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Ribosomal RNA genes of *Phaseolus coccineus*. I

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Abstract

rDNA fragments, including the whole intergenic spacer (IGS) region of *P. coccineus*, were cloned into dephosphorylated pUC 13 plasmid. Four clones of different insert size were analysed. Restriction patterns and physical maps of these length variants (pPH1, pPH2, pPH5, pPH6) were performed through complete *Eco* RI cleavage and partial digestion with *Hpa* II, *Hae* III, *Sau* 3AI, *Sma* I. Evidence was obtained that the length heterogeneity of the four genes was mainly due to a differing number of about 170 bp sub-repeating elements in the IGS. Indeed, there are 16 of these in pPH1, about 34 in pPH2, 10 in pPH5 and about 60 in pPH6. The sequence analysis of pPH6 sub-repeats revealed that there are two types of sub-repeats: short ones (S) of 162 bp and long ones (L) of 176 bp. The homology between S and L is high (93.8%). S and L elements are present in at least three of the four genes investigated, as shown by a restriction pattern obtained with *Hae* III digestion to completion. The relative frequency of S and L types, however, differs among the four genes. The possible functional meaning of the sub-repeat structure is discussed on the basis of the homology between the S and L sequences on the one hand and on the other the ribosomal sequences of: i) *Xenopus* promoter(s); ii) wheat block A sub-repeats; iii) presumptive promoter(s) of wheat.

Introduction

In previous investigations, Avanzi *et al.* [4] and Durante *et al.* [10] demonstrated that in *Phaseolus coccineus* cistrons coding for 18S and 25S rRNA are located on chromosome pairs I, II and V.

The behaviour of ribosomal cistron (r.c.)-bearing regions has been studied in relation to embryo development by Forino *et al.* [14] who have demonstrated that: (1) some r.c.-bearing chromosome segments (bands) are rarely involved in the nucleolus organization; (2) only certain r.c.-bearing bands are engaged in DNA puffing, a process considered to be the result of a disproportionate local DNA replication (DNA amplification). More recently, the methylation pattern of embryo suspensor polytene chromosomes engaged in nucleolar organization has been studied using the antibodies to 5-methylcytosine (5-mc) [15]. Among other things, the data collected indicate that: (1) 5-mc is not uniformly distributed in r.c.-bearing chromosome regions; (2) undermethylation and transcription are directly interconnected; and (3) only the r.c. of certain bands are engaged in RNA puffing activity

(nucleolus organization) thus supporting the results obtained by Forino *et al.* [14].

The data reported by Forino *et al.* [14] and Frediani *et al.* [15] indicate, therefore, a functional heterogeneity of r.c., an aspect that seems to be of particular interest in view of the fact that ribosomal DNA is structurally heterogeneous in both animal [32, 36, 46] and plant species [2, 11, 18, 20, 42].

Our previous results prompted us to verify the hypothesis of r.c. structural heterogeneity in *P. coccineus*.

Materials and methods

Plant material

200 seeds of *P. coccineus* were germinated and the shoots and roots were used for DNA extraction.

Nucleic acid preparation

Phaseolus coccineus nuclear DNA was extracted and purified as previously reported [24]. Total *Vicia faba* RNA was extracted from the post-mitochondrial fraction of a root homogenate, according to Ritossa and Spiegelman [37]; 18S and 25S rRNA were purified and labelled as previously described [26].

Restriction enzymes, electrophoresis and Southern blot hybridization

Ribosomal DNA restriction patterns, DNA electrophoresis and rRNA/DNA hybridization were performed as previously described [7]. *Hin*d III and *Hin*d III + *Eco* RI restriction fragments of lambda phage DNA and *Hae* III restriction fragments of pUC 8 plasmid were used as molecular weight markers.

