Theor Appl Genet (1988) 76:673-680

Structure of melon rDNA and nucleotide sequence of the 17-25S spacer region

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Received March 24, 1988; Accepted April 16, 1988
Communicated by H. F. Linskens

Summary. Restriction enzyme and hybridization analysis of melon nuclear DNA suggests a homogenous rDNA population with a repeat unit of 10.2 kb. Several full length Hind III rDNA repeat units were cloned and one of these is described in detail. The regions coding for 25S, 17S and 5.8S rRNAs were located by crossed-contact hybridization and R-loop mapping. Introns were not observed. The nucleotide sequence of the internal transcribed spacer and flanking regions was determined and compared with the corresponding region from rice rDNA by dot matrix analysis. In addition, the extent of gross sequence homology between cloned melon and pea rDNA units was determined by heteroduplex mapping.

Key words: Melon – Ribosomal DNA – R-loops – Heteroduplex analysis – 17-25S spacer sequence

Introduction

In eukaryotes, the genes for cytoplasmic ribosomal (r) RNA are present in multiple, tandemly repeated copies clustered at one or a few nucleolus organising regions of specific chromosomes. Many species of higher plants devote large proportions of their genomes (up to about 7% in cucumber) to coding for rRNA (Kavanagh and Timmis 1986).

It is likely, from measurements of transcription and accumulation rates and methylation patterns, that many rRNA genes are not transcriptionally active. Nevertheless, the nucleotide sequence of the regions of the repeat unit that code for mature rRNA products (25S, 17S and 5.8S rRNAs) are highly conserved in plants within the individual, the species and the plant kingdom as a whole (Ingle et al. 1975). This implies that a strong selection pressure acts to conserve these regions of the gene and suggests that each individual gene may be transcriptionally active at some stage of the plant life cycle, or that members of this multigene family have somehow evolved in concert (Dover and Flavell 1984). The non-transcribed and transcribed spacer regions of rDNA are, however, more variable. Information derived from cloned rDNAs indicates an unexpectedly large amount of sequence heterogeneity both within and between species.

In plants, repeat unit sizes range from 7.8 kb in soybean (Varsanyi-Breiner et al. 1979) to 18.5 kb in *Trillium kamschaticum* (Yakura et al. 1983). Length heterogeneity is present within many species (Yakura et al. 1983; Gerlach and Bedbrook 1979; Siegel and Kolacz 1983; Waldron et al. 1983; Kavanagh and Timmis 1986) but is apparently absent from others (Maggini and Carmona 1981; Goldsbrough and Cullis 1981; Rafalski et al. 1983; Varsanyi-Breiner et al. 1979). The origin of rDNA length heterogeneity has been examined in several plant species (Yakura et al. 1983; Siegel and Kolacz 1983; Kavanagh and Timmis 1986). This is of particular interest because of the highly redundant nature of plant rRNA genes and has implications for the control of gene expression and more generally for the evolution of rDNA sequences. Why some plants should maintain a heterogeneous population of rRNA genes while others contain only a single form of the repeat unit is unknown.

The following communication reports the cloning of rDNA repeat units from melon (*Cucumis melo*), a plant with an apparently homogeneous rDNA population that maintains a high redundancy of rRNA genes. Both the coding and non-coding regions of the repeat unit have been compared with those of pea by electron microscope

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analysis of heteroduplexed molecules and the nucleotide sequence of the 17S–25S spacer region has been compared with that of rice (Takaiwa et al. 1985).

Materials and methods

Preparation of nucleic acids

Honeydew melon (*Cucumis melo*) nuclei were isolated from 4-week-old cotyledons (Matsuda and Siegel 1967) and nuclear DNA prepared as described previously (Gross-Bellard et al. 1973). The DNA (200 µg) was digested to completion with Hind III and fractionated on isopycnic CsCl gradients (Ingle et al. 1975). Pooled gradient fractions enriched for rDNA sequences were identified by agarose gel electrophoresis, ethanol precipitated and further enriched for rDNA sequences by fractionation on stepped 10%–30% sucrose density gradients (Kavanagh and Timmis 1986). Marrow rRNA was prepared according to Lizardi and Engelberg (1979) and digested with RNAase-free DNAase I. Individual rRNA species were further purified by sucrose density gradient centrifugation (Kavanagh and Timmis 1986).

