

Nucleolar dominance does not occur in root tip cells of allotetraploid *Brassica* species

Robert Hasterok and Jolanta Maluszynska

Abstract: Using in situ hybridization and silver staining methods, the numbers of active and inactive rDNA loci have been established for three allotetraploid species of *Brassica* (*B. napus*, *B. carinata*, and *B. juncea*) and their diploid ancestors (*B. campestris*, *B. nigra*, and *B. oleracea*). The allotetraploid species have chromosome numbers equal to the sum of the numbers in their diploid relatives, but have fewer rDNA loci. All species investigated have lower numbers of active NORs (AgNORs, nucleolar organizer regions) compared with the numbers of rDNA sites revealed by in situ hybridization. The number of active rDNA loci of the allotetraploid species is equal to the number of AgNORs in their diploid ancestors, indicating the absence of nucleolar dominance in amphidiploid *Brassica* species, at least in root meristematic cells.

Key words: AgNOR, *Brassica*, FISH, nucleolar dominance, rDNA.

Résumé : À l'aide de l'hybridation in situ et de la coloration à l'argent, le nombre de loci d'ADNr actifs et inactifs a été déterminé chez trois espèces allotétraploïdes du genre *Brassica* (*B. napus*, *B. carinata* et *B. juncea*) et leurs ancêtres diploïdes (*B. campestris*, *B. nigra* et *B. oleracea*). Les espèces allotétraploïdes possèdent un nombre de chromosomes qui correspond à la somme des chromosomes présents chez leurs ancêtres diploïdes mais elles possèdent moins de loci d'ADNr. Toutes les espèces examinées montrent moins de NOR actifs (AgNOR) que de sites d'ADNr tels que révélés par hybridation in situ. Le nombre de loci d'ADNr actifs chez les espèces allotétraploïdes est égal au nombre d'AgNOR présents chez les espèces diploïdes ancestrales. Ceci suggère qu'il y a absence de dominance nucléolaire chez les espèces amphidiploïdes du genre *Brassica*, du moins dans les cellules méristématiques de la racine.

Mots clés : AgNOR, *Brassica*, FISH, dominance nucléolaire, ADNr.

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Introduction

Nucleoli are specific nuclear sites where rDNA transcription, rRNA processing, and ribosomal biogenesis take place. They are formed by the nucleolar organizer region (NOR) of one or more pairs of chromosomes bearing rRNA genes (18S–5.8S–25S). The number of nucleoli per nucleus can be equal to the number of NOR chromosomes, but in cells with small genomes, usually only one nucleolus is present.

The number of rRNA genes is considerably greater than that required to sustain ribosome synthesis. The rRNA genes in each NOR can have differential activity in the cell. Nucleolar volume and the size of the secondary constriction are proportional to the activity of rRNA genes (Flavell 1986). It is possible to estimate the activity of these genes by molecular and cytological observation, an advantage not available for many other genes.

In species with more than one rDNA locus, variability can be observed between nonhomologous NORs. Greater activity of one rDNA locus, nucleolar dominance, has been observed in many species but is especially well documented for species in the tribe Triticeae. In hexaploid wheat with 7 rDNA loci (Jiang and Gill 1994), the locus *1B* dominates *6B*, which is in turn dominant over *5D*. In a substitution line, with the single NOR chromosome of *Aegilops umbellulata* (1U), rRNA genes of added locus dominated over those of wheat (Martini and Flavell 1985). It is often observed in interspecific hybrids that ribosomal genes of one species are transcriptionally dominant over the ribosomal genes of another species. This phenomenon has also been called "differential amphiplasty" and was first described in some interspecific hybrids of *Crepis* (Navashin 1934), followed by the study of other hybrids or allopolyploid species (Lacadena et al. 1984; Reeder 1985; Gustafson et al. 1988). Recently, on the basis of molecular analysis, the hierarchy of nucleolar dominance in the *Brassica* genus has been demonstrated. The authors concluded that the rRNA genes of *B. nigra* were dominant over those of *B. campestris*, which were dominant over those of *B. oleracea*. Their analysis has shown that nucleolar dominance was controlled at the level of transcription and involved a mechanism by which one set of pre-rRNA transcripts was degraded preferentially (Chen and Pikaard 1997a).

