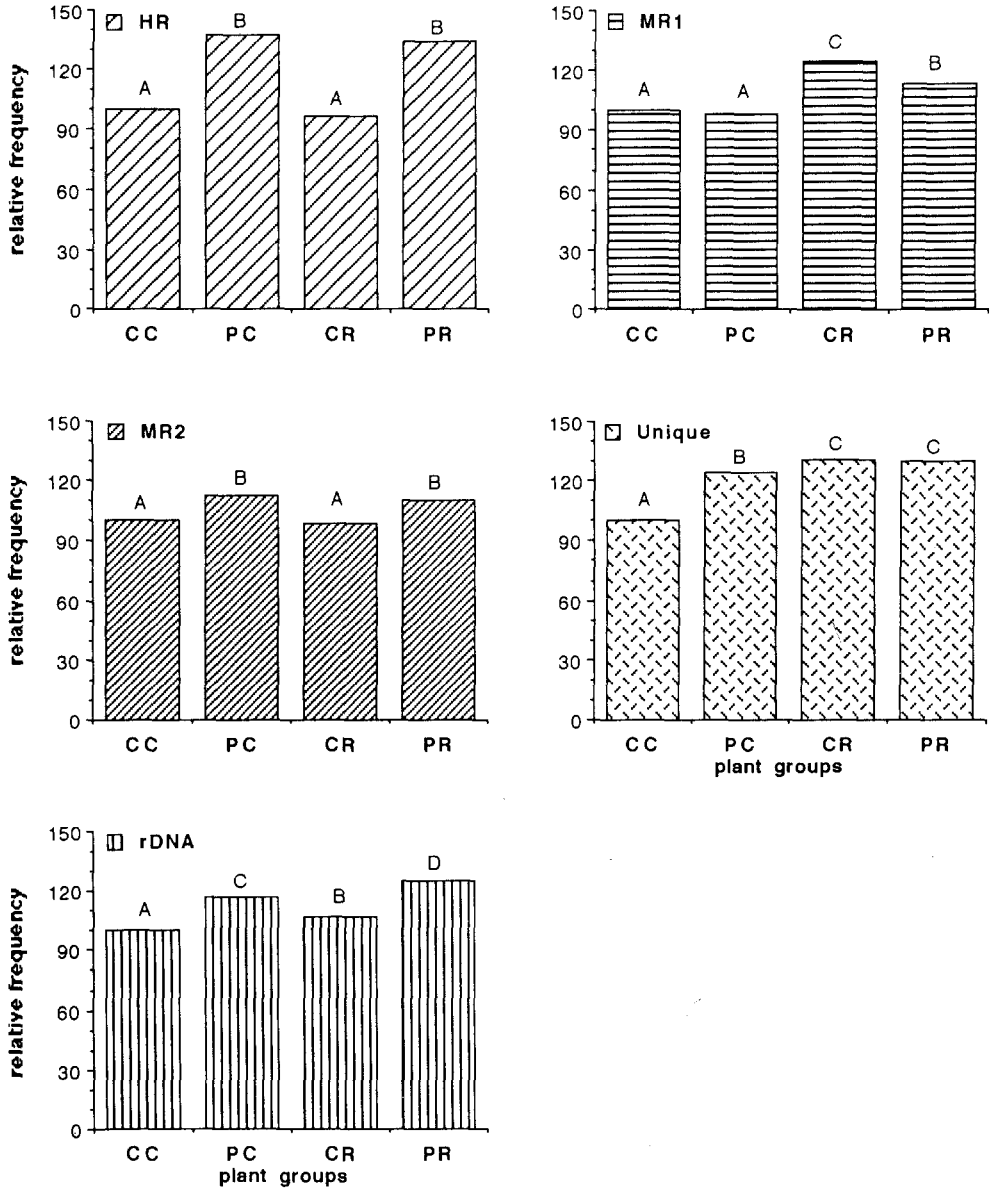

Fig. 1 Histograms representing the means of the values obtained by densitometrically scanning slot-blot filters loaded with DNA from single seedlings belonging to different groups of plants (*PC* plants from peripheral seeds; *CC* plants from central seeds; *PR* plants regenerated from cotyledons of peripheral seeds; *CR* plants regenerated from cotyledons of central seeds) and probed with different DNA fractions (see text). Values were normalized (CC = 100) to facilitate comparisons. *Letters* on each histogram indicate the significance of differences among plant groups: histograms with a common letter are not significantly different ($P \leqslant 0.01$, Scheffé method of multiple comparison)

Table 3 Analysis of variance for absorption values of slot blots probed with different DNA families. Values were obtained on six plants of *H. annuus* belonging to each of four groups: CC, PC, CR, PR (see text); experiments were repeated three times (*** $P \leqslant 0.001$; ** $P \leqslant 0.01$; * $P \leqslant 0.05$)

DNA family	Source of variation	Degrees of freedom	SS	MS	F
HR	Among groups	3	40205.84	13401.95	879.97***
	Among plants	5	289.42	57.88	3.80**
	Error	63	959.46	15.23	
	Total	71	41454.72	583.87	
MR1	Among groups	3	8581.11	2860.37	101.61***
	Among plants	5	413.14	82.63	2.94*
	Error	63	1773.62	28.15	
	Total	71	10767.87	151.66	
MR2	Among groups	3	10764.33	3588.11	168.69***
	Among plants	5	103.54	20.71	0.97
	Error	63	1339.80	21.27	
	Total	71	12207.67	171.94	
Unique	Among groups	3	13852.60	4617.53	220.72***
	Among plants	5	71.70	14.34	0.69
	Error	63	1318.20	20.92	
	Total	71	15242.50	214.68	
rDNA	Among groups	3	6906.99	2302.33	172.20***
	Among plants	5	3.15	0.63	0.05
	Error	63	842.23	13.37	
	Total	71	7752.37	109.19	

