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Characterisation of the nucleolar organising regions during the cell cycle in two varieties of *Petunia hybrida* as visualised by fluorescence in situ hybridisation and silver staining

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Abstract. The cell cycle-dependent spatial position, morphology and activity of the four nucleolar organising regions (NORs) of the *Petunia hybrida* cultivar Mitchell and the inbred line V26 have been analysed. Application of the silver staining technique and fluorescence in situ hybridisation on fixed root-tip material revealed that these interspecific hybrids possess four NORs of which only those of chromosome 2 are active during interphase, which implies that the NOR activity is not of parental origin. However, at the end of mitosis, activity of all NOR regions could be detected, suggesting that the high demand for ribosomes at this stage of the cell cycle requires temporal activity of all NORs. Using actin DNA probes as markers in fluorescence in situ hybridisation experiments enabled the identification of the individual petunia chromosomes.

Introduction

Nucleoli are specific nuclear domains in which rDNA transcription, rRNA processing and the assembly of ribosomes take place (Hadjiolov 1985; Trendelenburg et al. 1996). Ribosomal biogenesis lasts from the telophase stage of mitosis to the beginning of the next mitosis. During the second half of prophase, rDNA transcription is arrested and as a consequence the nucleolus disappears. A part of the nucleolar proteins dissolve into the nucleoplasm or join the perichromosomal sheath. Another part, together with inactivated rDNA, remain associated with the nucleolar organising region (NOR) (e.g. Schwarzacher and Wachtler 1993). It is assumed that the NOR-associated proteins are part of the transcriptional machinery, comprising RNA polymerase I, transcription factors UBF and SL1 (Jordan 1984; Roussel et al. 1996) and DNA topoisomerase I (Scheer and Weisenberger 1994; Medina et al. 1995). Reconstitution of the nucleolus at the site of the chromosomal NORs takes place when chromosomal decondensation progressively ensues throughout telophase (Schwarzacher and Wachtler 1993).

Not all NORs are associated with an active transcriptional machinery and therefore they will not form a visible nucleolus. The phenomenon that not all NORs are transcriptionally active during interphase is often seen in interspecific hybrids, and it has been named nucleolar dominance (Reeder 1985). As early as 1928 Navashin observed that in interspecific hybrids the NORs of one parent suppress the transcriptional activity of the ribosomal genes in the other parent and in consequence also the formation of nucleoli. Since then, nucleolar dominance has been reported in various organisms, such as *Xenopus* (MacLeod and Bird 1982), mouse-human somatic cell hybrids (Miller et al. 1976) and several plant species (Schubert and Wobus 1985, and references therein). Several models have been proposed to explain nucleolar dominance (Reeder 1985; Hilliker and Appels 1989).

From recent studies it has been known that the interspecific hybrids Mitchell and V26 of *Petunia hybrida* have four chromosomes with ribosomal gene clusters (Montijn et al. 1994; Fransz et al. 1996; ten Hoopen et al. 1996). They are localised on the chromosomes 2 and 3. Two NOR-containing chromosomes fuse and form a mature nucleolus, whereas the ribosomal genes of the other two NORs form condensed clusters. Statistical analyses of data from three-dimensional confocal microscopy demonstrated that during interphase the latter genes were randomly distributed in the spherical chromosomal shell bounded by the nucleolus and the nuclear envelope (Montijn et al. 1994). The condensed state of these NORs and the fact that they are not associated with the nucleolus suggests that the ribosomal genes within them are transcriptionally inactive.

Two points are unclear with respect to the active and inactive NORs. First, it is unknown whether the two active NORs belong to one pair of chromosomes (i.e. either

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2 or 3), or consist of the NOR of one chromosome 2 and one chromosome 3. The latter case would reflect the hybrid origin of *P. hybrida*. A second point of uncertainty concerns whether the NORs that are inactive during interphase remain so during the whole cell cycle. Previous observations (unpublished data, Montijn) suggested that there might be some activity during the resumption of ribosomal biogenesis shortly after the completion of mitosis.

The aim of the present study was to address these questions by the application of the technique of fluorescence in situ hybridisation (FISH) (to map the various NORs) and of silver staining (to monitor NOR activity) to mitotic and interphase cells of *P. hybrida* root. Silver staining is informative about the presence of proteins related to nucleolar transcriptional activity. These silver-precipitating proteins are thought to belong to the transcriptional machinery and they stay attached to the NOR during the cell cycle (Goodpasture and Bloom 1975; Schwarzacher and Wachtler 1993).

