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Organisation of the 5S RNA genes in flax

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SUMMARY

The 5S RNA genes of flax [Linum usitatissimum] are arranged as tandem arrays of a 0.35 - 0.37kb repeating sequence. The 5S DNA is extensively methylated at C^{5}CGG and C^{5}C^{5}GG. In contrast to the rDNA, the 5S DNA sequences exhibit both length and sequence heterogeneity. The number of copies of this sequence varies between 117,000 and 49,600 per 2C nucleus in different lines of flax, and does not correlate with the number of rRNA genes.

INTRODUCTION

In all eukaryotes there are multiple copies of the genes for 5S RNA, and in most the DNA coding for 5S RNA is physically separated from that coding for the large rRNAs [1]. The exceptions to this include yeast [2] and Dictyostelium [3] where the 5S DNA is linked to the rRNA cistrons. The genes for 5S RNA are normally present in a tandem array of repeating units, the coding sequence alternating with a non-transcribed spacer [4]. However the organisation of 5S DNA can be more complex. The repeat unit of the oocyte 5S DNA in Xenopus borealis can contain a number of coding sequences, pseudogenes and a spacer of variable length [5].

In the flax variety "Stormont Cirrus" differences in DNA content between a number of lines have been reported [6]. The only sequences that are known to be variable in amount and that have been characterised are those for rRNA [7]. The organisation of the 5S RNA cistrons of flax, and their copy number, are reported here. By comparing the organisation of the 5S DNA with that of the rDNA we may gain an insight into the processes that affect the variation of repeated DNA sequences in flax.

MATERIALS AND METHODS

Preparation of DNA

DNA was prepared from the leaves and apical shoots of plants grown in a heated glasshouse [8]. Actinomycin D/CsCl gradients, containing 0.2-1.0mg

of DNA, were run as described by Hemleben et al. [9].

Preparation of RNA

RNA was prepared from flax callus cultures [7]. ^{32}P-RNA was extracted from callus grown in the presence of 25μCi ml^{-1} ^{32}P-orthophosphate. The RNA was fractionated on a 10% polyacrylamide gel [10] and 5S RNA recovered from the relevant portion of the gel by elution in 6xSSC at 20° for 6 hours. The RNA was precipitated with ethanol, pelleted, redissolved in water and used without further purification for filter disc hybridisations. The 5S RNA had a specific activity of 38,000 cpm μg^{-1}.

Gel electrophoresis, Transfers and Hybridisations

EcoRI was obtained from Boehringer; MspI, HpaII and RsaI from New England Biolabs and all other enzymes from Bethesda Research Laboratories. The conditions for digestion were those suggested by the suppliers. DNA fragments were separated on either vertical 1.2%, or horizontal 4%, agarose gels run in 40mM Tris-acetate pH 7.9, 5mM sodium acetate, 1mM EDTA. DNA fragments were transferred to nitrocellulose by the method of Southern [11].

RNA was glyoxalated and fractionated on a 1.6% agarose gel in 10mM sodium phosphate buffer pH 7.0 [12]. RNA was transferred from the gel to nitrocellulose by the method of Thomas [13].

Nitrocellulose filters, carrying either DNA or RNA, were preincubated in 3 x SSC, 0.08% bovine serum albumin, 0.08% Ficoll, 0.08% polyvinylpyrollidone, at 65° for 4 hours [14]. Hybridisations were carried out in the same buffer, containing approximately 10^6 cpm of ^{32}P-labelled nick translated DNA [15], at 65° for 16 hours. After hybridisation the filters were washed extensively in 2 x SSC, 0.1% SDS at 65°.

Cloning of 5S DNA

1μg of DNA, from an Actinomycin D/CsCl gradient fraction, was digested with BamHI [Fig. 1, A] and mixed with 1μg of BamHI digested pAT153 [16]. The DNA fragments were ligated with 10 units of T4 DNA ligase at 10° overnight. $CaCl_2$ treated HB101 cells were transformed with this DNA. $Amp^R Tc^S$ colonies were selected and plasmid DNA prepared from these [17]. They were tested for the presence of 5S DNA inserts by hybridisation with a cloned wheat 5S DNA sequence [kindly provided by W.L. Gerlach]. A number showed positive hybridisation and two were further characterised. pBG6 contains a single insert of 0.35kb [Fig. 1, D] and pBG13 has a tandem array of ten repeats of 0.35kb [Fig. 1, B.C]. It is not known if pBG13 results from the ligation of more than a single fragment into pAT153. However all fragments are in the same orientation. To confirm that these are sequences coding for 5S RNA,

