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Nuclear DNA changes within *Helianthus annuus* L.: changes within single progenies and their relationships with plant development

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Summary. The variations in the basic nuclear DNA content, which previous results indicated to occur within one and the same progeny of *Helianthus annuus*, were studied in detail and correlated with certain developmental features of the plants. The size and organization of the genome of seedlings obtained from seeds (achenes) collected at the periphery (P-seedlings) or in the middle (M-seedlings) of the flowering heads of plants belonging to a line selfed for 10 years were compared. Cytophotometric determinations indicated that the nuclear DNA content of P-seedlings is 14.7% higher than that of M-seedlings. Thermal denaturation and reassociation kinetics of extracted DNAs showed that variations in the redundancy of repetitive DNA, in particular of a family of medium repeated sequences with a Cot range of 2–100, account for the differences in genome size. These findings were confirmed by the results of molecular hybridizations (slot blots), which also indicated a higher amount of ribosomal DNA in the P-seedlings than in the M-seedlings. Cell proliferation is affected by DNA content, and mitotic cycle time is 1h30′ longer in the P-seedlings. By studying mature plants, positive correlations were also found between genome size and both the surface area of leaf epidermal cells ($P \leq 0.01$) and flowering time ($P \leq 0.001$). It is suggested that the variations of nuclear DNA content and organization observed play a role in determining developmental variability in plant populations, which may be of importance in buffering the effects of changing environmental conditions.

Key words: *Helianthus annuus* – Intraspecific DNA changes – Cell proliferation – Cell phenotype – Plant development

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Introduction

It is now generally accepted that intraspecific changes in the basic amount of nuclear DNA do occur, since many reports to this effect can be found in the literature (reviewed, for plants, by Bennett 1985; Cullis 1990; Cavallini and Natali 1991).

Helianthus annuus is one of the plant species in which such changes have been found (Nagl and Capesius 1976; Olszewska and Osiecka 1983; Cavallini and Cionini 1986; Michaelson et al. 1991) and studied in some detail (Cavallini et al. 1986, 1989). Cytophotometric measurements, after Feulgen staining, in a number of cultivated varieties or lines have revealed large differences (up to 58.16%) in the basic amount of DNA. Changes in the number of chromosomes or alterations in chromosome structure do not account for these differences, which, instead, seem due to redundancy variations of repeated DNA sequences (Cavallini et al. 1986). Significant alterations in the amount of nuclear DNA, which are produced during early embryo development and then remain stable, cause the genome size to differ even within one and the same progeny of plants from a homozygous line. By means of a control mechanism that is based on the DNA content of the mother plant, a characteristic gradient is realized by which genome size increases from embryos developing at the centre of the head to those developing at its periphery. DNA fractions located in heterochromatic nuclear regions mainly account for these differences, which cover the whole range existing within the line (Cavallini et al. 1989).

Relationships between variations of nuclear DNA content and environmental parameters and/or plant phenotypic characters have been found in some cases. A well documented example is that of flax: in its plastic varieties, changes in the substrate, the genome size, and

the phenotype are all correlated (reviewed by Cullis 1990). However, the functional significance of intraspecific genome changes, remain at present a subject of debate. Likewise, the composition of the DNA fractions that vary has to be determined, although the observation of changes in heterochromatin, which was evident in several instances, is consistent with variations occurring in repetitive sequences, as was demonstrated by biochemical analyses in a few cases. Also the mechanisms by which such variations are produced and controlled have yet to be resolved (Cionini 1989; Cullis 1990; Cavallini and Natali 1991).

On the basis of the preceding, we planned further research in *H. annuus* in order to study, at the molecular level, the DNA changes occurring within single progenies of a line and to determine possible relationships between nuclear DNA amount and developmental features of plants. The results obtained are reported in this paper.

Materials and methods

Plant material

A *H. annuus* line selfed for 10 years was used. Plants were grown in the garden until they flowered, without applying nitrogen fertilizers, weedicides or pesticides; hand-weeding was performed when necessary. Organs were collected from these plants or from seedlings obtained by germinating achenes in damp vermiculite under sterile conditions at 25 °C.

