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T. Cuéllar · J. L. Bella · E. Belhassen

Intra-individual heterogeneity of rDNA allows the distinction between two closely related species in the Genus *Helianthus*

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Abstract The Ribosomal DNAs of *Helianthus annuus* and *H. argophyllus* were analysed. Total DNA from single individuals of six cultivated lines, one wild ecotype of *H. annuus*, and three ecotypes of *H. argophyllus*, were digested with various restriction enzymes. Hybridisation of Southern blots with sunflower ribosomal probes containing most of the interspacer regions (R3) or the 25 *s* coding region (R2) reveals different patterns from those expected: while no difference between *H. annuus* and *H. argophyllus* had been observed in previous rDNA RFLP analysis, our study clearly distinguished the two species on the basis of two different patterns when using R3 and *Bam*HI, *Bst*YI, or *Eco*RI/*Bam*HI. Furthermore, the sum of the fragment weights of the *Bam*HI restriction patterns was much greater than that of the rDNA entire unit-weight space. The co-existence of different rDNA units within single individuals is proposed as a model to explain these results. Four rDNA units were distinguished, which differed in their state of methylation and by the presence of mutations at two *Bam*HI restriction sites. *H. annuus* individuals displayed two types of rDNA units while *H. argophyllus* individuals displayed four types.

Key words Ribosomal DNA · Polymorphism · Methylation · *Helianthus annuus* · *Helianthus argophyllus*

Introduction

The analysis of genetic variability using molecular markers in plant genetics and breeding allows a better definition of the phylogenetic relationships between species. Such information can be of practical use in interspecific introgression programs. The ribosomal RNA genes (rDNA) have provided molecular markers for taxonomy, introgression and plant breeding studies (e.g. Guadet et al. 1989; Reiseberg et al. 1990; Belhassen et al. 1994).

The rDNA regions exist in tandemly repeated multiple copies. The 18*s*, 5.8*s* and 25*s* subunits, transcribed as a single RNA molecule, are separated by a transcribed intergenic spacer (IGS) and a short non-transcribed spacer (NTS). Variability of rDNA units exists among and within species and is generally due to punctual mutations, and or insertion/deletion events, in the IGS and NTS. The coding regions are considered to be stable in comparison with the non-coding regions (Rogers and Bendich 1987).

Sunflower (*Helianthus annuus* var. *macrocarpus*) is a crop which belongs to a complex of 62 species (Schilling and Heiser 1981)). The *annuii* section contains 14 annual diploid species including *H. annuus* var. *macrocarpus*, the sunflower, and *H. argophyllus*, its closest relative. This latter species is widely studied in plant breeding because of its drought-tolerance characteristics. The RFLP comparisons of rDNA between *H. annuus* and *H. argophyllus* did not result in their distinction (Choumane and Heizmann 1988; Rieseberg et al. 1990). More recently, two different RFLP patterns for *H. annuus* and *H. argophyllus* were observed (Belhassen et al. 1994). However, in the latter study the total fragment weight of the RFLP patterns for both species was unexpectedly greater than the total rDNA unit weight.

In the present paper, we show that the existence of rDNA differences between these two closely related species results from a combination of the presence or absence at two *Bam*HI restriction sites in the rDNA unit, due to methylation and mutation events. The co-existence of four types of rDNA units within a single individual could explain these observations. The two closely related species can be distinguished on the basis

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T. Cuéllar · J. L. Bella
Departamento de Biología. Unidad de Genética. Facultad de Ciencias, Universidad Autónoma de Madrid, E-28049. Spain

E. Belhassen (✉)
Génétique et Amélioration des Plantes, INRA. 2, Place Viala, F-34060 Montpellier. France

of the different extent of their intra-individual heterogeneity.

