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Nuclear DNA changes within *Helianthus annuus* L.: variations in the amount and methylation of repetitive DNA within homozygous progenies

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Abstract Complex alterations in the redundancy and methylation of repeated DNA sequences were shown to differentiate the nuclear genome of individuals belonging to single progenies of homozygous plants of the sunflower. DNA was extracted from seedlings obtained from seeds collected at the periphery of flowering heads (P DNA) or from seedlings obtained from seeds collected in their middle (M DNA). Three fractions of repeated sequences were isolated from genomic DNA: a highly repetitive fraction (HR), which reassociates within an equivalent Cot of about 2×10^{-1}, and two medium repetitive fractions (MR1 and MR2) having Cot ranges of about 2×10^{-1}–2 and 2–10^2, respectively. Denaturation kinetics allowed different sequence families to be recognized within each fraction of repetitive DNA, and showed significant differences in sequence redundancy to occur between P and M DNA, particularly as far as the MR2 fraction is concerned. Most DNA sequence families are more represented in P DNA than in M DNA. However, the redundancy of certain sequences is greater in the latter than in the former. Each repetitive DNA fraction was hybridized to Southern blots of genomic P or M DNA which was digested to completion by three pairs of isoschizomeric restriction endonucleases which are either insensitive or sensitive to the methylation of a cytosine in the recognition site. The results obtained showed that the repetitive DNA of *H. annuus* is highly methylated. Clear-cut differences in the degree of methylation of P and M DNA were found, and these differences were particularly apparent in the MR2 fraction. It is suggested that alterations in the redundancy of given DNA sequences and changes in their methylation patterns are complementary ways to produce continuous genotypic variability within the species which can be exploited in environmental adaptation.

Key words *Helianthus annuus* · Intraspecific DNA changes · Repetitive DNA · DNA methylation

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Introduction

Evidence is accumulating in the literature that intraspecific changes in genome size due to quantitative variations in certain nuclear DNA fractions can occur, particularly in plants. Therefore, the view that fluid domains may exist in plant genomes, in addition to more stable portions, is gaining ground within the scientific community.

Redundancy variations of certain DNA sequences, due to their differential replication or loss from the nucleus, may accompany developmental processes. Therefore, it has been suggested that these variations play a regulatory role in development (Cionini 1989; Bassi 1990; Nagl 1990; Frediani et al. 1994). Alterations in the basic amount of nuclear DNA have also been found to differentiate populations and/or individuals within species. Intraspecific changes in genome size may follow environmental clines, or may arise in response to stress (Bennett 1985, 1987; Cullis 1990; Cavallini and Natali 1991; Ceccarelli et al. 1992, 1995). Moreover, recent results have shown that variations of the basic DNA amount are able to affect certain phenotypic characters of the cell. Consequently, developmental modalities and the phenotype of the whole organism may be altered. On the basis of this knowledge, quantitative changes in the nuclear genome have been claimed to play a role in species evolution by allowing plants to bring adaptive strategies into play (Bennett 1987; Rayburn and Auger 1990; Cavallini et al. 1993; Cec-

carelli et al. 1993; Natali et al. 1993; Biradar et al. 1994; Graham et al. 1994; Frediani et al. 1994; Minelli et al. 1995).

Data from biochemical analyses (Cavallini et al. 1986; Ceccarelli et al. 1992, 1995; Natali et al. 1993; Frediani et al. 1994) and the observation of changes in heterochromatin (Cavallini et al. 1989; Cavallini and Natali 1990; Porter and Rayburn 1990; Rayburn and Auger 1990; Ceccarelli et al. 1992) are both consistent with intraspecific alterations of DNA amount involving repeated sequences. However, the mechanism(s) by which these genome changes are produced and controlled, how common they are, and the structure and chromosomal organization of the DNA fractions that alter are poorly understood.

An intriguing case of intraspecific variation in the basic amount of nuclear DNA occurs in *Helianthus annuus*. In this species, significant differences in genome size were found even within the progeny of homozygous plants. It was shown that these genomic changes are established during early embryo development. As a rule, a gradient is observed in which the genome size increases from embryos developing in the middle of the head to those developing at its periphery (Cavallini et al. 1986, 1989). Three fractions of repetitive DNA and the ribosomal cistrons have been shown to be involved, though to different extents, in these alterations of DNA amount, which are able to produce phenotypic variability by affecting certain aspects of plant development (Natali et al. 1993).

