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Structure and variability of nuclear ribosomal genes in the genus *Helianthus*

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Summary. The restriction map of the rDNA unit of *Helianthus annuus* was constructed using EcoRI, BamHI, HindIII, KpnI and SacI restriction enzymes. Variations in this map among 61 ecotypes representing 39 species of the genus *Helianthus* were analyzed. The sizes of the rDNA unit ranged from 9.8 to 11.0 kbp, due to a length-repeat heterogeneity of the external non-transcribed spacer by increments of 200 base pair segments. Length-repeat heterogeneity and restriction polymorphism were found to be characteristic of populations or species of *Helianthus*. Restriction patterns and thermal melting with probes of a cloned *H. annuus* ENTS segment allowed us to differentiate species from each other. However, most lines of the cultivated sunflower were found to be identical on the basis of the physical properties of their ribosomal DNA.

Key words: *Helianthus* – Nuclear rDNA unit – Variability – External non-transcribed spacer.

Introduction

Genes coding for rRNA occur universally in all organisms. In eucaryotes, they consist of tandemly repeated rDNA units composed of transcribed regions coding for the 18S, 5,8S and 25S rRNA and separated by nontranscribed regions or external nontranscribed spacers (ENTS). Due to strong functional constraints imposed on the highly integrated multicomponent assembly of the ribosome, and particularly on its rRNA backbone (Lake 1985), the coding regions of the rDNA units have evolved very slowly and are highly conserved among all living cells. As a result, sequence comparisons have been used to estimate phylogenetic relationships between very distant species (Field et al. 1988). The external nontranscribed spacers, on the other hand, have evolved much more rapidly in both animals (Fedoroff 1979) and plants (Rogers and Bendich 1987; Lassner and Dvorak 1986), even though these regions are endowed with important biological functions and seem to contain regulatory elements working like transcription enhancers (Reeder 1984; McMullen 1986). Variations in ENTS affect the structure and/or length of the rDNA units: they result from the reiteration in variable degrees of short, perfectly or imperfectly repeated sequences, as described for wheat, maize, barley, rye, broad bean and radish (Appels and Dvorak 1982; Flavell et al. 1986; McMullen et al. 1986; Saghai-Maroof et al. 1984; Appels et al. 1986; Yakura et al. 1984; Rogers et al. 1986; Tremousaygue et al. 1988). They may be used for differentiating species, populations, and even individuals (or inbred lines) as in the case of wheat (Appels and Dvorak 1982). They might thus be useful for estimating genetic distances in plant breeding and in evolutionary studies.

In this work, the nuclear ribosomal genes have been used as molecular markers to analyze the genetic variability of the genus *Helianthus*. The restriction map of the rDNA unit has been defined in *H. annuus*, line HA89. The structural variations of this unit among species or ecotypes of the genus *Helianthus*, and among inbred lines of *H. annuus* have been examined and compared with the taxonomy proposed for this group of plants (Schilling and Heiser 1981). We show that all species described can be distinguished from each other, especially those produced through interspecific crosses by Russian breeders.

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Materials and methods

Materials

Cultivated sunflower plants were grown under greenhouse conditions. *H. annuus* male fertile line HA89 and *H. tuberosus* (Jerusalem artichoke) line ID19 were obtained respectively as seeds (from Dr. Bervillé, INRA, Dijon, France) or tubers (from Pr. Courduroux, University of Clermont-Ferrand, France). Seeds of other lines of *H. annuus* were obtained from Dr. Serieys and Dr. Vincourt. Wild species material was collected as leaves harvested from plants (growing in the fields) of the sunflower collection of the INRA in Montpellier.

Methods

Nucleic acids extraction and analysis

Total DNA was extracted from frozen leaves ground in a mortar under liquid nitrogen. The powdered tissues were lysed in 25 m*M* *Tris* pH 7.5, 25 m*M* EDTA, 1% diethylpyrocarbonate and 20% ethanol, 1% β-mercaptoethanol, 3% SDS and 12% sucrose (w/w). The lysate was adjusted to 0.5 *M* NaCl, extracted with chloroform/phenol (v/v), and ethanol precipitated. The DNA was further purified by isopycnic CsCl/ethidium bromide gradient centrifugations.

Ribosomal RNA extractions were performed as described by Appels et al. (1980) from *H. annuus* HA89 embryos, and fractionated by sucrose velocity centrifugations.

Total DNA was digested with the restriction enzymes as recommended by the supplier (Boehringer Mannheim), using 5–10 units of enzyme per μg DNA. The restriction fragments were separated by horizontal agarose gel electrophoresis, photographed under UV illumination after ethidium bromide staining, and transferred to Schleicher and Schuell BA85 nitrocellulose filters according to Southern (1975). DNA probes were labeled by nick-translation with α[^{32}P]dCTP (Maniatis et al. 1982); rRNA probes were alkaline hydrolyzed and 5′-labeled with γ[^{32}P]ATP and polynucleotide kinase according to Maizels (1976). Hybridizations with labeled probes were performed according to Jeffreys and Flavell (1977) in Denhardt's mixture in 3 × SSC plus 40% formamide at 43 °C; washings were made in 0.1% SDS and 2 to 0.1 × SSC at 63 °C.

Molecular cloning

An EcoRI fragment library was constructed using phage λWES. Clones carrying the 6.1 kbp rDNA insert were selected using a radioactive probe of *Euglena gracilis* rDNA (Neyret-Djossou et al. 1986). The 6.1 kbp EcoRI ribosomal fragment and its BamHI digests (the 3.7 kbp Eco-Bam, 0.65 kbp Bam no. 1 and 0.65 kbp Bam no. 2, and 1.05 kbp Bam-Eco fragments (Fig. 4) were subcloned in the plasmid vector pEMBL8− (Dente et al. 1983).