Our results show that the NORs that are active during interphase are localised on the homologues of chromosome 2, and that the ribosomal genes on the other pair of NOR chromosomes (3) do show some activity at the end of mitosis when the interphase state of the nucleus is restored.

Materials and methods

Squash preparations. Plants were grown in a greenhouse. Squash preparations were made from root-tips of seedlings of the varieties Mitchell (Mitchell et al. 1980) and V26 of *P. hybrida*. Root-tips of 2 to 2.5 cm length were dissected after 4 weeks. A portion of these roots was treated with α-bromo-naphthalene for 70 to 90 min to accumulate mitotic cells in metaphase. All roots were fixed in a solution of absolute ethanol:glacial acetic acid (3:1, v/v). Squashes were made from fixed root-tips according to Montijn et al. (1994).

Probe labelling and in situ hybridisation. The following three plasmids were either labelled with biotin-16-dUTP (Boehringer Mannheim, Germany) or with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation: (1) pSR1 2B3; this plasmid contains a 1.05 kb internal fragment of 18S rDNA of soybean (Eckenrode et al. 1985). (2) pPAc 1 and pPAc 4; which contain a 2.7 kb fragment of actin 1 of petunia and a 3.6 kb fragment of actin 4 of petunia, respectively (McLean et al. 1988). All probes were kindly donated by R.B. Meagher, University of Georgia, Athens, USA. Hybridisation of the 18S rDNA, PAc 1 and PAc 4 DNA probes as done according to Montijn et al. (1994) and Fransz et al. (1996).

Immunocytochemical detection procedure. Immunodetection of the slides was performed by blocking in immunobuffer, which was 4×SSC (1×SSC is 0.15 mM NaCl, 0.015 M sodium citrate) containing 5% (w/v) non-fat dry milk (4 M) for biotin-labelled probes and TRIS-NaCl containing 0.5% (w/v) blocking buffer (Boehringer, Mannheim) (TNB) for digoxigenin-labelled probes.

Detection of biotin-labelled probes was performed with avidin-D-conjugated fluorescein isothiocyanate (FITC) (1:1500) (Vector Laboratories, Burlingame, USA) diluted in 4 M. The FISH signals were amplified with biotinylated goat anti-avidin-D (1:100) (Vector Laboratories), followed by a second layer of (1:1500) avidin-FITC. Detection of digoxigenin-labelled probes was performed using mouse anti-digoxigenin (1:1500) (Boehringer Mannheim), followed by incubation with FITC-conjugated rabbit anti-mouse (1:1500) (Sigma, St. Louis, USA). Amplification of the signal was done using FITC-conjugated goat anti-rabbit (1:1500) (Sigma). All dilutions were made in TNB. Simultaneous detection of the 18S rDNA and PAc probes was performed using the same concentrations as for the single detections. Biotin-labelled probes were either detected with amino-methylcoumerin-3-acetic acid (AMCA)-conjugated avidin-D (1:1500) (Vector Laboratories) or with avidin-D conjugated with FITC (Vector Laboratories). Digoxigenin labelled probes were detected using the same FITC-conjugated antibodies or with tetramethylrhodamin-isothiocyanate-conjugated antibodies (1:1500) (Sigma). All dilutions were made in TNB, except for the first incubation with AMCA-conjugated avidin-D or with FITC-conjugated avidin-D, which were incubated in 4 M. Slides with antibodies were incubated in a moist chamber at 37°C for 30 min.

Control experiments were performed in which the probes or antibodies were omitted to assure genuine detection of the 18S rDNA sequences or PAc sequences, respectively.

Silver staining. Squash preparations fixed in ethanol:glacial acetic acid (3:1 v/v) were stained with silver according to the method of Robert-Fortell et al. (1993). Briefly, 1 mg/ml of gelatine dissolved in 2% (v/v) formic acid, was mixed with one volume of a 50% (w/v) $AgNO_3$ solution. The solution was poured onto squash preparations and left for 30 min at room temperature in the dark. The slides were then washed with water and total DNA was counterstained with 500 ng/ml of 4′,6-diamidino-2-phenylindole (DAPI) dissolved in Vectashield (Vector Laboratories).