DNA extraction and fractionation

Seedling leaves were homogenized in liquid nitrogen and lysed at 60 °C for 15 min in 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.15 N NaCl, 2% sodium dodecyl-sarcosinate, 100 mM diethyldithio-carbamic acid. After incubation at 37 °C for 3 h in proteinase K (Boehringer; final concentration 250 µg/ml), the mixture was centrifuged at 20,000 g for 15 min and then solid CsCl and ethidium bromide were added to the supernatant up to final concentrations of 0.8 g/ml and 300 µg/ml, respectively. The solution was centrifuged at 44,000 rpm in a Beckman L5-65 ultracentrifuge using the 50 Ti rotor and the DNA band, visualized under long-wave UV illumination, was collected and recentrifuged. Ethidium bromide was then removed by gentle inversion of the solution with n-butanol.

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, allowed to reassociate according to Britten et al. (1974) up to desired Cot values, and fractionated by elution through a hydroxylapatite column equilibrated in the same buffer as above. Single-strand DNA was eluted with this same buffer and reassociated sequences were recovered by elution with 0.5 M phosphate buffer.

Thermal denaturation and reassociation kinetics

Thermal denaturation was performed in 0.1 × SSC using a Perkin-Elmer 576 ST spectrophotometer equipped with a temperature programme controller, and the increase in hyperchromicity at 260 nm was continuously followed by an X-Y recorder.

For reassociation kinetics, DNA was sheared by sonication as described above. Sedimentation in neutral sucrose gradients according to Clewell and Helinski (1969) revealed that the fragments of different DNA samples were constantly of a relatively homogeneous length of about 400 bp. Sheared DNA, dissolved in 0.12 M Na phosphate buffer at a concentration of 50 µg/ml was denatured and allowed to reassociate (see above). The reassociation process was monitored in a closed, thermostatically-controlled cuvette using the same equipment employed for the analysis of the thermal denaturation kinetics. *E. coli* DNA (Sigma) was used as a standard after shearing in the same conditions as above; its analytical complexity was assumed to be 4.5×10^6 base pairs. Reassociation experimental data were plotted according to the method devised by Marsh and McCarthy (1974; Scatchard-type analysis).

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 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 on 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 pUC13 vectors (Maggini et al. 1992), was also used. DNA probes were labeled with digoxigenin-11-dUTP by a DIG-DNA labeling kit (Boehringer). Hybridization was performed according to Maniatis et al. (1982). Filters were washed sequentially in 2×, 1×, and 0.3 × SSC containing 0.05% SDS at 65 °C, and 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. All hybridization experiments were repeated at least six times. As a control, all filters were rehybridized with *Pst*I maize alcohol dehydrogenase cDNA cloned into pBR322 vectors (Dennis et al. 1984) and labeled with a ^{32}P-dCTP Random Primer kit (Amersham). Filters were then autoradiographed and scanned as above.

Determination of mitotic cycle time

Seedling roots were treated for 30 min at 25 °C with a 5 µCi/ml aqueous solution of ^{3}H-thymidine (The Radiochemical Centre; specific activity 6.7 Ci/mmol) and, after a thorough washing, the seedlings were recovered in water. Roots collected after 2–30 h of recovery were fixed in ethanol-acetic acid 3:1 (v/v) and Feulgen-stained after hydrolysis in N HCl at 60 °C for 8 min. Root tips were then squashed in a drop of 45% acetic acid. The cover-slips were removed by the solid CO_2 method and the preparations were covered with Ilford L_4 emulsion, developed after 3 days of exposure, dehydrated and mounted in DPX (BDH Chemicals). The percentage of labeled mitoses after different recovery durations was determined, and the duration of the mitotic cycle and its phases was calculated according to Quastler and Sherman (1959) and Wimber (1960).

DNA cytophotometry

Root or shoot apices collected from seedlings fixed in ethanol-acetic acid were treated with a 5% aqueous solution of pectinase (Sigma) for 20 min at 37 °C and squashed. The preparations were then Feulgen-stained as described above. After staining, the slides were subjected to three 10-min washes in SO_2 water prior to dehydration and mounting. Preparations made to collect data which were to be directly compared were processed