Melting point determinations

Total plant DNA (5 μg) was denatured in 0.2 N NaOH for 120 min at 37 °C, then neutralized with 0.1 vol 2 N HCl + 2 M *Tris* pH 7.5 and brought to 10 × SSC. The mixtures were immediately loaded by filtration onto 25 mm discs of presoaked nitrocellulose, and the filters were then hybridized with radioactive probes of cloned *H. annuus* rDNA. Thermal melting of the hybrids was performed in 0.2 × SSC + 0.1% SDS as described by Appels and Dvorak (1982); the radioactivity eluted from the filters was counted by the Cerenkov effect. All determinations were performed in triplicate, and the ΔTm was expressed as the mean value of the three measurements.

Results

Restriction mapping of the rDNA unit in H. annuus

For the restriction mapping of rDNA, *H. annuus* genomic DNA (line HA89) was treated with the following restriction enzymes: EcoRI, BamHI, HindIII, SacI and KpnI. Ribosomal fragments were identified on the resulting Southern blots by hybridization with in vitro-labeled *H. annuus* rRNA (Fig. 1 A) and cloned *Euglena* rDNA (Neyret-Djossou et al. 1986). Unique 9.8 kbp fragments were produced by HindIII and KpnI digestions, thus giving the size of the basic rDNA unit in cultivated *H. annuus*. Two EcoRI sites, three SacI sites and five BamHI sites were also detected (Table 1). The relative positions of these restriction sites were determined by double digestions (Figs. 1–4).

EcoRI digestions carried out with 5 units of restriction enzyme per microgram DNA left small amounts of 9.8 kbp partial digests (Fig. 2, lane a). When 10 units/μg DNA were used, these partials were digested to 6.1 and 3.7 kbp fragments (Fig. 2, lane b); with25 units/μg DNA, two fragments of 5.5 and 0.6 kbp appeared in addition to the 6.1 kbp fragment (Fig. 2, lane c), indicating that some units are more resistant to EcoRI digestion and contain an additional polymorphic restriction site.

Overexposure of the Southern blots enabled the detection of minor 11 kbp units composed of 7.3 and 3.7 kbp EcoRI fragments that represented about 5% of the total rDNA. The ribosomal units are thus mainly homogeneous in *H. annuus* line HA89, but they show detectable levels of two different kinds of heterogeneity: a restriction polymorphism and a repeat-length heterogeneity. The minor units detected only after overexposure of the autoradiographs were not mapped, due to their very low quantities.

Table 1. Ribosomal restriction fragments resulting from simple and double digestions of total DNA from *H. annuus* with various restriction enzymes

Enzymes	No. of fragments	Size of fragments (kbp)
EcoRI	2	3.7, 6.1
HindIII	1	9.8
KpnI	1	9.8
BamHI	5	4.75, 2.55, 1.2, 0.65, 0.65
SacI	3	5.8, 2.45, 1.55
Eco/Bam	7	3.7, 1.5, 1.2, 1.05, 1.05, 0.65, 0.65
Eco/Hind	3	3.7, 3.3, 2.8
Eco/Kpn	3	4.35, 3.7, 1.75
Eco/Sac	5	5.75, 2.1, 1.55, 0.35, 0.05*
Hind/Bam	6	4.3, 2.55, 1.2, 0.65, 0.65, 0.4
Hind/Sac	4	3.35, 2.45, 2.45, 1.55