Microscopy. The squashes were mounted in Vectashield antifade medium (Vector Laboratories) containing either 500 ng/ml of DAPI or 1 μg/ml of propidium iodide. Fluorescence photographs were taken with an Olympus (BH2-RFC) microscope equipped with a 100 W mercury arc lamp, using Scotch 640 T ASA colour slide films. Silver staining was visualised using phase contrast microscopy. Digital images of FISH and silver staining, were recorded using a cooled CCD camera (Astromed, UK). A Hewlett Packard 700 series workstation with Scilimage software (ten Kate et al. 1990) and a Macintosh personal computer equipped with NIH-Image (Vischer et al. 1994; ftp://zippy.nihm.nih.gov) were used for image processing.

Results

Identification of the NOR-bearing chromosomes

According to Smith et al. (1973) the standard karyogram of *P. hybrida* can be divided into three groups based on the position of the centromere. In Fig. 1 an idiogram of petunia is depicted. The two chromosomes that were clas-

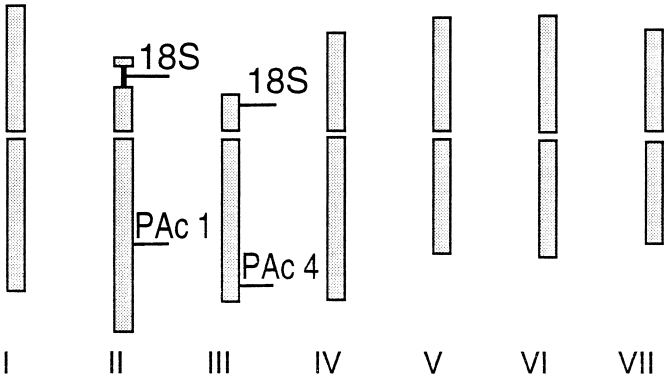


Fig. 1. Idiogram of the varieties Mitchell and V26. Shown are the seven chromosomes of *Petunia hybrida* with the ribosomal gene clusters (18S) and the actin genes (Pac 1, Pac 4) are depicted