We have already referred to the lack of knowledge about intraspecific changes in genome size and the DNA sequences that are involved. On account of this, we have carried out a further, detailed study of the fluid domains in the genome of *H. annuus*.

Materials and methods

Plant material

A *H. annuus* line selfed for 11 years was used. Seeds (achenes) collected at the periphery or in the middle of the head were germinated in damp vermiculite under sterile conditions at 25 °C. The seedlings were pricked out in Jiffy pots into a mould which had been sterilized by autoclaving and were used as the experimental material.

DNA isolation 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 diethyl-dithio-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 a 50 Ti rotor and the DNA band, visualized under long-wave UV illumination, was collected and re-centrifuged. Ethidium bromide was then removed by gentle inversion of the solution with n-buthanol.

For fractionating DNA at different Cot values, it was solubilized in 0.12 M sodium 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 15 min at 103 °C, allowed to re-associate according to Britten et al. (1974) up to the 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 re-associated sequences were recovered by elution with 0.5 M sodium phosphate buffer.

Thermal denaturation kinetics

Thermal denaturation of repetitive DNA fractions was performed in 0.1 × SSC using a Shimadzu UV-2101PC spectrophotometer equipped with a temperature programme controller, and the increase in hyperchromicity at 258 nm was continously followed. The first-derivatives curves of melting profiles were resolved into components and these were quantified, according to the method devised by Cuellar et al. (1979) and Yen and Blake (1980), using Igor Software (WaveMetrics).

Restriction endonuclease digestion and gel electrophoresis

Genomic DNA was digested with different restriction endonucleases in a five-fold excess according to the instructions of the suppliers (Boehringer and Bio Labs). That digests went to completion was checked by including unmethylated bacteriophage lambda DNA, which, when digested with *Eco*RI plus *Hind*III (DNA molecular size marker III; Boehringer), was also used as a fragment-size marker. DNA fragments were separated by electrophoresis performed on horizontal 1.0% agarose slab gels in 0.05 M Tris, 20 mM sodium acetate, 18 mM NaCl and 2 mM EDTA (pH 8.0) at 30 V overnight.

Southern-blot hybridization and densitometric evaluation

Southern blotting of digested DNAs and hybridization was performed according to standard protocols (Sambrook et al. 1989; Brown et al. 1991). Repetitive fractions of genomic DNA isolated as above were used as probes after having been labelled with digoxigenin-dUTP using a random primed DNA labelling kit (Boehringer). After hybridization, filters were washed sequentially in 2 × and 0.3 × SSC containing 0.05% SDS at 60 °C. Hybridization was detected with a DIG-DNA detection kit (Boehringer) by enzyme-linked immunoassay using an antibody conjugate (antidigoxigenin-alkaline phosphatase conjugate). Filters were then scanned using a UVP system 5000 equipped with GelBase-GelBlot software. To standardise differences between the quantity of DNA actually loaded onto gel slabs, the sum of the fragments was always normalized to 100.

Results

Thermal denaturation kinetics of DNA fractions

DNA was extracted from seedlings obtained from seeds (achenes) collected at the periphery of the flowering head (P DNA) or from seedlings obtained from seeds collected in its middle (M DNA). Three fractions of repeated sequences were separated from these DNAs according to previous results (Natali et al. 1993): a highly repeated fraction (HR), which reassociates within an equivalent Cot of about 2×10^{-1}, and two medium repetitive fractions (MR1 and MR2) having Cot ranges of about 2×10^{-1}–2 and 2–10^2, respectively. The first-derivative curves of the melting profiles of these three fractions of P or M DNA are shown in Fig. 1. From the