Radioactive labelling of rRNA

Ribosomal RNAs were labelled in vivo by culturing artichoke explants in culture medium containing ^{32}P-orthophosphate (Ingle et al. 1975). Purified rRNAs were labelled in vitro according to Maizels (1976).

Molecular cloning of rDNA

Gradient enriched Hind III repeat units of melon rDNA were ligated with Hind III digested, phosphatased pACYC184 (Maniatis et al. 1982). Competent *E. coli* cells were prepared and transformed as described in Maniatis et al. (1982). Positive clones were identified by colony hybridization (Thayer 1979) using in vitro ^{32}P-labelled rRNAs as probes.

Identification of rDNA restriction fragments

Restriction fragments containing rRNA coding regions were identified by filter hybridization (Southern 1975) or by crossed-contact hybridization (Rozek and Timberlake 1979). DNA and RNA samples were fractionated by agarose gel electrophoresis as described in Maniatis et al. (1982).

Electron microscopy

R-loops were formed in PIPES/formamide buffer as described previously (Kavanagh and Timmis 1986). Heteroduplexes were prepared in a final volume of 20 µl containing 10 m*M* Tris (pH 8.4), 1 m*M* EDTA, 0.3 *M* NaCl, 70% formamide and 2.5 µg/ml of Pvu II-linearized pML17 and pHA1. The DNAs were denatured at 75 °C for 15 min and reannealed at 24 °C for 1.5 h (Verbeet et al. 1983). Heteroduplexes were spread on a hypophase of distilled water, adsorbed onto Parlodion coated grids and stained with 0.1% acidic phosphotungstic acid (Thomas 1978). Grids were viewed in a Phillips EM201 electron microscope and analysed as described (Kavanagh and Timmis 1986).

DNA sequencing

DNA fragments were cloned into bacteriophage M13 vectors (Messing and Vieira 1982) and sequenced by the dideoxy chain termination procedure of Sanger et al. (1977).

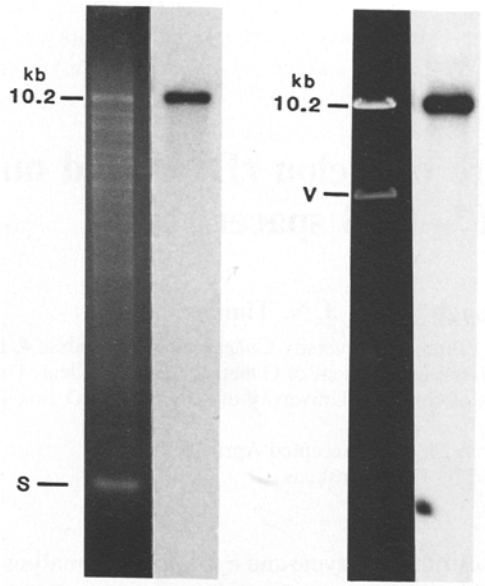

Fig. 1a–d. Comparison of Hind III digested melon genomic and cloned rDNAs. **a** 5 µg melon genomic DNA; **b** 0.25 µg pML17; **b** and **d** Southern transfer of **a** and **c** probed with in vivo ^{32}P-labelled artichoke rRNA and autoradiographed. Fragments were resolved in 0.8% agarose. *V* indicates the pACYC184 vector band. *S* indicates melon satellite DNA sequences

Results

Southern analysis of melon genomic DNA using a range of restriction endonucleases and summation of the molecular weights of restriction fragments hybridizing ^{32}P-rRNA suggested a homogenous rDNA repeat unit population of approximately 10.2 kb (results not shown). A Southern blot of genomic DNA digested with Hind III and probed with ^{32}P-rRNA yielded a single band of 10.2 kb, suggesting that melon rDNA is cleaved once in each repeat unit by this endonuclease (Fig. 1a and b).