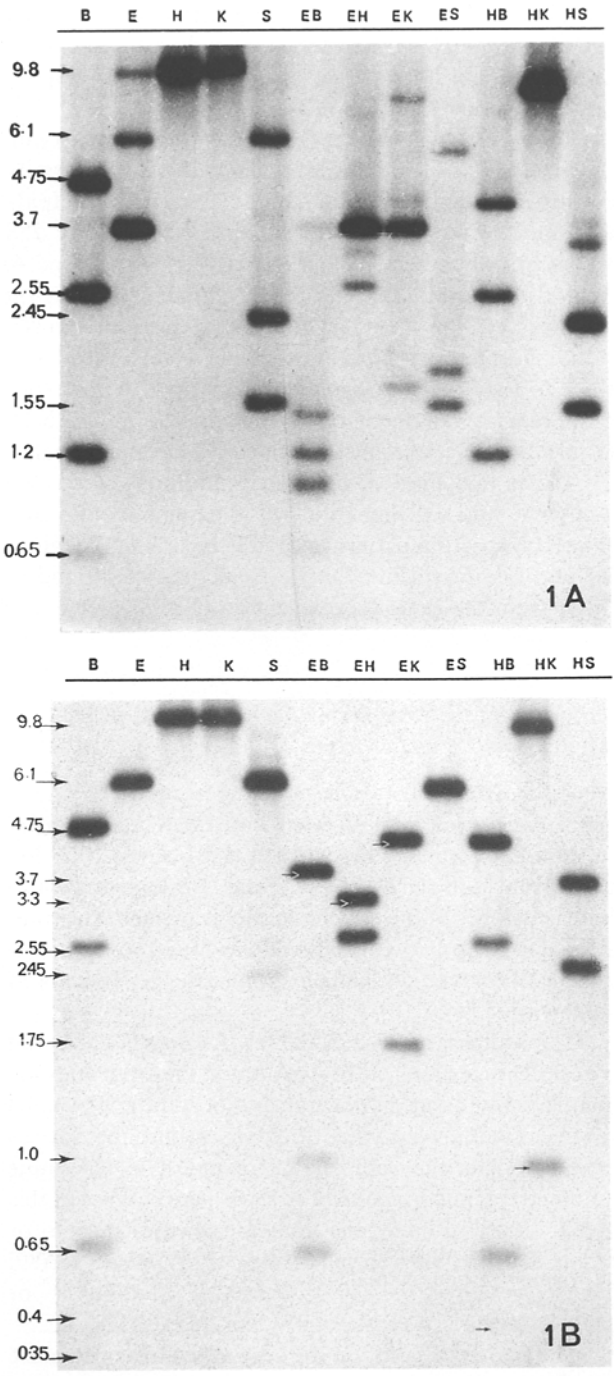

Fig. 1 A and B. Restriction patterns of *H. annuus* rDNA. Southern blots of total DNA from *H. annuus* were hybridized **A** with 18S+25S rRNA or **B** with cloned rDNA from *H. annuus* (6.1 kbp Eco fragment, in order to identify the coding and non-coding restriction fragments. The *arrows* indicate restriction fragments not detected with rRNA. *B*, BamHI; *E*, EcoR; *H*, HindIII; *K*, KpnI; *S*, SacI; *EB*, *EK*, *ES*, *HB*, *HK*, *HS*, double digests MW are in kbp

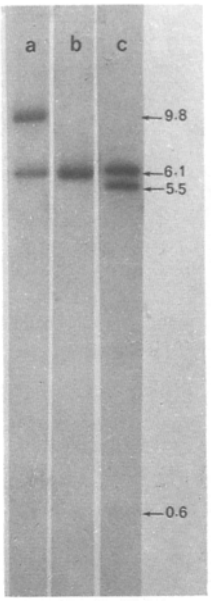

Fig. 2. EcoRI restriction polymorphism of *H. annuus* rDNA. Southern blots of total HA89 DNA digested with increasing amounts of enzyme (*a* 5 u/μg; *b*, 10 u/μg; *c*, 25 u/μg) were hybridized with the 3.7 kbp Eco-Bam rDNA cloned fragment of *H. annuus*. The hybridization between the 0.6 kbp Eco and the 3.7 kbp Eco-Bam probe localizes the polymorphic Eco site in the proximity of the 25S terminus

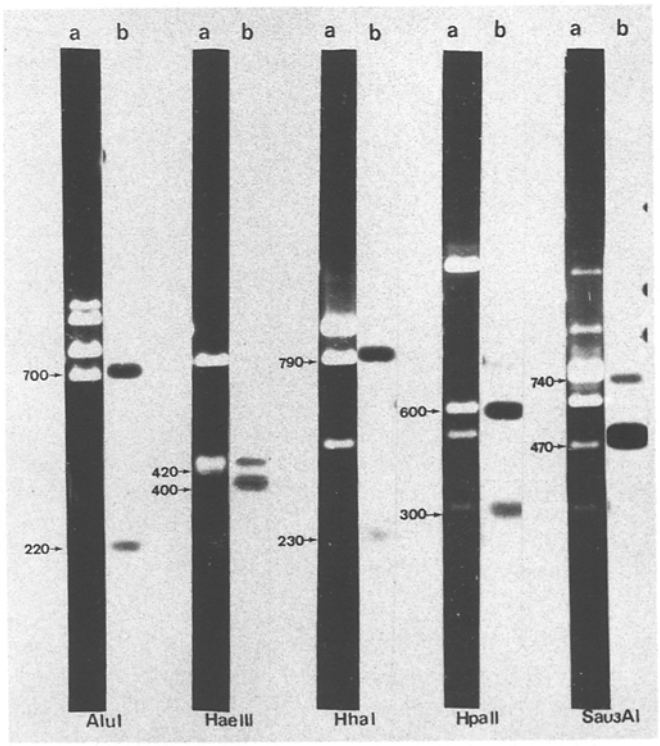

Fig. 3. Delimitation of the 25S coding region. Digestions of the cloned 3.7 kbp Eco-Bam fragment of *H. annuus* were performed with frequently cutting enzymes (AluI, HaeIII, HhaI, HpaII and Sau3A), and the hybridizations were carried out with 25S rRNA. *a*, Ethidium-bromide/UV photographs; *b*, rRNA hybridization patterns. MW are in kbp

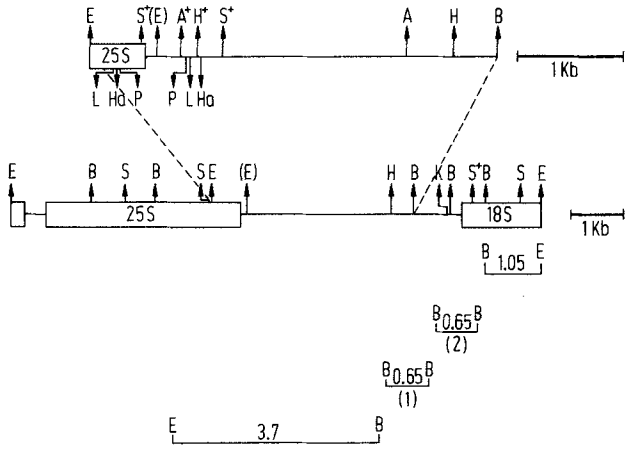

Fig. 4. Restriction map of the rDNA unit of *H. annuus* HA89. The BamHI subclones of the 6.1 kbp EcoRI fragment used in this work are represented as 3.7 kbp Eco-Bam, 0.65 kbp Bam fragments no. 1 and 2, and 1.05 kbp Bam-Eco fragments. The restriction sites of frequently cutting enzymes in the upper insert were determined by hybridization with 18S and 25S rRNA as in Fig. 3. E: EcoRI, B: BamHI, S: SacI, H: HindIII, K: KpnI, L: AluI, S^+: Sau3A, A: AccI, A^+: AvaII, H^+: HindII, Ha: HhaI, P: HpaII