Materials and methods

Genetic material

Cultivated lines RHA-274, RHA-265, AL-5–4, 89-B1, 89-B2, and HA-89, as well as a wild population from *H. annuus annuus* (ecotype 376 from the INRA *Helianthus* collection), were used, although most of the analysis was carried out with the HA-89 line employed in previous studies (Choumane and Heizmann 1988; Rieseberg et al. 1990; Belhassen et al. 1994). Three ecotypes of *H. argophyllus* were used (HAR-585, 92 and 93 from the INRA *Helianthus* collection). A minimum of five individuals per genotype were sampled and analysed.

RFLP experiments

DNA extraction and analyses were performed from leaves of single adult individuals using the method described by Dellaporta et al. (1983), modified by adding 1% soluble polyvinylpyrrolidone (PVP), 1% polyethylene glycol (PEG) and 0.2% β-mercapthoethanol to the grinding buffer. After the first isopropanol precipitation the DNA was recovered with a Pasteur pipette instead of by centrifugation. A further sample of DNA from the HA-89 inbred line, kindly provided by Dr L. H. Rieseberg, was used as a control.

Restriction-enzyme digestions (with *Bam*HI, *Bst*YI, *Eco*RI, *Hin*dIII, *Sac*I) were carried out following the manufacturer's (Boehringer-Mannhein) protocols. Spermidine was added to the digestion medium to a final concentration of 4 μM. Five to ten units of enzyme per μg of DNA were used for each standard digestion of 10 μg of total DNA. Double digestion (*Eco*RI-*Bam*HI) were made with equal amounts of the two enzymes, using the most efficient buffer following the manufacturer's recommendations. As a control, some digestions were conducted with an excess of restriction enzymes (15–25 units/μg of DNA).

Alkaline Southern transfers to Amersham nylon N membranes were carried out following the capillarity protocol of the supplier. Hybridisation was performed with two sunflower probes (kindly provided by Dr. P. Heizmann), which correspond to almost the entire 25*s* coding region plus a small part of the 18*s* rDNA (R2; 3.7 kb) and to the major part of the IGS spacer (R3; 3.7 kb) (see Fig. 1). These probes were labelled by random priming with ^{32}PdCTP (Boehringer-Mannhein kit). Hybridisations were respond in Denhardt's mixture under high-stringency conditions (2 × SSC, 65 °C). Membranes were washed twice in 0.1% SDS in 2 × SSC for 20 min at 65 °C, followed by 0.1% SDS in 0.5 × SSC at 65 °C for 20 min.

Results and Discussion

H. annuus

Results were identical for all individuals from the seven cultivated and wild *H. annuus* genotypes analysed, and for the sample of DNA from the HA-89 line provided by Dr. L.H. Rieseberg.

Hybridisation with R3 (IGS). The *Bam*HI restriction pattern of the total DNA obtained when hybridised with R3 gave two fragments of 4.7 kb and 5.7 kb (Table 1a; Fig. 2) which appeared in equal stoichiometry (Figs. 2 and 3). The total weight of the two fragments was 10.4 kb, which is close to the 9.8-kb weight of the total rDNA unit (Choumane and Heizmann 1988). Excess of *Bam*HI (up to 25 units/μg total DNA) did not suppress the additional 5.7-kb fragment (Fig. 3). This fragment has a similar weight to that of a B1–B3 fragment.

The digestion with *Hin*dIII, for which a unique restriction site exists in the ribosomal unit (Choumane and Heizmann 1988), gave rise to the expected 9.8-kb frag-

Table 1 Expected (following the restriction map of Choumane and Heizmann; 1988) and observed fragment weights (in kb) of (a) R3-hybridised total DNA of *H. annuus* (bold characters indicate unexpected fragments) and (b) of R2-hybridised total DNA of *H. annuus* (bold character indicate unexpected fragments)

