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Ribosomal RNA genes in soybean and common bean: chromosomal organization, expression, and evolution

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Abstract Ribosomal RNA (5S and 45S) genes were investigated by FISH in two related legumes: soybean [*Glycine max* (L.) Merr.] and common bean (*Phaseolis vulgaris* L.). These species are both members of the same tribe (Phaseoleae), but common bean is diploid while soybean is a tetraploid which has undergone diploidization. In contrast to ploidy expectations, soybean had only one 5S and one 45S rDNA locus whereas common bean had more than two 5S rDNA loci and two 45S rDNA loci. Double hybridization experiments with differentially labelled probes indicated that the soybean 45S and 5S rDNA loci are located on different chromosomes and in their distal regions. Likewise, the common bean 45S and 5S rDNA loci were on unique chromosomes, though two of the 5S rDNA loci were on the same chromosome. FISH analysis of interphase nuclei revealed the spatial arrangement of rDNA loci and suggested expression patterns. In both species, we observed one or more 5S rDNA hybridization sites and two 45S rDNA hybridization sites associated with the nucleolar periphery. The 45S rDNA hybridization patterns frequently exhibited gene puffs as de-condensed chromatin strings within the nucleoli. The other condensed rDNA sites (both 5S and 45S) were spatially distant from the nucleolus in nucleoplasmic regions containing heterochromatin. The distribution of rDNA between the nucleoplasm and the nucleoli is consistent with differential gene expression between homologous alleles and among homoeologous loci.

Key words Fluorescence in situ hybridization · *Glycine max* · *Phaseolis vulgaris* · Nucleolus · rDNA

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Introduction

Ribosome assembly requires a high level of ribosomal RNA gene expression which may explain the highly repeated nature of ribosomal RNA genes and their arrangement into different genomic locations. These non-translated rRNA species (5S, 5.8S, 18S and 25S) are produced from highly repeated (100s to 1 000s of copies) and tandemly arrayed genes. In plants, the 5S rRNA genes are arrayed by themselves while the 18S, 5.8S and 25S rRNAs are produced together from a 45S rRNA precursor gene. In addition to multiple genes within an array, there may be multiple arrays (loci) on different chromosomes. While there is a clear requirement for multiple gene copies, the selective use of a fraction of these in RNA production is problematic as is the control of which genes are being utilized for RNA production.

Recently developed molecular cytogenetic techniques such as FISH have been routinely used to localize repeated rDNA sequences both in interphase nuclei, or onto condensed chromosomes during mitosis, in plant species. The rDNA sequences were used as chromosome-specific DNA sequences to map and tag particular chromosomes (Ellis et al. 1988; Skorupska et al. 1989; Mukai et al. 1990, 1991; Griffor et al. 1991; Leitch and Heslop-Harrison 1992; Crane et al. 1993; Maluszynska and Heslop-Harrison 1993a, b; Pedersen and Linde-Laursen 1994; Schmidt et al. 1994; Fukui et al. 1994; and others). The spatial distribution of rRNA genes was used to investigate the relationship between function and the fine structure of the interphase nucleus, as well as the relationship between gene location and expression (e.g., Bauwens et al. 1991; Leitch et al. 1992; Highett et al. 1993; Pedersen and Linde-Laursen 1994; and others). Moreover, the chromosomal location, copy number, and conservation of rDNA sequences were determined towards clarifying the relationships and evolution of polyploid plant species in the Triticeae (Mukai et al. 1991; Linde-Laursen et al. 1992; Kim et al. 1993; and others), *Arabidopsis* (Maluszynska and Heslop-Harrison 1993a), *Gossypium* (Crane et al. 1993), and *Brassica* (Del-

seny et al. 1990; Maluszynska and Heslop-Harrison 1993b).

Soybean (2n=2x=40), an economically important crop species, is a diploidized tetraploid species (Hymowitz and Singh 1987; Zhu et al. 1994). Common bean (2n=2x=22) is a diploid relative of soybean that also has considerable world-wide economic importance. It has many primitive genomic features shared by the ancestors of soybean (Zhu et al. 1995a). In spite of their wide agronomic uses, cytogenetic studies are very difficult, and hence rare, in these two species due to the small size and similar morphology of their chromosomes (Lackey 1980; Hymowitz and Singh 1987; Zheng et al. 1991). Except for the soybean 45S ribosomal DNA array (Skorupska et al. 1989; Griffor et al. 1991), no molecular cytogenetic studies have been conducted in these two species.

