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AUTORADIOGRAPHIC AND ULTRASTRUCTURAL STUDIES OF THE EFFECT OF CHILLING ON SOYBEAN ROOT MERISTEM NUCLEOLI

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Root meristem nucleoli from soybean (*Glycine max*. cv. Aldana) seedlings germinated for 3 days at 25˚C and then for 4 days at 10˚C or still at 25˚C (control) were examined. Chill was observed to reduce root meristematic zone growth 15-fold. Nucleoli doubled their volume at 10˚C. Autoradiographic studies showed that after 20 min ^{3}H-uridine incubation at 10˚C, incorporation of this precursor (postincubation time 0) into nucleoli of chilled seedlings was 4.7 times weaker than in the control. After 80 min postincubation in nonradioactive medium, the cytoplasm became the most intensely labelled cell area in the control material, while in chilled roots the nucleoli were still most intensely labelled and the cytoplasm was 11 times less labelled than in the control. The increase in nucleoli volume at 10˚C is suggested to result from greater cold-induced inhibition of the dynamics of maturation and transport of ribosome subunits than of rRNA synthesis dynamics. Ultrastructural studies of chilled seedling nucleoli showed a significant decrease in the fibrillar component and an increase in the granular component, forming characteristic clusters. They are supposed to correspond to shortened and condensed pre-rRNA transcription complexes (compacted "Christmas trees").

Key words: ^{3}H-uridine incorporation, ultrastructure, nucleolus, meristematic soybean root cells.

INTRODUCTION

Although there is much recent data to show the plurifunctionality of nucleoli, their best-confirmed function is as the site of synthesis and maturation of ribosome subunits (Pederson, 1998 a,b; Olson et al., 2000; Visintin and Amon, 2000; Ospina and Matera, 2002). Nucleoli are labile structures; their architecture depends on the functional status and developmental stage of a cell (Scheer and Hock, 1999; Olson et al., 2000). Generally their sizes correlate with cell metabolic activity (Flavell and O'Dell, 1979; Martini et al., 1982; Guerrero et al., 1989; Fischer et al., 1991). However, even if typical nucleoli are lacking, ribosome synthesis may take place in cells equipped with rDNA, suitable enzymes and regulatory factors (Nierras et al., 1997; Oakes et al., 1998; Scheer and Hock, 1999; Olson et al., 2000). Moreover, big nucleoli do not unequivocally mean dynamic ribosome synthesis. For example, in *Chara vulgaris*, small nucleoli poor in the granular component incorporate ^{3}H-uridine as intensively as bigger ones abundant in the granular component present in the same nucleus. The former are more active in rRNA transport to the cytoplasm than the latter (Kwiatkowska and Maszewski, 1985). The size and structure of nucleoli result from the dynamics of two processes: (1) pre-rRNA synthesis and (2) its maturation and transport to the cytoplasm. These two systems can be modified independently in the cell (Olson et al., 2000; Verheggen et al., 2000; Yamamoto et al., 2000; Brown, 2001; Milkereit et al., 2001).

The present research uses ^{3}H-uridine autoradiography to assess changes in soybean root meristem nucleoli functioning in chilling conditions, and examines changes in nucleoli ultrastructure caused

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by chilling of seedlings. Chilling at 10˚C was employed, as this subtropical plant begins to degenerate at lower temperature. After 4–7 days of chilling, soybean seedlings are capable of fully regenerating metabolic activity and continuing growth (Stępiński, unpubl. data). There is a single nucleolus in a soybean meristematic interphase cell. NOR is localized only on chromosome 13 (Griffor et al., 1991).

MATERIALS AND METHODS

Seeds of soybean *Glycine max* (L.) Merr. cv. Aldana obtained from IHAR in Radzików were germinated for 3 days at 25˚C in darkness in Petri dishes on filter paper wetted with distilled water. Some of these seedlings continued growth at 25˚C (control) and some seedlings were transferred to cold (10˚C) for 4 days. Meristematic root tip cells were examined.

MORPHOMETRIC MEASUREMENTS

Nucleolar morphometric measurements were made on squash slides stained by two independent methods: (1) apical parts of roots were fixed in 0.0125 M Sörensen's phosphate-buffered (pH 7.2) 2% glutaraldehyde and stained using the Ag-NOR impregnation method described by Howell and Black (1980); (2) root tips were fixed in absolute ethanol:glacial acetic acid (3:1), rinsed with ethanol and then water, the material was treated with pectinase at 45˚C for 30 min and rinsed in McIlvain buffer (pH 5.0), squashed preparations were made and then stained with toluidine blue according to Smetana's method (Smetana et al., 1968).