Table 2. Restriction fragments resulting from the digestion of the 3.7 kbp Eco-Bam clone and the 0.65 Bam-Bam no. 2 clone of the ENTS, with frequently cutting enzymes. * and ** indicate the relative levels of hybridizations obtained with 25S rRNA on the 3.7 kbp clone or with 18S rRNA on the 0.65 kbp clone, respectively; these indications were used to order the fragments

Enzymes	Probes	No. of fragments	No. of fragments hybridizing		Fragment size (kbp)
			18S	25S	
AccI	3.7 E/B	2	0	1	2900
AluI	3.7 E/B	6	0	2	700 220
AvaII	3.7 E/B	4	0	1	800
HaeIII	3.7 E/B	9 or 10	0	2	420* 400*
HhaI	3.7 E/B	5	0	2	790 230
HindII	3.7 E/B	5	0	1	960
HpaII	3.7 E/B	5	0	2	600 300
Sau3A	3.7 E/B	8	0	2	740* 470**
Sau3A	0.65 B/B	2	2	0	400* 250**

Coding and nontranscribed spacer regions of the rDNA unit

The 18S and 25S rRNA were separated by sucrose gradient ultracentrifugation and used to identify their corresponding coding regions. The 3.7 kbp EcoRI fragment carries the sequences for most of the 25S rRNA and for a small part of the 18S rRNA. The 6.1 kbp EcoRI fragment gives a weak hybridization with the 25S rRNA and thus carries only a few terminal nucleotides (about 500) of this RNA species. It does carry most of the 18S rRNA coding sequences and the entire nontranscribed spacer identified as fragments hybridizing with cloned *Euglena* rDNA and/or *H. annuus* rDNA (=coding+non-coding sequences), but not with *H. annuus* rRNA (=coding sequences exclusively) (Fig. 1 B).

The digestion of the EcoRI 6.1 kbp fragment carrying the ENTS and of its BamHI subclones with frequently cutting enzymes (AccI, AluI, AvaII, HhaI, HindII, HpaII and Sau3A) was used to locate the 3′ end of the 25S rRNA and the 5′ end of the 18S rRNA (Figs. 3 and 4, Table 2). The codes for mature rRNA thus span a region comprising 5,700–5,900 bp. These enzymes also showed some short multiple fragments; i.e., HaeIII produced three to four 300 bp fragments, suggesting the presence of more or less perfectly repeated segments in the ENTS (Table 2).

Ten inbred lines of cultivated sunflower were also analyzed: nine of them showed the same EcoRI and BamHI restriction pattern as HA89, including the minor 7.3 kbp Eco fragments. Only a single line was found to differ from the general organization (Fig. 5).

Variations in the structure of the rDNA units in the different species and ecotypes of the genus Helianthus

Genomic DNA of various species or ecotypes of the genus *Helianthus* were digested with EcoRI and BamHI restriction enzymes. These digests were screened by hybridization with rRNA and with the Eco-Bam and Bam-Bam subclones of the 6.1 kbp EcoRI fragment. The transcribed 3.7 kbp EcoRI fragment was found to be invariable in all ecotypes; only the region of the ENTS showed some variability.

The rather large size of the few fragments generated by EcoRI digestions clearly visualized the variations accumulated by addition/deletion events in the rDNA units of the different ecotypes. It appears that the species *H. annuus* has the shortest and simplest rDNA units among the genus *Helianthus*: in the other ecotypes of this genus, the ribosomal gene units are always equal to or larger than the 9.8 kbp major unit of *H. annuus* HA89. They measure 9.8–10.0–10.2–10.4–10.6–10.8 or 11.0 kbp, with sizes differing by multiples of 200 bp.

The BamHI digests, on the other hand, revealed more details about the structural differences between the various ecotypes when hybridized with the 6.1 kbp Eco-Eco fragment containing the ENTS sequences. These variations were more specifically detected with the 3.7 kbp Eco-Bam subclone of the ENTS region (Fig. 6); with other subclones of the 6.1 kbp EcoRI fragment, we did not detect any variation, indicating that they represent a more conserved region of the ribosomal units among the various ecotypes. The 3.7 kbp Eco-Bam subclone was thus systematically used to check the variability of the rDNA units in the genus *Helianthus*.

