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The spatial localization of 18 S rRNA genes, in relation to the descent of the cells, in the root cortex of *Petunia hybrida*

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SUMMARY

The 3-D localization of transcription inactive 18 S rRNA genes was studied in interphase nuclei of *Petunia hybrida* root tip cells. To enable a cell type (i.e. cortex)-specific study in which also the orientation and descent of the cells could be taken into account, a method was developed to preserve the spatial organization of the root meristem. The ribosomal genes were detected by fluorescence in situ hybridization using a biotinylated cDNA probe. 3-D images of 81 nuclei, obtained by confocal scanning laser microscopy, were processed with newly developed computer software. 3-D nucleolar and nuclear dimensions, and the localization of the FISH-spots, were recorded interactively. We compared the absolute and relative position of the genes within and between files of cells of the cortex region of several roots, taking into account the genealogical relationship of the cells. Statistical analysis showed that both the relative and absolute positions of the inactive genes were random, also in more closely related cells within a file of cells. A 'relict telophase orientation' of the genes (i.e. the position of the genes in the daughter cells are mirror images of each other) could only be observed in the $G_{0/1}$ phase of 'true' daughter cells; the orientation was not preserved throughout the next cell cycle.

Key words: in situ hybridization, confocal microscopy, ribosomal gene, morphometry, *Petunia hybrida*

INTRODUCTION

Many aspects of gene expression in eukaryotic cells are readily understood at the molecular and biochemical levels, but relative little is known about the significance of the spatial positions of chromosomes and genes in the nucleus. Before anything can be said about a possible role for the chromatin organization, qualitatively and quantitatively well-documented data have to be collected. For the present, our knowledge from very different studies at the chromosome as well as the gene level in human, animal and plant material is still rather fragmentary and sometimes contradictory (see for instance reviews by Appels, 1989; Heslop-Harrison and Bennett, 1990; Haaf and Schmid, 1991).

One of the first reports about nuclear organization concerns the relict telophase arrangement (Rabl, 1885). The concept that chromosomes maintain their telophase orientation during the subsequent interphase and into the next prophase is supported by several more recent studies; for example: Agard and Sedat (1983); Avivi and Feldman (1980); Comings (1980); Fussel (1984). Since these studies are only informative about opposite positioning of telomeres and centromeres, it is not yet clear as to whether a relict telophase orientation is restricted to the overall chromosome orientation, or whether it is also applicable to the spatial positioning of genes or gene clusters.

The present paper deals with the question of to what extent the telophase orientation of the genes is preserved throughout the subsequent cell cycle(s). Therefore, we studied the ribosomal gene clusters in cortex cells of the *Petunia hybrida* root meristem, using a cDNA probe that hybridized with the 18 S rRNA genes. *Petunia hybrida* (2*n*=14) has four chromosomes with ribosomal gene clusters. The genes of two of them are involved in nucleolus formation, whereas the genes of the two other chromosomes are transcriptionally inactive and not associated with the nucleolus. An analysis of the spatial position of the latter gene clusters, in relation to the descent of the cells, is informative about gene localization in dividing cells, in interphase of the daughter cells, in dividing daughter cells and in more distantly related cells.

The analysis of a number of cell files in the cortex zone of *Petunia hybrida* roots, using 3-D fluorescence in situ hybridization, confocal microscopy and a specially developed computer program to measure the relative and absolute position of the ribosomal gene clusters, revealed that: (1) the concept of the relict telophase (Rabl) orientation is not applicable to the transcriptionally inactive ribosomal gene clusters, since soon after cell division random repositioning takes place; (2) the chromosomes with transcriptionally active ribosomal genes preferably enter the nucleolus parallel to each other.

MATERIALS AND METHODS

Squash preparations

Squash preparations and sections were made from seedlings of root-

tips of *Petunia hybrida* cultivar Mitchell (2*n*=14). Plants were grown in a greenhouse. After 4 weeks 1 to 1.5 cm long root-tips were dissected and then fixed for 2 hours in a solution of absolute ethanol/glacial acid (3:1, v/v).

For the squash preparations the root-tips were washed for 5 minutes in aqua dest., then rinsed in 15 mM NaAc, pH 4.3. This was followed by a maceration step for 1 hour in a 0.1% mixture of pectyolase, cytohelicase and cellulase (Sigma) dissolved in 15 mM NaAc, pH 4.3. Before squash preparations were made, the slides were rinsed in 15 mM NaAc and softened for 2 to 3 minutes in 45% acetic acid under a coverslip (18 mm × 18 mm). The slides were frozen in liquid nitrogen and the coverslip was gently removed with a razor blade.

Sectioning and embedding

The root-tips were embedded in a mixture of 2:1 (v/v) polyethylene glycol (PEG) 1500 and PEG 4000. First the root-tips were transferred to 100% ethanol, then to a 1:1 (v/v) solution of PEG 1500/4000 in absolute ethanol at 60°C; followed by a pure mixture of PEG 1500/4000 at 60°C; each step lasted 1 hour. For making longitudinal sections the root-tips were positioned on the bottom of a gelatine capsule, to facilitate cutting. The gelatine capsules were cooled down overnight in a fully dry oven, which resulted in solid blocks of PEG ready for trimming with a razor blade and cutting approximately 20 μm thick sections on a microtome, using a glass knife. Special care was taken to align the root-tip in such a way that the cutting of the knife was parallel to the root-tip.

Hybridizations on squash preparations

The squash preparations were treated with 120 μl RNase (Sigma; 100 μg/1 ml, in 2× SSC) under a coverslip (24 mm × 50 mm), upside down for 1 hour in a 37°C moist chamber. This was followed by rinsing the slides in 2× SSC for 3× 5 minutes. The squash preparations were digested with proteinase K (Boehringer Mannheim; 5 μl proteinase/100 ml proteinase K buffer, which is 20 mM Tris-HCl + 2 mM $CaCl_2$, pH 7.4) for 7.5 minutes at 37°C, followed by a rinse in 2× SSC. Subsequently, the slides were rinsed in PBS (10 mM Na_2HPO_4 + NaH_2PO_4) containing 50 mM $MgCl_2$, and fixed for 10 minutes by incubation in PBS containing 50 mM $MgCl_2$ + 3.75% formaldehyde. After a final wash in PBS the slides were dehydrated through an ethanol series (70%, 90% and 100%; 5 minutes each).

Hybridizations on sections

The sections were rinsed for three times in 2× SSC to wash out the PEG. RNase treatment was as described above, except that the incubation took place in a droplet on a siliconized glass slide. For digestion of the cell walls the sections were treated with 0.06% (w/v) pepsin (Boehringer Mannheim) in 15 mM HCl for at least 45 minutes (depending on the size and thickness of the section). Subsequently, the sections were rinsed three times in 2× SSC.