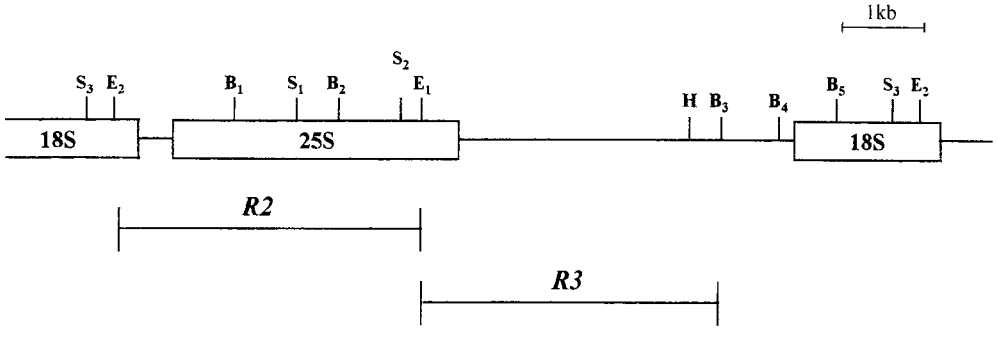
a

Enzyme	Expected fragment weights	Observed fragment weights
*Bam*HI	4.7	**5.7** + 4.7
*Hin*dIII	9.8	9.8
*Eco*RI	6.1	6.1
*Sac*I	6.1	6.1
*Bam*HI/*Eco*RI	3.7	3.7

b

Enzyme	Expected fragment weights	Observed fragment weights
*Bam*HI	4.7 + 2.5 + 1.2	**5.7** + 4.7 + 2.7 + 1.2
*Eco*RI	3.7	3.7
*Sac*I	2.5 + 1.6	2.5 + 1.6
*Bam*HI/*Eco*RI	1.6 + 1.2 + 1.0	**2.4** + 1.6 + 1.2 + 1.0

Fig. 1 Partial restriction map on the rDNA unit of *H. annuus* HA-89 (derived from Choumane and Heizmann 1988). Localisation of the probes used: R2 is an *Eco*RI/*Eco*RI fragment of 3.7 kb containing almost the entire 25*s* coding region; R3 is an *Eco*RI, *Bam*HI fragment of 3.7 kb containing the major part of the interspacer. These probes were kindly provided by Dr. P Heizmann B = *Bam*HI E = *Eco*RI S = *Sac*I H = *Hin*dIII



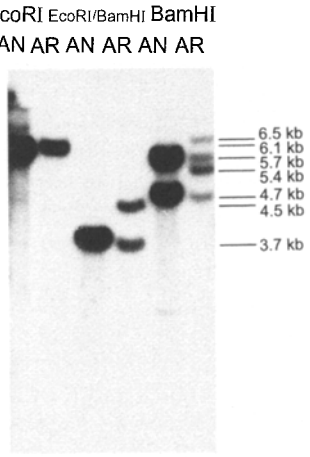


Fig. 2 Restriction patterns of *H. annuus* (*AN*) and *H. argophyllus* (*AR*) using *Bam*HI, *Eco*RI and both enzymes Southern blots of total DNA were hybridised with an R3 probe

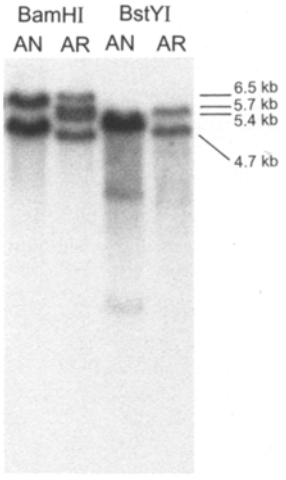


Fig. 5 Comparison of *Bam*HI and *Bst*YI restriction patterns of *H. annuus* (*AN*) and *H. argophyllus* (*AR*). Southern blots of total DNA were hybridised with an R3 probe