In the present study, we physically localized 5S and 45S rDNA loci simultaneously by FISH in both species. In order to explore the chromosomal organization and expression of soybean rRNA genes and their relations to the evolution of soybean duplicate genome, we compared the total loci number and nuclear location of the 5S and 45S rDNA loci, as well as the actively expressed locus number in the interphase nucleus in both species, using common bean as a diploid reference.

Materials and methods

Soybean cultivar "BSR-101" and common bean cultivar "Big Sweet Baker" (Gurney Seeds) were used for the FISH study. Preparation of root proptoplasts and chromosome spreads were as described previously (Zhu et al. 1995b).

DNA probe preparation

The two rDNA sequences used as probes for FISH were both derived from soybean genomic DNA and represent the entire repeat unit. The 5S rDNA probe was a 0.35-kb *Bam*HI DNA fragment in the plasmid vector pUC-9 (Quemada et al. 1987) while the 45S rDNA probe was an 8-kb *Eco*RI DNA fragment originally isolated as a lambda Charon 4A clone: RB115 (Jackson and Lark 1982).

The DNA probes were labelled with either biotin-16-dUTP or with digoxigenin-11-dUTP (Boehringer Mannhein) by the random primer labelling method according to manufacturer's specification.

In situ hybridization

In situ hybridization was conducted according to Pinkel et al. (1986) and Leitch et al. (1991) with modifications (Zhu et al. 1995b). Briefly, slides containing chromosome spreads were incubated in 70% formamide, 2×SSC for 2 min at 70°C, and quickly dehydrated through a −20°C ethanol series. Hybridization solution which consisted of 50% formamide, 2×SSC, 10% dextran sulphate, 50 µg/ml of de-graded herring sperm DNA and heat-denatured 2 µg/ml of biotinylated 45S rDNA probe and 2 µg/ml of digoxigenin-11-dUTP labelled 5S rDNA probe, was then applied to the slides. Hybridization was allowed to proceed overnight at 37°C.

Detection and visualization of hybridized probes

After hybridization, slides were rinsed through 50% formamide and 2×SSC, and PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% Nonidet P-40, pH 8.0) at 45°C (high stringency) or 37°C (low stringency). Slides were incubated in PNM buffer (5% non-fat dry milk, 0.1% sodium azide in PN buffer) with 5 µg/ml fluorescein Avidin DCS and 20 µg/ml of rhodamine anti-digoxigenin for 1 h, washed in PN buffer, then mounted in VECTASHIELD antifade medium (Vector Laboratories) containing 0.5 µg/ml of DAPI.

Observations were made with a Zeiss Axioplan epifluorescence microscope with appropriate filters. Double labelling signals were detected by the dual excitation and emission filters. Images were recorded with Kodak Ektachrome 400 color slide film.

Image processing

Image processing was used to superimpose the in situ hybridization signals onto the chromosome image (Maluszynska and Heslop-Harrison 1993a). Color slides were scanned with an Apple Macintosh computer-based image processing system using a Epson ES-600C scanner. Two images were then aligned and contrast-adjusted with the Adobe Photoshop software. Processed images were recorded on Fujichrome 200 color slide film with a Polaroid CI-5000 digital palette film recorder.