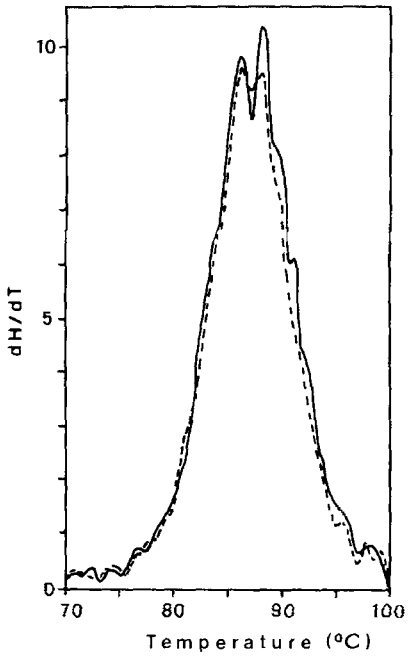

Fig. 1. First derivative curves of the melting profiles of the DNAs from seedlings obtained from seeds developed at the periphery (——) or in the middle (– – –) of the head. Graphs are obtained from the averaged values of four repetitions: two for each of two different DNA extractions

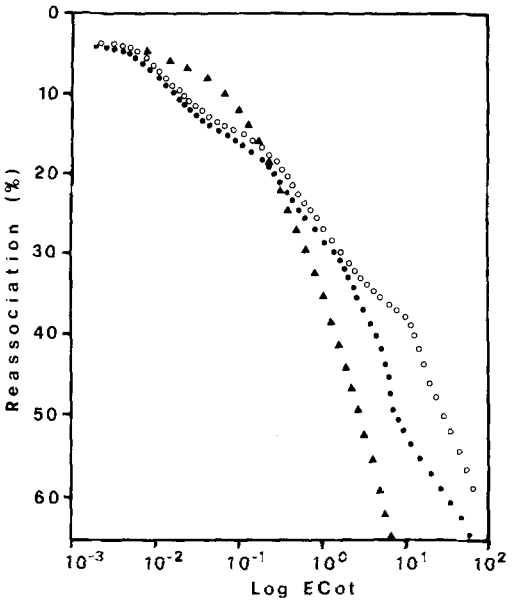

Fig. 2. Reassociation kinetics of the DNAs from seedlings obtained from seeds developed at the periphery (o) or in the middle (•) of the head. Each point is the mean of the values obtained in four repetitions, two for each of two different DNA extractions. *E. coli* DNA (▲) was used as a marker

simultaneously. Squashes made with root tips of *Vicia faba* (4C = 53.31 pg; Bennett and Smith 1976) were concurrently stained in order to convert relative Feulgen units into picograms of DNA. Feulgen/DNA absorptions in individual cell nuclei were measured at 550 nm using a Barr and Stroud GN5-type integrating microdensitometer.

Morphometry

Achenes collected from peripheral or central portions of the head were germinated in damp vermiculite and then transferred to the garden. Stem height was measured every 2 days during the first 2 weeks and then weekly.

Mature leaves were collected from plants with known DNA content and fixed in ethanol-acetic acid. Their upper epidermis was peeled off after treatment with a 5% aqueous solution of pectinase (Sigma) at 37 °C for 3 h, placed on gelatin-coated slides and stained with Delafield's haematoxylin (Merck). The mean surface area of cells was determined, after photocopying microphotographs on Wattman 3M paper, by cutting and weighing the shapes of 100 cells per plant.

Results

DNA analyses

Cytophotometric determinations of the Feulgen/DNA absorptions of early prophases in the root or shoot apices of seedlings obtained from seeds (achenes) collected at the periphery (P-seedlings) or in the middle (M-seedlings) of the flowering head were carried out. Five seedlings for each portion of the heads of five plants were analyzed. As expected from the results already obtained by studying other *H. annuus* lines (Cavallini et al. 1986), no significant difference was found either between root and shoot meristems of each seedling or within P- or M-seedlings. 4C nuclear DNA contents of 19.64 ± 0.27 pg and 17.12 ± 0.34 pg in P- and M-seedlings, respectively, were calculated from mean Feulgen/DNA absorptions of early prophases (14.7% difference with the latter value used as standard).

The DNAs extracted from P-seedlings (P-DNA) or M-seedlings (M-DNA) were characterized chemicophysically by means of thermal denaturation and reassociation kinetics. No significant difference occurred as far as the Tm values (87.2 ± 0.20 versus 86.8 ± 0.22), and hence the guanine+cytosine content, are concerned. However, differences are seen in the first derivative curves of the melting profiles, mainly on their heavy side (Fig. 1), suggesting that families of G+C-rich sequences are more represented in P-DNA than in M-DNA.