It was assumed that the nucleoli were not flattened during squash slide preparation and that the nucleolar profiles are circular. Their areas were measured with the computer-aided Imal-512 system. After the radiuses of the nucleoli were established the nucleolar volumes were calculated mathematically.

INCUBATION IN ^{3}H-URIDINE

Roots of seedlings growing 3 days at 25˚C were incubated in water containing ^{3}H-uridine (80 µCi/ml; 24.0 Ci/mM) for 20 min at 25˚C (control) or at 10˚C (3-day-old seedlings subjected to chill for 4 days). Half of the seedlings from each group were postincubated for 80 min in nonradioactive medium at 25˚C or 10˚C, respectively. Roots were fixed in absolute ethanol/glacial acetic acid (3:1) for 1 h at room temperature, rinsed with ethanol and kept in 70% ethanol for further procedures. The material was then treated with pectinase at 45˚C for 30 min and rinsed in McIlvain buffer (pH 5.0). Meristematic parts of roots were placed on microscope slides in a water drop and squashed. After freezing and removal of coverslips the slides were dried, covered with emulsion for high-resolution microautoradiography (Amersham-Pharmacia-Biotech) and exposed in darkness at 8˚C. After 14 days the autoradiograms were developed and stained with toluidine blue according to Smetana's method (Smetana et al., 1968) and embedded in Canada balsam. Silver grains were counted over the nucleoli, extranucleolar nucleoplasm and cytoplasm in 30 cells in preparations from 3 meristems from each variant.

ELECTRON MICROSCOPY

Selected root tips were fixed in 2% glutaraldehyde in 1% cacodylate buffer (pH 7.2–7.4) for 3 h at 4˚C. Roots were postfixed in 1% OsO_4 in the same buffer. After dehydration in an ethanol series, the material was embedded in medium consisting of Epon 812 and Spurr's resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate according to Reynold (1963). The sections were examined and photographed in a JEOL JEM 1010 transmission electron microscope.

RESULTS

EFFECT OF CHILLING ON GROWTH DYNAMICS OF ROOT MERISTEMATIC ZONE

Examination of a 2 mm root meristematic zone (marks on root surface labelled with activated charcoal) showed that during 4-day development at 10˚C the meristematic zone grew about 15 times slower than the control (Fig. 1). This means a decrease in cell elongation and/or a drastic decrease in cell division under chilling conditions but also that growth, though slowed, was not completely stopped.

EFFECT OF CHILLING ON SIZE OF NUCLEOLI IN ROOT MERISTEMATIC ZONE

Morphometric measurements of nucleoli from chilled seedlings showed that despite the significant decrease in growth activity in the root meristematic zone their volume was about twice that in the control (Fig. 2). However, the size of the nuclei in which the nucleoli were measured remained unchanged. This

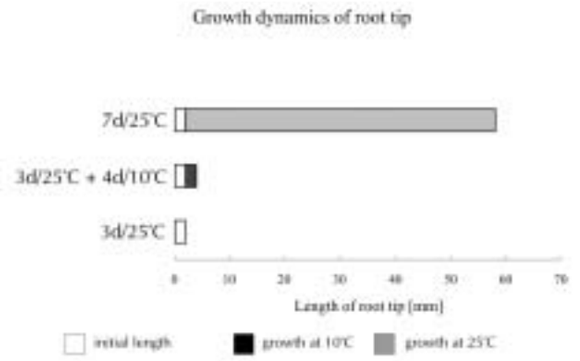


Fig. 1. Lengthening of 2 mm root tip of 3-day-old soybean seedling following 4 days of growth at 25˚C or 10˚C.

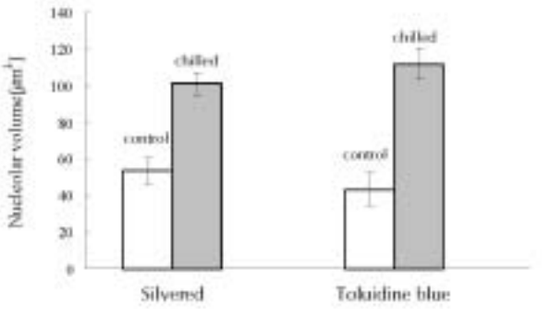


Fig. 2. Mean volumes of nucleoli in meristematic cells of control (grown at 25˚C) and chilled (10˚C) seedlings.

regularity was observed in preparations after Howell and Black's method (1980) as well as in those after Smetana's method (1968) with the use of toluidine blue staining RNA.