Probe labelling and in situ hybridization (ISH)

For detection of the 18 S ribosomal genes the pSR1 2B3 plasmid (kindly donated by R. B. Meagher, University of Georgia, Athens, USA) was used. This plasmid contains a 1.05 kb internal fragment of 18 S rDNA from soybean (Eckenrode et al., 1985). pSR1 1B3 was biotinylated with bio-11-dUTP (Sigma) by nick translation. The specimens were hybridized in a 10 μl assay containing 50% (v/v) deionized formamide/2× SSC, sonicated salmon sperm DNA (50-fold excess over probe DNA) and the probe (10 ng/μl). Squashes were hybridized under a coverslip (18 mm × 18 mm), whereas sections were hybridized in a droplet on a siliconized glass slide. The probe and target DNA were denaturated together for 5 minutes at 80°C. Hybridization was carried out overnight in a 37°C moist chamber consisting of 50% (v/v) formamide and 2× SSC. Post-hybridization washes were performed for 3× 5 minutes in 50% (v/v) formamide/2× SSC, pH 7.0, at 37°C.

Immunocytochemical detection procedure

To prevent aspecific antibody binding the squashes and sections were incubated with 4 M (4× SSC, 5% (w/v) non-fat dry milk) for 30 minutes. Detection of the single probe ISH was performed with 5 μg/ml avidine-D conjugated with FITC (fluorescein isothiocyanate) (Vector Laboratories) diluted in 4 M. The ISH signals were amplified with 5 μg/ml biotinylated goat anti-avidine-D (Vector Laboratories), followed by a second layer of avidine-FITC. Each step lasted 20 minutes in a moist chamber, in the dark. Between each incubation step the slides were rinsed for 3× 5 minutes in 4T (4× SSC/0.05% Tween-20). The squashes and sections were rinsed once in PBS and mounted in antifade reagent (0.2 M Tris-HCl, pH 7.5/glycerol (1:9, v/v) containing 2% (w/v) DABCO (1,4-di-azobicyclo-(2,2,2)-octane)) and counterstained with propidium iodide (PI). Fluorescence photographs were taken with an Olympus (BH2-RFC) microscope equipped with 100 W mercury arc lamp, using Scotch 640 ASA colour slide films.

Control experiments were performed on squash preparations. To confirm that our probe indeed detects rDNA sequences either the probe or one of the antibodies was omitted, or no RNase was applied.

Confocal microscopy and image analysis

3-D images were made using a confocal microscope as described by Brakenhoff et al. (1988). The two fluorochromes were excited using a 476.2 nm line of a krypton ion laser in combination with a 510 nm dichroic mirror. Dual detection was performed by using a 610 nm filter to detect PI signal and simultaneously a 525 DF 40 bandpass filter to detect the FITC signal. A Hewlett Packard/Apollo 425 series workstation with Scilimage software (ten Kate et al., 1990) was used for image processing. Depending on the size of the nuclei, pixel sizes of 100 to 150 nm lateral and 200 to 400 nm axial were used. An average of 30 z-scans per channel was performed. Each z-scan was the result of three times frame averaging to produce a high signal-to-noise ratio. Before the actual measurement took place the 3-D images were separated into an A-file (PI-signal) and a B-file (FITC-signal). A 3-D Gaussian (Niblack, 1986) filter was applied to the A- and B-files to remove noise components in the images. To enhance the voxel values on the edges of the objects to be measured a 3-D laplace (Niblack, 1986) was also used on the two separated images.

Morphometry

Morphometry was done according to an interactive software program IMAP3D (Interactive Measurement of Axes and Positioning in 3-Dimensions), as programmed in our department.

A 3-D cursor is used to indicate the position of the 18 S RNA genes within the FITC-channel of the two-channel 3-D confocal image. The cursor is present in three windows on the computer screen. The central window displays a front view (*XY*-projection) of the 3-D confocal data set. The windows on the side display a top view (*XZ*-projection) and a side view (*YZ*-projection). By moving the cursor to the *XY*-position of the FITC spots in the front view, and to the *Z*-position in the side views, the user accurately indicates the position of the genes within the 3-D image. By pressing the mouse button or a user-defined key the 3-D coordinates are stored.

The dimensions of the nuclei as well as of the nucleoli were determined using the 3-D cursor described above. First, the cursor was positioned at the nuclear centre (in the propidium iodide channel of the confocal image). Second, the cursor was rotated around its *Z*-axis, the *Y*- and *X*-axis were elongated, and the cursor position was adjusted in such a way that the cursor fitted the ellipsoid nucleus, making sure that the cursor *Y*-axis fitted the shortest nuclear axis. Finally, the cursor *Z*-axis was elongated to fit the nuclear axis oriented in the *Z*-direction of the image. The length of the three cursor axes (in voxels) as well as the degree of rotation were stored together with the 18 S RNA gene cluster positions.

The nucleolar dimensions were obtained in the same way except that, because of the spherical shape of the nucleolus, no rotation of

the cursor was necessary, and the *X*-, *Y*- and *Z*-axes were equally elongated.

Normalization of the position data

To compare the data from different cells, the dimensions of a 'model nucleus' were calculated in the following way: the mean lengths (in voxels) of the three nuclear axes were calculated, the *Z*-axis was set to the value of unity and the *Y*- and *X*-axes were set to their mean values relative to the *Z*-axis (where ||*Y*-axis|| < ||*X*-axis|| < ||*Z*-axis||). The positions of the 18 S RNA gene clusters were scaled to the coordinate system of this 'model nucleus'.

Statistical analysis of the position of the inactive 18 S rRNA genes

To test for regularities in the mutual position of inactive gene clusters, the distribution of the gene distances was compared with a random distribution of distances between points uniformly distributed in an ellipse with the dimensions of the model nucleus, taking into account the presence of a nucleolus (Houtsmuller et al., unpublished). The latter is important because the inactive genes are never located in the nucleolus. The Kolmogorov-Smirnov statistical test was used for comparison of the distributions. The interdistance random distribution was calculated by the Monte Carlo method.

To test for regularities in the positions of inactive genes independently of each other, the distribution of distances of the genes from the nuclear centre was compared with the random distribution in the same way as the distribution of the gene distances.

RESULTS

Identification of ribosomal genes in *Petunia hybrida*

To label the ribosomal genes, the technique of fluorescence in situ hybridization (FISH) was applied to squash preparations of *Petunia hybrida* root tips, using a biotinylated soybean ribosomal DNA probe. The pSR1 2B3 probe labels four clusters of 18 S ribosomal genes localized on four of the 14 chromosomes of *Petunia hybrida*. The 18 S rDNA target size is one of the genes of a tandem repeat unit consisting of 26 S, 18 S, 5.8 S and interspersed by noncoding sequences with a total length of approximately 6 to 7 kb. The frequency is not known for *Petunia*, but estimations are of the order of 800 copies per nucleolus organizing region (NOR) chromosome.

Control experiments on a series of squash preparations revealed that there was a large increase in ISH signals in the nucleolus and also in the cytoplasm, as RNase was omitted. Alternatively, no ISH signal was found, either because the probe was omitted or because none of the antibodies was applied.