These indications are confirmed by the results of the reassociation kinetics. The reassociation curves of both DNAs (Fig. 2) reveal three repetitive fractions. One of them, which reassociates within an equivalent Cot of about 2×10^{-1}, is considered by us to consist of highly repeated sequences (HR); two other fractions, having Cot ranges of about $2 \times 10^{-1} - 2$ and 2–100, respective-

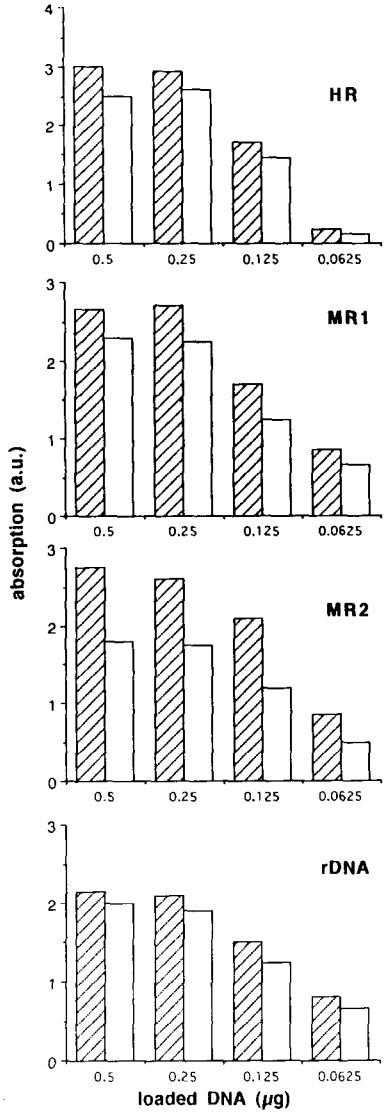

Fig. 3. Densitometric absorptions of slot-blot filters loaded with 0.5 μg, 0.25 μg, 0.125 μg or 0.0625 μg of DNA from seedlings obtained from seeds developed at the periphery (▨) or in the middle (□) of the head. Filters were probed with ribosomal DNA, the highly repetitive DNA fraction (HR), or two families of medium repeated sequences (MR1 and MR2)

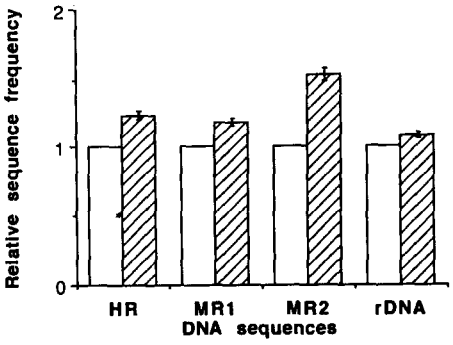

Fig. 4. Histogram representing the means of the values obtained by scanning densitometrically slot-blot filters loaded with DNA from seedlings obtained from seeds developed in the middle (□) or at the periphery (▨) of the head and probed at saturation in six repetitions (three for each of two different DNA extractions) with different DNA fractions as in Fig. 3. The values were normalized (□ = 1) to facilitate comparisons. Confidence limits at $P \leq 0.01$

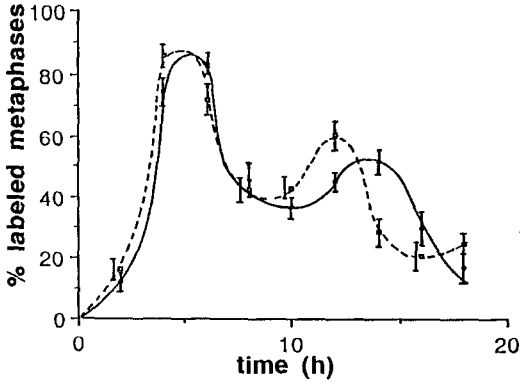

Fig. 5. Percentages of metaphases labeled after a 30-min feeding with ^{3}H-thymidine and different hours of recovery in water. Each point is the mean of the values obtained by analyzing the root meristems of five plantlets obtained from seeds developed at the periphery (●——●) or in the middle (□– – –□) of the head. Confidence limits at $P \leq 0.01$

ly, are considered to represent medium repeated sequences (MR1 and MR2). Scatchard-type analysis of the reassociation experimental data (Table 1) indicates that repeated sequences are more redundant in P-DNA than in M-DNA. In particular, the redundancy of MR2 sequences in the former DNA is more than twice that in the latter (5.26×10^2 versus 2.46×10^2 copies per 1C).