In this paper we present the results of a cytogenetic analysis of the nucleolar activity in six species of the *Brassica* triangle. It is demonstrated that the number of active rDNA

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loci is equal to the sum of NORs in diploid progenitors in all allotetraploid *Brassica* species investigated.

Material and methods

Plants used in this study were diploid species: *B. nigra* (var. *occidentalis*; BB; $2n = 2x = 16$), *B. oleracea* (var. *capitata*; CC; $2n = 2x = 18$), and *B. campestris* (var. *rapifera*; AA; $2n = 2x = 20$) and allotetraploid species: *B. carinata* (cv. Yellow Dodolla; BBCC; $2n = 4x = 34$), *B. juncea* (cv. Malopolska; AABB; $2n = 4x = 36$), and *B. napus* (winter oilseed rape cv. Leo; AACC; $2n = 4x = 38$). Seeds were germinated on moist filter paper. Seedlings (2–3 days old) were treated with 2 mM 8-hydroxyquinoline for 2 h at room temperature, fixed in an ethanol – glacial acetic acid (3:1) mixture and stored at -20°C until required. Chromosome preparations were made according to Maluszynska and Heslop-Harrison (1993) with some modifications. Fixed seedlings were washed in 0.01 M citric acid – sodium citrate (pH 4.8) for 15 min and digested in 2% (w/v) cellulase (Calbiochem) and 20% (v/v) pectinase (Sigma) for 1 h at 37°C . The root tips were squashed in a drop of 45% acetic acid. The quality of chromosome preparations was checked under a phase contrast microscope. Coverslips were removed after freezing and the preparations were air-dried.

CMA and DAPI staining

Double fluorescent staining with CMA (chromomycin A₃) and DAPI (4',6-diamidino-2-phenylindole) was made according to Schweizer (1976). Preparations were stained with CMA solution (0.5 mg/mL, Serva) for 1 h in the dark, briefly rinsed in distilled water and air-dried. Then slides were stained with DAPI solution (2 µg/mL, Serva) for 30 min in the dark, briefly rinsed in distilled water and mounted in antifade buffer (Citifluor, Ted Pella Inc.).

Silver staining

Silver staining was done using the modified method of Hizume et al. (1980). Slides were treated with a borate buffer (pH 9.2) for 15–30 min and a few drops of freshly prepared 50% silver nitrate in distilled water was applied to each preparation. Slides were covered with a nylon mesh and incubated in a humid chamber at 45°C for 1–2 h, washed in distilled water, and air-dried. The photographs were taken using Kodak Gold 100 ASA film. To remove the silver staining, preparations were placed in 30% hydrogen peroxide for 20–30 s, rinsed briefly in distilled water, air-dried, and then used for in situ hybridization.

Fluorescent in situ hybridization (FISH)

FISH followed the method described by Maluszynska and Heslop-Harrison (1993). The ribosomal DNA clone containing 18S–5.8S–25S rRNA genes isolated from *Arabidopsis thaliana* was directly labelled with Cy3 using nick translation. Chromosome preparations were pretreated with RNase (100 µg/mL, 37°C , 1 h), washed in $2\times$ SSC and air-dried. Slides were denatured together with a pre-denatured DNA probe (70°C for 5 min) in a humid chamber at 85°C for 7 min. Hybridization was performed overnight at 37°C . Stringent washing was performed in 50% formamide in $0.1\times$ SSC at 42°C followed by several washes in $2\times$ SSC. Preparations were counterstained with 2 µg/mL DAPI and mounted in antifade buffer (Citifluor, Ted Pella Inc.). Slides were examined with an Olympus AX-70 Provis epifluorescent microscope. Photographs were taken using 400 ASA film, scanned, and computer-processed using ANALYSIS 3.0 and PICTURE PUBLISHER 7.0 software, and finally printed using a high-resolution colour printer with CMYK technology.