^{3}H-URIDINE INCORPORATION INTO MERISTEMATIC CELLS AFTER CHILLING AND IN CONTROL

In the control after 20 min ^{3}H-uridine incorporation, the nucleolus was the most intensely labelled part of the cell (Fig. 3). After 80 min postincubation the cytoplasm was most intensely labelled (labelling was 4 times higher than at postincubation time 0), while the radioactivity of the nucleoli diminished.

In chill-treated plants after 20 min ^{3}H-uridine incubation, labelling of meristematic zone nucleoli was about 4.7 times weaker than in the control (Fig. 3). Extranucleolar nucleoplasm and cytoplasm labelling was proportionally lower. After 80 min postincubation, unlike in the control, nucleoli remained the most intensely labelled area, while labelling of cytoplasm was still low. Comparing the dynamics of the labelling increase in chilled and control material during postincubation, it can be seen that chilling decreased the rate of maturation and transport of ribosome subunits into the cytoplasm about 11-fold. This effect is more than twice stronger than the effect of chilling on ^{3}H-uridine incorporation into nucleoli.

ULTRASTRUCTURE OF NUCLEOLI IN CHILLED AND CONTROL MATERIAL

Central cross sections were analyzed, that is, the ones that were largest in serial sections of nucleoli of chilled and control seedlings.

In the control the structure of the nucleoli was dense. In the central part there was a distinct, abundant fibrillar component in which fibrillar centers (FC) could be seen, surrounded by a dense fibrillar component (DFC). The granular component (GC) was mainly peripherally situated (Figs. 4a, 5a).

After 4-day chilling, in the nucleoli, which were bigger than in the control, the fibrillar component significantly decreased while the granular component increased. Unlike in the control, the granules were not evenly spaced at the nucleolar periphery but formed characteristic clusters (Figs. 4b, 5b).

DISCUSSION

Autoradiographic studies showed that in the control the labelling of the nucleoli diminished while labelling of cytoplasm increased during postincubation. The increased radioactivity of the cytoplasm reflects mainly the activity of maturation and transport of ribosome subunits, since during mRNA splicing a significant part of the hnRNA becomes degraded in the nucleus (Moore, 2002).

In soybean plants, as in other species (Olszewska et al., 1985, 1987, 1988), chilling at 10˚C causes a drastic decrease in the activity of ^{3}H-uridine incorporation and transport of ribosome subunits to the cytoplasm. In chill-treated soybean seedlings the nucleoli enlarge because low temperature inhibits rRNA synthesis less than it inhibits ribosome subunit maturation and transport from the nucleolus to the cytoplasm. Uncoupling of these two processes may be part of soybean's acclimation to chilling. The arrest of pre-ribosomes in the nucleolus may protect them against the destructive effect of ribonuclease,