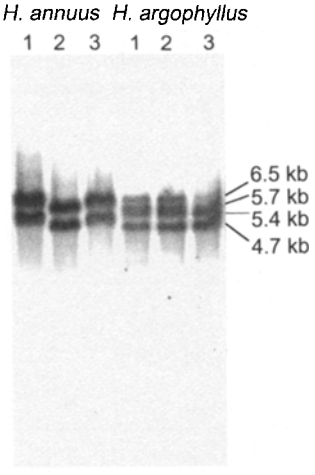


Fig. 3 Test of over-digestion with *Bam*HI Total DNA of *H. annuus* and *H. argophyllus* was digested with 5 (*1*), 15 (*2*) or 25 (*3*) units of enzyme/μg DNA. Southern blots of total DNA were hybridised with an R3 probe

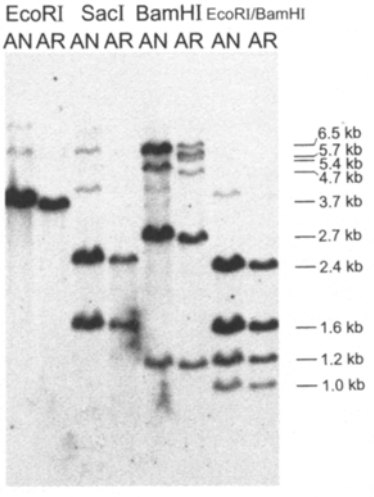


Fig. 6 Comparison of *Bam*HI, *Sac*I and *Eco*RI restriction patterns of *H. annuus* (*AN*) and *H. argophyllus* (*AR*). Southern blots of total DNA were hybridised with an R2 probe. Double digestions were obtained with *Eco*RI and *Bam*HI

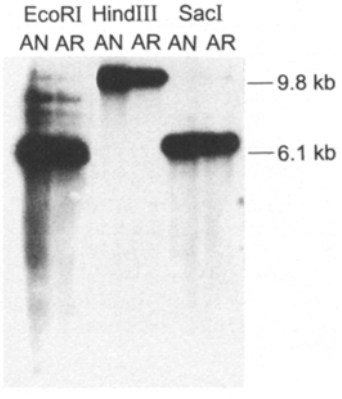


Fig. 4 Comparison of *Hin*dIII. *Sac*I and *Eco*RI restriction patterns of *H. annuus* (*AN*) and *H. argophyllus* (*AR*). Southern blots of total DNA were hybridised with an R3 probe

ment (Fig. 4; Table 1a) which corresponds to the entire unit weight. When total DNA was digested with *Eco*RI or *Sac*I, the single fragments detected were of the respective expected weights (6.1 kb each) in agreement with Choumane and Heizmann (1988) (Fig. 4; Table 1a). Double digestion with *Bam*HI and *Eco*RI gave the expected 3.7-kb fragment that presumably resulted from the restriction of the E1–B3 sites (Fig. 2, Table 1a). The *Bst*YI restriction pattern of *H. annuus* DNA displayed only one 4.7-kb fragment (Fig. 5) in comparison with the two observed with *Bam*HI.

Hybridisation with R2 (25*s*). The *Bam*HI-restricted total DNA hybridised with R2 displayed fragments of 5.7, 4.7, 2.7 and 1.2 kb (Table 1b; Fig. 6) in all genotypes tested. The total weight of the fragments was 14.3 kb, which is considerably larger than the total rDNA unit weight.

The digestion with *Eco*RI or *Sac*I yielded the expected fragments [3.7 kb (E2–E1) and 2.5 kb (S3–S1) + 1.6 kb (S1–S2), respectively; Fig. 6, Table 1 b)]. Double digestions with *Bam*HI and *Eco*RI enzymes show the three expected fragments plus an extra 2.4-kb fragment (Table 1b, Fig. 6), which is the same size as a B1–E1 fragment.

