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Different rRNA gene expression in primary and adventitious roots of *Allium cepa* L.

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Abstract: Sequentially used silver staining and *in situ* hybridization allowed to estimate the number of rDNA loci and their activity in meristematic cells of *Allium cepa* roots. In primary roots, obtained from germinated seeds, the rDNA probe hybridized with four chromosomes and showed four strong sites of hybridization. All of them displayed very clear positive silver staining. In cells of adventitious roots, from bulbs, only one pair of rRNA gene loci was active and after *in situ* hybridization showed strong signals while two other sites were very weak. The results indicate different transcriptional rRNA gene activity in meristematic cells of roots of different developmental origin. The reduction of the number of active rRNA loci can be the result of DNA methylation but the reduction mechanism of *in situ* hybridization sites in adventitious roots of *Allium cepa* remains an open question.

Key words: *Allium cepa* - AgNOR - FISH - nucleolus - rDNA

Introduction

Roots from *Allium cepa* (2n=16) bulbs are a very convenient source of dividing meristematic cells for chromosome analysis. Chromosomes are relatively big and easy for preparation and have been used in many different types of investigations. *Allium cepa* has been used in genotoxicity assays since 1938 [9]. During that time several karyotypes were presented but still the number and localization of rRNA genes is controversial. It was even demonstrated that *Allium cepa* NORs had no fixed position [21]. Ricroch *et al.* [19] applying *in situ* hybridization showed that rDNA loci were present on satellited chromosome No. 6 and the smallest chromosome No. 8. Panzera *et al.* [17] additionally detected a fifth site on a long arm of a single chromosome of pair No. 8. Nucleolar activity was investigated on the basis of a maximal number of nucleoli in root cells of *Allium cepa* bulbs but there was no uniform conclusion on transcriptional activity of rDNA loci [11]. Although much is already known about the structure and organization of rDNA in many plant species, the regulation of its expression in different gene clusters in species with more than one locus and changes of that expression during plant development have not yet been satisfactorily elucidated [15].

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The comparison of the rDNA loci number and their activity in two types of roots, which differ in their developmental origin, was the aim of our investigation. Sequentially applied silver staining and fluorescence *in situ* hybridization allowed the determination of active and inactive rDNA loci in the same cells of root meristem in primary and adventitious roots of *Allium cepa*.

Materials and methods

Materials. The bulbs and seeds of *Allium cepa* L. cv. Wolska from a commercial source were used. The primary roots were obtained from seeds germinated on moist filter paper in Petri dishes. The adventitious roots were obtained from bulbs growing in aerated tap water for 2-3 days. The root-tips were fixed in methanol-glacial acetic acid (3:1) after a 2-hour treatment with 2 mM 8-hydroxyquinolin. Root meristems were digested in an enzyme solution (2% cellulase and 20% pectinase in 0.01 M citrate buffer, pH 4.8) and squashed. The cover slip was removed after freezing and the preparation was air-dried.

Silver staining. Slides were incubated for 10 min in 0.01 M borate buffer (pH 9.2) then a few drops of freshly prepared 50% silver nitrate solution in distilled water were added to the preparation. Slides were covered with a nylon mesh [4], incubated in a moist chamber at 42°C for one hour, washed in distilled water and air-dried. The number of active rDNA loci was determined and the first chromosome photographs were taken. Then, for the next step, slides were destained with 30% H_2O_2 for 15-30 seconds at room temperature, briefly washed in distilled water and air-dried.

Fluorescence *in situ* hybridization. The *in situ* hybridization procedure was essentially that of Maluszynska and Heslop-Harrison

[13]. 45S rDNA directly labelled with Cy3 by nick translation was used as a probe. Slides were pretreated with RNase (100 µg/ml in 2×SSC) for 1h at 37°C, washed in 2×SSC, dehydrated in an ethanol series and air-dried. The hybridization mixture (consisted of 0.5 ng/µl DNA probe, 50% formamide, 10% dextran sulfate, 0.1% SDS, and 300 ng/µl salmon sperm DNA in 2 × SSC) was denatured for 5 min in 70°C and placed on ice for 10 min. Then 20 µl of hybridization mixture was added to preparations and denatured together on a hot plate (Hybaid Thermal Cycler PCR-*in situ*) at 85°C for 8 min. Hybridization was performed overnight at 37°C. A stringent wash was carried out in 50% formamide in 2×SSC at 42°C followed by several washes in 2xSSC at 37°C. Slides were counterstained with 2 µg/ml DAPI and mounted in an antifade solution (AF1, Citifluor). Slides were examined with an Olympus epifluorescence microscope with appropriate filter sets. Photographs were taken using Kodak Gold 400 ASA film. Digital overlaying of DAPI and FISH images was done using Picture Publisher® 5.0, and AnalySIS® computer programs.