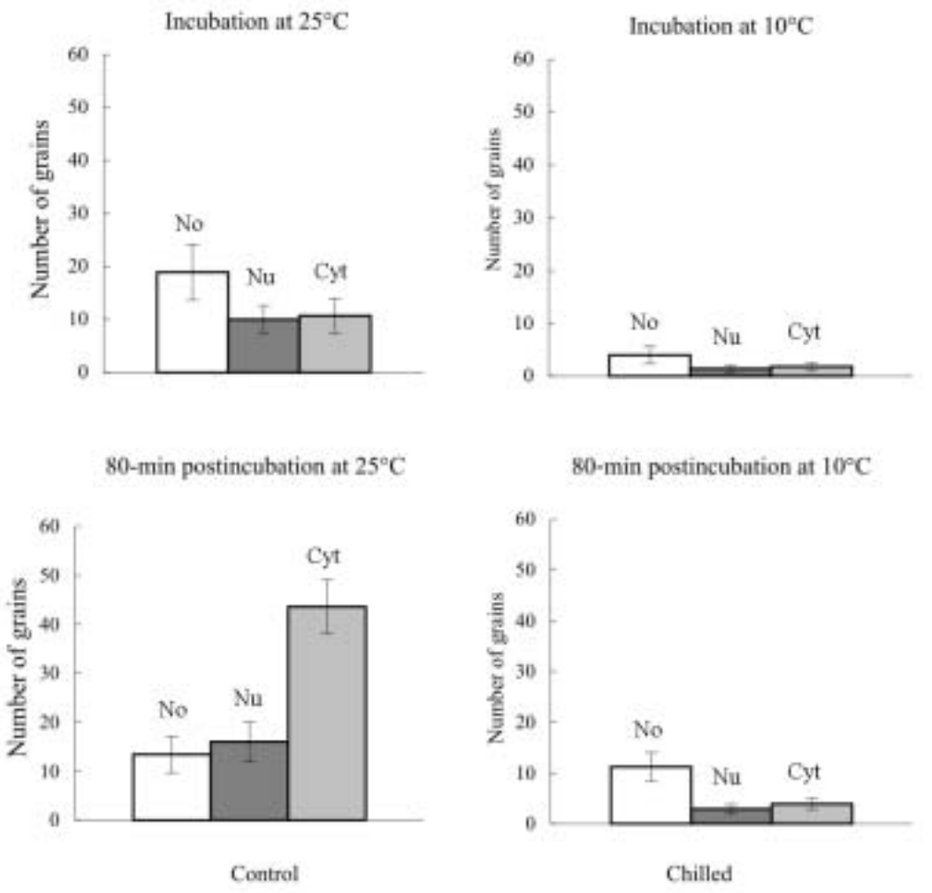
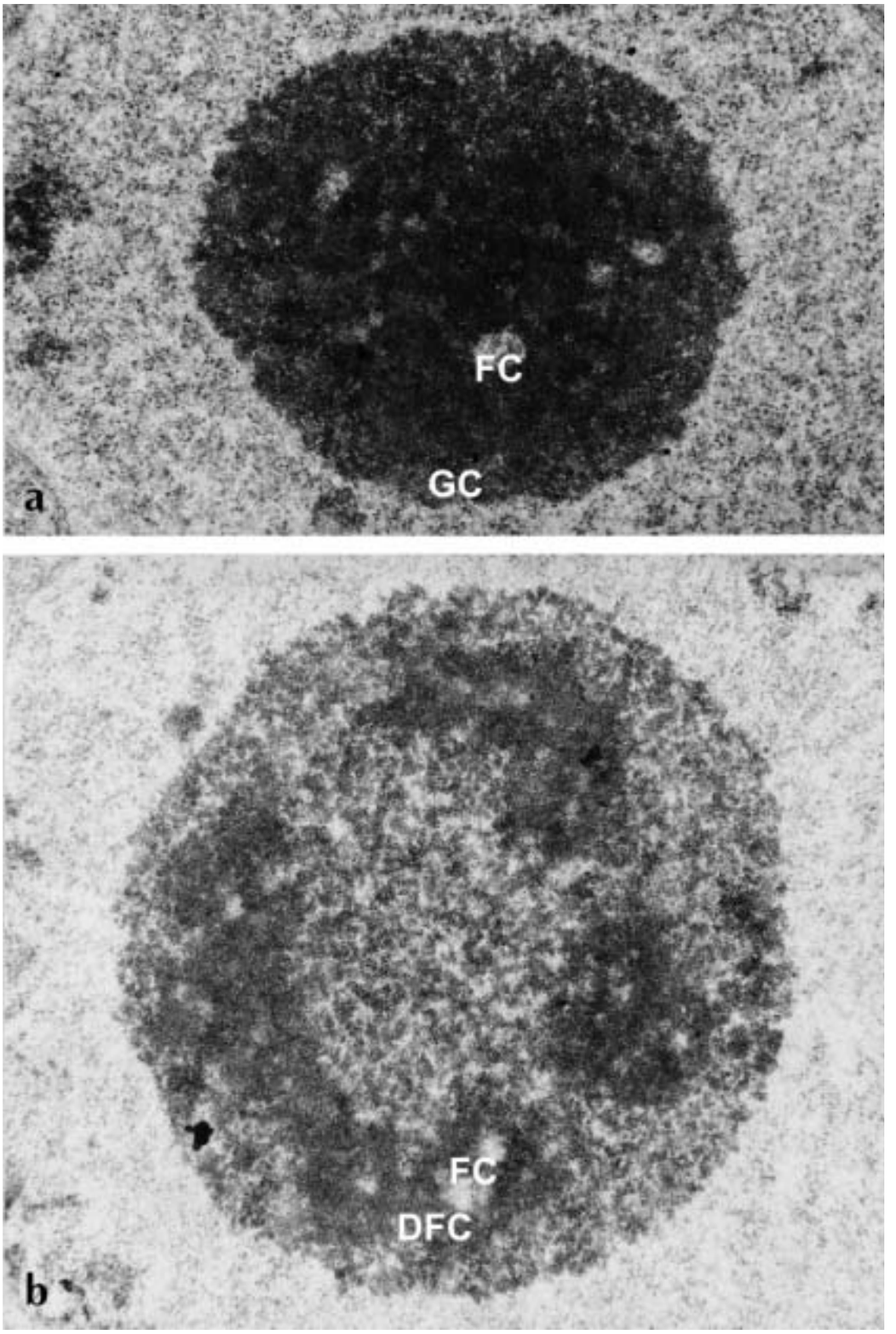