Results

Localization of rDNA in chromosomes of *Brassica* species

The number of rDNA loci has been determined in metaphase chromosomes using the in situ hybridization method and compared with results of CMA fluorescent staining for six species of the U-triangle (U 1935). The observed number of rDNA loci was similar to that reported earlier (Maluszynska and Heslop-Harrison 1993). *B. campestris* has 10, *B. nigra* 6, and *B. oleracea* 4 rDNA loci. In allotetraploid species, 14 rDNA loci were observed in *B. juncea*, 12 in *B. napus*, and 8 in *B. carinata*. All allotetraploid species investigated have one rDNA locus less than the sum of the loci in their ancestors. Metaphase of *B. napus* is shown in Fig. 1a and 1b as an example. These pictures comprise results of hybridization with rDNA and CMA staining, specific for GC-rich chromosomal segments. The pattern of positive CMA bands corresponds to the pattern of FISH with rDNA in all investigated species (Fig. 1a–c).

It is possible to distinguish three chromosomal positions of rDNA loci in *Brassica* genomes; secondary constriction and pericentromeric and terminal localization. The chromosomes with a satellite and rDNA at the secondary constriction are present in each genome. The pericentromeric localization of rRNA genes is observed in *B. campestris* (AA), *B. juncea* (AABB), and *B. napus* (AACC) chromosomes. This indicated that such a position is characteristic for the A genome only. The terminal localization of rDNA is typical for B and C genomes, because it is present in all species except *B. campestris* (AA) (Fig. 1c). The hybridization sites with rDNA at the secondary constrictions are usually significantly bigger than others.

Activity of the ribosomal gene cluster in diploid ancestral species

In situ hybridization provides essential information about the location of rRNA genes, which is complemented by silver staining as an indicator of transcriptional activity of these cistrons. Silver staining allowed determination of the number of active rDNA loci in each species investigated. All these species have a lower number of AgNORs compared with the number of in situ hybridization sites. Analysis of at least 15 metaphase plates for each species confirmed that active rRNA genes are located at the secondary constrictions of NOR-chromosomes. In *B. nigra* 4 out of 6 rDNA loci are active. Both pairs of AgNORs are similar in size (Fig. 2a). In *B. oleracea* among 4 rRNA loci, 2 are active (Fig. 2d); in *B. campestris* only 2 out of 10 rDNA are active (Fig. 2f). In the last species, AgNORs are more strongly stained and are much bigger than those of *B. nigra*.

Activity of the ribosomal gene cluster in allotetraploid species

The number of AgNORs of allotetraploid species is equal to the number of active loci in their diploid ancestors. *B. napus* has 4 active rDNA loci from the genomes of *B. campestris* and *B. oleracea*. Both pairs of AgNORs are similar in size and morphology. They are located on the chromosomes with distinct secondary constriction and satellites (Fig. 2e). *B. carinata*, containing genomes of *B. nigra* and *B. oleracea*,

Fig. 1. Localization of rDNA on *B. napus* chromosomes. (a) FISH sites of rDNA (pink). (b) Chromomycin A3 staining; six pairs of chromosomes with CMA⁺ bands. (c) Three types of rDNA position on *Brassica* chromosomes; at the secondary constriction (genome A, B, C), pericentromeric region (genome A), and terminal region (genome B, C). Each one presented by FISH, CMA⁺ band, and idiogram of the chromosome. (d, e) Metaphase plate after sequential silver staining and FISH with rDNA. Twelve sites of rDNA after FISH (pink) and four AgNORs (brown). Arrows indicate transcriptionally active rDNA loci. Magnification bars represent 5 μ m.