Ribosomal DNA cloning

Genomic *P. coccineus* DNA extracted from the roots was digested to completion with *Eco* RI restriction enzyme and subjected to electrophoresis on low-melting-point agarose gel. A fraction ranging from about 6 to 20 kb was isolated and cloned in the *Eco* RI-digested and dephosphorylated pUC 13 plasmid (Pharmacia). Utilizing *Escherichia coli* DH5 α mcr as host cells, the resulting clones were subjected to colony hybridization using ^{32}P-labelled 18 S RNA as a probe. Twelve clones with positive hybridization signal were analysed and four clones of different insert size were amplified for subsequent investigations.

Fine restriction mapping of the cloned ribosomal DNA

The DNA of pPH1, pPH2, pPH5 and pPH6, bearing an *Eco* RI-*Eco* RI segment including the intergenic spacer (IGS), was completely digested with *Eco* RI and partially digested with *Hpa* II, *Hae* III, *Sau* 3AI, *Sma* I restriction enzymes. It was then subjected to electrophoresis on a 1.2% agarose gel and blotted onto a nitrocellulose filter (Schleicher and Schuell). The filters were hybridized with two nick-translated probes obtained by subcloning of pPH5 DNA. The first probe bore the *Eco* RI-*Sac* I segment of the 18S RNA-coding region (350 bp long) and the second probe bore the *Eco* RI-*Sau* 3AI segment of the 25S RNA-coding region (450 bp long). Dehybridization was performed between the two subsequent hybridizations using a boiling solution of 0.1 × SSC, 0.1% SDS.

DNA sequencing

A DNA fraction of about 170 bp was recovered from a low-melting-point 1.2% agarose gel, cloned in *Sma* I-digested pBS plasmid. Fifteen recombinant clones were sequenced using the dideoxy method of Sanger *et al.* [43] as modified by Chen and Seburg [8] for double-strand plasmid DNA. Computer analysis of the sequence data and sequence comparison were carried out using the Pustell program (IBI).

Results

Single and double digestion of total DNA (extracted from root tips and shoots) with restriction endonucleases *Eco* RI, *Bam* HI and *Sac* I produced several discrete bands upon agarose gel electrophoresis (not shown). Some of these bands bear segments of rDNA, as demonstrated by their positive hybridization with *Vicia faba* 18S or 25S rRNA probes. The results of our experiment are shown in Figs. 1A (hybridization with 25S rRNA) and 1B (hybridization with 18S rRNA). In Fig. 1A it can be seen that two segments were obtained after *Eco* RI digestion. One segment, completely localized on the coding region (cf. Fig. 2.), is 3.85 kb long. The second one, which is larger, is highly variable, as seen in lane a. It includes nearly 0.5 kb of 25S rRNA-coding sequences, the whole IGS and about 1.5 kb of the 18S RNA-coding region (cf. Fig. 2). The variability in length of one portion of the rDNA replication unit is also evident after *Bam* HI and *Sac* I digestion. As the quantity of root and shoot DNA electrophorized in each lane of Figs. 1A and B is the same (1 μg) the figure also shows that rDNA segments are more represented in the root than in the shoot. The fact is in agreement with the finding that differences in the r.c. copy number are found not only among individuals of the same species, but also among tissues of one and the same plant [5, 23, 40].