Fig. 3. Mean number of silver grains above nucleolus (No), extranucleolar nucleoplasm (Nu) and cytoplasm (Cyt) of root meristematic cells of control and chilled seedlings after 20 min incubation in ^{3}H–uridine and 80 min postincubation in nonradioactive medium. Bars represent standard errors.

as its activity increases in chill-treated seedlings (Stępiński, 2002). During regeneration at optimal temperature (25˚C), high activity of synthesis and transport of ribosome subunits into the cytoplasm is restored (Stępiński, unpubl. data), while ribonuclease activity drastically decreases (Stępiński, 2002).

Ultrastructural studies show that chilling distinctly changes the organization of the nucleolus. In chilled seedlings the nucleoli become loose. The amount of the fibrillar component decreases while that of the granular component increases. Possibly the granular component represents ribosome subunits "frozen" at different developmental stages and those ready to leave the nucleolus (Risueno et al., 1982; Brown and Shaw, 1998; Bassy et al., 2000). It is worth noting that these granules form specific structures resembling the condensed and shortened pre-rRNA transcription complexes that Scheer et al. (1997) called "Christmas trees" in *Locusta migratoria* oocytes in situ. These structures have axes 6 times shorter (with a positive terminal deoxynucleotidyl transferase reaction to DNA), with a radial arrangement of tightly packed lateral fibrils (con-

Fig. 4. Nucleolus of seedling growing 3 days at 25˚C – control (**a**) and 3-day-old seedling subjected to chill for 4 days (**b**). FC – fibrillar centers; DFC – dense fibrillar component; GC – granular component. × 25 000.


FC
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a
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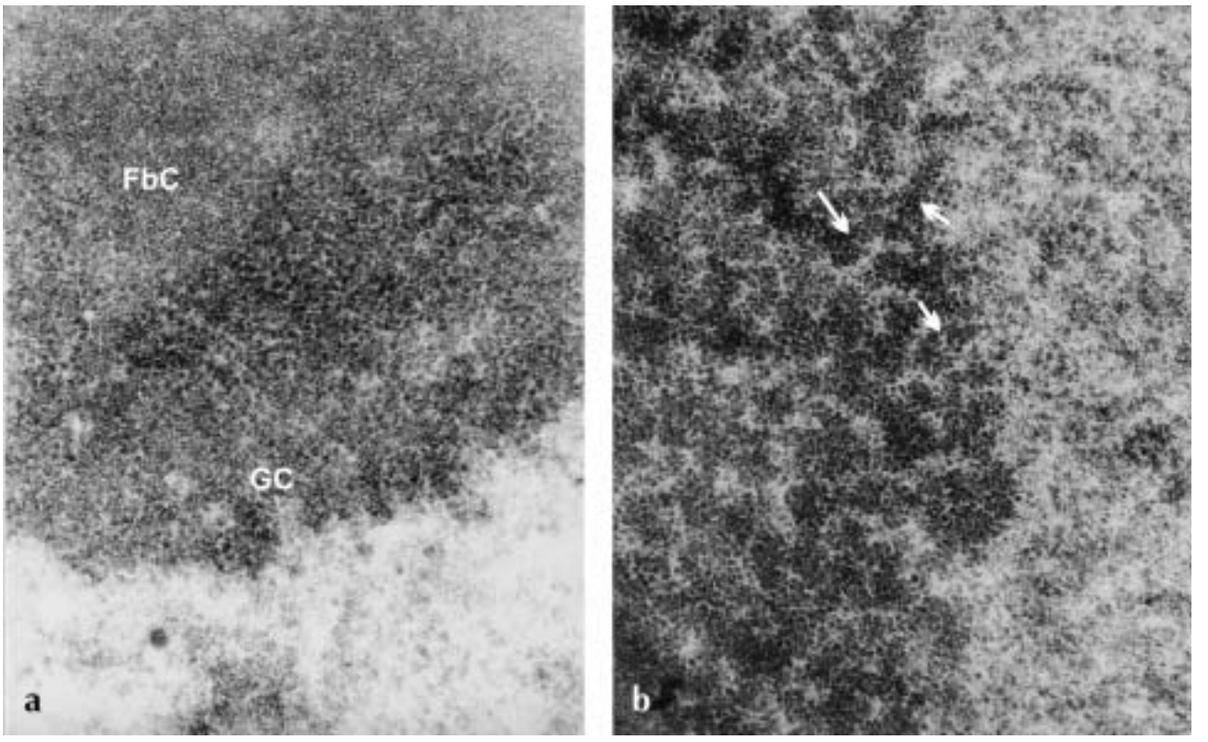