The expanded genes of two of the clusters are organized into one large nucleolus, whereas the genes on the other two chromosomes are condensed and located outside the nucleolus (Fig. 1). In view of the state and localization of the ribosomal genes on the latter chromosomes, they are presumably transcriptionally inactive. According to the standard karyogram of *Petunia hybrida* (Smith et al., 1973), chromosomes 2 and 3 possess NORs. The chromosomal localization of the two NORs are subtelomeric on the short arms. This was confirmed by ISH. The hybridization sites on metaphase chromosomes were restricted to the satellites of chromosomes 2 and 3. It is not yet clear which chromosomes are involved in nucleolus formation, and whether there are differences within and between cell types. In this paper we shall refer to the dispersed ribosomal genes inside the nucleolus as to (transcriptionally) active genes, and to the condensed clusters outside the nucleolus as inactive genes.

At interphase as well as prophase (Fig. 1) the nucleolus is characterized by a large number of small spots inside the nucleolus, and some larger spots (designated as perinucleolar ribosomal gene clusters) at the periphery. The number of perinucleolar gene clusters varies between zero and four, indicating that the complete NOR or only part of it is involved in nucleolus formation. When there are four clusters, two of them correspond to the proximal part and two to the distal part of the NOR (Fig. 1A). The distal perinucleolar clusters are usually larger than the proximal clusters. Since the preparations are treated with RNase prior to hybridization (see Materials and Methods), it is likely that the numerous small intranucleolar spots represents individual or several 18 rRNA *genes*. Measurements on spots, which are localized in tracks (see for example, Fig. 1B), revealed that the average inter-spot distance is approximately 1.1 μm. Comparison with electron micrographs of detergent-treated nucleolar chromatin spreads of *Zea mays* (Greimers and Deltour, 1984) suggests that an intranucleolar hybridization spot in our squash preparations corresponds to one or only a few ribosomal genes. Repeatedly we observed that one of the perinucleolar gene clusters was located several micrometres outside the nucleolus, while a thin track of spots connected the cluster with the mass of single spots in the nucleolus (Fig. 1B).

FISH on thick root-tip sections

To analyze the 3-D localization of 18 S ribosomal genes, *Petunia hybrida* root-tips are embedded in PEG as described in Materials and Methods. To ensure that at least one layer of nuclei was intact, approximately 20 micrometre thick longitudinal sections were cut from the embedded root tips. This method enabled the probe to reach the nucleus, because the cell wall of most cells has been partly cut away. The remaining (larger) parts of the cell wall ensured the preservation of the 3-D tissue organization. Fig. 2 shows part of a section after in situ hybridization. Only those cells in the cortex that are organized in longitudinal files were used for the 3-D localization of transcriptionally inactive 18 S ribosomal genes. Cells located in and near the apex as well as cells in the elongation zone have been omitted, to restrict the analysis to cells of a comparable developmental stage.

Size of the nucleus and nucleolus

To compare the positions of the gene clusters in cells with different sized nuclei and nucleoli, the size along the *X*-, *Y*- and *Z*-axis of each nucleus and nucleolus was measured, after which the normalized sizes were calculated. Fig. 3 shows a two-channel 3-D confocal image with (in A) the FITC-channel and the recording of the spots, and (in B) the PI-channel with the cursor measuring the nucleolar dimensions. The interactive IMAP3D program to record and re-scale the size of the three axes, and the position of the hybridization spots is shown in Fig. 4 (for further details about the procedure, see Material and Methods). The mean absolute size and the corresponding normalized size of the analyzed cortex nuclei and nucleoli are given in Table 1.