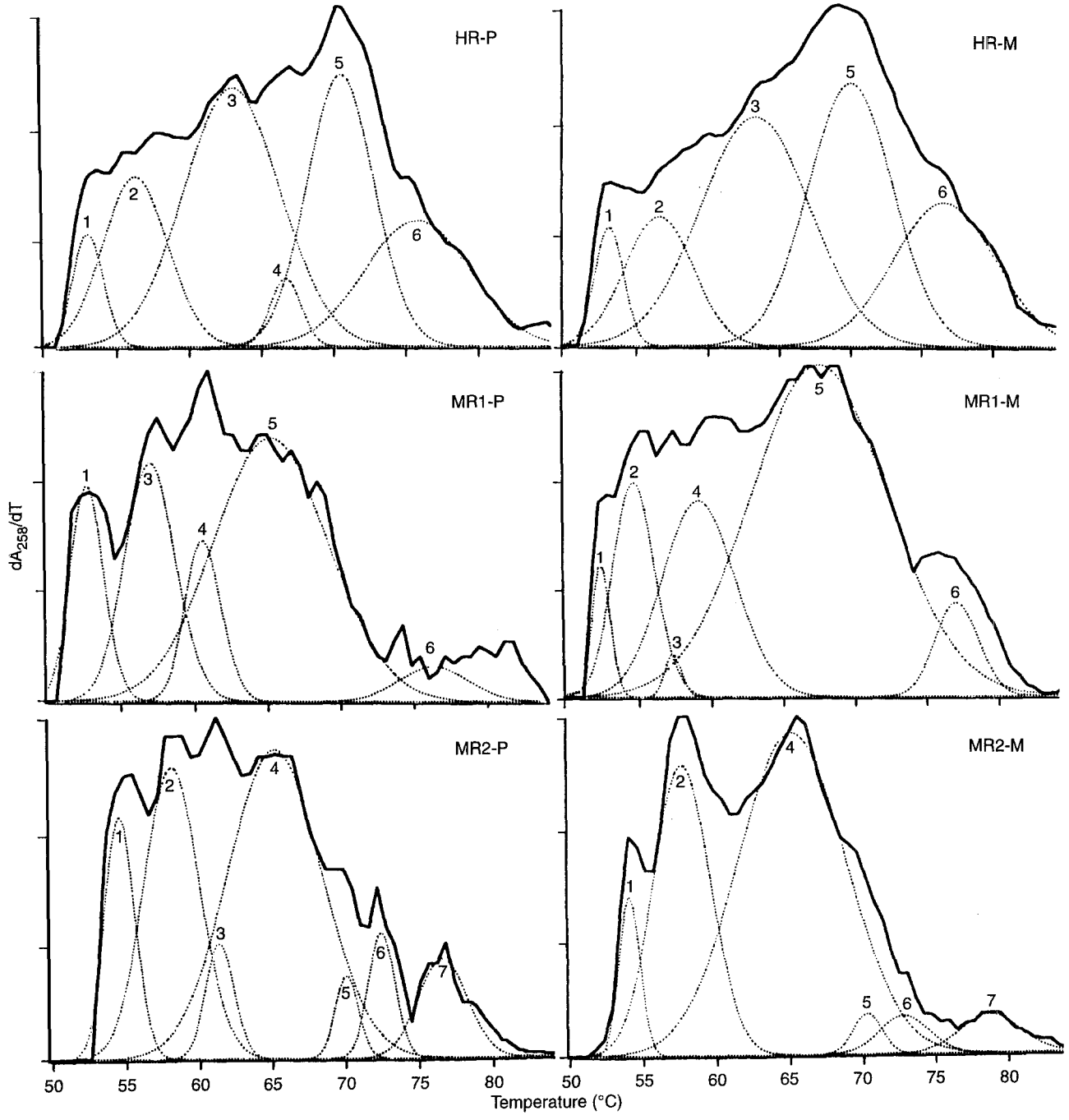

Fig. 1 First-derivative curves (——) of melting profiles of highly repeated DNA sequences (*HR*) and two fractions of medium repeated sequences (*MR1* and *MR2*, respectively) in the genome of seedlings obtained from seeds developed at the periphery (*P*) or in the middle (*M*) of the head. Graphs were obtained from the averaged values of four repetitions: two for each of two DNA extractions. The curves were resolved by using an iterative fitting procedure (--------). *Numerals* indicate DNA sequence families

resolution of these curves into components it appears that different sequence families, characterized by different temperatures of melting and hence by differing enrichments in G + C base pairs, can be recognized within each of the three DNA fractions.

It can be seem from Table 1, where the results of the denaturation kinetics have been quantified, that: (1) DNA sequence families are actually distinguishable from one another by their Tm values, since, as a rule, these differ significantly within each DNA fraction; (2) the Tm values identify the same DNA sequence families in both P and M DNAs, since pairs of values which do not differ significantly between them were found as a rule; (3) the percentage contributions of the components of the derivative curves of the melting profiles may differ significantly between P and M DNA.