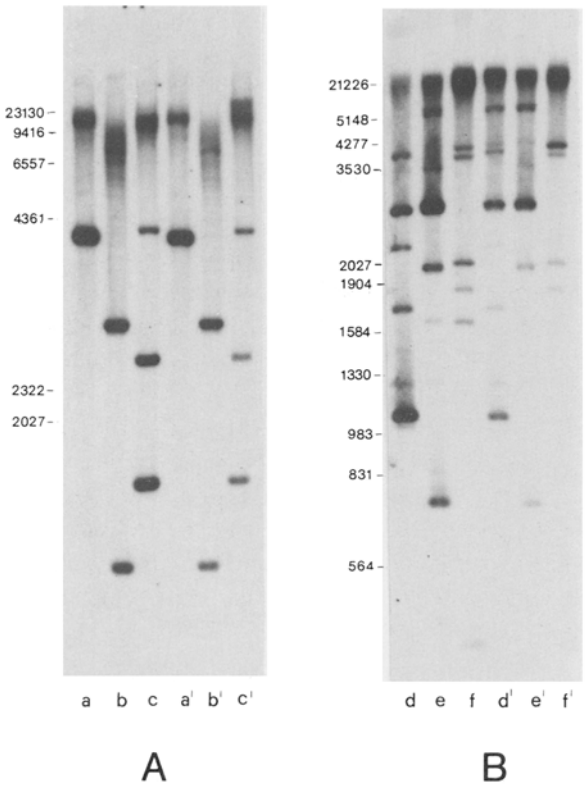

Fig. 1. Restriction pattern of ribosomal rRNA genes in *P. coccineus* after genomic DNA extraction from root tips (a, b, c; d, e, f) and from shoots (a′, b′, c′; d′, e′, f′). Digestion with: *Eco* RI (a, a′), *Bam* HI (b, b′), *Sac* I (e, e′). Double digestion: *Eco* RI-*Bam* HI (d, d′), *Bam* HI-*Sac* I (e, e′), *Sac* I-*Eco* RI (f, f′). A. Hybridization with *Vicia faba* 25S rRNA. B. Hybridization with *Vicia faba* 18S RNA. The numbers at the left of Fig. 1A indicate *Hin*d III digestion (in bp) of phage lambda; the numbers at the left of Fig. 1B are the result of phage lambda digestion (in bp) with *Eco* RI-*Hin*d III.

Figure 2 represents the simplest explanation of the results of the single and double digestion patterns reported in Figs. 1A and B. This figure shows the restriction map of *P. coccineus* rDNA and it can be seen that the size of the whole replication unit ranges approximately from 10 kb to 20 kb. This variability in length is the consequence of size changes of the segment delimited by the *Eco* RI site of 25S rRNA-coding sequences and the *Bam* HI site of the IGS. In Fig. 2 it is also seen that two methylated restriction sites are present in the region codifying for 25S rRNA, the second site being *Bam* HI and the first site *Sac* I. The methylation of *Bam* HI and *Sac* I sites, reported in several studies, is demonstrated in our system by the presence of the band ranging in length from 4600 to 14 200 bp (lanes b and b′ of Fig. 1A), of the band 3930 bp long (lanes c and c′ in Fig. 1A) and of the band 1930 bp long (lanes e and e′ of Fig. 1B).

In order to acquire further information as to the structure of the IGS, the *Eco* RI-*Eco* RI segment of four differently sized genes (pPH1, pPH2, pPH5, pPH6) was analysed by cloning them into pUC 13 plasmid. The DNA of the recombinant plasmids was digested completely with *Eco* RI and partially with *Hpa* II, *Hae* III and *Sau* 3AI. The DNA fragments were subjected to electrophoresis in agarose gel, transferred to nitrocellu-