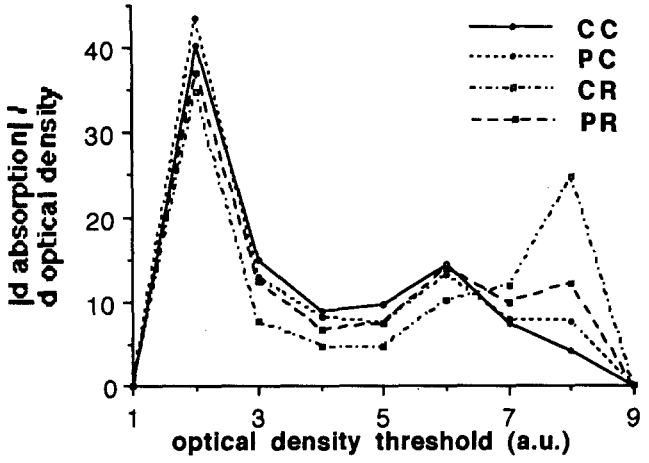

Fig. 2 First derivative curves (absolute values, mean) of optical density profiles of interphase 4C (G_2) nuclei of the four groups of sunflower plants as in Fig. 1. Standard errors not exceeding 1%

sunflower, even within the progeny of a presumably homozygous line (Natali et al. 1993). The variations occurring in vivo seem to affect the morphogenetic potential of the tissues: when cultured in vitro, cotyledons from peripheral seeds show a higher regeneration frequency than those from central seeds (Table 1). It is not possible to establish if the difference in morphogenetic potential is due to, or only correlated with, the difference in basic 4C DNA content between PC and CC plants. However, all regenerated plantlets, originating either from PC or CC cotyledons (i.e. PR and CR plants), show the same basic 4C DNA content, which is higher than that observed in vivo (Table 2).

The analysis of different DNA families by slot blotting (Fig. 1, Table 3) indicate that, in this sunflower line, HR, MR2, unique and rDNA sequences are involved in the variations in vivo; by contrast, only MR1 and unique sequences seem to vary consistently with plant regeneration. We are conscious that the slot-blot and hybridization techniques may be subject to some reservations, due to the difficulty in precisely quantifying the loaded and hybridized DNAs. However, since the experiments were repeated and the results analyzed statistically, this technique appears to provide good indications on the variations occurring for different genome fractions.

It is known that tissue culture can be responsible for producing a "genetic stress" that affects the differential replication of both repetitive and single-copy DNAs (Brown et al. 1991); in particular, quantitative variations have been reported in regenerated plants of tobacco (De Paepe et al. 1982; Dhillon et al. 1983), potato (Landsmann and Uhrig 1985), triticale (Brettell et al. 1986), rice (Brown and Lörz 1986; Kikuchi et al. 1987), flax (Cullis and Cleary 1986), *Scilla siberica* (Deumling and Clermont 1989), pea (Cecchini et al. 1992), maize (Brown et al. 1991; Peschke and Phillips 1992), *Aloe barbadensis* (Cavallini et al. 1993b), rye (Bebeli et al. 1993), alfalfa (Kidwell and Osborn 1993) and other species. In certain cases, it has been suggested that these variations can determine phenotypic changes in regenerated plants, possibly through alterations of nucleotypic parameters (see Introduction). Indeed, Price (1988) has claimed that even 5–10% differences in DNA amount may determine variations in nucleotypic expression between genetically homogeneous individuals.

The observed variations in unique sequences are of particular interest; analogous variations were reported for regenerated plants of *Scilla siberica* (Deumling and Clermont 1989) and pea (Cecchini et al. 1992) without, however, any apparent phenotypic changes. It may be hypothesized that the "unique" sequences involved in the variations are actually low sequence repeats, probably non coding, because, in the sunflower too, no particular phenotypic variation is observed in regenerated plants. These variable unique sequences might be pseudogenes or decaying relicts of former repeats, which often represent a consistent fraction of plant genomes (Smyth 1991).

In any event, the analysis of chromatin condensation (Fig. 3) indicated a higher heterochomatin content in regenerated sunflower plants than in the controls. It is conceivable that the amplified DNA is mostly maintained in a condensed and, hence, inactive state and affects phenotype merely through its mass, according to the nucleotypic hypothesis (Bennett 1972). In this sense, the increased "unique" sequences could also, at least in part, belong to heterochromatin.

Concerning the repetitive DNA sequences, it is worth noting that different fractions are involved in the in vivo and in vitro variations: while HR and MR2 change in vivo, only MR1 changes in vitro. A similar situation was described in the de-differentiation of the *Vicia faba* root (Natali et al. 1986): here, during root differentiation, a light (A-T rich) DNA satellite was under-replicated, while the same tissues showed amplification of a heavy (G-C rich) satellite when de-differentiation was induced.

In the sunflower, since the variations occurring in vivo involve different sequences from those varying in vitro, it may be hypothesized that in vitro culture stress activates different processes of differential DNA replication than those occurring during embryogenesis in vivo, when variations in intraspecific DNA amount are produced (Cavallini et al. 1989). Experiments are now in progress to determine if the DNA variations are transmitted to the progeny and also to characterize the sequences involved in these variations at the molecular, structural and functional levels.

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