Table 2 Expected (following the restriction map of Choumane and Heizmann: 1988) and observed fragment weights (in kb) of R3-hybridised total DNA of *H. argophyllus* (bold characters indicate unexpected fragments) and (b) of R2-hybridised total DNA of *H. argophyllus* (bold characters indicate unexpected fragments)

a

Enzyme	Expected fragment weights	Observed fragment weights
*Bam*HI	4.7	**6.5** + **5.7** + **5.4** + 4.7
*Hin*dIII	9.8	9.8
*Eco*RI	6.1	6.1
*Sac*I	6.1	6.1
*Bam*HI/*Eco*RI	3.7	**4.5** + 3.7

b

Enzyme	Expected fragment weights	Observed fragment weights
*Bam*HI	4.7 + 2.6 + 1.2	**6.5** + **5.7** + **5.4** + 4.7 + 2.7 + 1.2
*Eco*RI	3.7	3 7
*Sac*I	2.5 + 1.6	2.5 + 1.6
*Bam*HI/*Eco*RI	1.6 + 1.2 + 1.0	**2.4** + 1.6 + 1.2 + 1.0

The results of hybridization with R3(IGS) or R2 (25*s*) demonstrate the existence of additional fragments after *Bam*HI or *Bam*HI-*Eco*RI digestions of the *H. annuus* ribosomal unit. Their origin is not due to insertion events, as shown by other restriction enzyme patterns.

H. argophyllus

Results were identical for all individuals of the three ecotypes of *H. argophyllus* analysed.

Hybridisation with R3 (IGS). The *Bam*HI restriction pattern of the total DNA obtained when hybridised with R3 consisted of four fragments of 4.7, 5.4, 5.7 and 6.5 kb (Table 2a; Fig. 2) for all genotypes tested, as described in our previous study (Belhassen et al. 1994). The four fragments are in a comparable stoichiometry. Their total weight (22.3 kb) was considerably greater than the total rDNA unit weight. Excess *Bam*HI digestion (up to 25 units/µg total DNA) did not suppress any of the four fragments (Fig. 3). The four fragments have similar molecular weights to the B2–B3, B2–B4, B1–B3 and B1–B4 fragments, respectively.

The digestion with *Hin*dIII yielded the expected entire unit fragment weight (9.8 kb, Fig. 3, Table 2a). Comparable results to those of *H. annuus* were obtained when total DNA was digested with *Eco*RI and *Sac*I. The single fragments detected were of the expected respective weight (6.1 kb, Fig. 4).

Unlike the situation with *H. annuus*, *Bam*HI/*Eco*RI double digestions allow the detection of an additional 4.5-kb fragment (Fig. 2, Table 2a). The two species are easily distinguishable using this double digestion. The *Bst*YI restriction pattern of *H. argophyllus* DNA displayed only two fragments (4.7 and 5.4 kb, Fig. 6) in comparison with the four observed with *Bam*HI. This latter enzyme also permits the differentiation of these two species.

Hybridisation with R2 (25*s*). The *Bam*HI restriction pattern obtained when hybridised with R2 comprised six fragments of 6.5, 5.7, 5.4, 4.7, 2.7 and 1.2 kb (Table 2b; Fig. 6). giving a total weight of 26.2 kb which is much greater than the total rDNA unit weight. The digestion with *Eco*RI and *Sac*I yielded fragments of 3.7, 2.5 and 1.6 kb respectively (Fig. 6, Table 2b). Double digestion with *Bam*HI and *Eco*RI gave the three expected bands together with an extra 2.4-kb band (Table 2b, Fig. 6), this latter being the same size as a B1–E1 fragment (as observed in *H. annuus*).

The results from *H. argophyllus* show the existence of additional fragments after *Bam*HI or *Bam*HI-*Eco*RI digestion of the ribosomal unit. In each case, the RFLP patterns of *H. annuus* were included in those of *H. argophyllus*. The differences detected between the restriction patterns of the two species were always due to extra fragments found solely in *H. argophyllus*.

