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Nucleolar vacuolation in soybean root meristematic cells during recovery after chilling

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Abstract

The nucleolar vacuole formation in soybean root meristematic cells from seedlings grown 3 d at temperature 25 °C (control), 3 d at temperature 25 °C and then transferred to 10 °C (chilling) for 4 d, and after recovery for 1.5, 3, 6, 12 and 24 h at 25 °C were observed on semi-thin sections. Simultaneously, autoradiographic studies with ^{3}H-uridine on squashed preparations were carried out. During recovery of plants, the number of vacuolated nucleoli increased gradually from 24 % after 1.5 h up to 40 % after 24 h, while in the control there were 18 % of nucleoli with vacuoles and after 4-d chilling only 5 %. Labelling of cells during 20-min incubation in ^{3}H-uridine and during 80-min post-incubation in non-radioactive medium was increased in recovered plants in comparison with the control and chilled plants. The conclusion has been drawn that nucleolar vacuoles in soybean plants are formed as a result of migration of granular component accumulated in nucleolus during 4-d chilling.

Additional key words: *Glycine max*, ^{3}H-uridine incorporation.

Introduction

Nucleolus can show changes in activity, size, shape or ultrastructure depending on cell cycle phase (Jennane *et al*. 2000) or physiological and experimental conditions (Goessens 1984) – in which the transcription of rRNA genes, processing and maturation of ribosomes take place (Mineur *et al*. 1998).

In a nucleolus at the ultrastructural level three main areas are visible: fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC) (Olson *et al*. 2000). Apart from those, in some plant and animal cells one can observe different types of nucleolar vacuoles. They are not always present in a nucleolus, and their presence, size, number and morphology depend on the type of a nucleolus and many physiological cell factors (Rose *et al*. 1972, Goessens 1984).

There are two structurally different types of plant nucleolar vacuoles: *1*) vacuoles appearing in actively transcribing nucleoli, which are not associated with the intranucleolar chromatin; these vacuoles can contain precursors of ribosomes, *2*) vacuoles appearing during nucleolar activation which are associated with intra-nucleolar chromatin; they develop from the NOR-containing channel, and disappear, forming small vacuoles (Fakan and Hernandez-Verdun 1986). In spite of numerous studies the functions of nucleolar vacuoles have not been determined unequivocally so far. Presumably they play different roles depending on physiological needs (Mineur *et al*. 1998). It is thought that nucleolar vacuoles might be important in the initial phase of ribosomal gene dispersion and/or their activation by creating a favourable microenvironment, as it was suggested during the studies of an early stage of *Sinapis alba* germination (Deltour *et al.* 1986, Fakan and Hernandez-Verdun 1986). The presence of DNA was detected in large central vacuoles of nucleoli of *Pisum sativum* cotyledon quiescence cells (Nougarede *et al*. 1990). Also presence of pre-ribosomal particles distributed throughout the whole nucleolar vacuole as it was in cultured tobacco callus cells (Johnson 1969) and in pea root tip cells (Williams *et al*. 1985) and presence of some snoRNAs in nucleolar vacuoles of pea root cells (Beven *et al*. 1996) as well as nucleolar vacuole formation and its diminishing are closely related to rRNA biosynthesis and pre-ribosomal particle maturation in

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nucleolus. However, it is postulated more frequently that nucleolar vacuoles play a role in nuclear substance accumulation (Rose *et al*. 1972, Moreno-Diaz de la Espina *et al*. 1980) and can be essential for nucleolar-nucleoplasmic exchange as a response to increased output of nucleolar ribonucleoproteins (Moreno-Diaz de la Espina *et al*. 1980, Deltour and De Barsy 1985). Observations in living cells revealed characteristic 'pulsation' of nucleolar vacuoles, which were enlarging and then their content was moved out of nucleolus to cytoplasm (Johnson and Jones 1967, Rose *et al*. 1972).

Soybean, the subject of the present studies, is chill-sensitive plant. Our earlier studies showed ultrastructural changes in meristematic cell nucleoli of soybean roots subjected to 4-d chilling in comparison to nucleoli from plants growing at optimal conditions (Stępiński and Kwiatkowska 2003, Stępiński 2004). The nucleoli of chilled plants also exhibited lower transcriptional activity but bigger sizes than the control nucleoli (Stępiński 2003, Stępiński and Kwiatkowska 2003). In meristematic cells of plants subjected to chilling followed by 24-h recovery at optimal temperature nucleoli contained big, single centrally located vacuoles and incorporated ^{3}H-uridine even more intensively than the control nucleoli. Autoradiographic studies showed the presence of silver grains above nucleolar vacuole area. It can be suggested that nucleolar vacuoles of soybean meristematic cells may be involved in the mechanism that facilitates migration of ribosomal subunits to nucleus and then to cytoplasm (Stępiński 2004).