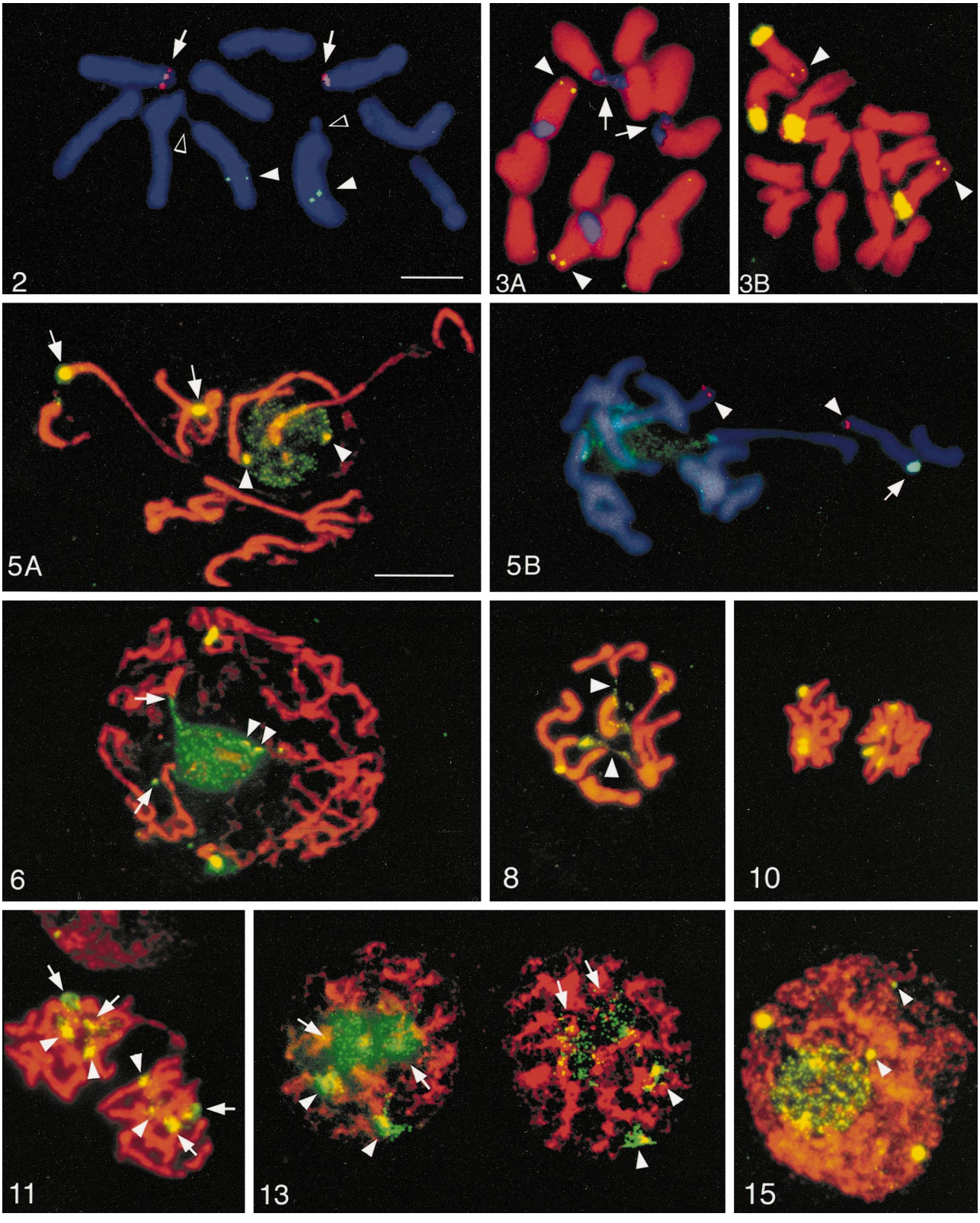


Fig. 2. Squash preparation of a subset of metaphase chromosomes of *P. hybrida* variety Mitchell after double fluorescence in situ hybridisation (FISH) with a biotinylated PAc 1 probe and a digoxygenin-labelled PAc 4 probe. The hybridisation signals were detected with fluorescein isothiocyanate (FITC)-conjugated avidin and tetramethyl rhodamine isothiocynate (TRITC)-conjugated antibodies. Total DNA is counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). The PAc 1 probe is centrally located on the long arm of chromosome 2 (green spots, *arrowheads*). The PAc 4 probe is located at the distal end of the long arm of chromosome 3 (red spots, *arrows*). The homologous chromosomes 2 are characterised by satellites at the distal end of the short arm (*open arrowheads*). *Bar* represents 10 μm in Figs. 2, and 3

sified as group B (chromosomes 2 and 3) have a sub-telocentric centromere. Chromosome 2 carries a satellite on the short arm. Cytological identification of the NOR-bearing chromosomes was done using the known position of two actin subfamilies (PAc 1 and 4). The subfamilies PAc 1 and PAc 4 had been mapped genetically on chromosome 2 and 3 of petunia, respectively (McLean et al. 1988). Cytological localisation of both PAc probes was performed by FISH on metaphase squashes of the Mitchell variety. The hybridisation sites of the actin probes were identified as fluorescent spots on both sister chromatids. Figure 2 shows a metaphase preparation after FISH with a PAc 1 and with a PAc 4 probe. From Fig. 2 it is evident that the PAc 1 signal is localised in the middle of the long arm of chromosome 2, which also possesses a satellite (open arrowheads), and that the Pac 4 signal is simultaneously detected at the distal end of the long arm of chromosome 3 (arrows). To verify the chromosomal localisation of the NOR a double FISH was performed on the varieties Mitchell and V26. Since the variety V26 of petunia is widely used, we also used this to analyse the versatility of the probes used (see for instance ten Hoopen et al. 1996 and references therein). Figure 3A shows a metaphase preparation of the Mitchell variety after double FISH with an 18S rDNA and a PAc 4 probe. In Fig. 3B the same set of probes were applied on a metaphase squash preparation of the V26 inbred line. Here, both hybridisation signals were detected with FITC-conjugated avidin or FITC-conjugated antibodies. Double FISH with the PAc 4 and 18S rDNA probes revealed that the PAc 4 cluster is simultaneously detected with one pair of ribosomal gene clusters. The other pair of ribosomal gene clusters, as can be seen in Fig. 3A, B, is localised on the satellited chromosome. Comparing Figs. 2 and 3 we can conclude that both varieties have NORs on chromosomes 2 and 3, and that the homologues of chromosomes 2 carry a satellite.