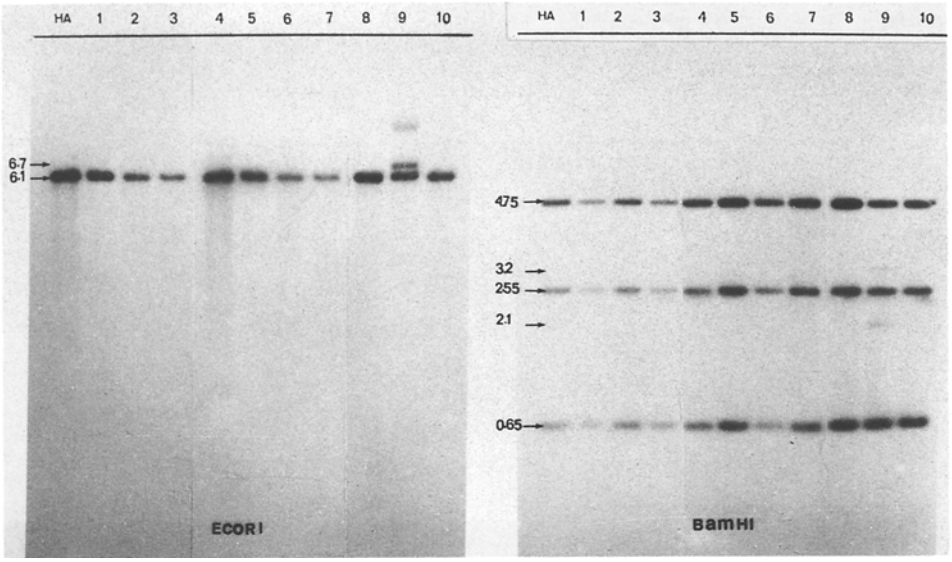

Fig. 5. Structural identity of most of the rDNA from various lines of sunflower. EcoRI and BamHI Southern blots of total DNA from various lines were hybridized as in Fig. 1 with the 6.1 kbp Eco-Eco cloned fragment of the ENTS. *Lanes 1* to *10*: lines 83R6, 125, 136, 85B6, 85B4, 84SR1, 85B31, 84R213, 82HR38 and 85B34

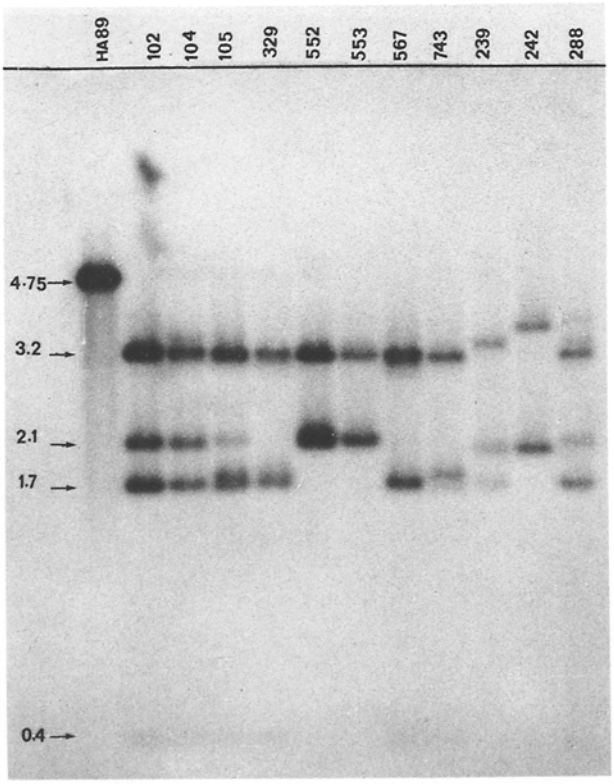

Fig. 6. Restriction fragment length polymorphism of the rDNA unit among various species of the genus *Helianthus*. Southern blots from total DNA of various species were digested by BamHI and hybridized with the 3.7 kbp Eco-Bam subclone of the ENTS from *H. annuus*. Identification of the species: *102, 552* and *553* = *H. giganteus; 104* and *567* = *H. maximiliani; 105, 242* and *288* = *H. californicus; 239, 329* and *743* = *H. nuttallii*. These results and others have been reported inTable 3

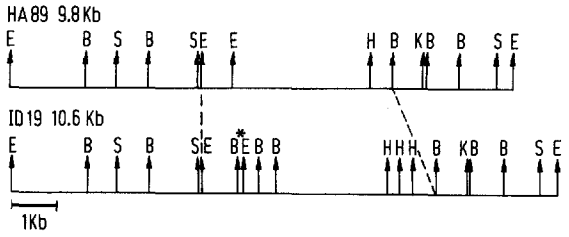

Fig. 7. Restriction maps of the rDNA units of *H. tuberosus* line ID 19 and of *H. annuus* line HA89. The restriction sites were determined and localized with the combined use of double digestions with EcoRI, BamHI, HindIII, KpnI, and SacI, and by hybridizations with the cloned 6.1 kbp fragment (and subclones) of the ENTS of HA89. The production of non-stoechiometric proportions of Bam fragments was interpreted as resulting from the presence of partially accessible sites; for instance the 1.7 kbp and 0.4 kbp Bam fragments result from the digestion of the 2.1 kbp Bam fragment. B*: Bam polymorphic site

An example of structural variation of the ribosomal unit was analyzed in the case of *H. tuberosus* (line ID19), whose unit is 10.6 kbp long (0.8 kbp longer than the major unit of *H. annuus*). While EcoRI, KpnI and SacI digestions indicated the same number of restriction sites in the two species (including a partial EcoRI site), HindIII gave two additional 250 bp fragments in *H. tuberosus*, and BamHI digestion produced eight fragments present in non-stoechiometric proportions. Eco + Hind and Bam + Hind double digestions enabled us to propose a restriction map for the ribosomal spacer of *H. tuberosus* (Fig. 7). The sequences added to the sim-