From the percentage contributions of the curve components, and previous data on the amount of repeated sequences in P or M DNA (Natali et al. 1993), it was

Table 1 Tm value and the percentage contribution of sequence families of the highly repetitive fraction (HR) and of two medium repetitive fractions (MR1 and MR2, respectively) of the DNA from seedlings obtained from seeds developed at the periphery (P) or in the middle (M) of the head. These data were calculated from the results given in Fig. 1

HR

Sequence family	P		M	
	Tm (°C ± SE)	Contribution (% ± SE)	Tm (°C ± SE)	Contribution (% ± SE)
1	53.1 ± 0.1	4.69 ± 0.4	53.1 ± 0.1	4.41 ± 0.4
2	56.3 ± 0.1	15.36 ± 0.4	56.5 ± 0.1	11.89 ± 0.3
3	63.0 ± 0.1	34.41 ± 1.3	63.3 ± 0.1	34.50 ± 1.7
4	66.7 ± 0.1	2.94 ± 0.2		
5	70.5 ± 0.2	24.65 ± 1.2	70.0 ± 0.2	29.91 ± 2.2
6	75.8 ± 0.2	17.94 ± 0.9	76.6 ± 0.2	19.28 ± 0.5

MR1

Sequence family	P		M	
	Tm (°C ± SE)	Contribution (% ± SE)	Tm (°C ± SE)	Contribution (% ± SE)
1	52.9 ± 0.2	12.41 ± 1.4	52.4 ± 0.1	3.16 ± 0.6
2			54.7 ± 0.2	11.66 ± 0.9
3	57.2 ± 0.1	20.88 ± 2.3	57.3 ± 0.1	0.90 ± 0.3
4	60.8 ± 0.3	10.09 ± 1.5	59.2 ± 0.4	18.73 ± 2.2
5	65.5 ± 0.5	52.32 ± 2.8	67.6 ± 0.6	60.20 ± 3.2
6	76.8 ± 0.3	4.29 ± 0.4	77.1 ± 0.3	5.33 ± 0.5

MR2

Sequence family	P		M	
	Tm (°C ± SE)	Contribution (% ± SE)	Tm (°C ± SE)	Contribution (% ± SE)
1	54.6 ± 0.2	11.34 ± 0.9	54.1 ± 0.2	5.75 ± 0.7
2	58.2 ± 0.2	24.26 ± 1.4	57.6 ± 0.2	27.33 ± 1.6
3	61.4 ± 0.3	5.03 ± 1.3		
4	65.2 ± 0.1	44.38 ± 2.9	65.1 ± 0.3	57.48 ± 3.1
5	70.1 ± 0.2	3.06 ± 0.4	70.3 ± 0.3	2.06 ± 0.3
6	72.5 ± 0.2	5.03 ± 0.6	72.8 ± 0.2	3.36 ± 0.5
7	76.6 ± 0.6	6.90 ± 0.9	78.7 ± 0.5	4.01 ± 1.0