The aim of the present studies was to check at which moment during 24-h regeneration of soybean seedlings, following 4-d chilling, nucleolar vacuoles appear and whether their presence is a result of increased transcriptional activity of nucleoli or more intensive translocation of pre-ribosomal particles from nucleolus to cytoplasm.

Materials and methods

Plants: Seeds of soybean [*Glycine max* (L.) Merr.] cv. Aldana (obtained from Plant Breeding and Acclimatization Institute in Radzików) were germinated for 3 d at 25 °C in darkness in Petri dishes on filter paper moistened with distilled water. Some of these seedlings continued growth under the same conditions (control) and some seedlings were transferred to cold (10 °C) for 4 d. Some of the chilled seedlings were then recovered for 1.5, 3, 6, 12 and 24 h at optimal temperature (25 °C). Ten plants were studied for each variant.

^{3}H-uridine incubation: Seedling roots from the control and recovered plants as well as from chilled ones were incubated in ^{3}H-uridine solution (2.96 MBq cm^{-3}; 888 GBq mmol^{-1}) for 20 min at 25 and 10 °C, respectively, then postincubated in a non-radioactive medium for 80 min at the respective temperatures. Roots were fixed in absolute ethanol + glacial acetic acid mixture (3:1) for 1 h at room temperature and rinsed in ethanol. After hydration the material was treated with pectinase solution [2.5 units mg^{-1}(protein) cm^{-3}; *Sigma*, Germany] in McIllvain buffer (pH 5.0) for 30 min at 45 °C. Squashed preparations from apical parts of roots were made on dry ice. Dry preparations were covered with light-sensitive emulsion (EM 1; *Amersham*, UK) and exposed at 8 °C. After 14 d autoradiograms were developed and stained with toluidine blue according to Smetana *et al*. (1968). Dry preparations were embedded in Canada balsam. Silver grains were counted in 30 cells over nucleoli, extra-nucleolar nucleoplasm and cytoplasm in preparations from 3 meristems from each variant.

Semi-thin section preparations: Root tips of each variant 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 ethanol series, the material was embedded in a medium containing the mixture of Epon 812 and Spurr's resin. Semi-thin sections were placed on microscopic slides and stained with toluidine blue. Nucleolar vacuoles were observed and counted in preparations from 3 meristems from each variant.

Observations were made by means of light microscope *Jenamed-2* (*Carl Zeiss*, Jena, Germany) and CCD camera *MTV-1801 CB* connected to a microscpoe and computer-aided *IMAL-512* system.

Results

^{3}H-uridine autoradiography: After 20-min ^{3}H-uridine incubation of soybean seedling roots the number of silver grains above the chilled plant nucleoli was 6 times lower than above the control nucleoli. During 24-h recovery the labelling of nucleoli was increasing gradually in relation to the labelling at chilling conditions and after 24 h of recovery the labelling was even 2 times higher than in the control. Labelling course of cytoplasm in the control, chilled plant cells and those subjected to recovery was similar to the labelling of nucleolus, however the silver grain number above cytoplasm was always lower than above nucleolus (Fig. 1*A*).

After 80-min post-incubation in non-radioactive medium the situation was opposite – labelling of cytoplasm was 2 fold higher than that of nucleolus with the exception of the cells of the plants subjected to 4-d chilling – in this case the nucleolus was more labelled than cytoplasm (Fig. 1*B*). This observation is in agreement with the earlier studies (Stępiński 2003, 2004, Stępiński and Kwiatkowska 2003) and proves intensive transport of pre-ribosomal particles from nucleolus to cytoplasm at the control conditions and during recovery, whereas at chilling – there is a stronger reduction of transport than of synthesis of pre-ribosomes. The number of silver grains was several times higher both above nucleolus and cytoplasm in the control and during recovery after post-incubation than after incubation which proves further incorporation of the labelled precursor of rRNA synthesis which penetrated into cells during incubation.