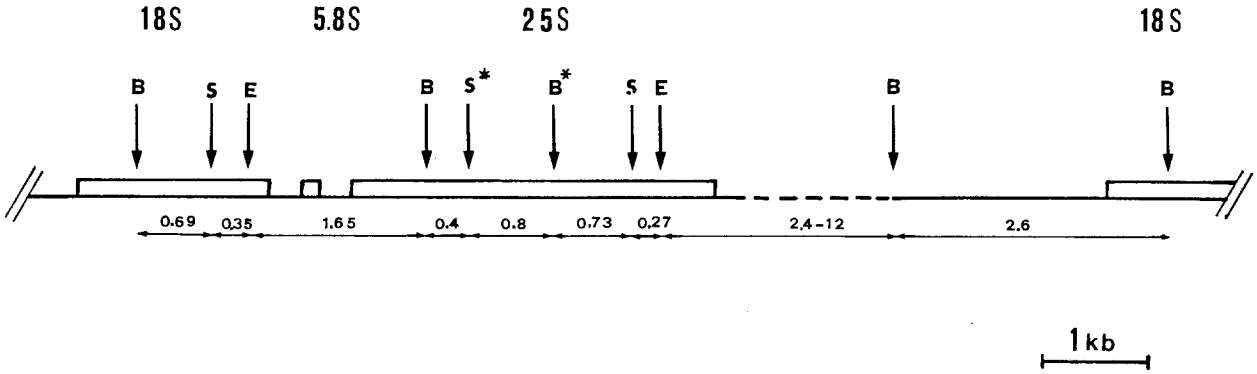

Fig. 2. Restriction map of a replication unit of *P. coccineus* ribosomal DNA. E, B and S represent *Eco* RI, *Bam* HI and *Sac* I restriction sites, respectively. * indicates methylated restriction sites.

lose paper and hybridized with two labelled probes of gene pPH5: (1) in Fig. 3A with the cloned segment, 350 bp long, delimited inside the 18S RNA-coding region by sites *Eco* RI and *Sac* I (cf. Fig. 4); (2) in Fig. 3B with the cloned segment, 450 bp long, delimited inside the 25S cod-