Fig. 5. Fragment of nucleolus of a plant growing 3 days at 25˚C (**a**) and 3-day-old plant subjected to chill for 4 days (**b**). GC – granular component; FbC – fibrillar component. Arrows show putative shortened and condensed transcription complexes (Christmas trees). × 75 000.

taining 28S RNA sequences observed in situ) terminated with granules (Scheer et al., 1997). Structures resembling "Christmas trees," thought to be transcription units, are also observed in nucleoli of onion root tip cells treated with cycloheximide. These structures are peripherally located on DFC and penetrate GC in nucleoli with a characteristic loose structure (Ghosh et al., 1993). Compacted "Christmas trees" in *Pisum sativum* plant nucleoli have been detected recently using a novel labelling technique followed by 3D electron microscopy with limited extraction of RNA and proteins (González-Melendi et al., 2001).

Localization of in situ ribosome synthesis on ultrathin sections is a very difficult, not entirely solved problem. Most scientists using precise molecular methods at ultrastructural level maintain that it takes place in the dense fibrillar component or between the fibrillar center and dense fibrillar component (Risueno et al., 1982; Raška et al., 1995; Brown and Shaw, 1998; Scheer and Hock, 1999; Bassy et al., 2000; Yano and Sato, 2000; Staněk et al., 2001; Koberna et al., 2002). However, Mais and Scheer (2001) and Cheutin et al. (2002) demonstrated recently that transcriptionally active rRNA genes are confined to the fibrillar centers of nucleoli.

Morphological criteria alone are not sufficient to unequivocally determine whether the clusters of nucleolar granules we observed in soybean are really condensed transcription complexes. Moreover, the physiological status of chilled soybean roots significantly differs from that of *Locusta migratoria*: the former exhibit low and the latter very high dynamics of ribosome synthesis. However, the observed further slowing down of ribosome subunit maturation and transport in soybean makes it highly probable that these are indeed transcription complexes, forming a very slow-moving "ribosome production line" which in a fixed preparation may look like one actively producing ribosomes. These structures in the control probably are masked in an abundant and densely packed fibrillar component containing nonactive rRNA genes as well. In plants under physiological conditions, 30–70% of rRNA genes are inhibited due to, among other things, rDNA cytosine methylation (Jacob and Ghosh, 1999). Stress, how-

ever, including low temperature, may cause cytosine demethylation and activation of these genes (Demeulemeester et al., 1999). Thus it seems probable that in soybean an increase in the granular component (presumably shortened and condensed transcription complexes) accompanied by a decrease in the fibrillar component may result from activation of additional rRNA genes, which makes the nucleolar structure looser. Activation of new genes when ribosome production is too slow seems necessary for synthesis of proteins produced at low temperature, enabling cells to adjust to unfavorable conditions (Hughes and Dunn, 1996). This interpretation seems supported by the results of these experiments with soybean roots transferred after 4 days of chilling to a favorable temperature. They displayed enhanced activity of synthesis and maturation of pre-rRNA, much higher than in the control (Stępiński, unpubl. data). This may be the result of the functioning of additional rRNA genes activated during chilling, promoting the formation of transcription complexes resembling shortened, condensed "Christmas trees."