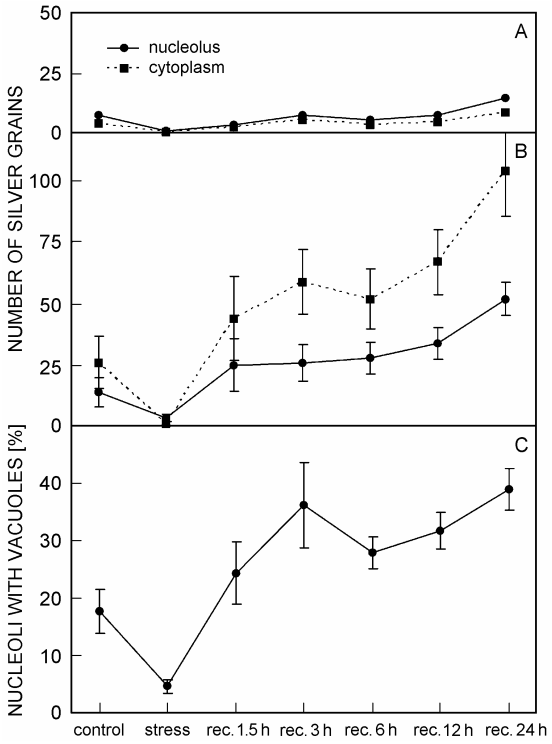

Fig. 1. Labelling of nucleolus and cytoplasm of root meristematic cells in control, stressed and recovered plants, after 20-min incubation in ^{3}H-uridine (*A*) and after 80-min postincubation (*B*); appearance of nucleolar vacuoles in root meristematic cells in control, stressed and recovered plants (*C*). *Bars* represent ± SD. Differences in labelling of compared cellular areas and number of vacuolated nucleoli between control, stress and recovery are statistically significant at $P = 0.05$.

Nucleolar vacuole analyses on semi-thin sections: In meristematic cells of the control plants there were 18 % vacuolated nucleoli (Fig. 1*C*) in which one big or small centrally located vacuole or a few small ones scattered throughout the nucleolus were visible (Fig. 2*A*). In the chilled plants nucleoli were bigger but only 5 % were vacuolated (Fig. 1*C*) and no big single vacuoles were observed only small ones scattered throughout nucleoli (Fig. 2*B*).

In the plants subjected to recovery (*i.e.* after 1.5, 3, 6, 12, and 24 h of recovery) the number of vacuolated nucleoli was higher than in the chilled plants and even than in the control plants. During recovery time the number of nucleoli with vacuoles increased from 24 % (1.5 h) to 40 % (24 h) (Fig. 1*C*). Along with recovery time the nucleoli, which were big at the beginning, were diminishing. After 1.5 h of recovery no nucleoli with large centrally located vacuoles were observed but only those with a few small ones (Fig. 2*C*). The nucleoli with small and mid-sized centrally located vacuoles, individual nucleoli with big vacuoles as well as nucleoli with centrally located vacuoles and a few small ones started appearing in plants after 3 h of recovery; nucleoli with a few small vacuoles predominated. As the recovery time became longer more and more nucleoli with one big centrally located vacuoles appeared (not shown). After 24 h of recovery the latter predominated (Fig. 2*D*). Centrally located vacuoles in the nucleoli of the recovered plants were bigger than those from the control plants.

Discussion

A lot of controversy concerning the role and presence of nucleolar vacuoles arise from the fact that their appearance is not strictly connected with any precise functional state of the cell and does not concern all nucleoli in a given type of cell. Some researchers think that nucleolar vacuolation may serve as a morphological indicator of nucleolar activity in plants (Moreno *et al*. 1989). Vacuolated nucleoli in tobacco culture cells Johnson (1969), *Allium cepa* meristematic cells and nucellus cells of *Pisum sativum* (Moreno-Diaz de la Espina *et al*. 1980) displayed higher ^{3}H-uridine incorporation than nucleoli without vacuoles. At early stage of *Zea mays* germination in root cells the appearance of vacuoles was correlated with the beginning of uridine incorporation into nucleoli (De Barsy *et al*. 1974). In turn, Barlow (1970) did not agree that vacuolation level of nucleoli could be related to the activity of RNA precursor incorporation in different *Zea mays* root tip regions. Also in artichoke slices in natural conditions the nucleolar vacuoles formed when nucleolar RNA metabolism was slowed down (Rose *et al*. 1972). It was suggested that vacuoles resulted from quantitative or

qualitative changes in processing of ribonucleoprotein precursors of ribosomes rather than being simply related to the rates of nucleolar RNA synthesis. Appearance of nucleolar vacuoles can also be induced by some inhibitors of rRNA synthesis (Moreno-Diaz de la Espina *et al*. 1980). However, treatment of *Zea mays* seeds with actinomycin D, 5-fluorouracil (5-FU), 2-thio-uracil (2-TU) during germination resulted in inhibition of rRNA synthesis, vacuolation process, migration of granular component and decrease in nucleolar sizes (De Barsy *et al*. 1974).