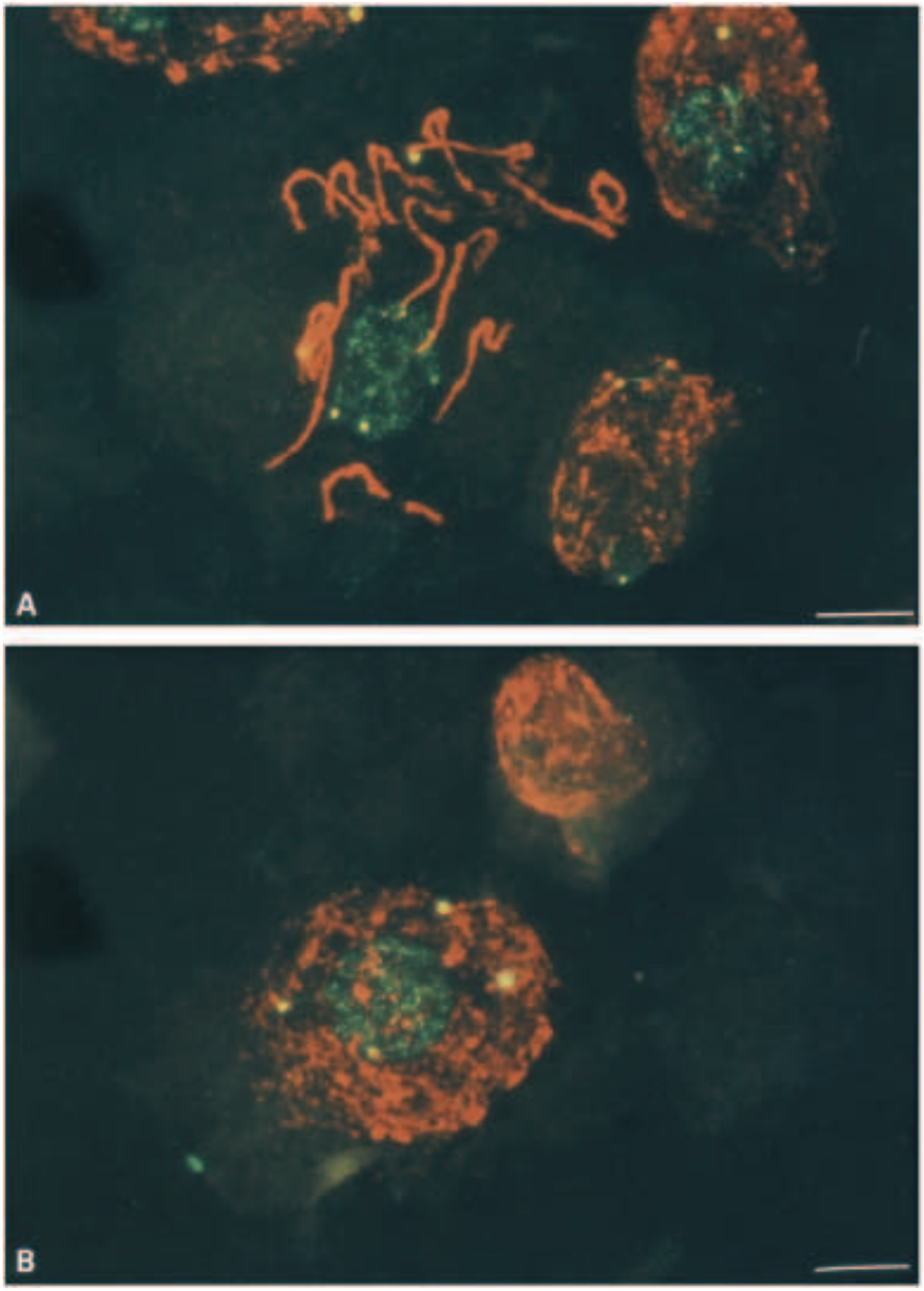


Fig. 1. Squash preparation of a *P. hybrida* at prophase (A) and interphase (B) after in situ hybridization with a biotinylated probe of 18 S rDNA. The probe is detected by avidin-FITC (yellow and green fluorescence), and DNA is counterstained with propidium iodide (red). The nucleolus is characterized by a large number of small hybridization spots, which correspond to one or a few 18 S rRNA genes. The gene clusters at the periphery of the nucleolus comprise proximal and distal (condensed) parts of the nucleolus organizer region (so called perinucleolar spots). Note that one of the small gene clusters in (B) is connected with the nucleolus by a track of spots. Two other chromosomes, not associated with the nucleolus, have large condensed clusters of transcriptionally inactive ribosomal genes (one of these large yellow spots is partly obscured by a chromosome). Bar, 10 μm.

Spatial position of the perinucleolar ribosomal gene clusters

Approximately 10% of the analyzed cortex cells had large enough perinucleolar gene clusters to allow a positive identification of the (smaller) proximal and (larger) distal regions (see also the first paragraph of Results). Usually the smaller spots (nos 1 and 2 in Fig. 5) are localized at the periphery of one hemisphere of the nucleolus and the larger spots (nos 3 and 4 in Fig. 5) are in the other hemisphere (nos 5 and 6 are the inactive ribosomal gene clusters). Also in the root sections we observed a few examples of a track of small hybridization spots protruding towards a somewhat shifted perinucleolar gene cluster (comparable to that in Fig. 1). As we do not have an explanation for this phenomenon, we are not sure whether these tracks are structures inherent in the nucleolus or artefacts.

The absolute spatial localization of inactive rRNA genes in sets of telophase chromatids

Twenty-five telophase nuclei were scanned to determine whether the arrangements of the ribosomal gene clusters in two sets of telophase chromatids were mirror images of each other. The mirror plane was defined as a plane halfway through a set of chromatids. During telophase all ribosomal genes are inactive, which results in two sets of four large gene clusters.

Table 1. Absolute and normalized sizes of 81 cortex nuclei and nucleoli

	X-axis (s.d.)	*Y*-axis (s.d.)	*Z*-axis (s.d.)
Mean absolute size, nucleus (μm)	5.6(0.84)	4.5(0.69)	5.9(1.1)
Normalized size, nucleus (μm)	0.98	0.77	1.0
Normalized size, nucleolus (μm)	0.35	0.41	0.45

All cells are smaller in the direction parallel to the root axis (*Y*-axis). Probably this is because of a reduced cell elongation in these rapidly dividing meristematic cells. For the statistical analyses of the spot positions (see Results) a mean radius of 0.405 for the nucleolus was used.

Fig. 2. Part of a 20 μm thick longitudinal section of a PEG-embedded *P. hybrida* root-tip after hybridization with biotinylated probe of 18 S rDNA. The 18 S ribosomal genes appear as white spots, DNA is counterstained with propidium iodide (grey). The analysis of the spatial position of ribosomal genes was restricted to cells in the cortex zone. Three examples of pairs of daughter cells are indicated by brackets.

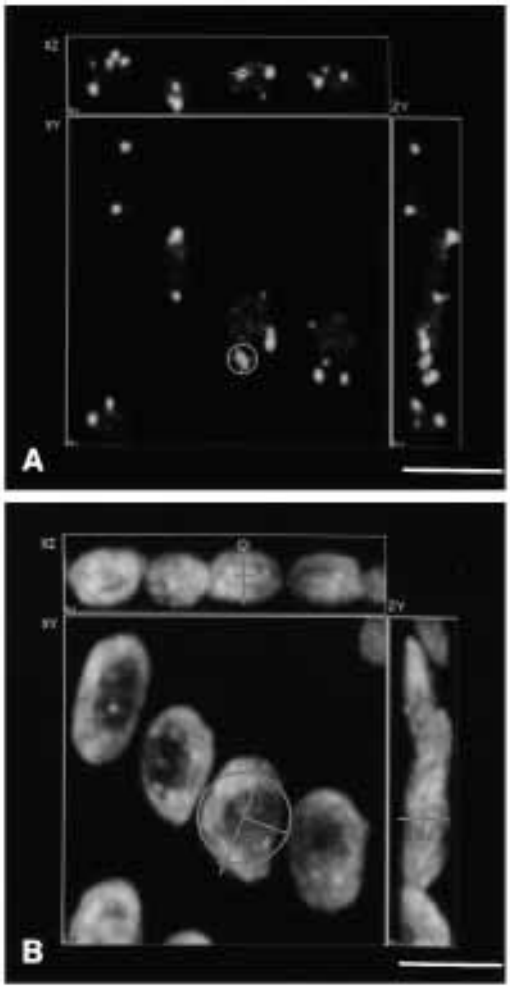


Fig. 3. Visualization of a two-channel confocal image of four interphase nuclei by the IMAP3D program. The large square window shows the *XY* (top) view, whereas the *XZ* and *YZ* (side) views are depicted at, respectively, the top and the right side. (A) Hybridization signal of the 18 S rRNA gene clusters (FITC fluorescence); (B) corresponding propidium iodide signal of the total DNA. In all windows a 3-D cursor is present to record the inactive 18 S rRNA gene clusters (A) and to measure the nucleolar and nuclear dimensions (B). Bar, 10 μm.

An example is shown in Fig. 6A,B. Repeatedly, we observed that two gene clusters were localized very close to each other. These double spots probably represent the ribosomal genes of the two NORs that are involved in nucleolus formation up to late prophase. Another characteristic of the double spots was that they were preferentially located close to a zone between the two groups of chromatids, whereas the spots corresponding to the inactive genes showed a more peripheral localization (i.e. near the region where the centromeres are localized). Analysis of a series of optical sections and stereoscopic images showed that the two sets of telophase chromatids, in several cases, were shifted in the *X*- and/or *Y*-direction to opposite sides along the mirror plane. Consequently, the two sets were no longer exact mirror images of each other. If the two sets of chromatids are shifted back to opposite positions on the computer screen, the spots in these telophases also proved to

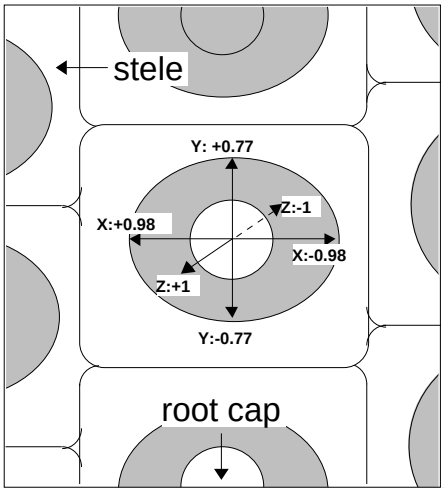


Fig. 4. Schematic representation of a top view of files of a cortex cell, indicating the orientation and mean value of the *X*, *Y* and *Z*-axes as recorded with the interactive computer program, IMAP3D.

be mirror images in both *X*- and *Z*-directions (lateral and axial). Taking the above mentioned shift phenomenon into account, the spot positions in all 25 telophases showed mirror images.