REFERENCES

BASSY O, JIMENEZ-GARCIA LF, ECHEVERRIA OM, VAZQUEZ-NIN GH, and MORENO DIAZ DE LA ESPINA S. 2000. High resolution detection of rRNA and rDNA in plant nucleoli with different activities by in situ hybridisation. *Biology of the Cell* 92: 59–70.

BROWN JD. 2001. Ribosome biogenesis: stripping for AAAction? *Current Biology* 11: 10–12.

BROWN JWS, and SHAW PJ. 1998. Small nucleolar RNAs and pre-rRNA processing in plants. *The Plant Cell* 10: 649–657.

CHEUTIN T, O'DONOHUE MF, BEORCHIA A, VANDELAER M, KAPLAN H, DEFÉVER B, PLOTON D, and THIRY M. 2002. Three-dimensional organization of active rRNA genes within the nucleolus. *Journal of Cell Science* 115: 3297–3307.

DEMEULEMEESTER MAC, VAN STALLEN N, and DE PROFT MP. 1999. Degree of DNA methylation in chicory (*Cichorium intybus* L.): influence of plant age and vernalization. *Plant Science* 142: 101–108.

FISCHER D, WEISENBERGER D, and SCHEER U. 1991. Assigning functions to nucleolar structures. *Chromosoma* 101: 133–140.

FLAVELL RB and O'DELL M. 1979. The genetic control of nucleolus formation in wheat. *Chromosoma* 71: 135–152.

GHOSH S, CHAKRABARTI A, and CHAUDHURI SK. 1993. Active ribosomal cistrons in plant nucleolus. *Cell Biology International* 17: 759–763.

GONZÁLEZ-MELENDI P, WELLS B, BEVEN AF, and SHAW PJ. 2001. Single ribosomal transcription units are linear, compacted Christmas trees in plant nucleoli. *Plant Journal* 27: 223–233.

GRIFFOR MC, VODKIN LO, SINGH RJ, and HYMOWITZ T. 1991. Fluorescent in situ hybridization to soybean metaphase chromosomes. *Plant Molecular Biology* 17: 101–109.

GUERRERO F, DE LA TORRE C, and GARCIA–HERDUGO G. 1989. Control of nucleolar growth during interphase in higher plant meristem cells. *Protoplasma* 152: 96–100.

HOWELL WM, and BLACK DA. 1980. Controlled silver-staining of nucleolus organizer method. *Experientia* 36: 1014.

HUGHES MA, and DUNN MA. 1996. The molecular biology of plant acclimation to low temperature. *Journal of Experimental Botany* 47: 291–305.

JACOB ST, and GHOSH AK. 1999. Control of RNA polymerase I–directed transcription: recent trends. *Journal of Cellular Biochemistry* 32/33: 41–50.

KOBERNA K, MALÍNSKÝ J, PLISS A, MAŠATA M, VEČEŘOVÁ J, FIALOVÁ M, BEDNÁR J, and RAŠKA I. 2002. Ribosomal genes in focus: new transcripts label the dense fibrillar components and form clusters indicative of "Christmas trees" in situ. *Journal of Cell Biology* 157: 743–748.

KWIATKOWSKA M, and MASZEWSKI J. 1985. Functional and structural heterogeneity of nucleoli: the dependence of the activity of transport of newly synthesised rRNA on nucleolar size and phase of the cell cycle. *Folia Histochemica et Cytobiologica* 23: 117–126.

MAIS C, and SCHEER U. 2001. Molecular architecture of the amplified nucleoli of *Xenopus* oocytes. *Journal of Cell Science* 114: 709–718.

MARTINI G, O'DELL M, and FLAVELL RB. 1982. Partial inactivation of wheat nucleolus organizers by the nucleolus organizer chromosomes from *Aegilops umbellulata*. *Chromosoma* 84: 697–700.

MILKEREIT P, GADAL O, PODTELEJNIKOV A, TRUMTEL S, GAS N, PETFALSKI E, TOLLERVEY D, MANN M, HURT E, and TSCHOCHNER H. 2001. Maturation and Intranuclear transport of pre-ribosomes requires Noc proteins. *Cell* 105: 499–509.

MOORE MJ. 2002. Nuclear RNA turnover. *Cell* 108: 431–434.

NIERRAS CR, LIEBMAN SW, and WARNER JR. 1997. Does *Saccharomyces* need an organized nucleolus? *Chromosoma* 105: 444–451.