Results

The sequentially used silver staining and *in situ* hybridization make it possible to analyse localisation and activity of rDNA loci in the same metaphase plate (Fig.1a, b, e, f). In primary root cells, rDNA hybridized with two pairs of chromosomes, at the secondary constriction of one pair and at the distal region of another chromosome pair in the complement (Fig. 1a). The size of the hybridization sites at the secondary constriction was slightly larger than at the distal region. rDNA loci displayed very clear positive Ag staining, which indicated activity of all NORs in the cells (Fig. 1b). In the interphase nuclei, a maximum of four nucleoli could be observed but most cells had one or two nucleoli. Nucleoli differed in size, two were small and two bigger or one smaller and one big (Fig.1d). After *in situ* hybridization, signals are dispersed over the nucleus (Fig. 1c).

In adventitious roots, *in situ* hybridization with rDNA showed the presence of rRNA genes in two pairs of chromosomes. Two sites, located at the secondary constriction and satellite, were very clear and large, while two minor sites could be seen at the telomeric position of the other chromosome pair (Fig. 1e). Silver staining revealed two AgNORs in all metaphase plates and a maximum of two nucleoli in interphase nuclei (Fig. 1f, h). Only rDNA located at the secondary constriction was active. Most interphase nuclei had one big nucleolus and three condensed signals of *in situ* hybridization. Usually two of them were associated with the nucleolus (Fig. 1g, h).

Discussion

The rRNA genes are the most accessible for analysis of their localization and gene expression at the cytogenetic level. *Allium cepa* rRNA genes are distributed between four NORs on satellited and the smallest pair of chromosomes of the complement [19; present study]. However there is still some controversy about the location of rRNA genes and their activity in the *Allium cepa* genome. Applying sequentially *in situ* hybridization for localization and silver staining for detection of activity, it is possible to distinguish active and inactive rDNA loci in exactly the same cell [3, 23, 24].

The number of hybridization sites after *in situ* hybridization with rDNA to *Allium cepa* chromosomes in the present study was similar to those reported earlier [19]. We did not detect the fifth locus on chromosome No. 8 described by Panzera *et al.* [17]. The lack of this locus can be due to different cultivars used for the study. In contrast to another result [21], we observed only fixed positions of rDNA loci in all analysed metaphases. We did observe variation in rDNA loci, one pair of chromosomes displays larger signals than the other ones. The size of signals is at great variance in adventitious roots while in primary roots this difference is not so large. Most studies on *Allium* chromosomes were carried out using adventitious roots obtained from bulbs and they also showed differences in the size of NORs [17, 19]. *In situ* hybridization is not a quantitative method but size of signals can reflect the copy number of rRNA genes in the locus. Different sizes of NORs have been described in many species with multiple rRNA gene loci [13, 14, 16]. The number of rDNA repeats can significantly vary between the loci. Wide variations in ribosomal gene number in particular loci were demonstrated for wheat and barley [2, 8]. There is no information about rDNA distribution among chromosomes in *Allium cepa* genome. However it was shown that rDNA unit is about 12.7 Kb long and number of repeats varies from 5,000 to 12,500 genes between individual onion plants [12].

Very little is known about rRNA gene numbers in different plant tissues and about changes in rDNA loci during plant development. Different numbers of rRNA genes in leaves and roots of *Vicia faba* were reported by Rogers and Bendich [20]. Amplification of rRNA genes during the maturation of metaxylem in *Allium cepa* roots has been shown by radioactive DNA-rRNA hybridization [1]. Similarly, the amplification of rDNA repeats was reported during vascular cambium reactivation in *Abies balsamea* [10]. In the latter case, the increase in relative content of rDNA was attributed to the reactivation of the metabolism. It is difficult to explain the size reduction of rDNA sites in adventitious *Allium* roots in comparison to the sites in primary roots. One reason can be lost copy number. Such mechanism was found in long term wheat cell suspension cultures [7] but this could be due to *in vitro* culture conditions. The other explanation could be the heterochromatization of these loci associated with the suppression of their activity. Since in adventitious roots synthesis of rRNA is not as active as during seedling development in primary roots, the majority of rDNA can be located in the NOR heterochromatin. The heterochromatin segment in metaphase chromosome is strongly condensed and may be seen as