Repeat units were cloned into the Hind III site of pACYC184 (Maniatis et al. 1982) using rDNA enriched fractions prepared from Hind III digested melon genomic DNA by centrifugation on neutral CsCl and sucrose density gradients. Clones containing rDNA repeat units were identified by colony hybridization (Thayer 1979). Southern analysis of a Hind III digest of 1 of 33 positive clones obtained, pML17, is shown in Fig. 1c and d.

Digestion of pML17 with Sst II yields six well resolved restriction fragments (Fig. 2a). Fragments coding for the 17S and 25S rRNAs were identified by crossed-contact hybridization with electrophoretically resolved in vivo ^{32}P-labelled artichoke rRNAs and autoradiography (Fig. 2b). Two Sst II fragments of 6.6 and 0.7 kb hybridized exclusively with 17S rRNA and two frag-

ments of 3.7 and 0.9 kb hybridized exclusively with 25S rRNA. A single fragment of 2.1 kb hybridized to both mature rRNAs suggesting that this fragment also contained the 17S–25S internal spacer (IS) region encoding the 5.8S rRNA. This fragment was gel purified, rendered blunt using Klenow polymerase and cloned into pUC19 to facilitate further analysis of the 17S–25S IS region. The resulting clone was designated pMS1.

A restriction map of pML17 was constructed using Sst II and five other restriction endonucleases (Fig. 3a). Coding regions were localized on this map using crossed-contact hybridization data and by R-loop and heteroduplex mapping; pML17 was linearized with Pvu II, which cuts at two sites 400 bp apart in the vector but not in the rDNA insert and hybridized under R-loop conditions with purified marrow rRNAs. Representative R-loops are shown in Fig. 4a and b together with a line drawing interpretation (Fig. 4c). Each contains two uninterrupted R-loops of 3270 ± 190 bp and 1570 ± 140 bp corresponding to the regions encoding 25S and 17S rRNAs, respectively. The IS region containing the 5.8S rRNA gene is approximately 680 ± 102 bp in length.

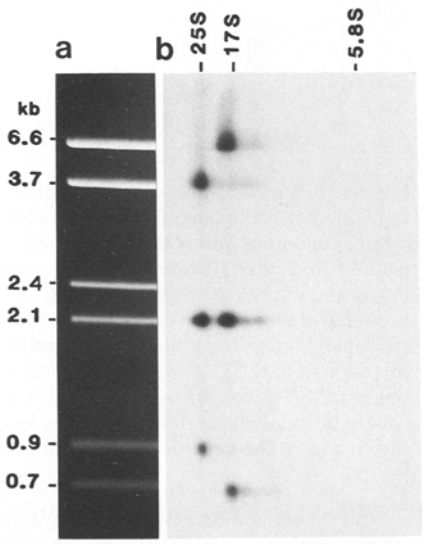

Fig. 2a and b. Crossed-contact hybridization analysis of cloned melon rDNA. **a** pML17 was digested with Sst II and the fragments resolved on a preparative wide-welled 0.8% agarose gel (a narrow section of the gel is shown). Artichoke rRNAs, labelled in vivo with ^{32}P-orthophosphate, were separated on a urea-agarose gel (not shown) and blotted, under conditions allowing hybridization, through a nitrocellulose filter onto which the fragments resolved in gel **a** had been transferred (Southern 1975)

The R-loop data obtained for pML17 corresponds closely with that obtained by Jorgensen et al. (1987) for a cloned Hind III rDNA repeat unit (pHA1) of pea (*Pisum sativum*). The similarities in the physical organization of the rDNA clones from pea and melon prompted an investigation of the extent of gross sequence homology between the two rDNA inserts by electron microscope analysis of heteroduplexed molecules. Both plasmids contain inserts in the same site in pACYC184 and both inserts are in the same orientation. This, combined with the fact that each may be linearized with Pvu II, allowed the use of the entire plasmids in the construction of heteroduplexes. The annealing of the molecules and the subsequent interpretation of electron micrographs was facilitated by the fact that heteroduplexed inserts were flanked by homologous vector sequences (Verbeet et al. 1983).