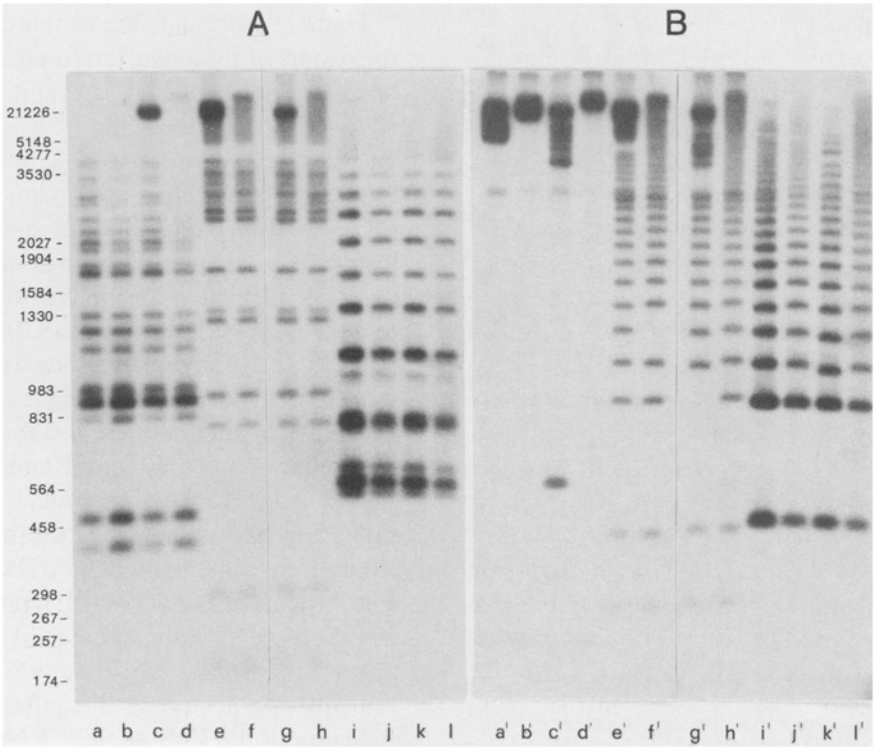

Fig. 3. Restriction pattern of *Eco* RI-*Eco* RI (including IGS) segments of ribosomal DNA of *P. coccineus*; a, b, c, d, and a′, b′, c′, d′ represent the segment of pPH1, pPH2, pPH5 and pPH6, completely digested with *Eco* RI and partially digested with *Hpa* II; e, f, g, h, and e′, f′, g′, h′ indicate the identical portion of the same genes completely digested with *Eco* RI and partially digested with *Hae* III; i, j, k, l, and i′, j′,k′; l′ show the restriction patterns of these four gene fractions completely digested with *Eco* RI and partially digested with *Sau* 3AI. A. Hybridization with segment *Eco* RI-*Sac* I (350 bp) of sequences coding for 18S rRNA (cf. Fig. 4). B. Hybridization with the *Eco* RI-*Sau* 3AI (450 bp) segment of sequences coding for 25S RNA (cf. Fig. 4). Markers: the first one, bearing bands (in bp) higher than 458, is phage lambda digested with *Hin*d III-*Eco* RI; the second one, including the 458 bp band and bands of less bp, is plasmid pUC 8 digested with *Hae* III.

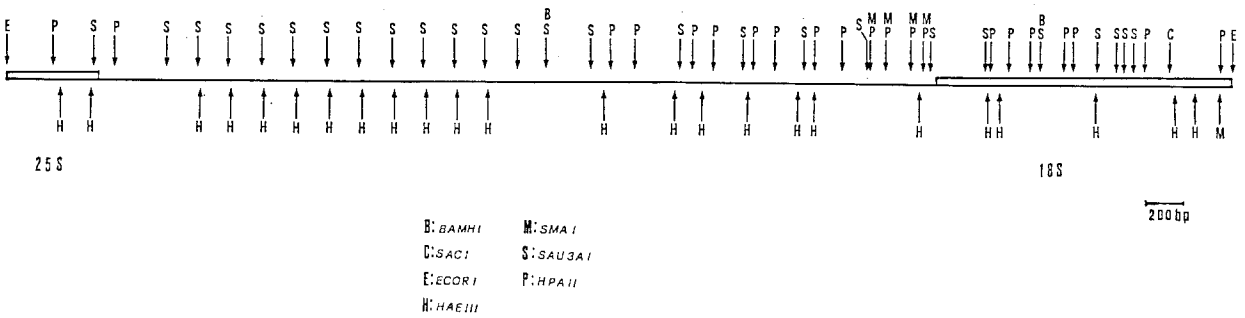

Fig. 4. Restriction map of the clone pPH5 including IGS *Eco* RI-*Eco* RI fragment of gene pPH5 (cf. Fig. 3 after partial digestion with *Hae* III, *Hpa* II and *Sau* 3AI. *Bam* HI and *Sac* I restriction sites are indicated on the basis of the results of Fig. 1; the *Sma* I sites were determined by partial digestion (not shown).

ing region by sites *Eco* RI and *Sau* 3AI (cf. Fig. 4). The two hybridizations, subsequent in time, were separated by a dehybridization process as described in Materials and methods. This type of hybridization gives a higher resolution in the band pattern analyses than that resulting from hybridization with the whole segments coding for 18S RNA and 25S rRNA, respectively.

Figure 3 shows that (a) the gene length is not homogeneous and (b) some bands are not present in certain genes. Case a is observable in both Figs. 3A and 3B; case b is to be found in Fig. 3B where, after cleavage with *Hpa* II, one band (about 630 bp long) is present only in pPH5 and two other bands are not present in genes pPH2 and pPH5 after *Hae* III cleavage.

Figure 4 contains the physical map of the *Eco* RI-*Eco* RI fraction including the whole IGS of pPH5 (data of Fig. 3). The figure shows that one IGS portion is the result of the reiteration of a segment about 170 bp long (sub-repeat). The number of sub-repeats (s.r.) is, however, not constant from one gene to another. Indeed, clone pPH5 is seen to have 10 s.r. (Fig. 4) while pPH1 has 16 s.r. and genes pPH2 and pPH6 have about 34 and 60 s.r., respectively (Table 1).