Table 3. EcoRI and BamHI ribosomal restriction fragments detected in total DNA from various ecotypes of the genus *Helianthus* by hybridization with the 3.7 kbp Eco fragment of the *H. annuus* ENTS, as illustrated in Fig. 6. The relative hybridization intensities of each fragment have been grouped into three classes; 1: low intensity; 3: high intensity. *Column 1*: series of species: 1 = annui; 2 = ciliares; 3 = pumili; 4 = divaricati; 5 = angustifolii; 6 = atrorubentes; 7 = gigantei; 8 = microcephali; 9 = *H. porteri*; 10 = ecotypes of Russian origin. *Column 2*: the ecotype numbers are those of the sunflower collection of the INRA station in Montpellier. *Column* 4: the ΔTm have been determined as in Fig. 9 using the 3.7 kbp Eco-Bam fragment as radioactive probe

Serie	Eco-type	Species	Δ Tm	EcoRI fragments (kbp)											BamHI fragments (kbp)																		
				7.3	7.1	6.9	6.7	6.5	6.3	6.1	5.9	4.2	1.9	0.8	4.7	4.3	4.0	3.7	3.5	3.4	3.2	3.1	3.0	2.9	2.3	2.1	1.9	1.8	1.7	2.0	1.4	0.6	0.4
1		*annuus* HA89	0	1					3						3																		
1	519	*annuus*	–	1					3					1	3																		
1	521	*annuus*	–	1					3						3																		
1	741	*argophyllus*	1	1					3						3																		
1	667	*bolanderi*	–						3		2	3			1							3				2						
1	671	*exilis*	2						3													3				2						
1	213	*niveus*	1.5			3																			3			3		3		
1	737	*petiolaris*	4	1				3										1		1					1			3		3		
1	219	*praecox*	3.5			3																					3	3		3		
2	203	*arizonensis*	–			3													3		3				3							
2	594	*arizonensis*	3.5			3													3		3				3							
3	226	*gracilentus*	–			3			1				1											3				2	3			
3	227	*pumilus*	3.5			3			1				1					3		3					3			3				1
4	100	*decapetalus*	4			3		2												3					3			2				2
4	551	*decapetalus*	–			3		2										3		2					3			2				1
4	232	*divaricatus*	3			3														3		1						3		2		2
4	234	*egertii*	–			3												3							2			2			3	1
4	237	*grossesseratus*	3.5			3			3				2							3					1		1	2				1
4	672	*hirsutus*	4			3		3												3		2			1			2				
4	230	*mollis*	–			3														3								3				
4	285	*mollis*	–			3			2				1					1		3					2			2				2
4	600	*mollis*	3			3														3								2				
4	231	*occidentalis*	1			3												3		1					2			3				2
4	243	*resinosus*	–			3												2		3					3			2				1
4	555	*rigidus*	–			3												3		1					3			2		1		1
4	294	*strumosus*	–			3			1				1					3		3					2							2
4	527	*strumosus*	1.5			3		2	1				1					2		3		1			2			2				2
4		*tuberosus* ID19	4			3			1				1					2		3					3			2				2
4	289	*tuberosus*	–			3			1				1					2		3					3			2				2
4	326	*tuberosus*	4			3			1				1					2		3					3			2				2
5	111	*angustifolius*	–			3			2				1				3		3					2	2			3				
5	529	*angustifolius*	2.5	3											3													3				
5	564	*simulans*	4			3										1	1			3					2			3				1
5	596	*simulans*	5			3										3												2		1		
6	601	*atrorubens*	3.5		3													3		1					1			3		1		
6	531	*silphioides*	3			3										1		3		2					1			3		2		
7	105	*californicus*	–			3			3				2							3					2		2	2				1
7	242	*californicus*	–		3												3								3							
7	288	*californicus*	4			3			2				1				1	1		3					2			3				1
7	102	*giganteus*	3			3														3					3			3				1
7	552	*giganteus*	–			3														3					3							
7	553	*giganteus*	–			3														3					3							
7	554	*giganteus*	–			3			1				2							3					1			2				2
7	104	*maximiliani*	–			3			2				1					2		3					3			3				2
7	567	*maximiliani*	–			3			2				1							3		1						3				2
7	569	*maximiliani*	3.5		3														3						3			3				
7	239	*nuttallii*	–		3													3							3			3				
7	329	*nuttallii*	–			3			3				2							3								3				2
7	743	*nuttallii*	3.5			3			3				2							3							3	2				1
7	599	*salicifolius*	4			3			3				2							3			3					3				2
8	246	*glaucophyllus*	4		3	3										3				3					3			3				
8	560	*laevigatus*	5			3												3							3			1		1		
8	562	*laevigatus*	–			3										1		3							3			1		3		
8	563	*longifolius*	4.5	3		3										3			3								2	2				1
8	261	*microcephalus*	5.5	2		3												3		3					3			1				
8	247	*smithii*	4.5			3												3		1					1			1		2		
9	676	*porteri*	5			3									2		3											3				1
10	107	*macrophyllus*	–			3			1				1					3		3			2		3			3				3
10	106	*micranthus*	–			3			3				1					2		3		1			2		2	3				1
10	108	*orgyalis*	–				3													3				3	3			3		3		1
10	287	*tomentosus*	1			2			3		2		1		3					2					2						1	