The absolute spatial localization of inactive rRNA genes in cell files

To investigate whether the inactive rRNA genes preserve their telophase mirror-image orientation (see above), spot positions in files of cortex cells were examined. All cells in a file are genealogically related to each other. On the basis of the following morphological criteria it was often possible to identify true daughter cells: (1) the separating cell wall is thinner compared to more distantly related cells (Rawlins et al., 1991); and (2) often there is an alternating shift (along the *X*-axis) in the position of the nucleus in pairs of daughter cells (Oud and Nanninga, 1992). We analyzed 10 cell files, which contained 7 to 15 successive cells each. An example of part of a cell file, with a pair of true daughter cells, is given in Fig. 7A-C. Top and side views of drawings of the normalized nuclei were used for a visual inspection of the spot positions (Fig. 7C). The separating cell wall between two daughter cells was defined as the mirror plane. In some pairs of cells, the top view image of the spot positions might give the impression of a mirror image. However, when the axial positions are also taken into consideration no convincing indication of any similarity are found, either in true daughter cells, or in less closely related cells in the same file. We also did not find any preference in relation to the overall tissue organization, such as a tendency to be localized on the part of the nucleus facing the root apex, periphery etc.

The relative spatial localization of inactive rRNA genes

As well as analyzing the absolute positions of the inactive rRNA gene clusters (see above), the spatial positions of these clusters with respect to each other and with respect to the centre of the nucleus were analyzed. We analyzed the 10 files of cells mentioned in the previous paragraph, as well as the overall sample of 81 cortex cells taken from different roots. Fig. 8 summarizes the data about spot-to-spot and spot-to-centre distances in the nuclei of three cell files. In Fig. 8A the mutual distance between the inactive ribosomal gene clusters, and in Fig. 8B the distance from a ribosomal gene cluster to the nuclear centre of three files of cells, are graphically presented. Also the expected random density functions are given for both parameters (see Materials and Methods). Because of the inevitably small sample sizes, a statistical analysis is not very meaningful. A comparison of the random distribution with each of the observed patterns of distances did not show noticeable preferences regarding the intergene distance and the distance from gene to nuclear centre.

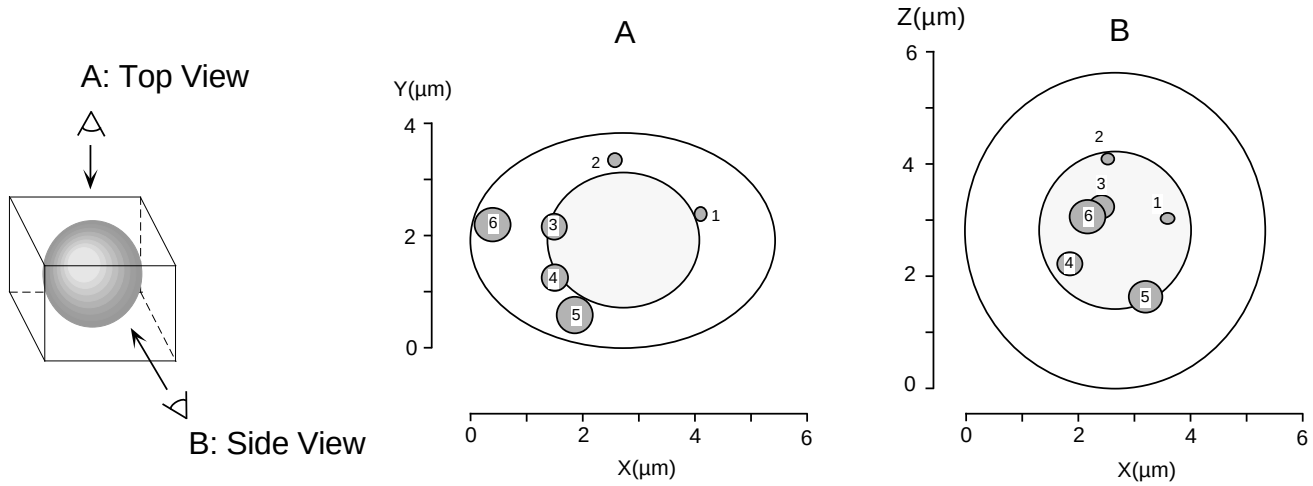


Fig. 5. Schematic representation of the ribosomal gene clusters in a nucleus of a *P. hybrida* cortex cell in (A) top view and (B) side view, showing the proximal (1 and 2) and distal (3 and 4) parts of the active NORs (spots inside the nucleolus are omitted), and the large transcription inactive gene clusters outside the nucleolus (5 and 6); compare with Fig.1. In this representative nucleus, the smaller proximal and larger distal perinucleolar spots are localized at the periphery of opposite hemispheres of the nucleolus. The drawing is based on a confocal data set of 128×128 pixels of 64 (2×32) optical sections with a step size of 280 nm.

Finally, a statistical evaluation of the relative spot positions in all 81 interphase cortex cells has been performed. The observed cumulative frequency distribution of the inter-spot distance was compared with the theoretically generated random distribution. For the theoretically generated random distribution the size and the localization of the nucleolus, where inactive rRNA genes are never localized, was taken into account. The Kolmogorov-Smirnov test (K-S test) was applied (Young, 1977) to compare the two distributions. The critical K-S value is 0.51 for n=81, with a 5% confidence level. A maximum deviation (D) of 0.064 was calculated, which indicates that the distribution of the inactive genes is not significantly different from the random distribution.

The distance of the inactive gene clusters from the nuclear centre and the theoretical random distributions were also compared. In this case, the critical K-S value was 0.11 for n=162, with a 5% confidence level. The calculated D_{max} was 0.058. Consequently, the observed frequency distribution could not be distinguished from the random distribution.

DISCUSSION

The analysis of gene positions

A meaningful study about the question of whether genes have preferential positions in the nucleus has to fulfil a number of conditions, concerning the following factors.