The physical map of pPH5 as well as the physical map of pPH1, pPH2 and pPH6 (not shown) indicates that the IGS is divided into 3 regions: one (constant in length and about 400 bp long) close to the 3′ end of the 25S RNA coding sequences, one variable in length (s.r. region), and one third constant in size in our system, approximately 2000 bp long. This region, delimited by the *Bam* HI restriction site and by the 5′ end of 18S RNA-coding sequences, contains four sub-regions (islands) each including one *Sau* 3AI and two *Hpa* II restriction sites (cf. Fig. 4). In pPH2, however, the first *Hpa* II site in the third island is lacking. The heterogeneity in length (in bp) of the *Eco* RI-*Bam* HI IGS segments is clearly seen in Fig. 5. This IGS organization is very similar to that described by Flavell *et al.* [13] in *Triticum*.

Table 1. Number of s.r. ca. 170 bp in length included in the four analysed clones.

Clone	Number of s.r.
pPH1	16
pPH2	ca. 34
pPH5	10
pPH6	ca. 60

The length of one single s.r. is however approximated in Fig. 4. To evaluate the exact extention (in bp) of one single repetition unit, the s.r. of the longest pPH6 gene were subcloned, and 15 of them were sequenced. This showed that, at least in pPH6, two types of s.r. are present, as reported in Fig. 6. The first type is 176 bp long (L) and the second type 162 bp long (S). The relative frequen-

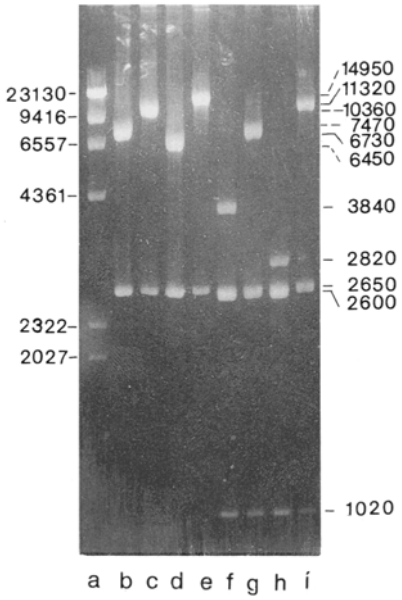

Fig. 5. Agarose gel (1.5%) electrophoresis of pPH1, pPH2, pPH5, pPH6 after digestion with *Eco* RI (b, c, d, e, respectively) and double digestion *Eco* RI-*Bam* HI (f, g, h, i, respectively). Marker: phage lambda cleaved with *Hind* III (a). Ethidium bromide staining. -------: *Eco* RI cleavage; ———: *Eco* RI-*Bam* HI cleavage.

cies of L and S in the pPH6 clone are about 60% and 40% respectively. There is a high degree of homology between the two s.r. types. The comparison reported in Fig. 6 indicates, in fact, 93.8% of matching nucleotides.

In order to establish whether all four clones include both L and S s.r. types, pPH1, pPH2, pPH5 and pPH6 DNAs were digested with *Hae* III restriction enzyme. The results of this experiment are given in Fig. 7, which shows that both s.r. are present in at least three genes (pPH1, pPH5, pPH6), but to a variable degree.

Figure 8 contains the following sequence comparisons: (1) promoter of *Xenopus* [28] to s.r. of *P. coccineus*; (2) block A s.r. of wheat [6] to s.r. of *P. coccineus*; (3) presumed promoter of wheat [13] to s.r. of *P. coccineus*.

In all three cases, sequences of *P. coccineus* s.r. were chosen among nucleotides matching between L and S. The first comparison shows a homology of 47%; the second of 54% and the third of 61%.

Discussion

Variation in length of rDNA replication units within single plants is well documented in the literature. *Vicia faba*, for instance, has only one chromosomal locus for rDNA [22, 44] and an enormous heterogeneity of the r.c. [41], while in *Pisum sativum*, which has two loci for rDNA, many IGS size classes are present in one locus, and the second contains only one or two IGS size classes [11, 31]. Heterogeneity in length of the IGS is, however, clearly absent in other species [9, 18, 25, 33, 51].