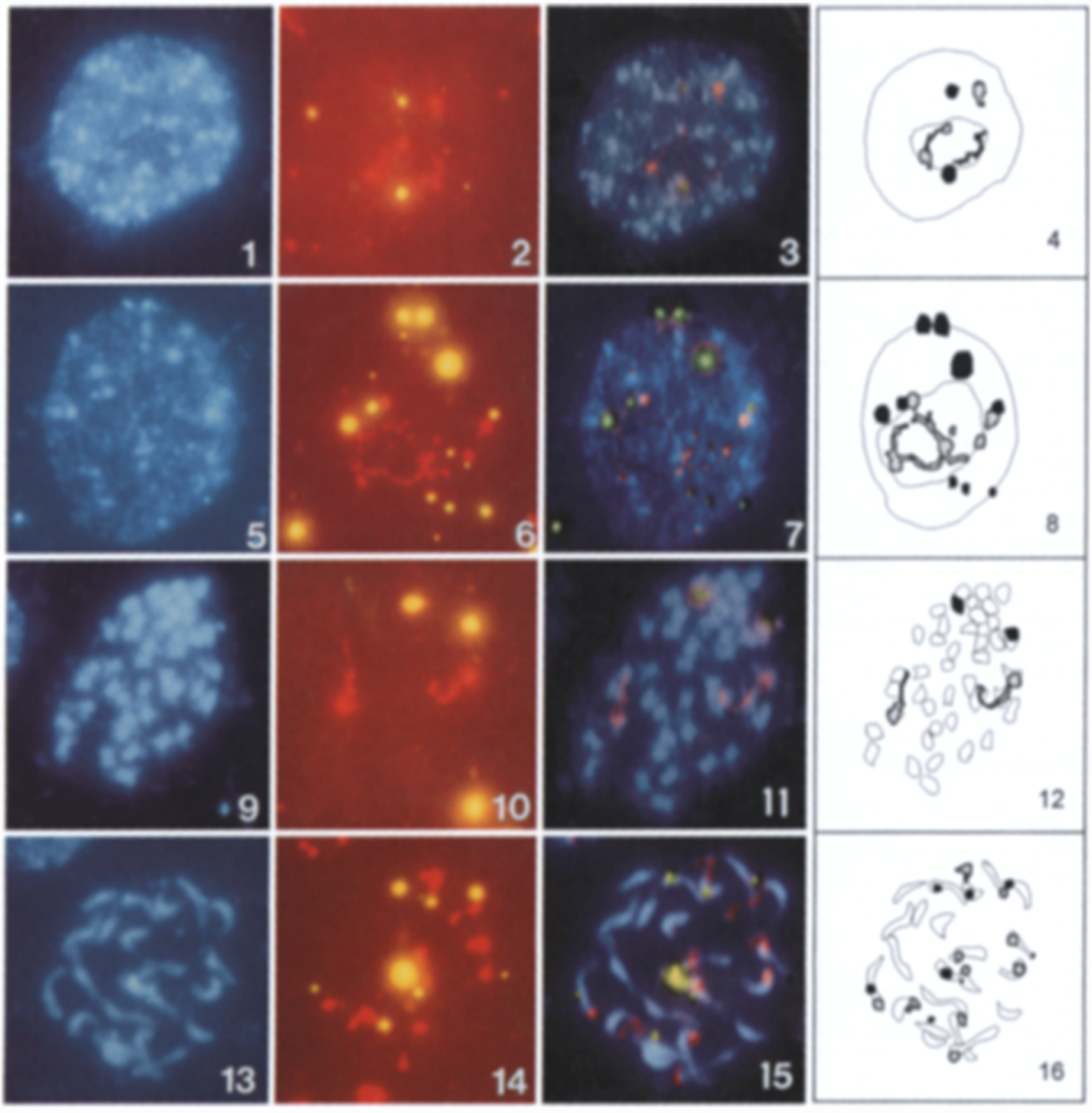
Results

Interphase nuclei and metaphase chromosomes of both soybean and common bean are effectively detected by counterstaining with DAPI (Figs. 1, 5, 9, 13). While interphase heterochromatin can be observed by DAPI, nucleoli are best observed by negative DAPI staining (Figs. 1, 5) or by phase contrast microscopy. The chromosome numbers of both species agreed with the previous determinations of Lackey (1980), where soybean is 2n=2x=40 and common bean is 2n=2x=22 (Figs. 9, 13).

5S rDNA loci

In soybean, we observed only one 5S rDNA locus (two hybridizing sites) in both interphase and metaphase nuclei when high-stringency washes were used (see Materials and methods). This 5S rDNA locus could be observed in the distal part of a middle-sized chromosome pair in metaphase chromosome spreads (Figs. 9–12); in interphase nuclei, the hybridization signals appeared as two sites. In all cases, at least one of the 5S rDNA sites was associated with the periphery of the nucleolus (Figs. 3–4), and very often both spots were located there. When lower-stringency washes were used, two additional sites of 5S rDNA were observed at the distal regions of a different pair of chromosomes, and were not associated with the periphery of the nucleoli in the interphase nuclei (data not shown). It is unclear from these results if this represents an additional 5S locus or, perhaps, a partially homologous satellite sequence; however, an analysis from CHEF electrophoresis argues for a single locus (Danna et al. 1996).