Representative pHA1:pML17 heteroduplex molecules are shown in Fig. 5a and b together with a line drawing interpretation (Fig. 5c). Nine distinct regions were observed in these molecules consisting of five homologous double-stranded regions and four non-homologous single-stranded regions. Their identity was deduced by comparing the mean lengths of the various regions with their expected counterparts in R-looped molecules of pHA1 and pML17 (Table 1). The two double-stranded ends of the heteroduplex correspond to the long and short Pvu II-Hind III arms of the vector, pACYC184. Located immediately adjacent to each vector arm is a loop consisting of nonhomologous DNA

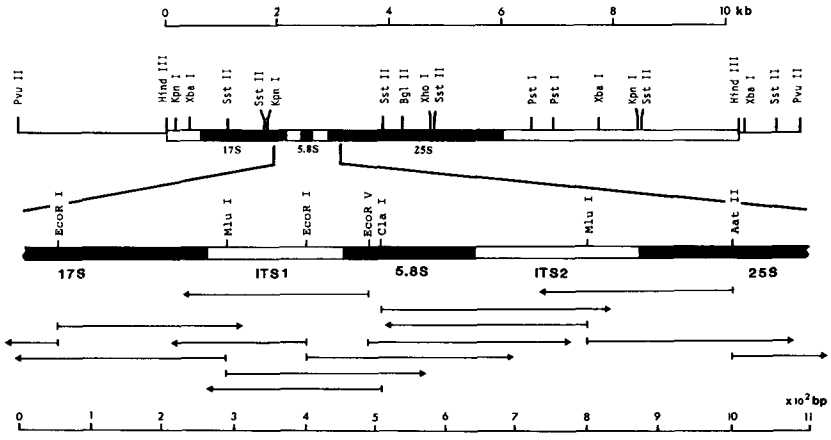

Fig. 3. Restriction map of pML17 and the strategy adopted for sequencing the 17S-25S intergenic region. *Double lines* represent the 10.2 kb melon rDNA insert and *single lines* represent vector sequences. The coding regions (shown in *black*) were assigned using the data derived from crossed-contact hybridization, R-loop and heteroduplex mapping experiments. The *horizontal arrows* indicate the direction and extent of the DNA segments sequenced

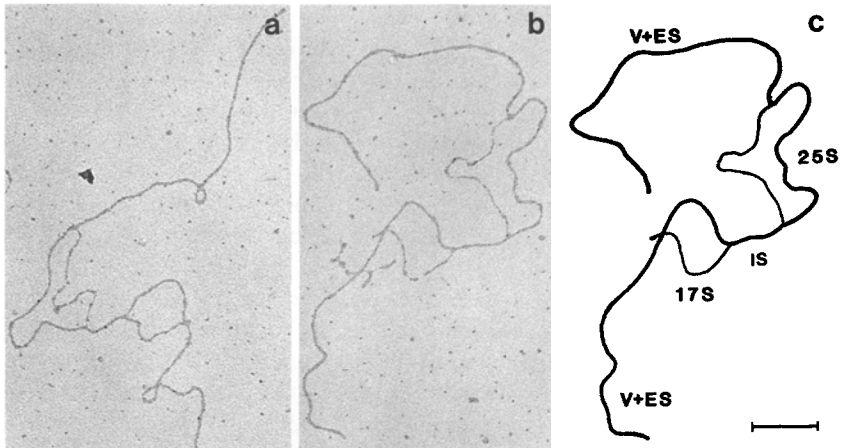

Fig. 4a–c. Electron microscope visualization of R-looped molecules of pML17. pML17 was linearized by digestion with Pvu II and hybridized under R-loop conditions with purified marrow 17S and 25S rRNAs. **a, b** representative R-looped molecules; **c** line drawing interpretation of **b**; *V* vector; *IS* internal spacer; *ES* external spacer; *17S* and *25S:* regions encoding the mature rRNAs. The *bar* in **c** represents 1 kb