Cell type and tissue specificity

If preferential gene positions do exist, we have to take into account the possibility that such preferences are (among others) dependent on cell type, developmental stage and metabolic state of the cell (Nanninga et al., 1992). In that case, no preferences will be detected in a large sample comprising cells of different origin, whereas a non-random distribution of genes might be present in a smaller, well-defined group of cells. If we apply this notion to the root meristem, in which the tissue organization (i.e. cell files) is informative about the descent of cells, we have to analyze the localization of the genes in relation to the position of cells.

The method used to record gene positions

Gene localization can be defined in relative as well as absolute terms. The determination of relative gene positions is informative about the mutual position of homologous genes, the position with regard to the nuclear centre etc. Recording the absolute localization of genes gives valuable additional information; for example, about the question of whether gene positions in daughter cells are mirror images of each other (assuming that the telophase orientation will be preserved throughout the cell cycle). One can think of two methods to ascertain the absolute position of genes. Firstly, one can use an intracellular marker with a proven fixed position such as the centrosome (see for instance, Hulspas et al., 1993). Alternatively, one can employ preparations in which the tissue organization is preserved. The latter method has the additional advantage that gene positions can be determined in relation to the orientation of the cells. For example in roots, the position with respect to the tip (apex) or periphery of the root can also be taken into consideration.

Sample size and statistical evaluation

Generally, the larger the sample size, the more powerful the statistical analysis can be. However, sometimes (very) small sample sizes are inevitable. For example, in the case of two daughter cells, or when there is a limited number of cells in a file. In such cases the analysis often has to be restricted to a 'visual' comparison of the observed and expected (random) gene positions. Concerning the calculation of a random distribution, care has to be taken to estimate the average form as well as possible and to exclude all areas in the nucleus in which the genes cannot be found. In the case of *Petunia hybrida* root cortex cells it is important to realize that the nucleolus occupies up to 50% of the volume of the nucleus. Except for the ribosomal genes, which are actively involved in the process of ribosomal subunit formation, all other genes are localized in the shell between the nucleolus and the nuclear membrane.

The (transcriptional) activity of the gene

In several studies the idea has been put forward that genes that are actively transcribed are localized in specific nuclear domains, for example close to a group of nuclear pores (Blobel, 1985), or more generally in the peripheral part of the nucleus (Hutchinson and Weintraub, 1985; Manuelidis and Borden, 1988). If the concept of domain specificity being related to gene activity is correct, then it is of course important to know the state of expression of the appropriate gene(s), to avoid a heterogeneous sample consisting of (transcriptionally) active as well as inactive genes.

In the present study of the localization of 18 S rRNA genes, we restricted the spatial analysis, for the reasons mentioned above, to the transcriptionally inactive gene clusters in files of cortex cells of the *Petunia hybrida* root meristem.

Do ribosomal gene clusters conform to a Rabl orientation?

In telophase nuclei, pairs of daughter cells and files of genealogically related cells yielded the following results:

(1) In the two sets of telophase chromatids the positions of all four ribosomal gene clusters are mirror images of each other (Fig. 6).

(2) In the nuclei of true daughter cells no convincing mirror images could be observed with respect to the inactive gene clusters (Fig. 7).

(3) A comparison of the observed absolute as well as the relative positions of the inactive gene clusters in sequences of related cells, with a theoretical random distribution (Figs 7-8), also proved that soon after cell division random repositioning of the gene clusters takes place. At the periphery of the nucleolus a specific pattern of condensed gene clusters might be present, corresponding to the proximal and distal parts of the NORs (Fig. 5).

The observed loss of the telophase orientation of the ribosomal gene clusters seems to be in contradiction to the concept of relict telophase orientation as was first described by Rabl (1885). Rabl's observation, which has been confirmed by several more recent studies (for example: Anamthawat-Jonsson and Heslop-Harrison, 1990; Avivi and Feldman, 1980; Fussel, 1984; Rawlins et al., 1991; Schwarzacher and Heslop-Harrison, 1990), only concerns the overall organization of only some chromosomal features, i.e. centromeres and telomeres