In *P. coccineus*, comparison of pPH1, pPH2, pPH5 and pPH6 shows that the four genes differ in length (Figs. 3A and B; Fig. 5; Table 1) as well as in the presence or absence of certain restriction sites (Fig. 3B). Furthermore, Fig. 5 gives the length of the four *Eco* RI-*Eco* RI and the four *Eco* RI-*Bam* HI segments, each including the variable portion of IGS (cf. Fig. 2), showing that the size of pPH1, pPH2, pPH5 and pPH6 is not homogeneous. The data reported in Figs. 3 and 5 and in Table 1 are, therefore, in agreement with the results of the first experiment (Figs. 1 and 2)

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176 CC      AAGTTCCATGATTTTGGTTTTTTGGCTTGATTTTCTGACTTGGTGGTGTGTGGGGTGAAAAACGAAAATCTTGGTTTTGGAAATC
                                                  *
162 CCCGATAAAGTTCCA                  TTGATTTTGTGACTTGGTGGTGTGTGGGGTGAAAAACGAAAATCTTGGTTTTG  AATC

176 TGGCTGCACTCAATGCTAACAAATGCTTAGAAAGTGATGTGGGAGAAGTTGGG AGCAAAAGGAATTGATCT GACATAGTTGGTA ATGGG
                                                                                          *
162 TGGCTGCACTCAATGCTCACAAATGCTTAGAAAGTGATGTGGGAGAAGTTTGGGAGCAAAAGGAATTGATCTTGACATAGTTGGTCCATGG
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Fig. 6. Nucleotide sequences of the two consensus sub-repeats of *P. coccineus*: 176 bp long = long (L); 162 bp long = short (S); * indicates not matching nucleotides.

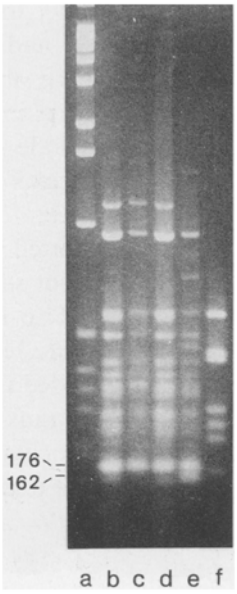

Fig. 7. Agarose gel (1.5%) electrophoresis after *Hae* III digestion of pPH1 (b), pPH2 (c), pPH5 (d), pPH6 (e). Markers: a = 1 kb ladder (BRL); f = pUC 8 cleaved by *Hae* III (bp). Ethium bromide staining.

and with a number of findings concerning animal and plant systems.

As to the loss or gain of restriction sites (Fig. 3B), the situation resembles that described in certain plants, such as *Raphanus sativus* [9], flax [18], *Allium cepa* [25], *Lupinus luteus* [33] and *Carduus nutans* [52] which show sequence heterogeneity among r.c. In the case of *P. coccineus*, as in many other plant systems, we find both replication unit length and sequence heterogeneity.

The molecular basis for IGS length heterogeneity has been explained by several investigators. A variable number of small repetitive segments within IGS have been described for wheat [1, 13, 40], rice [29], pumpkin [45], broad bean [20, 53], maize [27, 38, 39, 50], mungbean [17], carrot [48], cucumber [16], *Cucurbita maxima* [21] and *P. coccineus* (present data), and this accounts for length heterogeneity. Jorgensen *et al.* [19] and Tremousaygue *et al.* [51] recently demonstrated that these short repeating units are species-specific as they do not hybridize, at least at a standard criterion, to closely related species.

In *P. coccineus* at least two different s.r. sequences (Fig. 6) are repeated to a variable extent. The situation resembles that described in wheat [6] in which two series of repeats are present: A (repeats about 135 bp long) and B (repeats about 150 bp long).