To discriminate the chromosomes bearing the active NORs from those with the inactive NORs silver staining was applied to squash preparations of root-tips. Figure 4A shows a metaphase preparation of the variety Mitchell after staining with silver nitrate. In Fig. 4B the chromo-

Fig. 3A, B. Squash preparation at metaphase of *P. hybrida* variety Mitchell and inbred line V26 after double FISH with a biotinylated 18S rDNA probe and digoxigenin-labelled PAc 4 probe. (**A**) The hybridisation signals were detected with aminomethylcoumerin-3-acetic acid (AMCA)-conjugated avidin and FITC-conjugated antibodies. Total DNA is counterstained with propidium iodide (PI, red). The 18S rDNA FISH signals are visible as blue spots at the distal end of the short arm of chromosomes 2 and 3. The 18S FISH signals of the homologous chromosomes 2 are discriminated from the 18S FISH signal of the homologous chromosomes 3 by a more expanded appearance. The site at hybridisation of the PAc 4 probe is located at the distal end of the long arm of chromosome 3 (yellow spots; *arrowheads*) and is visible as a double spot on both sister chromatids. (**B**) Both hybridisation signals were detected with FITC-conjugated avidin and FITC-conjugated antibodies. Total DNA is counterstained with PI (red). See (**A**) for a description of the FISH signals. The lower FISH signal intensity of the 18S rDNA spots in (**A**) is due to more rapid fading of the AMCA signal in comparison with the FITC signal

Fig. 5A, B. Two squash preparation at mid-prophase of the variety Mitchell after FISH with biotinylated 18S rDNA (**A**) and (**B**) and PAc 4 probe labelled with digoxigenin (**B**). The 18S rDNA probe is detected by FITC-conjugated (green/yellow spots) avidin (**A**) and (**B**). The PAc 4 probe is detected with TRITC-conjugated antibodies (**B**). In both panels the nucleolus is characterised by a large number of small hybridisation spots. The NORs of the two homologous chromosomes 2 are still expanded into the nucleolus. In (**A**) the proximal regions of the NORs of the homologous chromosomes 2 are clearly visible (*arrowheads*). The two homologous chromosomes 3 are not associated with the nucleolus and have large condensed clusters of transcriptionally inactive ribosomal genes (*arrows*). (**B**) PAc 4 localises at the distal end of chromosome 3 (*arrowheads*) opposite the inactive 18S rDNA cluster (*arrow*) on the same chromosome. Note that one 18S rDNA cluster is obscured by the bulk of DNA in (**B**). *Bar* represents 10 μm in Figs. 5, 6, 8, 10, 11, 13 and 15

Fig. 6. Squash preparation at early prophase of the variety Mitchell after FISH with biotinylated 18S rDNA. The probe is detected by FITC-conjugated (green/yellow spots) avidin and total DNA was counterstained with PI (red). The nucleolus is characterised by a large number of small hybridisation spots. The gene clusters at the periphery of the nucleolus are the perinucleolar knobs and represent the proximal region of NOR (*arrowheads*). The gene clusters denoted by the *arrows* are representatives of the distal region. Two large spots are not associated with the nucleolus and represent the transcriptionally inactive ribosomal gene clusters