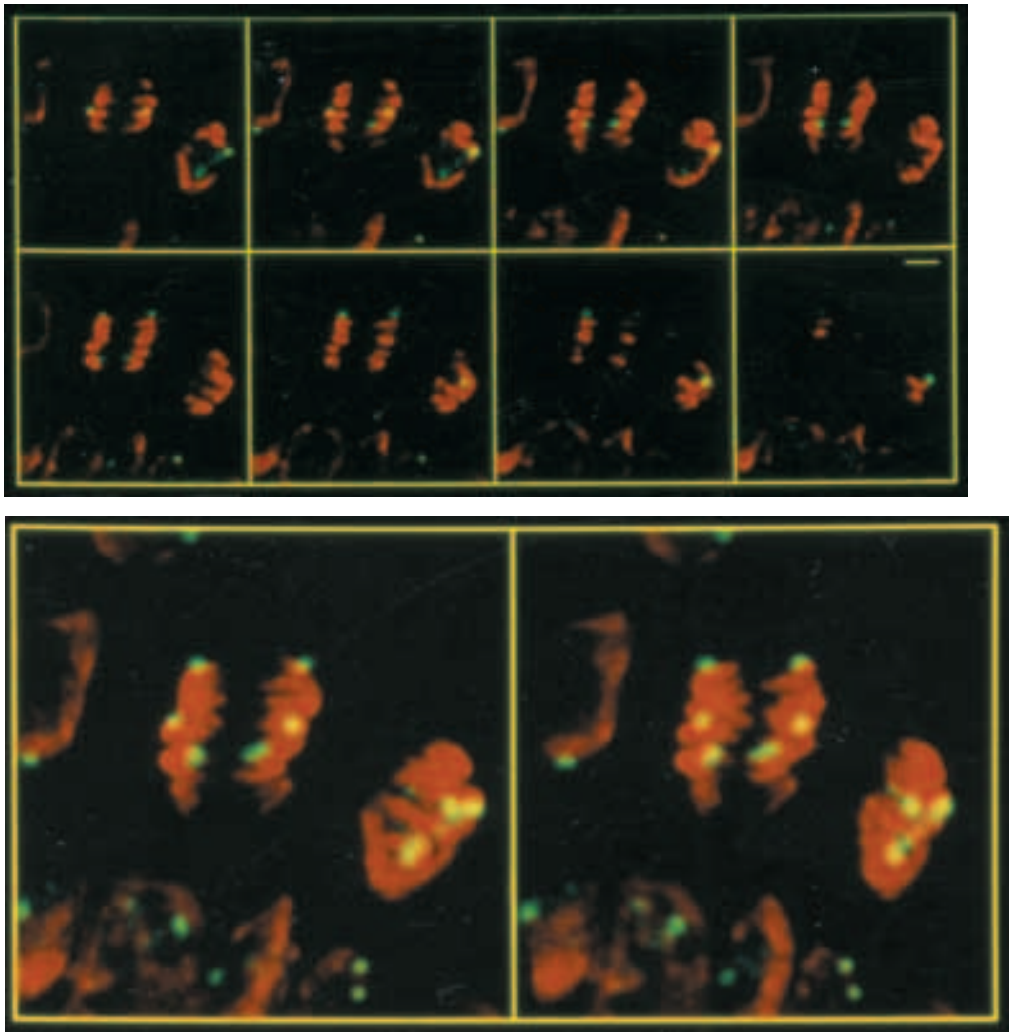


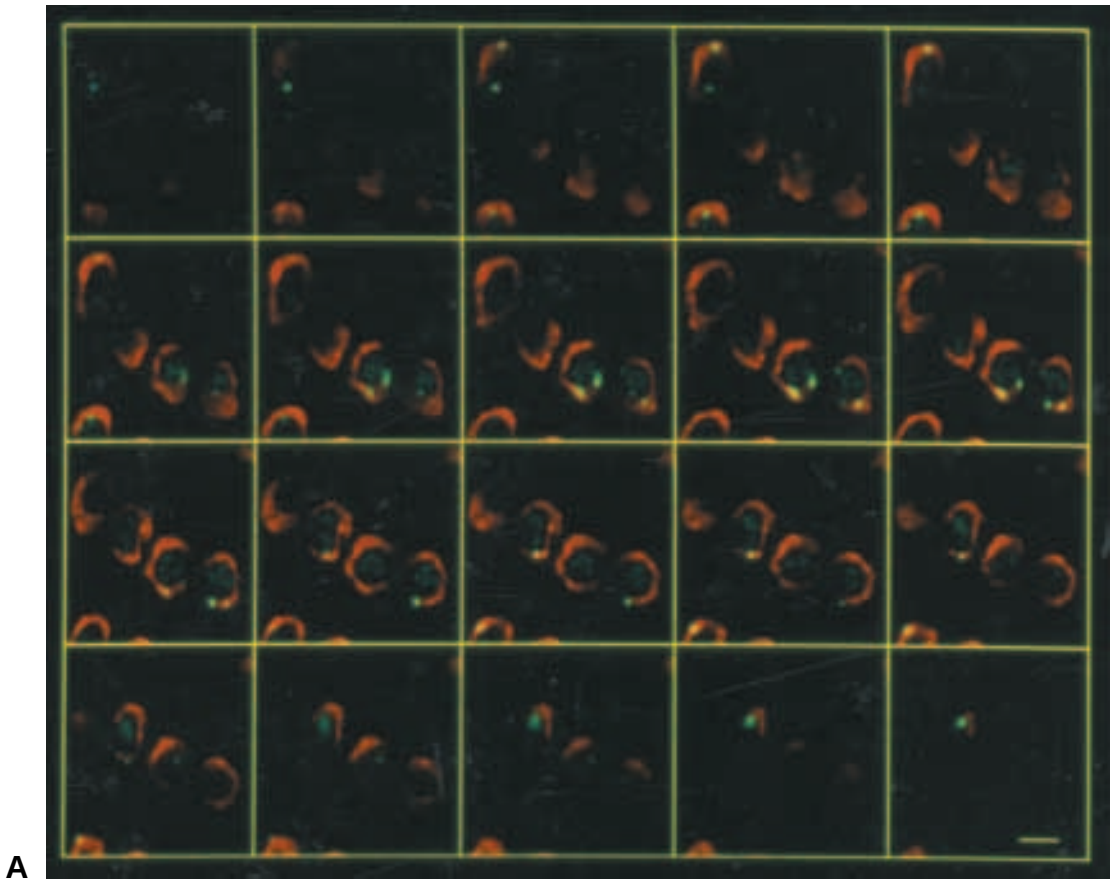
Fig. 6. Detection of the ribosomal gene clusters by 3-D fluorescence in situ hybridization and confocal microscopy of *P. hybrida* root cortex nucleus in telophase. Red colour corresponds to DNA staining by propidium iodide; yellow/green colour corresponds to ribosomal gene clusters, detected with an 18 S rDNA-specific probe, labelled with avidin-FITC. (A) Eight successive optical sections; (B) stereo image obtained by combining all 16 sections. Note the mirror-image orientation of the ribosomal gene clusters in the two sets of telophase chromatids. Bar, 10 μm.

remain localized in opposite hemispheres of the nucleus throughout the whole cell cycle. However, the persistence of such an overall organization does not exclude the repositioning of (parts of) the decondensed chromatin between the chromosome ends and centromeres during interphase. Certainly, if processes like DNA transcription, splicing and replication only take place in discrete nuclear domains, chromatin redistributions are necessary (for review see Haaf and Schmid, 1991).

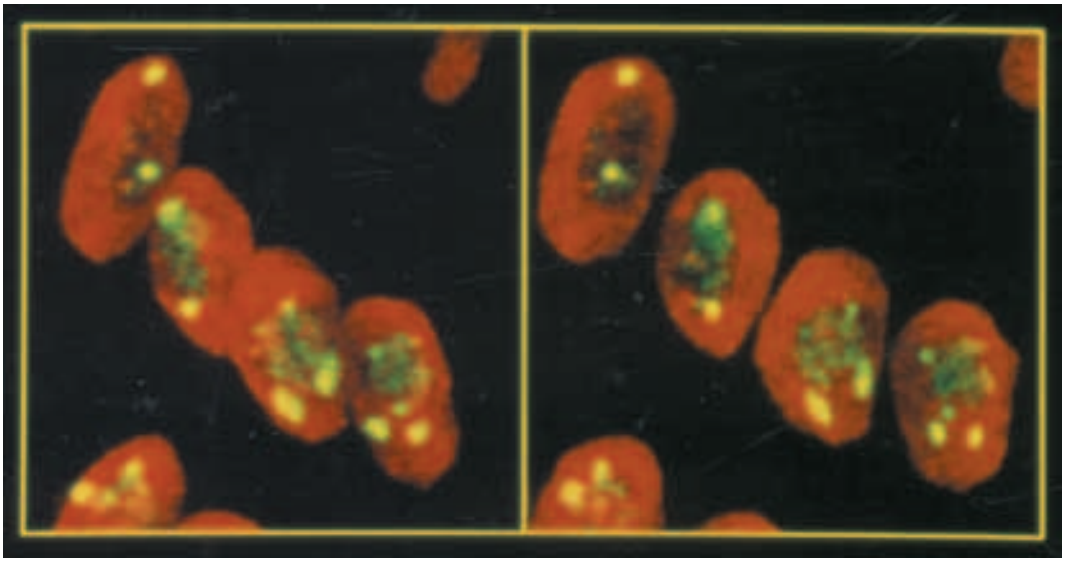
The spatial position of 18 S rRNA genes in *Petunia* root cortex cells

The nucleolus-associated ribosomal gene clusters show a two-by-two organization of the proximal and distal parts of the (active) NORs, indicating that the NORs enter the nucleolus parallel to each other at one side and leave the nucleolus at the opposite side. This interphase chromosome arrangement is in accordance with the preference for an association of the NOR-bearing homologous chromosomes during mitosis as observed, among others, in *Crepis capillaris* (Oud et al., 1989), *Zea mays* (Heslop-Harrison et al., 1988) and man (Sele et al., 1977). The absence of such a preference, as reported for *Aegilops* and *Hordeum* species (Heslop-Harrison et al., 1988), can be explained by assuming that a somatic association of NOR chromosomes is restricted to those chromosomes that are actively involved in nucleolus formation (see also comments by Appels, 1989; Hernandez-Verdun, 1991).