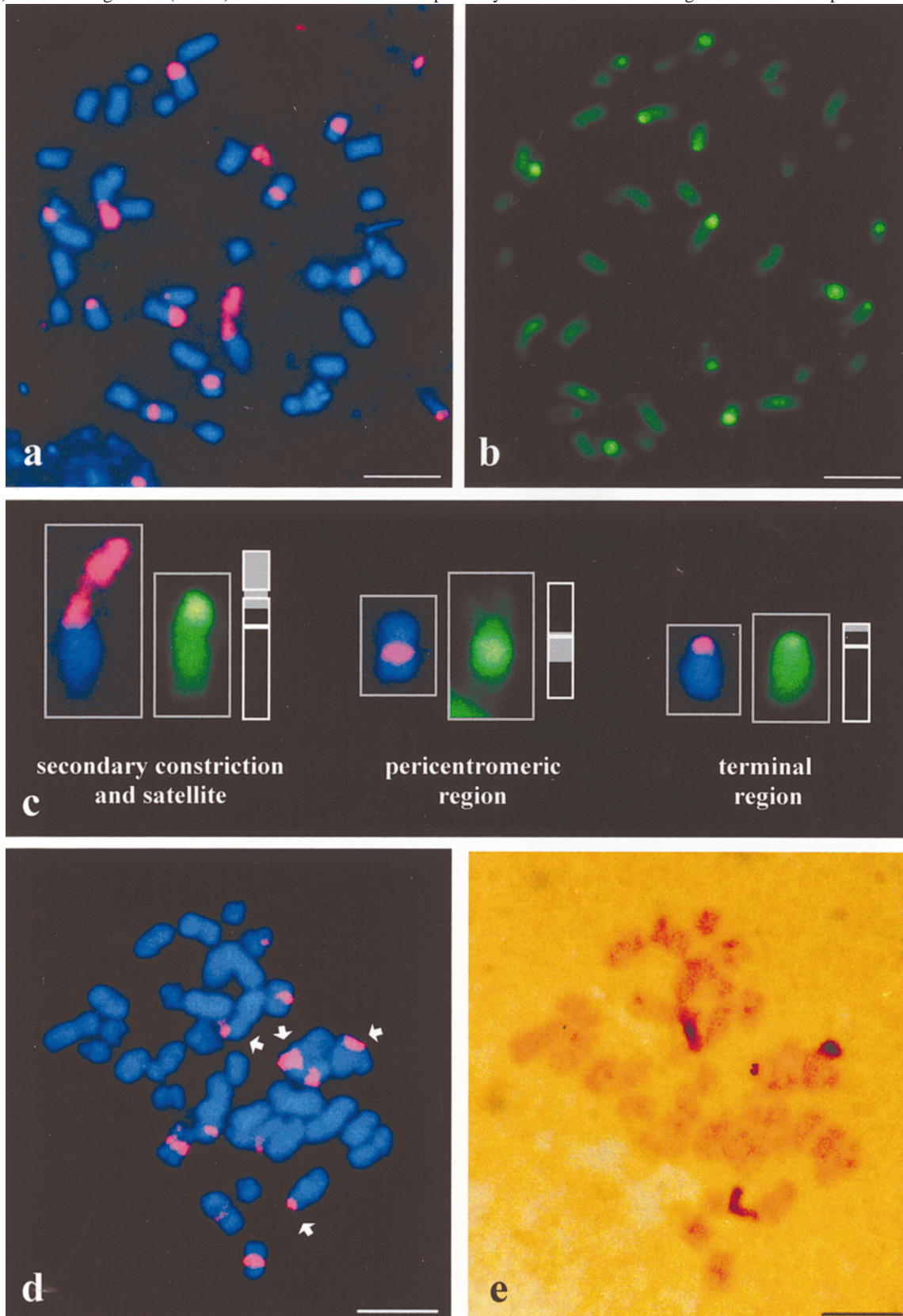