In contrast to soybean, common bean usually had 3–4 5S rDNA loci (6–8 sites) in its interphase and metaphase nuclei (Figs. 5–8, 13–16), though ten sites (five loci) in a single nucleus were occasionally evident. Among these 6–8 hybridization sites, the intensity of the hybridization



Figs. 1–16 **1–4** The 5S and 45S rRNA genes in soybean, ×2400. **1** The morphology of the interphase nucleus is revealed by DAPI staining with the nucleolus shown by negative staining. **2** In situ signals of 5S and 45S rDNA using the dual filter set. Gene puffs can be observed as rhodamine stained (*red*) extension fibers from one 45S rDNA site associated with the nucleolus, while 5S rDNA sites are represented by the *yellow-green* color of FITC. **3** Processed image of both the DAPI-stained interphase nucleus and the hybridization signals. Note the spatial association of 5S and 45S rDNA sites with the nucleolus. **4** An interpretative drawing of the hybridization pattern, showing the outline of the nucleolus, and 45S rRNA gene puffs within the nucleolus. *Black dots* 5S rDNA signal; *shaded dots and extensions* 45S rDNA signal. **5–8** The 5S and 45S rRNA genes in common bean, × 2400. **5** Interphase nuclear morphology revealed by DAPI staining, and the nucleolus shown by negative staining. **6** In situ signals of 5S and 45S rDNA using the dual filter set. Gene puffs can be seen as rhodamine-stained (*red*) extension fibers from the 45S rDNA sites, while 5S rDNA sites are represented by the *yellow-green* color of FITC; **7** Processed image from the hybridization signals and the DAPI-stained interphase nucleus. Note that multiple sites of both 5S and 45S rDNA are present; however, only one 5S and two 45S rDNA sites are associated with the nucleolus. **8** An interpretative drawing of **7**: the outline of the nucleolus, and 45S rRNA gene puffs within the nucleolus are shown; *black dots* 5S rDNA signal; *shaded dots and extensions* 45S rDNA signal. **9–12** The chromosomal locations of 5S and 45S rDNA in soybean, × 2400. **9** Soybean metaphase chromosome morphology revealed by DAPI staining. **10** Hybridization signals of 45S rDNA (*red*) and 5S rDNA (*yellow-green*). **11** Overlaid processed images from DAPI staining and rDNA hybridization show the locations of 5S (*yellow-green*) and 45S (*red*) rDNA sites on soybean metaphase chromosomes. Note that the 5S and 45S rDNAs are distributed on different chromosomes. **12** Interpretative drawing showing the chromosome spread and the rDNA hybridization signals: *black dots* 5S rDNA signal; *shaded dots and extensions* 45S rDNA signal. **13–16** Common bean metaphase chromosomal locations of the 5S and 45S rDNA. **13** The chromosome morphology of common bean revealed by DAPI staining, ×2400. **14** Hybridization signals of 45S rDNA (*red*) and 5S rDNA (*yellow-green*). **15** Overlaid processed images from DAPI staining and rDNA hybridization to show the locations of 5S (*yellow-green*) and 45S (*red*) rDNA sites on soybean metaphase chromosomes. Note that most 5S and 45S rDNA loci are distributed at different chromosomes. **16** An interpretative sketch of rDNA chromosomal locations in common bean: *black dots* 5S rDNA signal; *shaded dots and extensions* 45S rDNA signal

signal varied. The intensity differences of hybridization signals among major and minor sites could be as large as ten-fold (Figs. 5–8, 13–16). In the interphase nucleus, two weak sites were associated with the nucleolus while the other sites, including the very intense hybridization sites, were often associated with the heterochromatin, which stains strongly with DAPI counterstaining (Figs. 5–7). In the metaphase nucleus, 5S rDNA loci were on four different chromosome pairs. One pair of highly condensed chromosomes had very intense-fluorescing 5S rDNA spots, which often obscured the entire chromosomes (Figs. 13–16). In early metaphase nuclei, we have observed that each of these intense spots actually represents two hybridization sites on different arms of the same chromosome, which may explain the variation in the number of hybridizing sites between different nuclei.