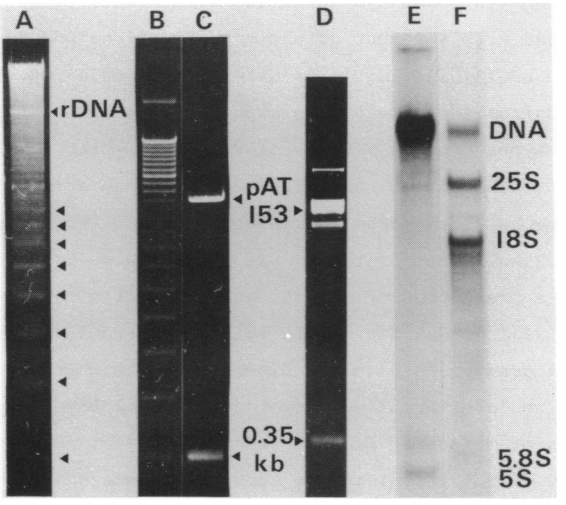

Figure 1. A, BamHI digest of DNA from an Actinomycin D/CsCl gradient fraction; 5S DNA fragments are marked ◀ . B, partial; C, complete BamHI digests of pBG13. D, partial BamHI digest of pBG6. E, F, fractionated RNA hybridised with pBG6 [E] or pBG35, a plasmid containing a complete flax rDNA repeat [F].

^{32}P-pBG6 was hybridised to a filter carrying glyoxalated, fractionated RNA. pBG6 hybridised to an RNA slightly smaller than 5.8S rRNA [Fig. 1, E].

Filter Disc Hybridisation

DNA prepared from five lines of flax was bound to nitrocellulose discs [Millipore, HAWP 013 00] at a concentration of approximately 5µg per disc [18]. Six filters from each line, with control filters carrying heterologous Streptomyces coelicolor DNA, were hybridised with ^{32}P-5S RNA in 3mls of 6 x SSC at 70° for 5 hours at an RNA concentration of 5µg ml^{-1}. These conditions had been previously determined to be saturating for 5S DNA sequences. After hybridisation the filters were washed and treated with RNase by the batch method [19]. The filters were counted and the amount of DNA bound to the filter determined [20]. The number of genes for 5S RNA was calculated based on a size of 7.0 x 10^8 base pairs for the 2C flax genome [21] and 120 nucleotides for 5S RNA.

RESULTS

Organisation

The organisation of the genes for 5S RNA in flax has been examined by

restriction enzyme analysis and hybridisation with cloned 5S DNA. Total flax DNA was digested with a number of enzymes, fractionated, transferred and hybridised with pBG13 [Fig. 2]. The size of the basic repeat unit is 0.35kb, as expected from the size of the cloned fragments. Partial digests with a number of enzymes produce a range of fragments which are multiples of this repeat unit. This is characteristic of a tandem array of a repeated sequence. No enzyme has been found that digests the 5S DNA to give a single fragment size of 0.35kb. Most of the 5S DNA repeats have a single site for RsaI and TaqI, but a proportion of repeats have two sites for these enzymes. Although BamHI and TaqI digest the 5S DNA to a considerable extent, both leave tracts of DNA greater than 15kb undigested. From a series of digests we have calculated the cleavage frequency in a terminal BamHI digest [22]. If the distribution of sites for BamHI is random then, at this extent of digestion, we would expect less than one copy per genome of 5S DNA fragments greater than ten repeat units in length. That fragments larger than this are found indicates that the distribution of these sites within the 5S DNA is not random. The sequences that are undigested by BamHI are not the same as those resistant to TaqI as shown by a digest with both enzymes.

HaeIII leaves most of the 5S DNA uncut. However it does produce fragments of all the observable repeat sizes. EcoRI has a similar effect but has

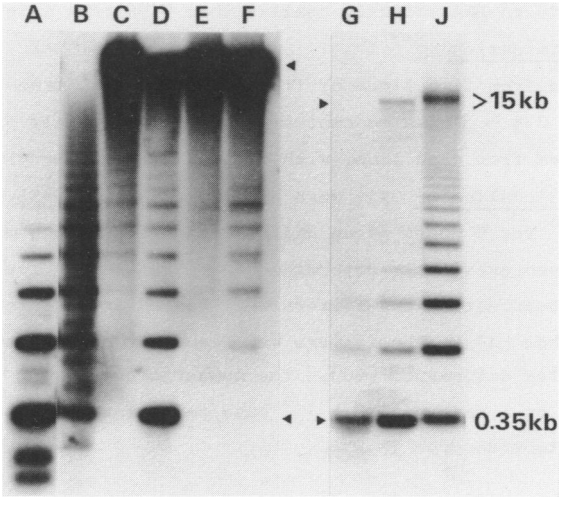

Figure 2. Total flax DNA digested with a number of enzymes and hybridised with pBG13. A, RsaI; B, MspI; C, HpaII; D, HaeIII; E, HindIII; F, EcoRI; G, BamHI and TaqI; H, TaqI; J, BamHI.

fewer sites within the 5S DNA. There are also some fragments produced by EcoRI that are not multiples of the 0.35kb repeat. HindIII digests the 5S DNA to a very limited extent; minor bands are visible only at the six- and nine-repeat positions on the ladder. It is known that the activity of these three enzymes [HaeIII, EcoRI and HindIII] is not affected by DNA methylation in the rDNA of flax [7]. It is reasonable to assume therefore that these patterns of digestion are the result of sequence divergence. This is further reinforced by the finding that there are no sites for EcoRI in the inserts of pBG6 and pBG13.