OAKES M, ARIS JP, BROCKENBROUGH JS, WAI H, VU L, and NOMURA M. 1998. Mutational analysis of the structure and localization of the nucleolus in the yeast *Saccharomyces cerevisiae*. *Journal of Cell Biology* 143: 23–34.

OLSON MOJ, DUNDR M, and SZEBENI A. 2000. The nucleolus: an old factory with unexpected capabilities. *Trends in Cell Biology* 10: 189–196.

OLSZEWSKA MJ, KURAN H, DAMSZ B, and MARCINIAK K. 1985. Transport of rRNA into cytoplasm required for initiation of S phase in root meristem cells in *Helianthus annuus* L. *Protoplasma* 127: 110–118.

OLSZEWSKA MJ, KURAN H, DAMSZ B, and MARCINIAK K. 1987. Autoradiographic, ultrastructural and interferometric analyses of the temperature effect on the synthesis and migration of ribosomes in root meristem cells of *Helianthus annuus* L. Relation to S phase initiation. *Folia Histochemica et Cytobiologica* 24: 69–78.

OLSZEWSKA MJ, KURAN H, and MARCINIAK K. 1988. Relationship between resumption of rRNA transport into cytoplasm after cold treatment and progression through the cell cycle in root

meristem cells. *Environmental and Experimental Botany* 28: 375–380.

OSPINA JK, and MATERA AG. 2002. Proteomics: The nucleolus weighs in. *Current Biology* 12: 29–31.

PEDERSON T. 1998a. Growth factors in the nucleolus? *Journal of Cell Biology* 143: 279–281.

PEDERSON T. 1998b. The plurifunctional nucleolus. *Nucleic Acids Research* 26: 3871–3876.

RAŠKA I, DUNDR M, KOBERNA K, MELČÁK I, RISUENO MC, and TÖRÖK I. 1995. Does the synthesis of ribosomal RNA take place within nucleolar fibrillar centers or dense fibrillar components? A critical appraisal. *Journal of Structural Biology* 114: 1–22.

REYNOLDS SS. 1963. The use of lead citrate of high ph as an electron-opaque stein in electron microscopy. *Journal of Cell Biology* 17: 208–212.

RISUEÑO MC, MEDINA FJ, and MORENO DÍAZ DE LA ESPINA S. 1982. Nucleolar fibrillar centers in plant meristematic cells: ultrastructure, cytochemistry and autoradiography. *Journal of Cell Science* 58: 313–329.

SCHEER U, and HOCK R. 1999. Structure and function of the nucleolus. *Current Opinion in Cell Biology* 11: 385–390.

SCHEER U, XIA B, MERKERT H, and WEISENBERGER D. 1997. Looking at Christmas trees in the nucleolus. *Chromosoma* 105: 470–480.

SMETANA K, LEJNER J, and POTMESIL M. 1968. A further contribution to the demonstration of RNA and nucleoli of blood cells in smear preparations. *Folia Hematologica* 91: 381–393.

STANĚK D, KOBERNA K, PLISS A, MALÍNSKÝ J, MAŠATA M, VEČEŘOVÁ J, RISUEÑO MC, and RAŠKA I. 2001. Non–isotopic mapping of ribosomal RNA synthesis and processing in the nucleolus. *Chromosoma* 110: 460–470.

STĘPIŃSKI D. 2002. Changes in ribonuclease activity in roots of soybean seedlings subjected to chilling and recovery. *Acta Physiologie Plantarum* 24: 297–301.

VERHEGGEN C, ALMOUZNI G, and HERNANDEZ–VERDUN D. 2000. The ribosomal RNA processing machinery is recruited to the nucleolar domain before RNA polymerase I during *Xenopus laevis* development. *The Journal of Cell Biology* 149: 293–305.

VISINTIN R, and AMON A. 2000. The nucleolus: the magician's hat for cell cycle tricks. *Current Opinion in Cell Biology* 12: 372–377.

YAMAMOTO K, KOGA A, YAMAMOTO M, NISHI Y, TAMURA T, NOGI Y, and MURAMATSU M. 2000. Identification of a novel 70 kDa protein that binds to the core promoter element and is essential for ribosomal DNA transcription. *Nucleic Acids Research* 28: 1199–1205.

YANO H, and SATO S. 2000. Ultrastructural localization of transcription sites, DNA, and RNA reveals a concentric arrangement of structural and functional domains in plant nucleolonema. *Protoplasma* 214: 129–140.