Conclusion

Choumane and Heizmann (1988) and Rieseberg et al. (1990) described the rDNA unit of several species of the genus *Helianthus*. In both studies, RFLP analysis of genomic DNA with *Bam*HI/R3 did not allow the two closest related species of the genus, *H. annuus* and *H. argophyllus*, to be distinguished. In the present study, we have found clearly repeatable differences in their RFLP patterns. While two fragments are found in *H. annuus*, *H. argophyllus* presents four fragments (Fig. 2; Tables 1a and 2a).

The single *Bam*HI/R3 4.7-kb fragment observed by Choumane and Heizmann (1988) and Rieseberg et al. (1990) was also present in both species in our results. The other fragments observed in all the genotypes analysed (with a total of more than 100 individuals) were always present in a similar stoichiometry. The total weight of all *Bam*HI fragments was greater than that of the entire unit (9.8 kb; op. cit) with both R2 and R3. However, the additional fragments were not generated by insertion events, since the *Hin*dIII restriction pattern shows the entire unit weight of rDNA to be conserved (Fig. 4). The possibility of additional fragment generation through partial digestion was rejected because massive concentrations of enzymes did not produce different patterns (Fig. 3). The results obtained with a sample of the total DNA of the HA-89 line donated by Dr. L. H. Rieseberg also demonstrate that distinct extraction methods cannot account for the differences that we observe. These differences could arise from yet unexplained events during digestion (such as the use of spermidine in our digestion medium) and/or hybridisation. Another explanation would be that the Choumane and Heizmann

RFLP map was constructed with cloned cDNA consisting of the A unit and thus did not allow the observation of intra-individual heterogeneity (see below).

In *H. annuus*, the 5.7-kb additional fragment was larger than the "classical" 4.7-kb fragment after R2 and R3 Southern hybridisation (Table 1a, b). Since the B1 and B2 *Bam*HI sites (Fig. 1) are separated by 1.2 kb, it is possible that the B2 site was not digested. This hypothesis was confirmed by the double digestion. The *Bam*HI/*Eco*RI total DNA digestion and hybridisation with R2 showed an additional 2.4-kb band which corresponds to the molecular weight of a B1–E1 fragment (Fig. 1). To explain the simultaneous existence of the two fragments in the *Bam*HI/R3 RFLP patterns of single *H. annuus* individuals, two types of rDNA units must co-exist: those with the B2 site (unit A, Fig. 7) and those without it (unit B, Fig. 7).

*Bam*HI/R3 RFLP patterns of *H. argophyllus* also displayed the two fragments found in *H. annuus*. These results are consistent with the hypothesis proposed above for *H. annuus* rDNA. The two extra fragments (5.4 and 6.5 kb) are about 0.7 kb larger than those of *H. annuus* (4.7 and 5.7 kb). Since the *Bam*HI sites B3 and B4 (Fig. 1) are separated by 0.65 kb, we can assume that the B3 site had not been digested. This hypothesis was confirmed by the double digestion. The *Bam*HI/*Eco*RI patterns of *H. argophyllus* (using R3 as a probe) showed an additional 4.5-kb band which must correspond to the molecular weight of a E1–B4 fragment (Fig. 1). To explain the simultaneous existence of the four fragments in the *Bam*HI/R3 RFLP patterns of *H. argophyllus*, there must be four types of co-existing rDNA unit (Fig. 7):

unit A: B2 and B3 are digested
unit B: B2 is not digested
unit C: B3 is not digested
unit D: B2 and B3 are not digested.

The similar stoichiometry suggests that these four units are present in similar quantities (Figs. 2, 3 and 5). The variation of the B2 and B3 restriction sites can be explained by mutations or methylations. As indicated above, the possibility of noticeable insertion/deletion events generating this hetrogeneity was discarded because the entire unit weight did not change.