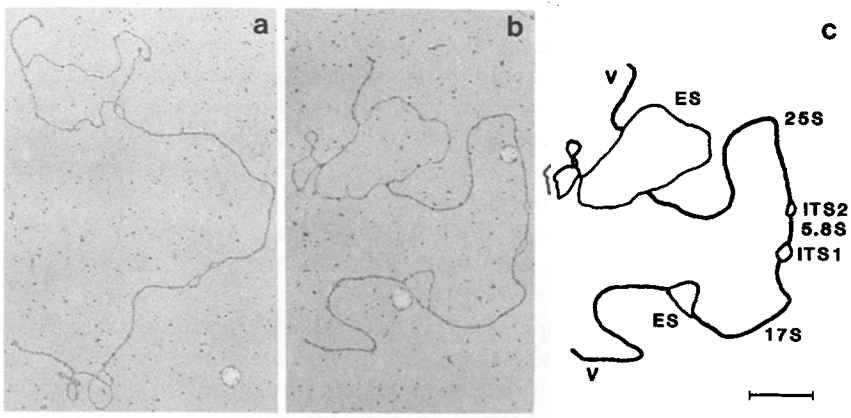

Fig. 5a–c. Electron microscope visualization of heteroduplexs formed between cloned melon and pea rDNA repeat units. pML17 and pHA1 were linearized with Pvu II and hybridized as described by Verbeet et al. (1983). **a, b** representative heteroduplex molecules; **c** line drawing interpretation of **b**; *ITS 1, 2* internal transcribed spacers; *5.8S* region encoding 5.8S rRNA. Other abbreviations as described in the legend to Fig. 3 The *bar* in **c** represents 1 kb

Table 1. A comparison of the mean lengths of duplex and single-stranded regions of pHA1:pML17 heteroduplexes and their counterparts in R-looped molecules. Molecular lengths without standard deviations were calculated by difference. R-loop data for pHA1 is from Jorgensen et al. 1987

Region	Heteroduplex data (bp)		R-loop data (bp)	
			pHA1	pML17
Vector (long arm)	2,830±200		2,730	2,700
ES, pHA1	760± 90		876	—
pML17	450± 90		—	550
17S	1,780±130		1,664±145	1,570±140
ITS 1	240± 50			
5.8S	220± 40	(IS) 690±80	676± 78	680±102
ITS 2	230± 50			
25S	3,600±230		3,484± 88	3,270±190
ES, pHA1	2,520±220		2,216	—
pML17	3,730±260		—	4,280
Vector (short arm)	890± 40		1,000	1,100

ES = external spacer; *ITS* = internal transcribed spacer; *IS* = internal spacer, comprising the region encoding the 5.8S rRNA and ITS1 and 2; *17S* and *25S* = regions encoding the 17S and 25S mature rRNAs, respectively

strands of different lengths. These represent the external spacer regions (ES) of pea and melon rDNA. The lengths of the two major double-stranded regions internal to the ES loops closely correspond to the observed lengths and positions of the 17S and 25S coding regions in R-looped molecules (Table 1). Length discrepancies observed between corresponding regions of R-looped and heteroduplexed molecules reflect the greater stability of rDNA:rDNA over rRNA:rDNA hybrids and the effect of this stability difference on the determination of molecular lengths by electron microscopy.