In soybean plants subjected to recovery at optimal temperature after 4-d chilling, vacuolated nucleoli appeared already at the beginning of recovery (1.5 h), even more numerous than in the control. These vacuoles appeared just during recovery because in the chilled plants there was only an insignificant number of vacuolated nucleoli. Perhaps this fact can be explained by an increased need for ribosomes in cytoplasm in order to restore metabolism in the cells after unfavourable conditions. The simultaneous autoradiographic studies with the use of ^{3}H-uridine showed increased RNA precursor incorporation during incubation in the isotope and increase in labelling during postincubation in non-radioactive medium during recovery in relation to the control. It seems to be obvious that nucleoli were more

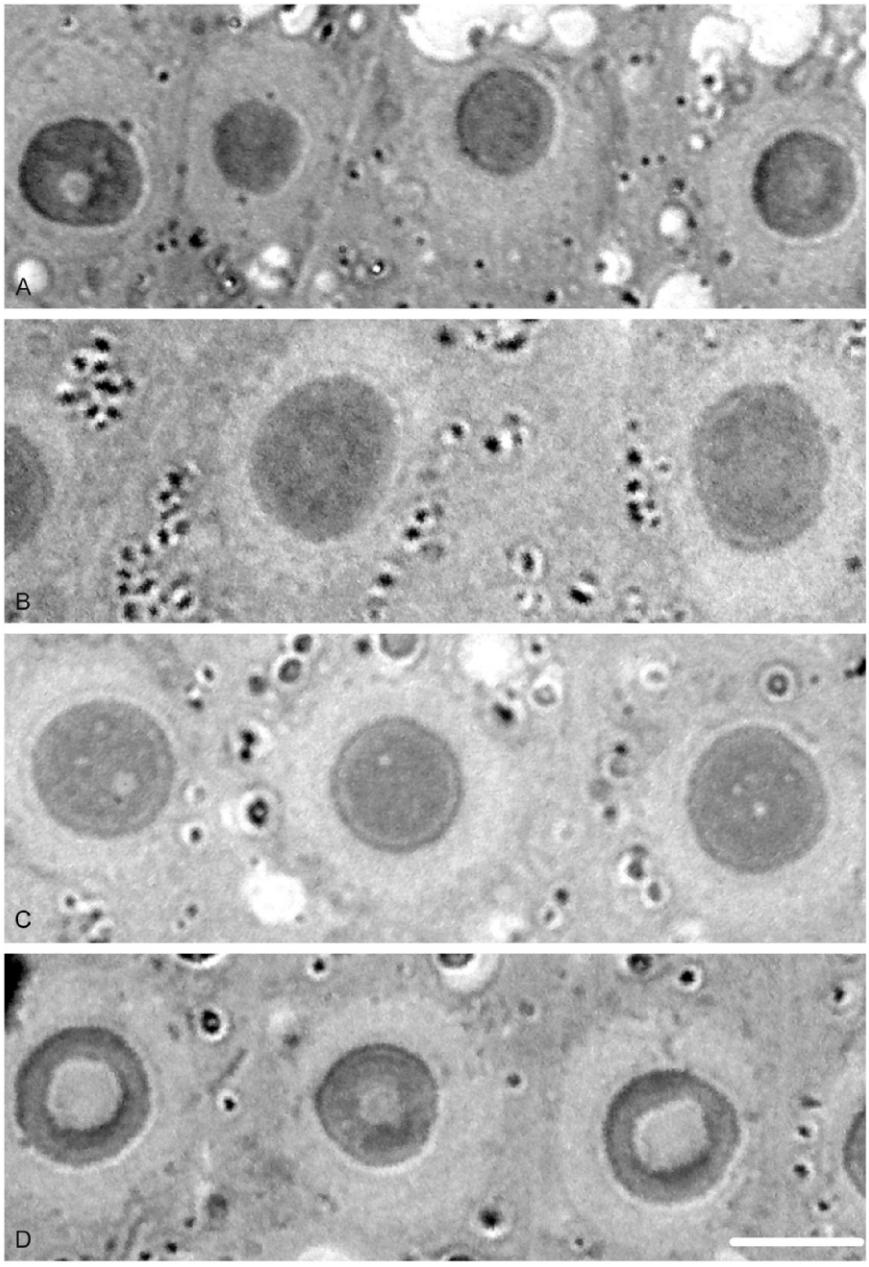

Fig. 2. Images of nucleoli in root meristematic cells, *A* - control, *B* - stress, *C* - 1.5 h recovery, *D* - 24 h recovery. *Bar* = 10 μm.