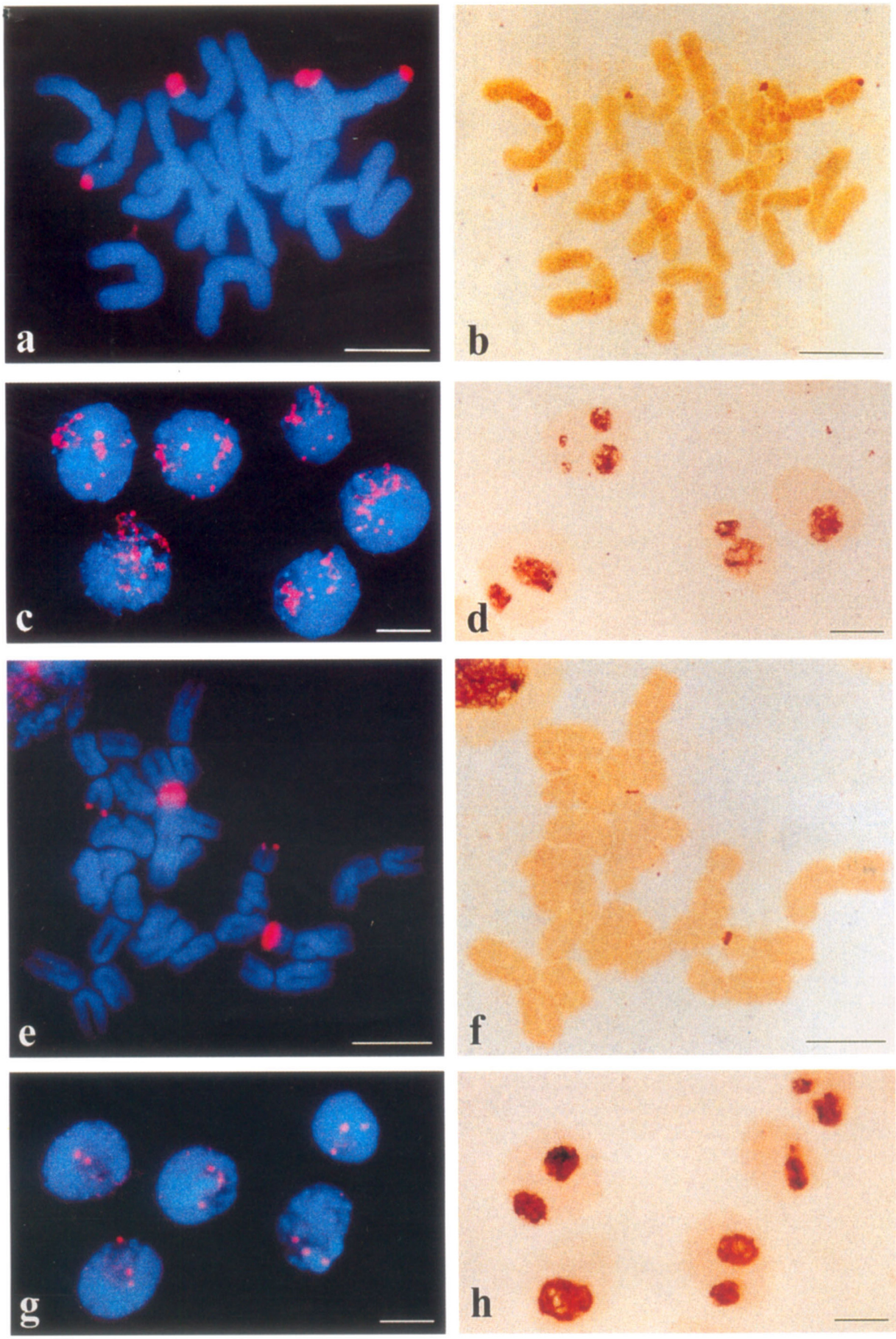

Fig. 1. Loci and activity of rDNA in meristematic cells of primary and adventitious roots of *Allium cepa* after sequential silver staining and *in situ* hybridization; (a-d) primary roots, (e-h) adventitious roots; (a, c, e, g) - signals of *in situ* hybridization with rDNA (red), (b, d, f, h) - silver staining (brown); a and b; e and f - present the same metaphase cells. a, b, e, f - bar = 10 μm; c, d, g, h - bar = 20 μm.

smaller signals. This suggestion is confirmed by silver staining. In primary roots, all rDNA loci are active but in adventitious roots only those at secondary constriction are. Those two large signals of *in situ* hybridization collocate with AgNORs in cells of adventitious roots.

Differences in rDNA distribution and activity were also observed in interphase nuclei of the investigated types of roots. In primary roots, decondensed signals of rDNA were dispersed in the nucleus, while in adventitious roots, three condensed signals were present and two of them were associated with the nucleolus. The expression of rRNA genes has been correlated with dispersion of the locus during interphase [22]. Investigations on cereals have shown that expressed rRNA genes are decondensed and occur inside the nucleolus [6]. The number and size of nucleoli demonstrate rDNA activity in the cell. The maximum number of nucleoli per nucleus is equal to the number of chromosomes with nucleolar organiser regions. During interphase nucleoli can fuse and very often one nucleolus is seen in the cell. [5]. In *Allium cepa*, a maximum of four nucleoli were observed in interphase but most cells showed two or only one large nucleolus. In adventitious roots, cells with one or maximum two nucleoli dominated, which can indicate lower rDNA expression. Different distribution and activity of rDNA observed in primary and adventitious roots of *Allium cepa* indicate significant changes in expression of rRNA genes during plant development. Decrease in rDNA activity of one pair of loci in adventitious roots can result from methylation of DNA, one of the common mechanisms in gene activity regulation [18].

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