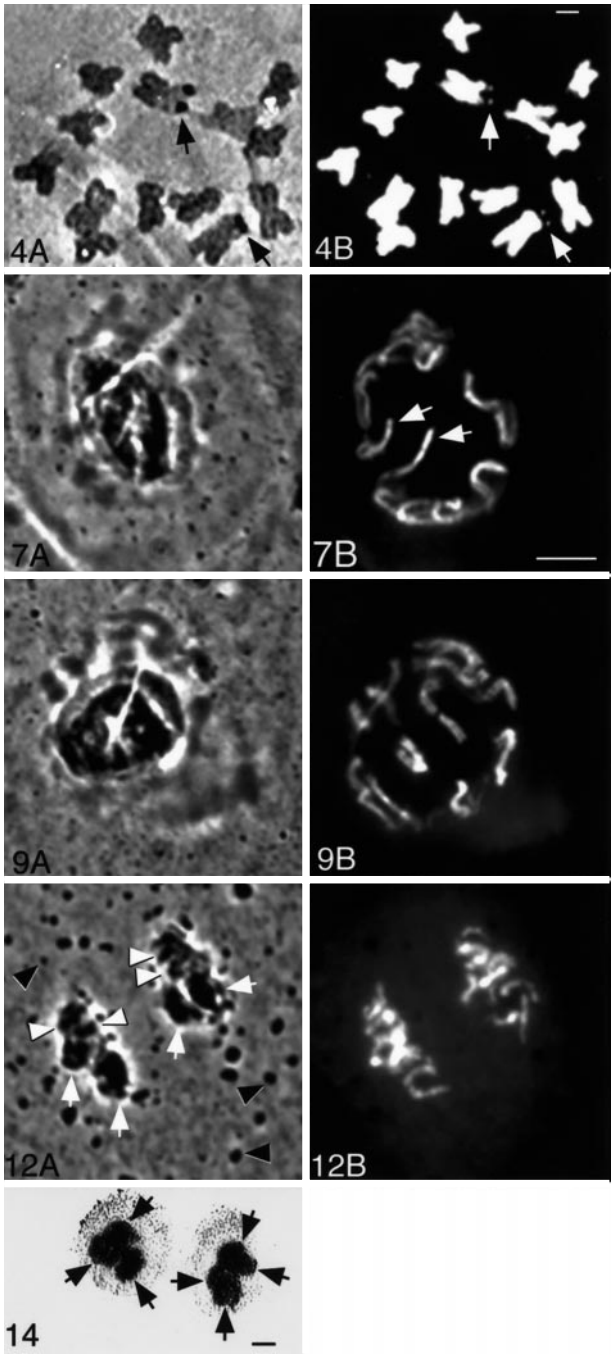
Fig. 8. Squash preparation at late prophase of the variety Mitchell after FISH with biotinylated 18S rDNA. The probe is detected by FITC-conjugated (green/yellow spots) avidin and total DNA was counterstained with PI (red). Some remnants of the nucleolus are still visible as thin tracks of 18S rDNA FISH spots (*arrowheads*). Only one inactive ribosomal cluster on chromosome 3 is visible

Fig. 10. Squash preparation at anaphase of the variety Mitchell after FISH with biotinylated 18S rDNA. The probe is detected by FITC-conjugated (green/yellow spots) avidin and total DNA was counterstained with PI (red). The individual chromosomes are visible. Four condensed ribosomal clusters per daughter nucleus are visible

Fig. 11. Squash preparation at late anaphase of the variety Mitchell after FISH with biotinylated 18S rDNA. The probe was detected with FITC-conjugated (green/yellow spots) avidin and total DNA was counterstained with PI (red). The individual chromosomes are still visible. Four expanding ribosomal clusters per daughter nucleus are visible. There are four expanded clusters, two large (*arrows*) and two minor (*arrowheads*), per nucleus

Fig. 13. Squash preparation at transition of telophase to early interphase of the variety Mitchell after FISH with biotinylated 18S rDNA. The probe was detected with FITC-conjugated (green/yellow spots) avidin and total DNA was counterstained with PI (red). The individual chromosomes are not visible. Four expanding ribosomal clusters per daughter nucleus, showing two large clusters (*arrows*) and two minor clusters (*arrowheads*), are visible

Fig. 15. Squash preparation at interphase of the variety Mitchell after FISH with biotinylated 18S rDNA. The probe was detected with FITC-conjugated (green/yellow spots) avidin and total DNA was counterstained with PI (red). One nucleolus is visible and is characterised by a large number of small hybridisation spots. Two small spots, representing the perinucleolar knobs of the distal region are visible (*arrowheads*). Two large spots are not associated with the nucleolus and represent the transcriptionally inactive ribosomal gene clusters



somes have been counterstained with DAPI. From Fig. 4 it is clear that silver precipitates (black dots) around the secondary constriction at the NOR. From the FISH experiments and the silver staining experiments we conclude that the satellited chromosomes (i.e. chromosomes 2) possess the actively transcribed ribosomal genes. The results of a double FISH experiment using 18S rDNA and PAc 4 probes, revealing that PAc 4 localises at the distal end of chromosome 3 opposite to the inactive 18S rRNA cluster (Fig. 5B), confirms this. Note that one 18S rRNA cluster in Fig. 5B is obscured by the bulk of the DNA.