labelled than cytoplasm after 20-min incubation in ^{3}H-uridine, which suggested prevalence of rRNA synthesis in comparison to its transport. After 80-min post-incubation the situation was reversed – the labelling of cytoplasm increased in comparison to the nucleolar labelling and thus more intensive transport of pre-rRNA out of nucleolus than rRNA synthesis. The ratios of the number of silver grains above nucleoli to the number of silver grains above cytoplasm after post-incubation both in the control and during recovery are similar which means that during recovery the transport of nucleolar substances to cytoplasm did not change in relation to its synthesis. It should be emphasized that in the regenerated plant cells the processes of ^{3}H-uridine incorporation and trans-location of labelled rRNA precursors proceeded more intensively than in the control. Moreover, the present and earlier autographic studies (Stępiński and Kwiatkowska 2003) showed that during 4-d chilling there was accumulation of the granular component in nucleolus due to more handicapped pre-rRNA transport than its synthesis. Both higher activity of pre-ribosome synthesis and their transport during recovery, and migration of the granular component accumulated during plant chilling seem to prove that nucleolar vacuoles arise rather owing to pre-ribosomal particle migration from nucleolus to cytoplasm than to their synthesis in nucleolus. The results of the present studies support the opinion of many authors that the appearance of nucleolar vacuoles is related to rRNA transport from nucleolus to cytoplasm (Barlow 1970, De Barsy *et al*. 1974, Deltour and De Barsy 1985, Olszewska *et al*. 1985, Jennane *et al*. 2000).

It is worth noticing that in meristematic cells of soybean roots the type of vacuolated nucleoli changed together with regeneration time. At the initial stage of recovery there were only nucleoli with a few small vacuoles whereas after 24 h there were almost exclusively nucleoli with one big centrally located vacuole. Formation of only small vacuoles in a nucleolus at the beginning of recovery may result from the change of nucleolar structure during 4-d chilling. Nucleoli of the chilled plants had loose structure in comparison to the control ones (Stępiński and Kwiatkowska 2003, Stępiński 2004) which might facilitate formation of a few small vacuoles in a nucleolus. With recovery time the nucleolar structure stabilized and nucleoli with centrally located vacuole appeared. In meristematic root cells of *Allium cepa* nucleoli with a few vacuoles were observed which then fussed producing one big centrally located vacuole (Moreno-Diaz de la Espina *et al*. 1980). The same situation might happen in soybean cells during 24-h recovery where the number of nucleoli with a few small vacuoles decreased and of those with one central vacuole increased with time.

Numerous studies confirm the relation between nucleolar size, nucleolar vacuole size, their number, activity and morphological state of the cell or organism. Decrease in nucleolar volume and nucleolar vacuole number as well as nucleolar activity in aging and in weaken tobacco cultured cells were observed (Johnson and Jones 1967). However, in *Zea mays* root tip cells the number of vacuoles in a nucleolus was inversely related to the nucleolar volume – appearance of nucleolar vacuoles resulted in nucleoli diminution, the smaller nucleoli correlated with the highest vacuolation which in turn depended on the activity of RNA production in a nucleolus (Barlow 1970, De Barsy *et al*. 1974). On the contrary, in the case of nucleoli of soybean subjected to low temperature the nucleolar volume increased significantly and vacuolation level decreased drastically in comparison to the control nucleoli. Simultaneously, chilled nucleoli also showed a considerable decrease in activity of ^{3}H-uridine incorporation and much more handicapped transport of radioactive particles out of a nucleolus which was reflected by a lower level of cytoplasm labelling. Inhibition of the process of nucleolar vacuole formation under chilling (6 °C) that inhibited rRNA synthesis was also observed by De Barsy *et al*. (1974) in *Zea mays* seedlings and by Olszewska *et al*. (1985) in *Helianthus annuus* roots treated with 10 °C for 12 h.

On the basis of the present experimental system one can conclude that the appearance of greater number of vacuolated nucleoli during recovery than in the control may result from migration of the granular component accumulated during plant chilling. Gradual increase in the number of nucleoli with vacuoles during recovery may prove that pre-rRNA accumulated in the chilled plant was released during whole recovery period. Moreover, formation of new vacuoles during recovery may be an element that promotes the intensification of rRNA synthesis and transport as suggested Rose *et al*. (1972).

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