The functional aspects of IGS have not yet been closely investigated in plants, but it is well known that in several animal systems IGS contains at least six control elements [35]. Although they have not yet been localized within the IGS of many species, Reeder [35] argues that 'they

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   A

-60 to -10    TCGGGCCCCCCGCACGACGCCTCCA.TGCTACGCTTTTTTGGCATGTGCGGG
              || ||| | | ||  | | | |||| | | | || | | |
86 to 132     TCTGGCTGCACTCAATGCTAA.CAAATGCTTAGAAAGT..GATGTG.G.GAGAA


   B

73 to 109     GCGCGCCATGGAAA.A.CTGGGCAAAACCACGTAC.GTGG
              || | | ||| || | || | | || ||||
93 to 129     GCACTCAATGCTAACAAATGCTTA..GAAA.GTGATGTGG


   C

-120 to -91   TGG.CAGCGAAAACATG.TCTCA.TGGCAAAAAA
              ||| | |  | || |||| || || |||
68 to 114     TGGAAATCTGG..C.TGCACTCAATGCTAACAAA
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Fig. 8. Sequence comparison of *Xenopus* promoter (Fig. 8A), wheat s.r. (Fig. 8B) and wheat promoter (Fig. 8C) to the s.r. of *P. coccineus*.

appear to constitute a basic set that will eventually be found in most eukaryotic ribosomal genes'. Some of these elements function as enhancers, others as gene promoters (present in one single copy per replication unit of rDNA) and as spacer promoters [34, 35].

Spacer promoters and enhancers occur either in the form of single elements, or in the form of sub-repeats as in *Xenopus* [47]. According to Flavell *et al.* [13], the A type s.r. sequences of wheat may be enhancers and spacer promoters; more recently, developmental data concerning pea and genetic data obtained from wheat indicate that the s.r. of these systems may also function as enhancers [49]. Moreover, according to Barker *et al.* [6], examination of the DNA sequences suggests promoter duplication in the IGS of wheat. Sequences of presumably additional promoter regions are also found in the s.r. of rye [3], maize [27, 50] and other plant systems. It is therefore tempting to conclude, in line with Flavell [12, 13], that the intergenic repeats of plant rDNA also act as additional promoters or enhancers.

To test this assumption in *P. coccineus* we made the sequence comparisons reported in Fig. 8. The first comparison (*Xenopus* promoter with s.r. of *P. coccineus*) shows 47% of related sequences with dyad symmetry in the two systems (Fig. 8a). The sequences of *Xenopus* promoter compared with *P. coccineus* s.r. are 'sequences whose deletion results in a non functional promoter' [28]. Their homology with s.r., though not high, might be therefore of particular significance.

Considering that (i) in spite of its species specificity the protein-binding element has been strongly conserved across species in the gene promoter [35]; (ii) gene and spacer promoters share many nucleotide sequences [36]; (iii) the more enhancers are present in the IGS, the stronger the promoter becomes [35]; (iv) s.r. include sequences of enhancers and/or spacer promoters, the reported homology might be an indication that also s.r. of *P. coccineus* function as enhancers or spacer promoters. The other two sequence comparisons (the s.r. and promoter of wheat with the s.r. of *P. coccineus* (Figs. 8b and 8c respectively) seem to support this postulate. Indeed, in these cases, the homology is 54% and 61%, respectively. In connection with the above considerations, the fact that in all cases investigated, except yeast, the enhancers include sequences also found in the promoter [35] seems worthy of particular attention.

On the basis of the reported postulate our present results seem to account satisfactorily for the functional heterogeneity of r.c. in *P. coccineus*. In agreement with the literature [13, 35], in fact, the more enhancers are included in the IGS, the more active the r.c. are in the transcription. DNA methylation, among other processes, would further modulate ribosomal gene transcription.

Acknowledgements

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