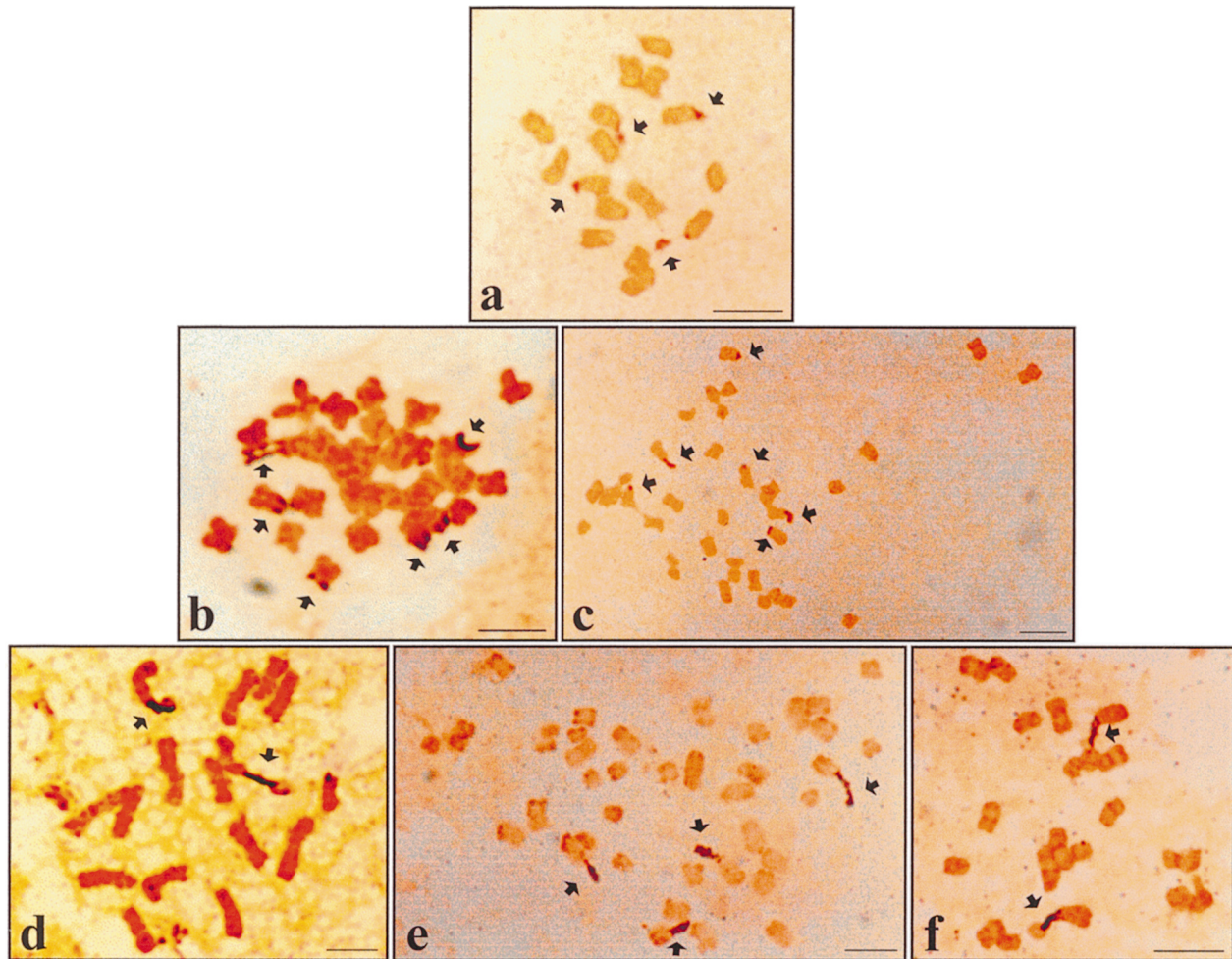