The degree of methylation in the 5S DNA of flax has been examined by comparing the extent of digestion produced by the isoschizomers MspI and HpaII [23]. MspI digests the 5S DNA more completely than HpaII, showing that methylation of the sequence C$\overset{*}{C}$GG [where * indicates the methylated base] occurs in flax 5S DNA. It has been calculated that 5% of the flax 5S DNA repeats carry a susceptible HpaII site, whereas 30% of the repeats have a site for MspI [22]. Therefore between 83% and 95% of the sites are methylated at C$\overset{*}{C}$GG. Addition of HpaII to MspI digested DNA results in no further digestion of the 5S DNA. There is a second MspI site, present in a small proportion of the 5S DNA repeats, that is not detectably cleaved by HpaII.

Length variation in 5S DNA

When pBG13 was digested with BamHI and analysed on a polyacrylamide gel it was found that one of the ten 5S DNA repeats in the insert was slightly larger [~0.37kb] than the others. To show that this larger repeat was present in flax, flax DNA and pBG13 were digested with BamHI, separated on a 4% agarose gel, transferred and hybridised with pBG13 [Fig. 3]. The major fragment in flax DNA to hybridise was of 0.35kb. This comigrated with the single insert in pBG6 and all but one of those in pBG13. However in flax DNA there was also a discrete band at 0.37kb that had the same mobility as the large 5S DNA insert of pBG13. Similar results have been observed with TaqI digests of flax DNA. In contrast to these results, digestion of flax 5S DNA with RsaI produces a single fragment in the region of 0.35kb. However three smaller fragments are detected [Fig. 3, A]. From their sizes it appears that a small proportion of the 0.35kb repeats, and all those of 0.37kb, contain two sites for RsaI. There is a fragment of 0.15kb common to both types. The 0.22kb fragment is derived from the 0.37kb repeats, the 0.20kb fragment from those of 0.35kb.

In some experiments using 4% agarose gels two bands have been clearly observed at both the dimer and trimer positions on the ladder. However

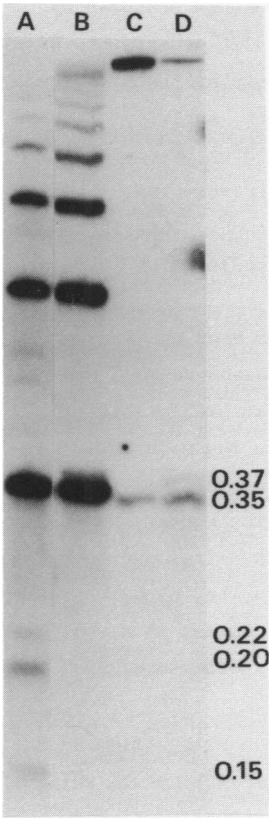

Figure 3. Total flax DNA digested with RsaI[A], BamHI[B] and pBG6[C], pBG13[D] digested with BamHI, separated on a 4% agarose gel, transferred and hybridised with pBG13.

accurate determinations of the difference in size between these double bands has not been possible. Therefore we cannot say to what extent the two size classes of 5S DNA are interspersed.

Redundancy of 5S genes

The number of genes for 5S RNA in five flax lines has been determined by saturation hybridisation with ^{32}P-5S RNA to DNA immobilised on nitrocellulose discs [Table 1]. Each determination is the mean of six filters. The number of genes for rRNA are listed for comparison. The correlation coefficient between the numbers of genes for rRNA and 5S RNA is 0.19.

The difference in amount of 5S DNA between L1 and S1 is clearly seen when pBG13 is hybridised to equal amounts of L1 and S1 DNA digested with BamHI [Fig. 4, A]. However when TaqI digests are compared, the amount of hybridisation to the monomer band at 0.35kb is similar in L1 and S1 [Fig. 4, B]. The only difference that can be seen between these DNAs is in the amount of 5S DNA larger than 10kb produced by TaqI, there being more in L1 than in

Table 1. Numbers of genes for 5S RNA per 2C nucleus in five flax lines, determined by saturation hybridisation. Each determination is the mean of six filters. The numbers of rRNA genes are taken from [32].