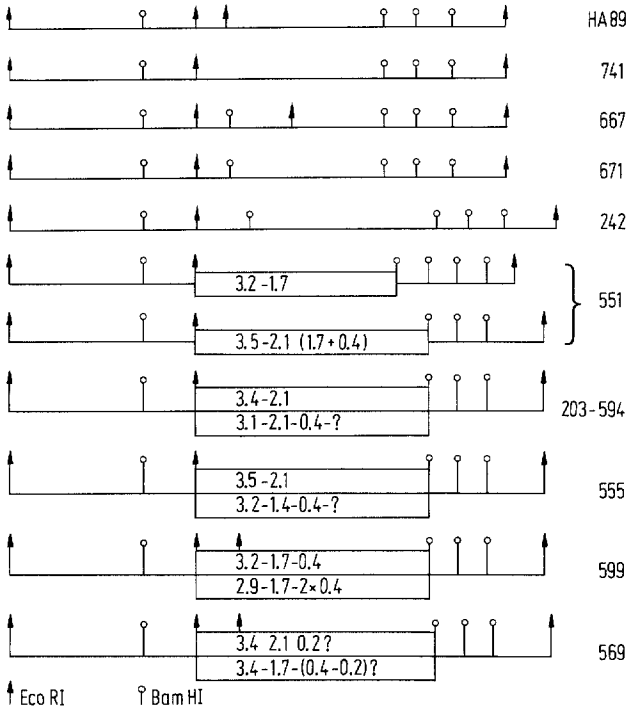

Fig. 8. EcoRI and BamHI restriction maps proposed for a few ecotypes of the genus *Helianthus* based on data from Table 3 and Fig. 6. In the case of the BamHI restriction site polymorphism, several maps are proposed

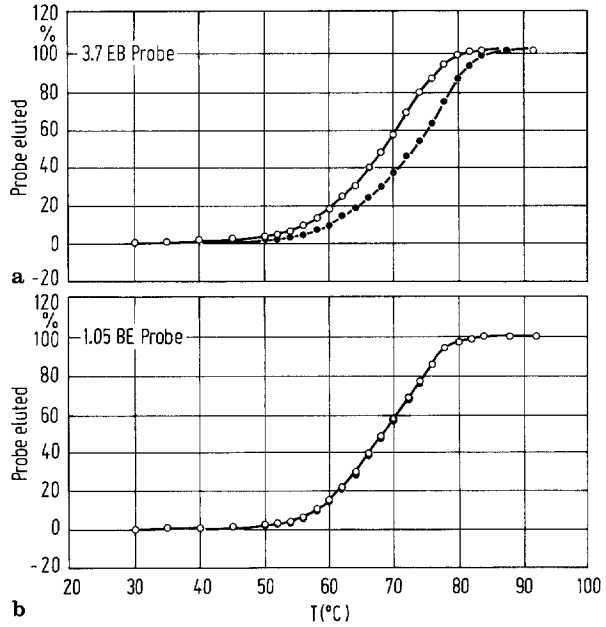

Fig. 9. a Thermal melting of duplexes produced by hybridization between the 3.7 kbp Eco-Bam fragment of the ENTS of *H. annuus* used as radioactive probe and total DNA of *H. annuus* (● = homoduplex) and *H. tuberosus* (○ = heteroduplex) fixed on nitrocellulose filters. **b** Same experiment run with a probe of the conserved region coding for the 18S rRNA (1.05 kbp Bam-Eco fragment; see Fig. 4)

ple basic map of *H. annuus* span between the 3′ region of the 25S rRNA and the HindIII site.

Among the 61 ecotypes representing 39 different species, analyzed, 48 have one single size rDNA unit; 13 (including the two ecotypes 519 and 521 and the cultivated line HA89 of *H. annuus*) have two units of different size occurring in various proportions, and thus present various levels of repeat-length heterogeneity (see Table 3). In a few species, these rDNA units are homogeneous with respect to the distribution of their EcoRI and BamHI restriction sites; they produce stoechiometric amounts of Bam restriction fragments, and this enabled us to propose tentative restriction maps for the rDNA of these plants (Fig. 8). The majority of the ecotypes, however, show a restriction site polymorphism reproducibly characteristic of each ecotype. In some instances, the Bam restriction fragments can be grouped in two or three families showing the same stoechiometry and the same molecular sizes matching those of the corresponding EcoRI fragments. Restriction polymorphism was also shown to be superimposed to length heterogeneity in some ecotypes (no. 246, 261, 287, 563 and 737; Table 3). Taking account of the presence or absence of EcoRI and BamHI restriction fragments and of their relative abundance, most of the species or ecotypes analyzed can be differentiated from each other.

The sequence divergence between the ENTS of *H. annuus* and those of other species was estimated by thermal melting: total DNAs from *H. annuus* and from other species were immobilized on nitrocellulose filters and hybridized with the radiolabeled 3.7 kbp Eco-Bam subclone of the variable ENTS segment of *H. annuus* HA89. The ΔTm between homo- and heteroduplexes is reported in Table 3, column 3. In comparison, similar thermal fusion experiments were performed with a probe of the conserved 18S rRNA coding region (the 1.05 kbp Eco-Bam fragment). In this case, homo- and heteroduplexes showed perfectly identical melting curves (Fig. 9). Depending on their phylogenetic distances from *H. annuus*, the various species gave ΔTm ranging from 1 °C (+0.5 °C) to 5.5 °C (+0.5 °C). The present data match fairly well with the classification proposed for the genus *Helianthus* by Schilling and Heiser (1981).

Discussion

Structure of rDNA units in H. annuus

The rDNA units of *H. annuus* HA89 are mainly homogeneous as 9.8 kbp repeated segments. However, two types of low level heterogeneity were regularly observed:

(1) a repeat-length heterogeneity in about 5% of the rDNA that resulted from the presence of 10.6 kbp units. The superposition of the restriction products originating

from the 9.8 kbp units prevented us from mapping these minor units.