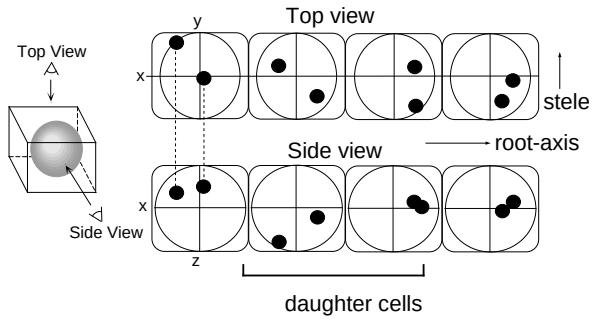
Other plant species in which the spatial localization of ribosomal gene clusters has been studied comprise several grass species and pea. *Triticum aestivum* (wheat) and *Pisum sativum* (pea) have, like *Petunia hybrida*, more than one pair of NOR chromosomes. In wheat, which has five pairs of NOR chromosomes, the unexpressed rDNA is localized at extranucleolar, perinucleolar and intranucleolar sites (Leitch et al., 1992). Except for the condensed gene clusters inside the nucleolus (which have not been studied in detail in *Petunia*) the situation in wheat is comparable to our observations. A plant species with an apparently different spatial distribution of the ribosomal genes is *Pisum* (Rawlins and Shaw, 1990). Here, the two pairs of NOR chromosomes showed two larger and two smaller gene clusters, all of which were invariably adjacent to the nucleolar periphery. The four spots often lay at the apices of a regular tetrahedron. Apparently, in *Pisum* both pairs of chromosomes with ribosomal genes are actively involved in nucleolus formation, which might explain their non-random localization (as in *Petunia*). However, the pattern of the perinucleolar gene positions differs between the two species: a tetrahedon arrangement has not been observed in *Petunia*. A simple explanation for this is the difference in the



A



B



C

Fig. 7. Detection of ribosomal gene clusters in four *P. hybrida* interphase nuclei in a file of root cortex cells. Red colour corresponds to DNA staining by propidium iodide; yellow/green colour corresponds to ribosomal gene clusters, detected with a 18 S rDNA-specific probe, labelled with avidin-FITC. (A) 20 subsequent optical sections obtained by confocal microscopy; (B) stereo image; (C) schematic drawing of a top view and a side view of the four interphase nuclei in which the normalized positions of the inactive ribosomal gene clusters outside the nucleolus are indicated by black dots. Note the absence of a pattern of mirror images with respect to the *X*, *Y* and *Z* positions of the gene clusters, even in daughter nuclei. Bar, 10 μm.

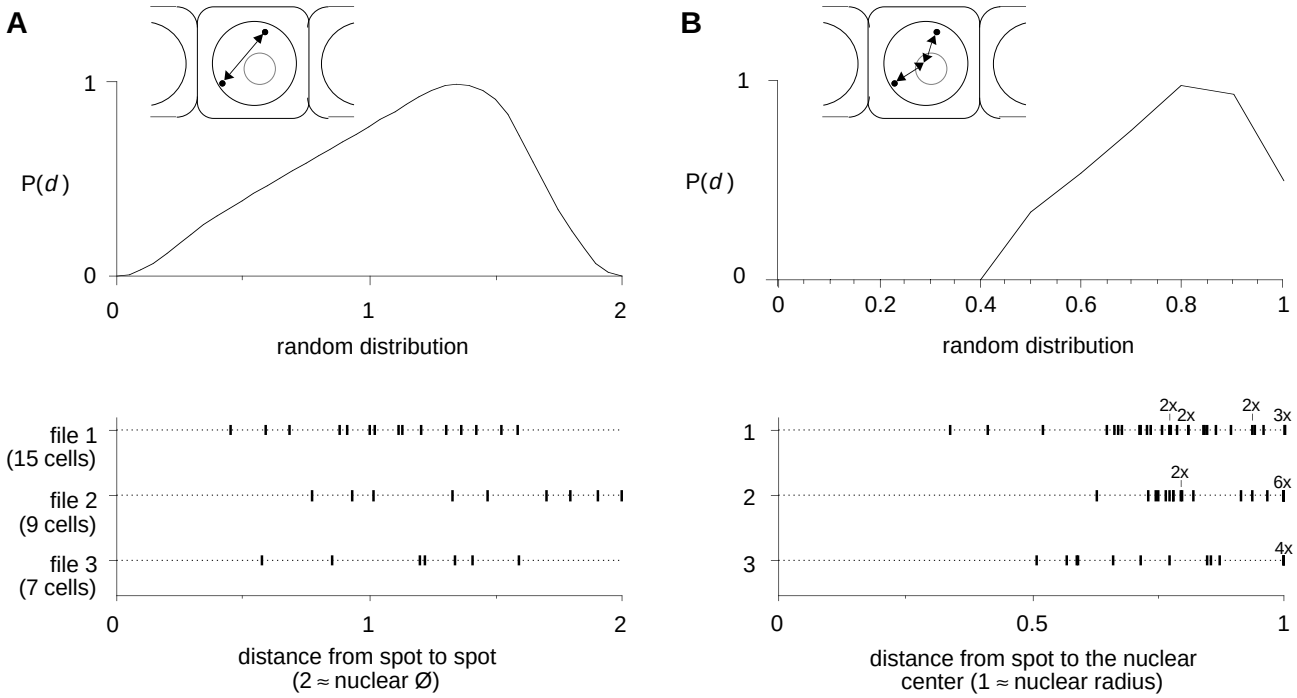


Fig. 8. The relative positions of the inactive 18 S ribosomal gene clusters in three files of *P. hybrida* cells. (A) The mutual distance between the inactive genes clusters; (B) the distance from an inactive gene cluster to nuclear centre. Both graphs are presented with their random density function as was calculated with the Monte Carlo procedure, see Materials and Methods. The nuclei shown in Figs 4 and 7 are a subset of file no. 3.

number of chromosome pairs involved: two for *Pisum* and one for *Petunia*.

The present study supports the concept that the interphase nucleus, at least when in a non-stationary phase, is a dynamic structure with respect to the spatial positioning of genes and chromosome domains. There is no doubt that the nucleolus influences nuclear organization. To what extent other factors, like gene expression and DNA replication, are also involved is less clear. Further research, preferably at the single-copy gene level, comparing cell types with different patterns of development and of metabolic activity, is necessary.

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