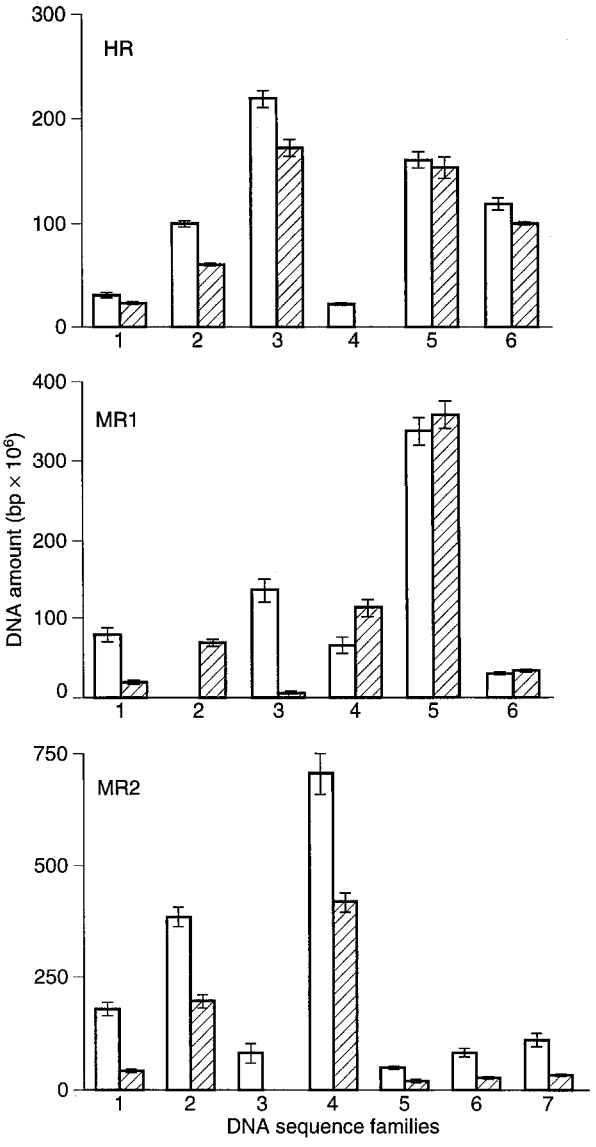

Fig. 2 Representation of repeated sequences belonging to different families in the DNA of seedlings obtained from seeds developed at the periphery (P seedlings; □) or in the middle (M seedlings; ▨) of the head. The values were calculated on the basis of the data given in Table 1 and of previous data on the amounts of repeated sequences in the DNA of P and M seedlings, respectively (Natali et al. 1993). Confidence limits at $P \leq 0.01$

possible to calculate how much of each family of repeated sequences is represented in the two genomes. The results are given in Fig. 2. It can be seen from this figure that: (1) significant differences between P and M DNA may be found within all three repetitive genomic fractions though these differences are more marked in the MR2 fraction; (2) in certain instances, the differences in sequence redundancy between P and M DNA are remarkable, thus, while sequence family 4 in the HR fraction, family 2 in the MR1 fraction, and family 3 in the MR2 fraction are all represented to an appreciable extent in the one DNA, they cannot even be detected in the other and family 3 in the MR1 fraction is about 27-times greater in P DNA than in M DNA; (3) most DNA sequence families are more represented in the P than in the M genome, but the redundancy of certain sequences is greater in the latter than in the former; it may be worth noting that all these sequences are in the MR1 fraction of DNA.

Restriction endonuclease digestions and Southern-blot hybridizations

The results of gel electrophoresis of genomic P and M DNAs after digestion to completion by *Bst*NI or *Eco*RII restriction endonucleases are shown in Fig. 3. In both DNAs, the cleaving capability of *Eco*RII, which cannot cleave when methylation has occurred in the recognition site, is much reduced when compared to that of its

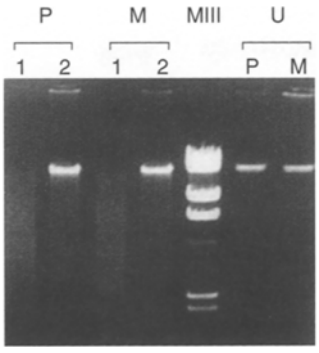

Fig. 3 Gel electrophoresis of genomic DNA from seedlings obtained from seeds developed at the periphery (*P*) or in the middle (*M*) of the head. The DNAs were digested to completion by *Bst*NI (*1*) or *Eco*RII (*2*) restriction endonucleases. Undigested controls (*U*) are shown. The molecular-size marker (*MIII*) is lambda DNA which was digested by *Eco*RI plus *Hin*dIII

isoschizomere *Bst*NI, which is not affected by methylation in the target sequence.

The three fractions of repetitive DNA as described above were hybridized to Southern blots of genomic P or M DNA digested to completion by three pairs of isoschizomeric enzymes. No significant difference in the restriction patterns of P and M DNA was shown after digestion by *Sma*I or *Xma*I (both recognizing the CCCGGG sequence) and hybridization with any repetitive DNA fraction. By contrast, differing restriction patterns were found when the three DNA fractions were hybridized to P or M DNA after digestion with *Msp*I (target sequence CCGG) or *Bst*NI [CC($\frac{A}{T}$)GG] and their respective isoschizomeres *Hpa*II and *Eco*RII.

These differences can easily be observed in Fig. 4, where the results of densitometric scans of the Southern-blot hybridizations after DNA digestion by these enzymes are given. It can be seen from this figure that: (1) in both P and M DNA the activity of methylation-insensitive enzymes is much greater than that of their methylation-sensitive isoschizomeres and this holds after hybridization with any repetitive DNA fraction; (2) clear-cut differences are apparent when the enzyme activity on P and M DNA is compared and, while this occurs with methylation-insensitive restriction endonucleases, it is evident to a greater extent in the case of the methylation-sensitive enzymes; (3) as well as in the case of the thermal denaturation kinetics, differences in the restriction patterns between P and M DNA are particularly striking when the MR2 fraction is taken into account.