Fig. 2. Active rDNA loci in *Brassica* species (U triangle) detected by silver staining. (a) *B. nigra*, (b) *B. carinata*, (c) *B. juncea*, (d) *B. oleracea*, (e) *B. napus*, (f) *B. campestris*. Arrows indicate AgNORs. Magnification bars represent 5 μ m.



has 6 active rDNA loci, similarly to *B. juncea*, containing the genomes of *B. nigra* and *B. campestris* (Fig. 2b, 2c). In both these species, one pair of AgNORs is significantly bigger than two others, and is located on the chromosomes with prominent secondary constriction. These loci are similar to those of *B. oleracea* and *B. campestris*. Two pairs of chromosomes with smaller AgNORs are typical for genome B of *B. nigra*. It should also be emphasized that secondary constriction in NOR-chromosomes is very sensitive, and satellites might often be lost during preparation. It decreases the size of the NOR and AgNOR is limited to distal part of the short arm of the chromosome only.

Sequential silver staining and in situ hybridization with rDNA allows accurate identification of active and inactive loci. In *B. napus*, four chromosomes with active and eight with inactive rRNA genes can be distinguished in the same metaphase (Fig. 1d, 1e).

Discussion

Chromosomes of *Brassica* species are relatively small and morphologically little differentiated, which makes them difficult to identify in the complement. However, they are widely investigated. The number of chromosomes in *Brassica* was determined more than 75 years ago (Karpechenko

1922; Morinaga 1928) and several karyotypes have been presented (Olin-Faith and Heneen 1992; Snowdon et al. 1997b; Fukui et al. 1998). Cytogenetic studies of chromosome pairing indicated that the diploid species are in fact the secondary polyploids (Röbbelen 1960). U (1935) first presented the phylogenetic relationship between diploid and allotetraploid *Brassica* species in his triangle. This has been confirmed by molecular comparative genome analysis (Song et al. 1991; Kianian and Quiros 1992; Truco et al. 1996).

Development of in situ hybridization (FISH) with repetitive sequences of DNA (Maluszynska and Heslop-Harrison 1993; Harrison and Heslop-Harrison 1995) and total genomic DNA (GISH) (Snowdon et al. 1997a; Skarzhinskaya et al. 1998) has opened new possibilities in cytogenetic studies. The number of rDNA loci in *Brassica* species estimated by in situ hybridization varies slightly in published results. *B. nigra* has two (Maluszynska and Heslop-Harrison 1993) or three pairs of loci (Snowdon et al. 1997b; Fukui et al. 1998, and present study). Similarly, *B. carinata* containing the genome BB has either four (Maluszynska and Heslop-Harrison 1993) or five (Snowdon 1997) pairs of rDNA loci. The data for the two species *B. napus* and *B. campestris* are in full agreement. These differences can be due to variation between varieties of the species used for investigation. Technical differences such as chromosome preparation or the de-

tection system for in situ hybridization can also affect the size of rDNA signals.

The nucleolar activity of rDNA loci has been described for *B. campestris*, *B. nigra*, and *B. oleracea* (Cheng and Heneen 1995; Cheng et al. 1995). In the present study, the number of AgNORs has been determined for the six species of the U triangle. Active rDNA loci in all of these species are located at the secondary constriction of chromosomes, while those at pericentromeric or terminal locations are inactive or their activity is below detection. In contrast to the number of rDNA loci, the number of AgNORs in all amphidiploids is equal to the sum of active NORs in diploid ancestral species. This indicates that there is no nucleolar dominance in root meristematic cells of *Brassica* species.

The phenomenon of nucleolar dominance has been investigated in different taxonomic groups of plants and animals and recently in the *Brassica* genus. The genus offers a very convenient system for these investigations due to availability of polyploids formed in various combinations from the same diploid progenitors. Chen and Pikaard (1997a) have demonstrated nucleolar dominance in young leaves of allotetraploid species of *Brassica*. They suggested that rRNA gene promoters and the rRNA Pol I transcription system evolve rapidly and as a consequence, rRNA gene promoters from one species are often not recognized in another genetic background because of incompatibility of the Pol I transcription factors. They have shown that DNA methylation and histone modification were also involved in rRNA gene silencing. Our cytogenetic observation seems to be contradictory to these molecular results. However, we investigated meristematic cells of primary roots with high mitotic activity. The rRNA gene expression can be different in different organs and developmental stages of the plant. Meristematic cells have greater activity than differentiated ones and may be endopolyploid cells of dormant tissues. This conclusion can be confirmed by the observation that rRNA genes silent in vegetative tissues of *B. napus* were expressed in all floral organs (Chen and Pikaard 1997b). Their molecular study delivered data contesting the hypothesis that the passage through meiosis is necessary to reactivate suppressed genes. However, a cytogenetic investigation of rDNA activity can be done only on dividing cells. The estimation of the number of active rDNA loci in interphase nuclei is especially difficult in *Brassica* cells where, despite numerous rDNA loci, usually only one nucleolus is present. As it is impossible to undertake cytogenetic investigations in the leaves, it would be desirable to corroborate our results by molecular analysis of transcriptional activity in root meristematic cells.

Organ-specific regulation of the expression of rRNA genes was also observed in *Allium cepa* (Hasterok and Maluszynska 1998). In primary roots, more loci of active rRNA genes were observed than in adventitious ones. This can indicate that greater transcriptional activity of rDNA is required at the beginning of seedling development than in later phases of plant growth.

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