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NUCLEOLAR ACTIVATION AND VACUOLATION IN EMBRYO RADICLE CELLS DURING EARLY GERMINATION

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SUMMARY

The activation of the nucleolus of primary root cells of *Sinapis alba* embryos during the first 72 h of germination was monitored by autoradiographic, ultrastructural and microstereological methods. Autoradiographs showed that within 48 h, the nucleolus progressively resumed the capacity to synthesize pre-rRNA molecules at a high rate. In quiescent embryos the nucleolus was small, compact and composed of mixed granular and fibrillar components. Within the first 6 h of germination a strong nucleolar vacuolation occurred, accompanied by a decrease in the volume of the nucleolus and a concomitant high loss of its ribonucleoproteins (RNPs). From 6 to 24 h, nucleolar vacuolation decreased to reach a stable level. During this last period the volume of the nucleolus increased by the accumulation of the fibrillar component resulting from a slow pre-rRNA processing. At 24 h the nucleolus presented a predominantly fibrillar texture. After 24 h, nucleolus growth continued but was due to the accumulation of the granular component, indicating that pre-rRNA processing occurred at a higher rate than during the first day of germination. From 48 h the nucleolus was composed of well-delineated granular and fibrillar areas. Dense nucleolus-associated chromatin as well as fibrillar centres were always observed during the whole period of observation. In addition, previous studies on the nucleolus of radicle cells of *Zea mays* embryo during early germination were completed by studying changes in the nucleolar volume and in the density of pre-ribosomal subunits of the granular component. On the basis of the data obtained with both species we suggest that a possible function for the nucleolar vacuoles is the increase in the nucleolus–nucleoplasm exchange interface in response to a rapid increase in the output of nucleolar RNPs. The nucleolar growth pattern during early germination is also discussed.

INTRODUCTION

The germination of higher plant seeds is characterized by a rapid metabolic activation. This is accompanied by ultrastructural changes in cell organelles and, in particular, by a spectacular modification of the nucleolus (Rose, 1974; Deltour, Gautier & Fakan, 1979). In maize embryos, a large and transient vacuolation of the nucleolus of radicle cells occurs a few hours after the start of germination when the amounts of pre-rRNA synthesis and processing are still very low (Deltour & Bronchart, 1971; de Barsy, Deltour & Bronchart, 1974; Van de Walle, Bernier, Deltour & Bronchart, 1976). At the ultrastructural level, the nucleolar vacuoles or nucleolar interstices (Goessens, 1984) are less electron-dense areas located inside the nucleolus, containing a few granules and fibrils that remain contrasted after the Bernhard's preferential

Key words: nucleolar activation, nucleolar vacuolation, germination.

stain for ribonucleoprotein (RNP) (Deltour *et al.* 1979; Moreno-Díaz de la Espina, Medina & Risueño, 1980; Jordan, 1984). They appear distinct from the nucleolus-organizing region (NOR) or fibrillar centres, but are sometimes observed in association with them (Chouinard, 1982).

Nucleolar vacuoles are generally small and not numerous. However, large nucleolar vacuoles have been reported in several tissues and physiological situations very different from those of germinating plant embryos. In plant cells intense vacuolation occurs after the release of inhibition of RNA synthesis (Johnson, 1969), during the reactivation of quiescent cells (Rose, Setterfield & Fowke, 1972), during growth of the cotton fibre (De Langhe, Kosmidou-Dimitripoulou & Waterkeyn, 1978), in cultured tobacco and *Rhoeo* cells (Johnson & Jones, 1967; Kohlenbach, 1967) and in root cells during interphase (Chouinard, 1964). In animal cells, it has been reported during axon regeneration (Pannese, 1963), in nerve cells (Chouinard, 1965; Busch & Smetana, 1970), and in oocytes (Esper, 1965; Zybina, 1968). Furthermore, observations on living cells have shown that nucleolar vacuoles are growing, moving and contracting structures that can open at the surface of the nucleolus (Johnson & Jones, 1967; Kohlenbach, 1967; Rose *et al.* 1972; Erdelská, 1973). De Langhe *et al.* (1978) suggest that the formation of nucleolar vacuoles could be controlled by growth factors.

The function of nucleolar vacuoles is controversial. They may be involved in the transport and storage of nucleolar material (Chouinard, 1964; Johnson & Jones, 1967; Rose *et al.* 1972; Moreno-Díaz de la Espina *et al.* 1980). Johnson (1969) and Zybina (1968) suggested that vacuolated nucleoli are very active in pre-rRNA transcription but others (Barlow, 1970; Moreno-Díaz de la Espina *et al.* 1980) deny this. From these observations we cannot at present attribute with certainty a precise origin or a general function to nucleolar vacuoles. We have thus started a study to determine whether, during early germination: (1) nucleolar vacuolation occurs in plant embryo cells from different species; and (2) particular changes in ultrastructure during activation of the nucleolus are related to the formation of nucleolar vacuoles.