Discussion

Mutually supporting each other, the results of thermal denaturation kinetics and Southern-blot hybridizations indicate that complex changes in the amount and methylation of repeated DNA sequences may differentiate the nuclear genome of individuals belonging to single progenies of homozygous plants of the sunflower. Our findings reinforce others already quoted in the Introduction, and shed some light on certain features of the sequences that make up the fluid domains in the nuclear DNA of *H. annuus*.

The results of denaturation kinetics indicate that sequences in different fractions of repetitive DNA may undergo quantitative variations within a progeny (Table 1, Figs. 1 and 2). It may be argued that certain quantitative differences between DNA families are due to the increased methylation of some sequences of another DNA family. Indeed, it has been reported that cytosine methylation increases the melting temperature of DNA (Pivec et al. 1974). However, other authors assume that melting temperature is not affected by base methylation (Kemp and Sutton 1976). Apart from this, the most striking differences between P and M DNA occur in A + T-rich sequence families, obviously less prone to methylation in cytosine residues. DNA sequences in the MR2 fraction appear to be the most fluid (Figs. 1 and 2). The same indication comes from the results of Southern blots after digestion of P or M DNA with methylation-insensitive endonucleases, since differences in their activity (Fig. 4) may be interpreted as the result of a differential redundancy of target sequences in the genome. This agrees with results already obtained by studying the reassociation kinetics of P and M DNA (Natali et al. 1993). Most of the DNA sequence families are more represented in the former than in the latter. This agrees also with the observation that seedlings obtained from seeds developed at the periphery of the head have a larger genome than seedlings obtained from seeds developed in its middle (Cavallini et al. 1986, 1989). However, the differences between P and M DNA appear to be more complex than those due to amplification of given sequences in the former genome. Indeed, there are sequence families that are more represented in M DNA than in P DNA. Remarkably, all these sequences belong to the MR1 DNA fraction (Fig. 2).

These results suggest that quantitative changes in the nuclear DNA within *H. annuus* may represent specific responses to certain stimuli. Therefore, variable DNA sequences can hardly be considered as selfish (Doolittle and Sapienza 1980; Orgel and Crick 1980). That is, redundancy variations cannot be hypothesized as due simply to differences in cell environment which only allow, or only inhibit, the synthesis of certain DNA sequences. It has already been reported in the Introduction that quantitative variations in the nuclear DNA within sunflower progenies can affect certain developmental features of plants. A proposed view (Natali et al. 1993) is that this result is obtained through nucleotypic effects (Bennett 1985), i.e. through changes that alterations in the amount of DNA produce in nuclear mass and volume. The finding that different sequences are specifically either under-represented or amplified in P or M DNA might also render less conceivable the hypothesis that DNA sequence fluidity in *H. annuus* has a role mainly in altering the nucleotype.

The repetitive DNA of the sunflower is highly methylated. This is indicated by the finding that the cleaving activity of methylation-insensitive restriction endonucleases, in both P and M DNAs, was as a rule largely greater than that of their methylation-sensitive