Cytological characterisation of ribosomal gene activity

The activity of the rDNA genes throughout the cell cycle was visualised by means of FISH with a biotinylated 18S rDNA probe detected with FITC-conjugated avidin and by silver staining. In the successive descriptions and the accompanying Figs. 6–15 we show the results obtained with the Mitchell variety. The same results (not shown) hold for the V26 variety.

In early prophase, two inactive NORs are visible, which are not associated with the nucleolus (Fig. 6). Also visible are the perinucleolar knobs, representing the distal regions (arrowheads), as well as the proximal regions of the active NORs (arrows). In Fig. 5, two mid-prophases are shown. In both panels the condensed clusters, representing the inactive NORs of homologous chromosomes 3 (arrows), are not associated with the nucleolus (for a description of Fig. 5A see previous paragraph). The arrowheads in Fig. 5A denote the perinucleolar knobs of the homologous chromosomes 2.

Silver staining of a mid prophase (Fig. 7) shows a large nucleolus, which is visible as a dark precipitate. The DAPI stain in Fig. 7B shows that two chromosomes enter the nucleolus side by side (arrows). These chromosomes are associated with the nucleolus, and are presumably the chromosomes bearing the active NORs.

In late prophase the nucleolus dissolves and the rDNA appears contracted into the secondary constrictions (Fig. 8). Some remnants of the nucleolus are still visible (arrowheads in Fig. 8). Figure 9 shows silver staining of a

Fig. 4A, B. Squash preparation of the variety Mitchell after silver staining (**A**) showing silver deposits at the satellite regions of the homologous chromosomes 2 (*arrows*). The chromosomes are counterstained with DAPI (**B**). The satellites located at the distal end of the short arm of the homologous chromosomes 2 are visible by DAPI staining (*arrows*). *Bar* represents 10 μm

Fig. 7A, B. Squash preparation at early prophase after silver staining (**A**): total DNA was counterstained with DAPI (**B**). The nucleolus is differentially stained with silver, showing a lighter round precipitate with darker stained substructures. No silver staining of the NORs was detected on the homologous chromosomes 3. DAPI staining (**B**) shows the two homologous chromosomes 2 entering the nucleolus in a parallel arrangement. *Bar* represents 10 μm in Figs. 7, 9, 12

Fig. 9A, B. Squash preparation at late prophase after silver staining (**A**): total DNA was counterstained with DAPI (**B**). The nucleolus is differentially stained with silver, showing a lighter round precipitate with darker stained substructures. No silver staining of the NORs was detected on the homologous chromosomes 3. DAPI staining (**B**) shows the individual chromatids resulting from the condensing chromosomes. The two homologous chromosomes 2 are parallel and enter the nucleolus side by side

Fig. 12A, B. Squash preparation at late anaphase after silver staining (**A**): total DNA was counterstained with DAPI (**B**). The silver staining shows numerous roundish black speckles of different sizes (*black arrowheads*) and four expanding clusters of different sizes per nucleus. Small clusters are denoted by *white arrowheads*, and large clusters are denoted by *white arrows*

Fig. 14. Squash preparation of two nuclei at the transition from telophase to early interphase after silver staining, showing at least three expanded ribosomal clusters per daughter nucleus. *Bar* represents 10 μm

late prophase, in which the nucleolar proteins still seem to be assembled in a nucleolus-like structure.

In anaphase the hybridisation spots of the ribosomal genes, have a condensed appearance (Fig. 10). No silver staining could be detected during this stage of mitosis (result not shown). In late anaphase the FISH signals have a dispersed appearance (Fig. 11). Frequently we observed that two of these clusters (arrows in Fig. 11) were more expanded than the other two clusters (arrowheads in Fig. 11). All expanded clusters also stained silver positive (Fig. 12). We observed two large clusters (white arrows in Fig. 12) and two minor clusters (white arrowheads in Fig. 12), indicating four areas with pairwise different transcriptional activity. During this stage of anaphase we also observed numerous round speckles of different sizes spread over the cell (black arrowheads in Fig. 12). These speckles are only visible with silver staining, and they presumably represent the prenucleolar bodies (PNBs; Ploton et al. 1987).

A telophase/G1 nucleus with four expanding NORs is visible in Fig. 13. Clearly two clusters are more expanded (arrows in Fig. 13) than the other two clusters (arrowheads in Fig. 13). The application of silver staining to late telophase/early interphase nuclei revealed several nuclei with at least three areas of silver precipitation (arrows in Fig. 14). Other nuclei, larger and presumably at a later stage of interphase, show only one nucleolus, together with two inactive NORs (Fig. 15). At this stage the PNBs have disappeared.