Flax line	5S RNA Number of genes	S.E.M.	Number of rRNA genes	5S RNA / rRNA
P1	117,000	± 4,037	2,660	44.0
L1	80,300	± 6,034	2,370	33.9
S1	52,300	± 3,983	1,430	36.9
LH	49,600	± 3,372	2,570	19.3
SH	101,900	± 4,231	1,570	64.9

S1. Although there is relatively little hybridisation to these high molecular weight fragments, in comparison with those of 0.35kb, the transfer to nitrocellulose of such large fragments will be at a lower efficiency than for small DNA molecules. This is the only difference in the organisation of 5S DNA in L1 and S1 that has been observed in a number of experiments.

DISCUSSION

The 5S DNA of flax is organised in long tandem arrays, separate from the genes coding for rRNA, as found in most eukaryotes. There is both length

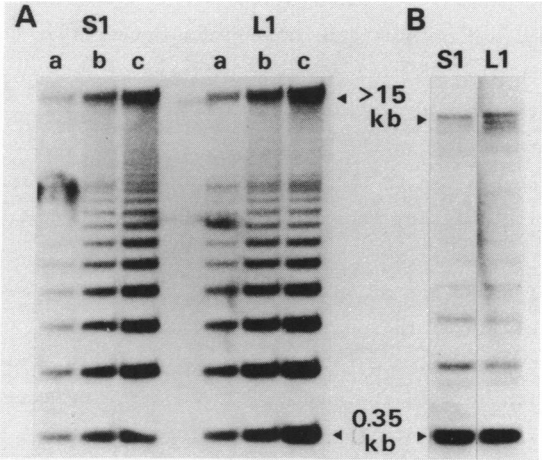

Figure 4. A: a, ½ µg; b, 1 µg; c, 2 µg of DNA from S1 and L1 digested with BamHI and hybridised with pBG13. B: 1 µg of DNA from S1 and L1 digested with TaqI and hybridised with pBG13.

heterogeneity and sequence divergence between repeat units. The rDNA of flax is, in contrast to the 5S DNA, very homogeneous, exhibiting neither of these forms of variation. It is clear that there must be a mechanism to produce such a uniform population of a repeated sequence and experimental evidence supports unequal crossing over [24]. Why then is the 5S DNA so variable in comparison with the rDNA? Ohta [25] has suggested that the rate of fixation of a sequence variant in a tandem array is a function of the number of repeats in that array. As there are more repeats of 5S DNA than of rDNA, fixation of a repeat class may be less likely for 5S DNA than for rDNA. Furthermore it is not known how many chromosomal locations there are for the 5S genes while only one chromosome pair has a nucleolus organiser region [26].

The existence of sequence divergence has a bearing on the methylation of flax 5S DNA. There are three reasons for MspI not digesting the 5S DNA to leave a single major class of 0.35 - 0.37kb. First there may not have been sufficient enzyme; this seems unlikely as a 20-fold excess was used and the enzyme had a normal activity on non-methylated DNA. Secondly the recognition sequence for MspI may be absent in a proportion of the repeats, as found with EcoRI. However each of the eleven cloned 5S DNA repeats that have been examined contain both sites for MspI. Lastly the recognition sequence may be present, but protected from digestion by both MspI and HpaII - $\overset{**}{C}CGG$ [27]. There is evidence that this modified sequence is present in flax rDNA [P.B.G., unpublished observations]. It seems likely that both sequence divergence and methylation at sequences other than $\overset{*}{C}G$ are involved at the MspI site in flax 5S DNA. Similar patterns of sequence divergence and methylation have been reported for the 5S DNA of maize [28]. However in Xenopus laevis methylation in the 5S DNA is found only at the sequence $\overset{*}{C}G$ [29], and in sea urchin the 5S DNA is not detectably methylated [30]. This supports recent findings that the organisation of methylated nucleotides in the DNA of higher plants is more complex than that found in animals [31].

The number of genes for 5S RNA in flax is very high. The 5S DNA comprises approximately 3% of the nuclear DNA in the line PL. This may in part be a reflection on flax being a tetraploid species [21]. The lack of any positive correlation between the numbers of genes for rRNA and 5S RNA within a single species implies that the rate of synthesis of these RNAs cannot be governed solely by the numbers of their genes.

There is a large amount of variation between different flax lines in the number of genes for 5S RNA. L1, S1, LH and SH are derived from PL after

growth under different environmental conditions [32]. However there is no evidence that the changes in 5S RNA gene number are caused by the environment. The observed variation may be a consequence of the selection from PL of individuals that vary in their number of genes for 5S RNA and their maintenance by inbreeding. An examination both of the variation in PL and the level of 5S DNA in the progeny after growth under different conditions is required to determine the effect of the environment. Comparisons of TaqI digests show differences between the 5S DNA of L1 and S1. Variation in the 5S gene number [33] and the organisation of the 5S gene cluster [34] have also been observed in stocks of Drosophila.

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