(2) an EcoRI restriction site polymorphism affecting the 9.8 kbp units. High enzyme concentrations were necessary to obtain the cleavage of all the Eco sites of these variant units. The particularly low sensitivity of these rDNA units towards EcoRI might be the result of a higher degree of methylation, generally proposed as a potential way of regulating the transcriptional activity of rDNA units (Gerlach and Bedbrook 1979). The cloned 6.1 kbp EcoRI fragment did not show any internal Eco site made accessible after replication in *E. coli*, an indication that the majority of the 9.8 kbp units do not have such sites.

The same rDNA organization was also found in the two wild-type ecotypes, 519 and 521, of *H. annuus*, although these ecotypes are rather genetically distant from HA89 since they induce cytoplasmic male sterile progeny when crossed with cultivated sunflower (Dr. Serieys, personal communication). A minor difference was observed at the rDNA level of ecotype 519, which consisted of the presence of low amounts of 0.8 kbp EcoRI fragments.

Among ten inbred lines of cultivated sunflower, nine had rDNA units with stricly identical EcoRI and BamHI sites; only line 82HR38 differed from the general BamHI pattern described by an important structural rearrangement, although this line is not particularly distant from the nine others on the basis of quantitative genetics (Drs. Serieys and Vincourt, INRA Montpellier). Nuclease S1 digestion of heteroduplexes formed between the cloned spacer fragment of HA89 and total DNAs from the cultivated sunflower lines did not indicate more structural differences than those already demonstrated in line 82HR38 by restriction analysis (data not shown). Thus, as already observed by Riesberg et al. (1988), the variability of the ribosomal cistrons among ecotypes and cultivars in the species *H. annuus* appears to be rather low.

Structure of rDNA units in other species of the Helianthus genus

The various rDNA units described in species of the genus *Helianthus* differ from each others by size increments of 200 bp and are localized in the proximal region of the ENTS. The distal part of the ENTS and the coding regions remain much more conserved, showing no variation in EcoRI and BamHI restriction sites. It seems likely that the higher conservation of the distal part of the ENTS compared to that of the proximal one is due to constraints imposed by the presence of transcription promotors of the ribosomal cistrons (Labhardt and Reeder 1987).

The intraspecific variability differs from species to species; while the ecotypes of *H. arizonensis, H. giganteus* and *H. tuberosus* are similar within each species, those of *H. angustifolius, H. californicus, H. maximiliani, H. mollis, H. nuttallii, H. simulans* and *H. strumosus* are more variable. This illustrates the remark made by Schilling and Heiser (1981), that the exact delimitation of each species is often difficult to define in the genus *Helianthus*, due to frequent interspecific fertility. In fact, larger numbers of ecotypes should be analyzed to ascertain the variability within each species.

Moreover, ecotypes of American origin have usually been maintained as pure populations in various collection sites. On the contrary, the ecotypes of Russian origin result from deliberate interspecific crosses between various and unreported species (Dr. Serieys, personal communication). The complexity of their ribosomal Bam patterns illustrates these facts: ecotypes 106, 107 and 108, for instance, are phenotypically very close to *H. tuberosus*, although they show additional Bam fragments of 3.0, 2.9, 2.3, 1.8 or 1.4 kbp, probably a result of these crosses. *H. tomentosus* is a particular perennial diploid ecotype: it shows a mixed pattern of restriction fragments very typical of *H. annuus* and its closely related species (namely the 4.75 kbp BamHI fragment), in addition to perennial type fragments.

The melting behaviour of the heteroduplexes formed between the cloned ENTS fragment of *H. annuus* HA89 and the total DNA from the various ecotypes is consistent with the classification of the genus *Helianthus* proposed by Schilling and Heiser (1981): the homology of *H. annuus* with other annual species is generally high, although the ΔTm observed with *H. petiolaris* and with *H. niveus* is important. *H. argophyllus* and *H. annuus* are very similar on the basis of their EcoRI and BamHI restriction patterns, but they can be differentiated by the ΔTm test. In opposition, the perennial species *H. tomentosus* has a very complex restriction pattern, but is very close to *H. annuus* on the basis of the ΔTm. The ΔTm increases with more distant species (divaricati and microcephali series) and is particularly high with *H. porteri* (ecotype 676): this species had formerly been classified in the genera *Rudbeckia, Gymnolomia* or *Viguiera*, and was more recently proposed as a species of the genus *Helianthus* (Yates and Heiser 1979).

The length variations observed in the rDNA of the genus *Helianthus* occur by increments of 200 bp. In at least 27 ecotypes, the BamHI digests display multiple bands of 400 bp (Table 3). The existence of 200 bp segments is suggested by molecular weight calculations but could not be demonstrated, probably because they have only little homology with *H. annuus* rDNA. Nucleotide mismatches demonstrated by thermal melting experiments indicate that these fragments have about a 1%–5% sequence divergence from those of *H. annuus* (taking the figure of 1% mismatch per °C of ΔTm; Bonner et al. 1973). They are probably species specific, as

was shown for the family of cruciferae (Tremousaygue et al. 1988).

It is thus apparent from these observations that structural variations observed at the level of the ribosomal spacer are fairly well correlated with the systematic classification in the genus *Helianthus*. However, within the species *H. annuus* itself, the genetic distances estimated by biometric analysis (as differences of allele frequency distributions between lines) do not correlate with the few restriction and structural changes shown at the level of the ribosomal genes. Genes evolving more rapidly than rDNA spacers should thus be analyzed in order to establish such correlations.

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