The region encoding the 5.8S rRNA is clearly visible in heteroduplexed molecules as a short double-stranded region flanked on either side by small non-homologous loops of approximately equal size (the internal transcribed spacers, ITS1 and 2) located between the 17S and 25S genes (Fig. 5). The 5.8S gene, while too small to form a stable rRNA:rDNA hybrid in R-looped molecules, is clearly large enough to form a stable double-stranded DNA region in heteroduplexed molecules. The mean length of this region (220±40 bp) closely matches the length of the 5.8S rRNA gene of yellow lupine (Lupinus luteus), derived from nucleotide sequence analysis (164 bp) (Rafalski et al. 1983).

The data derived from analysis of pHA1:pML17 heteroduplexes suggested very high sequence homology between regions coding for the mature rRNA from disparate plant species and very low homology between the intervening spacer regions, even between the short ITS regions transcribed as part of the precursor rRNA molecule. The nucleotide sequence of the 17S–25S IS region of rice, however, constitutes the only such plant sequence published to date (Takaiwa et al. 1985). We therefore sequenced the corresponding region of melon rDNA in order to determine the extent of sequence homology. The sequencing strategy adopted is detailed in Fig. 3b. Restriction fragments were gel purified from digests of pSM1 and cloned into M13 vectors. All of the sequence, except that at the extreme termini, was confirmed by sequencing in opposite directions. The nucleotide sequence of the 17S–25S IS region is shown in Fig. 6. Regions encoding the 17S, 5.8S and 25S rRNAs were delimited by comparing the melon sequence with that of rice (Takaiwa et al. 1985).

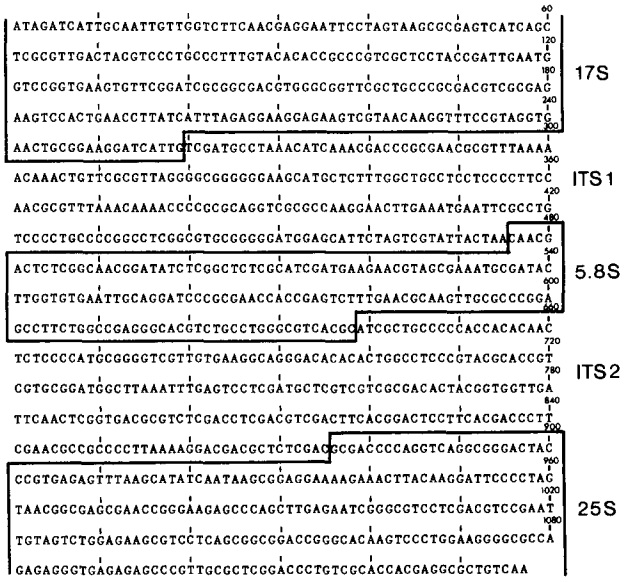

Fig. 6. Nucleotide sequence of the melon 17S-25S IS region. The noncoding strand (RNA-like sequence) is shown. Coding regions are *boxed*

The nucleotide sequence of the melon coding regions shows very high homology with that of rice (Takaiwa et al. 1985): 93% for both the 25S and 5.8S genes and 98% for the 17S coding region. This contrasts markedly with sequence homology in the ITS regions. ITS1 and ITS2 of melon are longer by 24 and 4 nucleotides, respectively, than the corresponding rice sequences with a 'best fit' homology of 40% for ITS1 and 29% for ITS2. This low level of homology is partly due to the large G+C difference between melon and rice ITS sequences. ITS1 and ITS2 of melon have a G+C value of 56% and 60%, respectively, while those of rice have values of 73% and 77%, respectively. These sequence differences are strikingly illustrated when compared by dot matrix analysis (Fig. 7). A positive score, indicated by a dot, is obtained when 14 nucleotides within a scanning 21 nucleotide window are identical. The very high dot density obtained when comparing melon and rice coding sequences contrasts sharply with the very low dot density obtained when the corresponding ITS regions are compared.