*Bst*YI shares with *Bam*HI an identical target sequence for the four central bases. The sequence cut by the restriction enzyme *Bam*HI (G↓ GATC*C) is included in those cut by *Bst*YI (Pu↓GATCPy). However, an inhibition of cleavage by 5-methylcytosine occurs in *Bam*HI (indicated by C*), whereas methylation does not influence *Bst*YI cleavage. The digestion of total DNA with *Bst*YI helps the methylation and mutation hypotheses to be distinguished in *H. argophyllus*. The *Bst*YI pattern displayed two fragments (4.7 and 5.4 kb, Fig. 6) when hybridised with R3; if B3 were methylated, a single 4.7-kb fragment pattern would have been found with *Bst*YI. The two fragments observed must correspond to the fragments B2–B3 of unit A and B2–B4 of unit C. The presence of the B2–B4 fragment indicates that the B3 site is not cut by *Bst*YI. Thus, the B3 site must be modified by mutation rather than methylation. Definitive evidence will be provided by sequence comparisons of different 25*s* units. The 5.7-kb additional fragment observed with *Bam*HI in *H. annuus* disappeared with *Bst*YI. In our model, it could be interpreted as methylation at a number of ribosomal units at the cytosine of the B2 site, which inhibits digestion with *Bam*HI but not with *Bst*YI (unit B, Fig. 7). This would also account for the absence of the 5.7-kb fragment in the *H. argophyllus Bst*YI pattern. However, we cannot discard transitions in the external nucleotide positions of this *Bam*HI site (B2) which may also explain this observation.

The stability of patterns observed here has been tested among generations. Interspecific crosses of *H. argophyllus* with *H. annuus* yielded F_1 individuals that

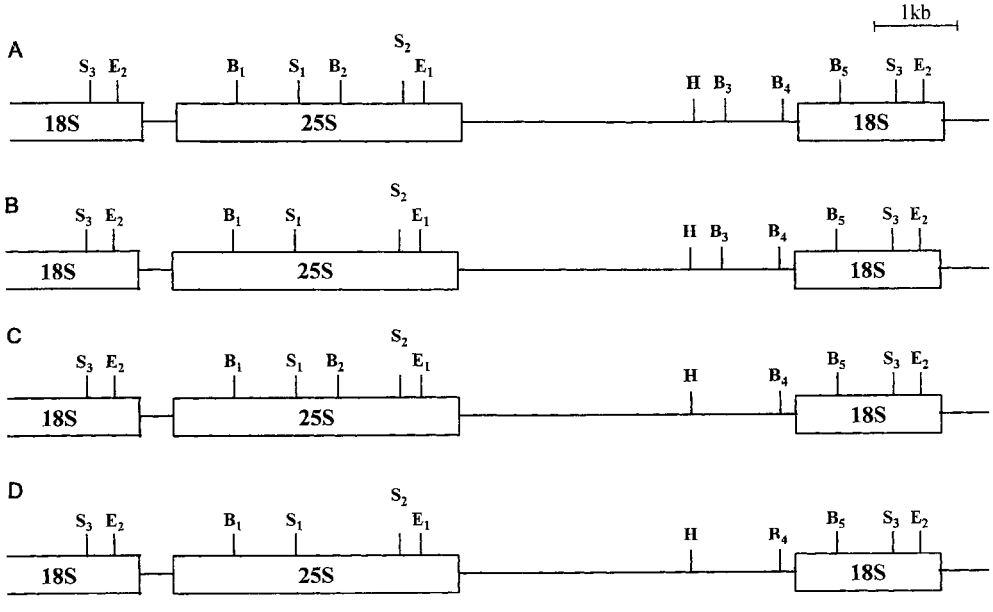


Fig. 7 Intra-individual variability model of the rDNA of *H. annuus* and *H. argophyllus*. Four rDNA units differing for B2 (which can be methylated) or B3 (whose sequence can be modified) *Bam*HI sites In *H. annuus*, the **A** and **B** units co-exist. In *H. argophyllus*, **A**, **B**, **C** and **D** units co-exist

showed all four *Bam*HI/R3 RFLP bands of *H. argophyllus*. As would be expected for simple mixing of the two genotypes, there was a higher stoichiometry of the two bands which the species share (data not shown). A backcross with *H. annuus* as the recurrent parent showed Mendelian segregation with a 50% of *H. annuus*-type patterns and 50% F_1 patterns. Stability among organs was also investigated. DNA from seedling cotyledons and bracts presented similar patterns to those observed in adult leaves (data not shown).