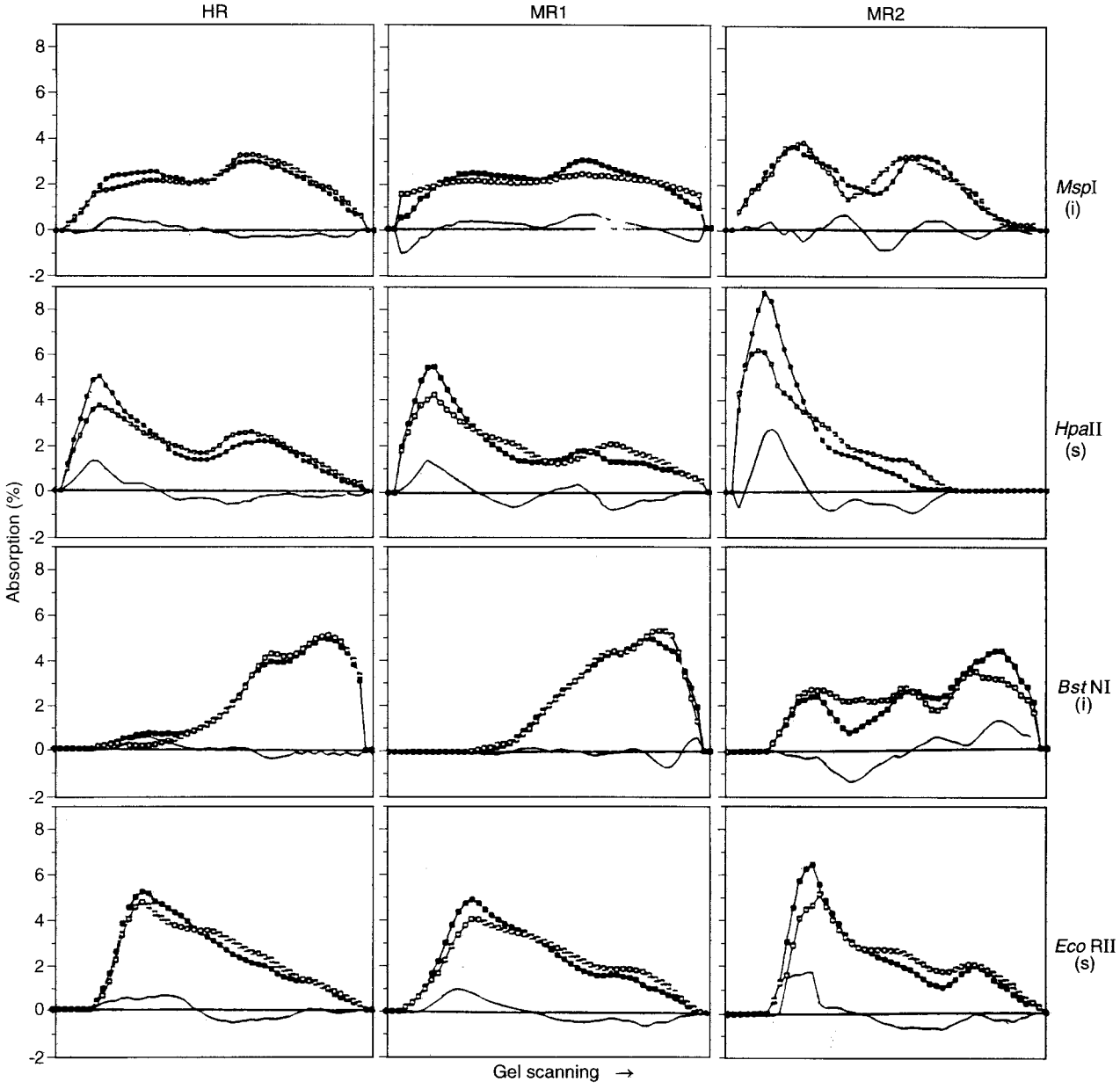

Fig. 4 Densitometric scans of Southern blots of genomic DNAs from seedlings obtained from seeds developed at the periphery (■) or in the middle (□) of the head. The DNAs were digested to completion by isoschizomeric restriction endonucleases which are either insensitive (*i*) or sensitive (*s*) to the methylation of a cytosine in the recognition site. The blots were hybridized to digoxigenin-labelled, homologous highly repeated DNA sequences (*HR*) or two fractions of medium repeated sequences (*MR1* and *MR2*, respectively). The gels were scanned starting at the highest molecular weights. Each point represents the average of the values obtained in three complete repetitions of the experiments, and standard errors never exceeded 5% of the means. *Continuous lines* indicate differences between scans

isoschizomeres (Fig. 3). Moreover, the restriction patterns of P and M DNA often differ significantly (Fig. 4). In presenting our results, we have already emphasised that these differences may be larger after digestion with methylation-sensitive enzymes than after digestion with methylation-insensitive isoschizomeres. Therefore, at least in part, the difference in methylation patterns between P and M DNA does not depend on alterations between the two genomes in the redundancy of sequences that may undergo methylation. Results obtained with other material also suggest that differential DNA replication and DNA methylation are potentially independent events (Arnholdt-Schmitt 1993).

In conclusion, changes in the patterns of DNA methylation are added to changes in sequence redundancy in differentiating individual genomes within a sunflower progeny. Evidence has been obtained that DNA methylation may be correlated with gene expression in eukaryotes (Frediani et al. 1992). Thus, extra synthesis or under-replication of DNA sequences and differential DNA methylation may be complementary ways to produce continuous genotypic variability within a population which may then be exploited in environmental adaptation.

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