45S rDNA loci

We consistently observed only one soybean 45S rDNA locus in interphase and metaphase nuclei as demonstrated by two spots (Figs. 1–4, 9–12). In interphase nuclei, at least one hybridization site was associated with the nucleolar peripheral regions (Figs. 1–4). Frequently, the two hybridization sites were very close together and even, occasionally, fused. In some interphase nuclei containing relatively large nucleoli, gene puffs (de-condensed chromatin strings) extended into the nucleoli (Figs. 1–4). The hybridization signals on these gene puffs were fainter, and more diffuse with the exception of some bright minor sites along the chromatin extension strings. At metaphase, this de-condensed locus is located in the distal region of one pair of chromosome arms (Figs. 9–12). When 5S and 45S rDNA loci were examined simultaneously with different fluorescent labels, it was clear that the hybridization sites for these two genes are located on different chromosomes (Figs. 9–12).

In the common bean nucleus, we observed multiple 45S rDNA hybridizing sites. Three or four pairs of hybridization sites, including one pair of minor sites, were frequently observed (Figs. 5–8, 13–16). In interphase, two punctate sites were associated with the periphery of the nucleolus, while other sites were distributed in the heterochromatic regions of the nucleoplasm as shown in the processed DAPI image (Figs. 5–8). As in soybean, 45S rDNA gene puffs emanate from punctate sites on the nucleolus periphery in the larger nucleoli. Occasionally, two or three nucleoli with labelled gene puffs were present in the same interphase nucleus (data not shown). The de-condensed chromatin extensions in these nucleoli are normally shorter, and connected to the large, more condensed sites on the nucleolus periphery. At metaphase, these loci appear to be mostly telomeric and on different chromosomes (Figs. 13–16). By labelling 5S and 45S rDNA probes with different fluorescent dyes, we observed that no chromosome contains major sites for both 5S and 45S RNA genes (Figs. 13–16). However, the possibility that minor sites are located on the same chromosome could not be excluded.

Discussion

Locus number and evolution of rDNA

In this study, we report strikingly different loci numbers for ribosomal genes in two related legumes: soybean and common bean. Our data indicate that soybean has less than two loci for 5S rDNA and a single locus for 45S rDNA. In contrast, common bean appears to have multiple 5S (four to five) and at least two 45S rDNA loci. Our results fit well with previous studies on the 45S rDNA of soybean (Skorupska et al. 1989; Griffor et al. 1991), as well as with pulsed-field gel electrophoresis studies in which three 5S arrays were observed for common bean compared to only one in soybean (Danna et al. 1996).

Soybean is considered a diploidized tetraploid species with common bean as one of its diploid relatives (Lackey 1980; Zhu et al. 1995a). One tetraploid model of soybean evolution suggests that this species was derived from diploid ancestors containing 2n=22 chromosomes, followed by chromosomal doubling and a reduction to 2n=40. This hypothesis is supported by the high percentage of the soybean genome that is duplicated (Zhu et al. 1994). Diploidization involves the progressive loss of duplicated genes and it has been estimated that approximately 25% of the duplicated genome has been lost (Zhu et al. 1994). Previously, it was suggested that the single 45S rDNA locus is evidence of diploidization and that soybean would have had two loci in the tetraploid state (Skorupska et al. 1989). Indeed, loss of duplicated sequences, including rDNA genes, has been documented in many diploidized polyploid species, such as in wheat (Mukai et al. 1991) and *Brassica* (Maluszynska and Heslop-Harrison 1993b). However, in the present study the diploid relative actually has multiple rDNA loci compared to the single rDNA locus found in soybean, which confounds this simple model. It is evident that 5S and 45S rDNA evolution is more complex than represented in a simple diploid-to-tetraploid model. In fact, different rDNA loci numbers have been reported in related diploid species of the same genus (Errico et al. 1991; Fukui et al. 1994), documenting the alteration of the rDNA loci number without changes in ploidy. On the other hand, disproportional numbers of rDNA loci in diploid and polyploid nuclei have been documented in both plant and animal systems (Linde-Lausen et al. 1992; Becker and Nagl 1994). Further in situ hybridization studies on other close soybean relatives within the subtribe Glycininae will be conducted to better understand the gain and loss of rDNA loci in the evolution of these species.