Discussion

The regions coding for 17S and 25S rRNAs were initially located on specific restriction fragments of pML17 by crossed-contact hybridization (Rozek and Timberlake 1979). This technique is limited, however, because it does

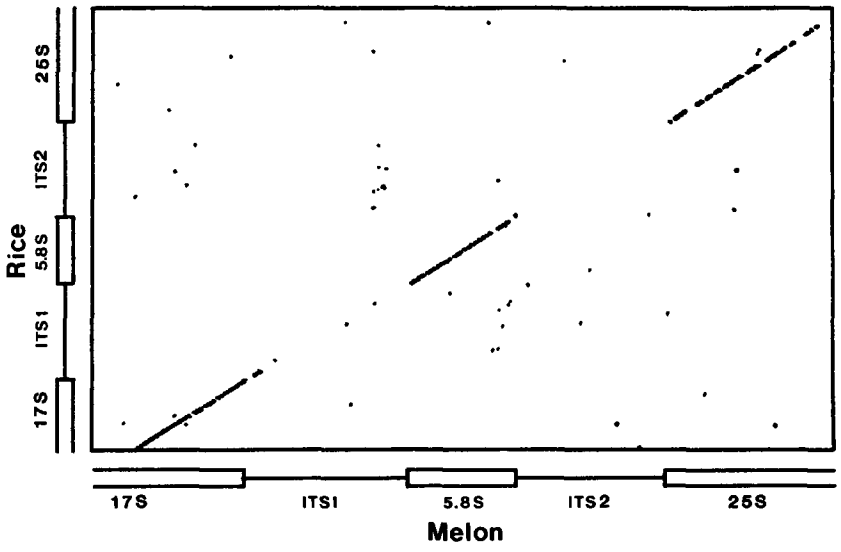

Fig. 7. Dot matrix comparison of the nucleotide sequence of the 17S-25S IS region of melon with the corresponding sequence from rice

not allow precise localization of the termini of the coding sequences, permitting only the identification of restriction fragments that hybridize with mature rRNAs. More precise mapping of coding sequences was accomplished by R-loop analysis (Thomas et al. 1976). In melon and pea (Jorgensen et al. 1987), uniform populations of R-loops were observed ranging from 1570–1660 bp for the 17S and 3270–3480 bp for the 25S coding sequences. Introns were not found in the rDNAs examined here and were also absent from cucumber rDNA (Kavanagh and Timmis 1986). Appels et al. (1980), using uncloned actinomycin D-purified rDNA, obtained very similar results (1550–1610 bp and 3130 bp for the 17S and 25S coding regions, respectively) and Kavanagh and Timmis (1986) measured corresponding R-loops in a heterogeneous population of cucumber rDNA repeat units where the 17S regions ranged from 1450–1620 bp and the 25S regions varied from 3180–3270 bp. All these R-loops were shorter than the length estimates of flax 17S and 25S coding sequences obtained by analysis of S1 nuclease digestion products (1850 bp and 3600 bp, respectively) (Ellis et al. 1984). They were also shorter than the lengths of the duplex regions in pML17:pHA1 heteroduplexes that correspond to the 17S and 25S coding regions. Other investigators have also reported R-loop sizes that are shorter than estimates of the lengths of hybrids obtained by alternative means or direct measurement of rRNAs. For example, White and Hogness (1977) obtained values for the lengths of 18S and 28S R-loops of *Drosophila melanogaster* that were approximately 6% shorter than those based on electrophoretic and hybridization criteria. It has been suggested (Wellauer and Dawid 1977) that the use of duplex DNA as a length standard in such studies may be responsible for the discrepancies. A duplex DNA length standard (ΦX174 RFII DNA) was used in this study. In addition to providing information on both the size and location of coding sequences, R-loop analysis allows determination of the size of both internal and external spacers and the detection of introns. Appels et al. (1980) observed internal spacers of 610–740 bp in R-looped barley rDNA, a range that closely matches the range obtained in this study.