That repetitive DNA is more represented in P-DNA than in M-DNA was confirmed by the results of slot-blot analyses (Fig. 3). These also indicate that ribosomal DNA sequences are amplified in P-DNA as compared to M-DNA. Indeed, both HR, MR1 and MR2, and rDNA sequences hybridize to a larger extent in the former than in the latter. That these differences are significant can be seen from Fig. 4, which provides a comparison of the mean values at saturation obtained in the two DNAs in six repetitions of slot blots carried out using the four probes.

Cytology

Figure 5 gives the results obtained by feeding P- and M-seedlings with ^{3}H-thymidine and then scoring labeled metaphases in the root meristems after different dura-

Table 1. Scatchard-type analysis of the reassociation curves (Fig. 2) of the DNAs from seedlings obtained from seeds developed at the periphery (P) or in the middle (M) of the head

DNA sequences	DNA sample	Cot range	Cot 1/2 pure (moles × sec/l)	Reassociation constant (l/moles × sec)	Kinetic complexity (base pairs)	No. of copies per 1C-genome[a]	Δ bp
Highly repeated	P	<0.22	3.22×10^{-3}	43.5	3.67×10^{3}	1.72×10^{5}	1.38×10^{8}
	M	<0.23	3.53×10^{-3}	35.7	4.02×10^{3}	1.23×10^{5}	
Fast medium repeated (MR1)	P	0.22–2.1	9.65×10^{-2}	1.47	1.10×10^{5}	5.81×10^{3}	0.50×10^{8}
	M	0.23–2.4	1.20×10^{-1}	1.25	1.37×10^{5}	4.30×10^{3}	
Intermediate medium repeated (MR2)	P	2.1–100	2.64×10^{0}	0.133	3.01×10^{6}	5.26×10^{2}	8.60×10^{8}
	M	2.4–100	2.58×10^{0}	0.714	2.94×10^{6}	2.46×10^{2}	

[a] Calculated on the basis of 1C values of 4.91 pg (4.50×10^{9} bp) and 4.28 pg (3.93×10^{9} bp) for P- and M-seedlings, respectively

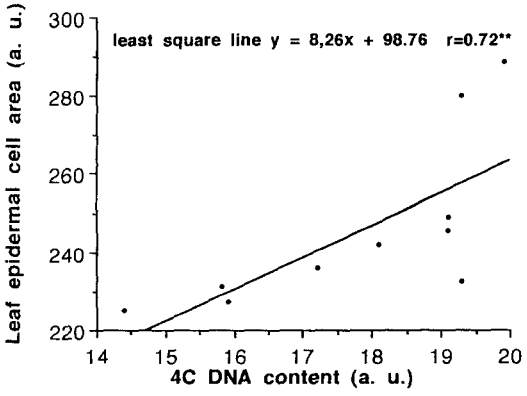

Fig. 6. Relationship between the basic nuclear DNA content and the surface area of leaf epidermal cells in plants obtained from seeds collected from one and the same head

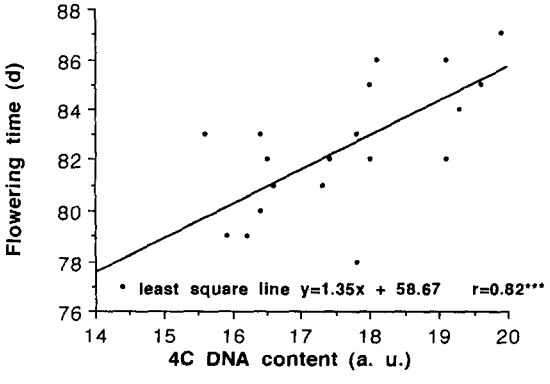

Fig. 7. Relationship between the basic nuclear DNA content and the flowering time in plants obtained from seeds collected from one and the same head

tions of recovery in water. Using these results and others obtained by analyzing the same preparations, the durations of the mitotic cycle and its phases in the meristems of the two groups of seedlings were calculated according to Quastler and Sherman (1959) and Wimber (1960). It can be seen from Table 2 that the mitotic cycle time is longer in P-seedlings than in M-seedlings. The mitotic indices in the root meristems are also reported in Table 2. No significant difference occurs between P- and M-seedlings; the same result was obtained when analyzing shoot meristems (data not shown). This indicates that, while cell proliferation is faster in P- than in M-seedlings, no clear-cut difference exists in the proportion of cells entering mitosis in the meristems of the two groups of plants.