Our interpretation of the present results involves the co-existence of several ribosomal units in the same individual (two units for *H. annuus* and four units for *H. argophyllus*). Variability of the *Eco*RI restriction rDNA sites has been noticed in the HA-89 line; however, this heterogeneity only involves 5% of the rDNA (Choumane and Heizmann 1988). In *Raphanus* sp., heterogeneity in 18*s* and 25*s* ribosomal subunits was described for a higher proportion of units; the four *Eco*RI and the single *Bam*HI restriction sites being variable. The hypothesis of the co-existence of four major ribosomal units was also proposed, although it was not possible to distinguish between mutation and methylation events as the mechanism generating the different unit types (Delseny et al. 1983). Further analysis demonstrated that *Eco*RI sites were mutated, while *Bam*HI ones were methylated (Delseny, personal communication). It is noteworthy that the localisation of the varibale *Bam*HI site is the same as that of *Helianthus* (B2 site; Fig. 1). This site has also been observed to be variable in three *Brassicae* species (Delseny et al. 1990). The other *Bam*HI restriction sites of the coding subunits (B1 and B5) are relatively constant in several angiosperm families (e.g. Doyle and Beachy 1985; Takiawa et al. 1985; Jorgensen et al. 1987; Faivre-Rampant et al. 1992; Santoni and Berville 1992), except in some monocotyledons (maize, sorghum and sugarcane) (Springer et al. 1989) in which B1 is missing. Sequence analysis of three domains of the 25*s* ribosomal subunit demonstrates the existence of variable 'hot spots' inside the coding regions in many plant and animal species (Guadet et al. 1989; Kolosha and Fodor 1990; Michot et al. 1990).

Ribosomal RNA genes are present in multiple copies to support high rates of transcription. This redundancy would allow mutations causing functional inactivation to persist. On the other hand, the overall excess of the ribosomal cistrons makes the regulation of their activity necessary. Gene methylation is one of the processes involved in this (Martienssen and Richards 1995). Variation in the molecular organisation of rRNA genes may be associated with an adaptative advantage in higher plants (Flavel et al. 1986; Allard et al. 1990; Rocheford 1994). *H. annuus* has about 7000 copies of the rDNA unit per cell (Choumane 1988) and it has been estimated that only some 300 copies of the rDNA genes are transcriptionally active in plants (Lapitan 1992). The intra-individual heterogeneity found in *H. annuus* and *H. argophyllus* may be involved in the regulation of rDNA. Chromosomal 'in situ' hybridisation with a rDNA probe revealed the existence of three pairs of chromosomes bearing rDNA clusters in *H. annuus* and *H. argophyllus*. However, the silver staining of mitotic chromosomes of meristem cells shows these six NORs always to be active during the preceding interphase (Cuéllar et al. 1996), indicating that this heterogeneity does not inactivate any NORs.

The results presented in the present paper are consistent with a model wherein intra-individual heterogeneity for the rDNA units is generated by modification of a *Bam*HI restriction site in the 25*s* coding region, combined with a mutation of another *Bam*HI restriction site in the IGS. This latter modification is a further case of variability in the interspacer region of the rDNA units. The variability reported here allows the distinction of the two most closely related *Helianthus* species by RFLP analysis. These markers will be of use in introgression programs using *H. argophyllus* wild species as a drought-tolerant parent (Belhassen et al. 1995).

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