Ribosomal DNA coding regions are among the most conserved genomic sequences with respect to the accumulation of base-pair substitutions. Here, as assessed by EM analysis of R-looped molecules, complete homology was observed between marrow 17S and 25S rRNAs and the corresponding coding regions of melon rDNA. Although short rRNA tails were frequently observed at the 5′ end of the 17S R-loop adjacent to the external spacer, these probably represent branch migration rather than non-homology at this region. Furthermore, a comparison of the nucleotide sequence of the 5.8S gene and the flanking 3′ and 5′ ends of the 17S and 25S coding regions (respectively) of melon rDNA with the corresponding sequences from rice (Takaiwa et al. 1985) extends this general observation of very high sequence homology between the rDNA coding regions of disparate plant species.

Analysis of heteroduplexed molecules of cloned pea and melon rDNAs revealed little or no homology outside the regions coding for the mature rRNAs. Neither the internal transcribed spacers nor the external nontranscribed spacer regions formed stable duplexes in hybrid molecules. Furthermore, a dot matrix comparison of the nucleotide sequence of the internal transcribed spacers of melon and rice (Takaiwa et al. 1985) revealed marked differences in overall sequence and base composition. Comparison of spacer sequences from all but the most closely related species consistently shows that these regions of rDNA repeat units have evolved much more rapidly than the coding regions (Brown et al. 1972; Appels et al. 1986).

Determination of the extent of homology between the external spacers of wheat and rye rDNA by analysis of the melting profiles of homoduplex and heteroduplex molecules (Appels and Dvorak 1982) suggests at least 21% sequence divergence between these two species. The probe used in these experiments consisted of a single 130 bp repeat unit from the external spacer of wheat rDNA. This estimate of sequence divergence is a minimum estimate because the Tm of the hybrid formed between the probe and rye genomic DNA was only 8.5 °C above the hybridization temperature (37 °C) and therefore a significant proportion of poorly matched hybrids were probably not assayed. It would be of interest to examine the extent of sequence homology in these species by electron microscope analysis of heteroduplexed rDNA repeat units.

A realistic claim of length homogeneity for the rDNA of a species requires analysis of a large number of clones such as those included in this work on melon where 33 clones were obtained. Length homogeneity has been suggested, however, for several species, notably flax (Goldsbrough and Cullis 1981), onion (Maggini and Carmona 1981), yellow lupine (Rafalski et al. 1983) and soybean (Varsanyi-Breiner et al. 1979). A more detailed analysis of the apparent homogeneity of the rDNA repeat unit in *Bombyx mori* (Manning et al. 1978) revealed the presence of a minor fraction of repeat units containing introns in the 28S rRNA gene (Beckingham 1982). Length heterogeneity appears to be the rule rather than the exception in plants and in broad bean (Yakura et al. 1984), pea (Jorgensen et al. 1987) and wheat (Appels and Dvorak 1982; Appels et al. 1986; Flavell et al. 1986). The majority of rDNA length variation has been accounted for by the presence of different numbers of subrepeats in the external spacers.

The sequence of the subrepeat is related to the sequence that initiates transcription by RNA polymerase I

(Moss and Birnsteil 1979; Coen and Dover 1982). Hence, despite the fact that spacer sequences have diverged to such an extent that they are largely or completely non-homologous between even closely related species, their molecular organization as internally repetitive subunits is essentially similar. Little homology can be detected between PolI initiation regions of *Xenopus* (Reeder 1984), *Drosophila* (Long et al. 1981) or mouse (Urano et al. 1980). Consistent with the general finding of low homology between species in the intergenic regions is the fact that cell-free extracts from a particular species will only transcribe rDNA from the same species and not that from a different species (Grummt et al. 1982; Miesfeld and Arnheim 1984; Mishima et al. 1982). Surprisingly, a comparison of rDNA promoter sequences in wheat and *Xenopus* has revealed considerable homology between sequence elements located close to the rRNA transcription start site (Flavell et al. 1986).

Acknowledgements. We wish to thank D. Higgins for help with the computer analysis of sequence data.

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