Table 2. Mitotic index and duration of the mitotic cycle and its phases in the root meristems of seedlings obtained from seeds developed at the periphery or in the middle of the head

Head portion	Mitotic index ±SE	Mitotic cycle time (h)				
		Total length	G_1	S	G_2	Mitosis
Peripheral	4.84±0.25	8.75	1.52	3.83	2.80	0.60
Central	4.13±0.79	7.25	0.96	3.25	2.45	0.59

Plant development

From Fig. 6, it can be seen that a significant ($P\leq0.01$) positive correlation exists in mature plants between their basic nuclear DNA content and the surface area of leaf epidermal cells. Thus, in plants with lower DNA content cells proliferate faster but, when differentiating, remain smaller than in plants with higher genome size and vice versa. Possibly due to this combination of events, no difference exists between P- and M-plants either in their growth rate or their final height (data not shown).

In contrast, a highly significant ($P\leq0.001$) correlation was observed between genome size and flowering time, the larger the former the longer the latter (Fig. 7).

Discussion

Confirming other findings already obtained (Cavallini et al. 1986, 1989), our results point to the presence in the

nuclear DNA of *H. annuus* of unstable sequences, which, by rapidly altering their redundancy, give rise to genotypic variability even within the progeny of single plants from an inbred line. Our results also show that these genome changes are able to produce phenotypic variability by affecting some aspects of plant development.

It appears from our present data that the DNA sequences which mainly vary within a line belong to a medium repetitive genome fraction and comprise ribosomal RNA cistrons (Figs. 2, 3 and 4; Table 1). The results of a previous investigation indicate, by contrast, that DNA changes between different sunflower lines are restricted to highly repeated sequences, since significant redundancy variations concerning medium repeated sequences were not observed (Cavallini et al. 1986). That sequences undergoing quantitative variation are not the same in the two instances might suggest that the mechanism(s), and possibly the functional significance(s), which underlies the alterations in genome size and organization between lines might not be the same as those underlying the alterations within the progeny within a line.

The latter cannot be explained in terms of preexisting genetic variability carried by the parental genomes. The most conceivable hypothesis is that DNA changes are caused by micro-environmental variations, possibly due to differences in the availability of water and nutrients in different portions of the head and/or to differences in the times at which reproductive events take place, since they occur within the head in centripetal succession. Thus, our findings support the view that environment can have a direct effect on the generation of quantitative changes in nuclear DNA fractions (Cullis 1990).

An open question (Cionini 1989) is whether DNA changes of this kind represent programmed responses to alterations in the environment or whether the latter simply allow, or inhibit, the synthesis of DNA sequences to be regarded as selfish (Doolittle and Sapienza 1980; Orgel and Crick 1980). In *H. annuus*, at least, the degree of repetitivity of the sequences that mainly vary, and the involvement of ribosomal DNA in the variations, seems to render the latter view less plausible.

An intriguing point concerns the role(s) of the nuclear DNA changes we have observed. It was previously suggested that they might play a part in buffering the effects of variations in the microenvironment in which each embryo develops, so as to avoid or to limit their influence on its growth (Cavallini et al. 1989). The correlations between nuclear DNA content and plant developmental features which we have found (Figs. 5, 6 and 7; Table 2) may suggest another possible significance. DNA changes within progenies might, by generating continuous genotypic variability, have the function of allowing different developmental modalities and reproduction times to occur within plant populations. This may be of importance in buffering the effects of changing environmental conditions.

Changes in sequence redundancy might, in part at least, affect development simply by producing alterations in the nucleotype, i.e., in the DNA mass and volume (Bennett 1972). That genome size per se can influence many cellular and organismal phenotypic characters has been suggested after study in many angiosperms, although conflicting results were obtained, probably due to the genetic diversity occurring between species (reviewed by Bennett 1987). The differences in DNA content between P- and M-plants range around 14.7%, and it has been suggested that 5–10% differences may be sufficient to determine changes in nucleotype expression (Price 1988). In fact, effects on such parameters as mitotic cycle time, cell size, or generation time, are all nucleotypic and so may be produced by different kinds of variation in the nuclear DNA, such as the addition of B chromosomes (cf., Jones and Rees 1982). In the very few instances in which a search for correlations between intraspecific changes in the basic nuclear DNA content and cell phenotypic characters has been carried out, relationships similar to those reported here have also been found (Karp et al. 1982; Cavallini et al. 1992).

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