Expression sites of the rDNAs

Ribosomal RNA gene regulation can occur by the suppression of the whole locus (all the genes in an array), as well as by the suppression of just some of the genes within an individual locus which is accompanied by increased methylation (Ellis et al. 1989). In a number of plants, rRNA

gene expression is associated with the spatial distribution of these genes in the interphase nucleus (Rowlins and Shaw 1990; Bauwens et al. 1991; Leitch et al. 1992; Highett et al. 1993).

Although the rDNA loci numbers varied between the two species studied here, only one or two 5S and two 45S rDNA hybridization sites were actively expressed in each case.

In both soybean and common bean, the sites of actively transcribed 45S rDNA are observed by: (1) association with the nucleolar periphery of the interphase nucleus (Fig. 1–6), as commonly noted (Rogers and Bendich 1987; Flavell et al. 1993); (2) de-condensation of the hybridization sites (smaller size with less intensive signal, Figs. 1–6); and (3) hybridized gene puffs associated with the peripheral sites and extending into large nucleoli (Figs. 1–8). This is similar to the terminal de-condensation pattern of actively expressed 45S rDNA observed in rye (Leitch et al. 1992) and sugar beet (Schmidt et al. 1994).

In soybean, all of the 45S rDNA hybridization sites were associated with the interphase nucleolus (Figs. 1–4), in contrast to common bean where 2–6 additional interphase sites were spatially distinct from the nucleolus (Figs.5–8). The non-nucleolar sites were larger and gave a stronger hybridization signal than those associated with the nucleolus. These hybridization sites were spatially associated with heterochromatin in the nucleoplasm. We believe that these sites may not be transcriptionally active because , as previously suggested (Leitch et al. 1992; Flavell et al. 1993; Schmidt et al. 1994), they are highly condensed and distant from the nucleolus.Therefore, the spatial distribution pattern, the presence of de-condensation, and the gene puffs of rDNAs may be direct evidence that only a subset of the total rDNA genes in an array is active. What factors control the expression of multiple loci and whether the same locus is always used from cell to cell have not been determined.

While the spatial relationship between active 45S rRNA loci and the nucleolus has been well established (Rogers and Bendich 1987), the transcription activities of 5S rRNA genes were thought to be located elsewhere in the nucleus (Rogers and Bendich 1987). Recently, the spatial proximity of the 5S rDNA locus to the nucleolus has been noticed in pea (Ellis et al. 1988) and wheat (Mukai et al. 1991).

In both soybean and common bean, the association between the nucleolar periphery and at least one of the 5S rDNA sites was so frequently observed (Figs. 1–8) that it could not be due to coincidence. The 5S rDNA sites associated with nucleoli may be transcriptionally active, since such a location is not associated with the heterochromatin, and the intimate spatial relationship between active 5S rRNA genes and 45S rRNA genes may facilitate transcriptional regulation between these two rRNA genes and the transport of 5S rRNA gene transcripts into the nucleolus. A similar idea has been proposed for wheat, where 5S rRNA genes have been observed near the nucleolus (Mukai et al. 1991).

In common bean, where there are eight to ten 5S rDNA sites, multiple 5S rDNA sites were found distant from the nucleolus and spatially located with heterochromatin in the nucleoplasm (Figs. 7–8). In some soybean nuclei, one 5S rDNA site was also observed distant from the nucleolus. Such sites may not be actively transcribed. The 45S rRNA products (18S and 25S rRNAs) and the 5S rRNA are needed in a 1:1 stoichiometry in the ribosome. However, the 5S RNA is more readily produced from a smaller number of genes due to its small size (120 nucleotides in soybean) and the ability of RNA polymerase III to processively transcribe 5S rRNA following a single initiation. We have never observed more than two 5S rDNA sites associated with the common bean nucleolus suggesting that multiple loci are not required for adequate 5S rRNA production.

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