Discussion

In this study we examined the position, morphology and activity of ribosomal genes in two varieties of *P. hybrida*, in order to answer the following questions: (1) which of the four NORs are responsible for nucleolus formation, and (2) does the transcriptional inactivity of the remaining NORs persist during the whole cell cycle.

Karyotyping of rRNA genes

The subfamilies PAc 1 and PAc 4 were used as markers to map the NOR-bearing chromosomes of the varieties Mitchell and V26. We found that one pair of NORs was located on the satellite-bearing chromosome 2, whereas the other NOR was located at the distal end of chromosome 3. Based on the genetic results of McLean et al. (1988) and on cytological mapping by FISH, an idiogram was constructed in which the physical localisation of the NOR and the actin gene loci are depicted (Fig. 1). Double FISH experiments (Fig. 5B) and staining metaphase chromosomes of the Mitchell variety with silver nitrate revealed that the NORs localised on chromosomes 2 are heavily stained with silver (Fig. 4). Because the silver-reducing proteins are believed to belong to the transcriptional machinery and to stay associated with the NORs during mitosis (Roussel and Hernandez-Verdun 1994; Weisenberger and Scheer 1995), these sites have probably been active during the preceding interphase. No obvious silver staining was encountered in the NOR sites of chromosome 3. Because these two varieties are of hybrid origin one might expect that at least one of the NORs of a particular parental origin would be active. This, however, is clearly not the case.

Activity of the ribosomal gene clusters on chromosomes 2

During the interphase and prophase stages of the cell cycle a large nucleolus with regions of condensed chromatin adjacent to the nucleolus, the so-called perinucleolar knobs, (Jordan 1984) is visible (i.e. chromosome 2). In some nuclei we observed four knobs, two large ones and two small ones (Fig. 6). These knobs are assumed to represent the distal and the proximal regions of an active NOR, respectively. The knobs are not visible with silver staining and they are presumably inactive. An explanation for the fact that the smaller knobs are not always visible with FISH is that these proximal regions are expanded into the nucleolus because of their higher activity. Although the chromosomes shorten from early prophase to late prophase, it is not until late prophase that rDNA is contracting (Fig. 8). The low frequency at which such late prophase stages can be seen in squash preparations indicates that the duration of the process is very short during mitosis (Montijn, unpublished data).

Activity of the ribosomal gene cluster on chromosomes 3

The majority of interphase nuclei, as well as prophase nuclei, show two transcriptionally inactive ribosomal gene clusters, which have been mapped to chromosomes 3 (Figs. 5, 6 and 15). At metaphase (Fig. 4), the NORs of chromosomes 3 do not show silver staining (in contrast to the NORs of chromosome 2). The lack of silver precipitation not only suggests that the NORs of chromosome 3 do not possess an active transcriptional machinery, but also that they remain inactive during a large part of the cell cycle. During the transition from telophase to interphase, however, we observed decondensation of all four ribosomal gene clusters (Fig. 13), which coincides with the appearance of three or four silver-positive areas (Figs. 12, 14). These observations suggest that there is a relatively short period during which one or both NORs of chromosome 3 become active. Afterwards, the NORs of chromosome 3 condense and no associated silver staining is detectable.

Since transcription has ceased during mitosis it seems reasonable to assume that the temporal activity of the ribosomal genes on chromosome 3 is related to the demand for additional ribosomes at this stage of the cell cycle. Note that the large ribosomal gene clusters of (presumably) chromosome 2 at early interphase (Fig. 13) lack peri-nucleolar knobs. Also this feature points to maximum activity with respect to ribosomal gene expression.

Little is known about the mechanism that suppresses the activity of the NORs. According to Reeder (1985),

the dominance that occurs in several interspecific hybrids of different species might be due to an enhancer element located in the non-transcribed spacer region, the so called enhancer-imbalance mechanism (see also Sardana and Flavell 1996). Support for this mechanism comes from studies on the variety Mitchell done by Waldron et al. (1983). They reported that there is a size heterogeneity of the rDNA repeating units, which results from length differences in the non-transcribed spacer region. Probably the NOR localised at the satellite has the largest non-transcribed spacer region, and will therefore be the actively transcribed NOR during interphase.

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