For these purposes the previous studies with germinating *Zea mays* embryos (Deltour & Bronchart, 1971; de Barsy *et al.* 1974; Deltour *et al.* 1979) have been completed and observations extended to radicle cells of germinating *Sinapis alba* embryo, a species taxonomically very different from maize. The seeds of the two species are morphologically different: *S. alba* (dictotyledon) has an exalbuminous seed whereas the maize kernels (monocotyledon) are albuminous. To facilitate the temporal comparison between *Sinapis* and maize, the physiological conditions of germination were chosen to obtain the same rate of radicle protrusion in seeds of both species. Changes in [^{3}H]uridine incorporation, ultrastructure, percentage of vacuolated nucleoli, volumes of nucleolar vacuoles, numerical densities of pre-ribosomal subunits of the granular component and volumes of the nucleoli have been studied.

MATERIALS AND METHODS

Unless specified otherwise, the methods were applied to both species.

Germination procedure

Batches of 100 seeds of *Sinapis alba* and *Zea mays* var. CiV2 were germinated in Petri dishes over cotton wool and filter paper saturated with distilled water. They were incubated in darkness in an Ehret temperature chamber at 9 °C and 16 °C, respectively. Germination began when the seeds were placed in contact with water. The criterion of germination was the protrusion of the radicle from the seed coat. The maximum percentage of germinated seeds was always over 90% and the rates of germination were very similar for both species.

*Histoautoradiography (*Sinapis*)*

After various periods of germination, 12 *Sinapis* embryos were isolated and incubated at 9 °C for 2 h in 200 μl of [5-^{3}H]uridine (250 μCi/ml, sp. act. 25 Ci/mmol; Radiochemical Centre, Amersham). At the end of the incubation time embryos were fixed and embedded as described below, except that after fixation they were washed for 48 h in 0·1 M-sodium cacodylate buffer (pH 7·0) to eliminate the non-incorporated precursor (Monneron & Moulé, 1969). Cross-sections 1 μm thick were cut at 1 mm from the radicle apex. The sections were dipped for a few seconds in diluted Ilford L4 emulsion. After a 10-day exposure at 4 °C, the autoradiographs were developed in Microdol X and stained with 0·2% Toluidine Blue. Alternatively, at the end of the labelling period the embryos were fixed in formalin/acetic acid/ethanol for 12 h, washed in running water for 24 h, embedded in paraffin wax, and sectioned longitudinally at 5 μm. The dewaxed sections were again washed in running water for 24 h and were covered with autoradiographic stripping film Kodak AR-10. After a 10-day exposure, the films were developed and the sections then stained with 0·2% Toluidine Blue.

For quantitative autoradiography counts were performed on 5–10 radicles for each experimental batch. The silver grains were counted over 10–15 randomly chosen cortical cells. The background was estimated by counting the grains over 10 areas the size of a mean cell section and chosen randomly near the radicle section. For background corrections the areas of the cytoplasm, nucleoplasm and nucleolus were estimated as 70, 18 and 12% of the total cell area, respectively (Deltour, 1972).

Epon embedding material

Embryos were excised from ungerminated or germinated seeds and immersed rapidly in a cold solution of 4% glutaraldehyde buffered at pH 7·0 with 0·1 M-sodium cacodylate. Half an hour later, the first millimetre of the root tip was cut off and dipped in fresh, cold fixative for a further 3 h. The samples were washed in 0·1 M-sodium cacodylate buffer, dehydrated in a graded series of ethanol and embedded in Epon.

*Electron microscopy (*Sinapis*)*

Ultrathin cross-sections 90 nm thick were cut in the cortex 1 mm from the root tip, contrasted for 3–5 min with aqueous uranyl acetate and then destained for 25–30 min with an aqueous solution of 0·2 M-EDTA and finally stained for 3 min with Reynold's lead citrate according to the technique of preferential stain for nuclear ribonucleoproteins (Bernhard, 1969). The sections were observed in a Siemens Elmiskop 101 at 80 kV.

On electron microscope micrographs we consider nucleolar vacuoles as poorly contrasted areas of varying size containing variable amount of granular and fibrillar material not bleached after EDTA treatment of the ultrathin section (Figs 6, 8, 9, 10).

According to Risueño, Medina & Moreno-Díaz de la Espina (1982) we consider that homogeneous fibrillar centres are small and less electron-opaque areas immersed in the fibrillar component of the nucleolus (Fig. 7), whereas heterogeneous fibrillar centres show, in addition, a central core of condensed nucleolar chromatin (Fig. 8). Fibrillar centres and nucleolar vacuoles are frequently associated (Fig. 7) to form an apparent entity.

Determination of the percentage of vacuolated nucleoli, the number of vacuoles per nucleolus and the relative volume of the nucleolar vacuoles

Semithin cross-sections 1 μm thick were cut 1 mm from the root tip, stained with Toluidine Blue and observed with a Wild M20 microscope at a magnification of × 1250. Vacuolated and non-vacuolated nucleoli were counted in the cortical cells of one section per radicle and in six radicles for each experimental batch. A nucleolus was recorded as vacuolated when at least one completely unstained area was clearly visible within it. A minimum of 800 nucleoli were recorded for each germination time.

In 1 μm thick semithin sections, 40 nucleoli per radicle were photographed randomly in the cortex of six to eight radicles per germination time and projected at a final magnification of × 12 000 on a lattice of 1395 equally spaced points. The relative volume of the nucleolar vacuoles (Vv_{vac}) was estimated by recording the number of test points enclosed within the profiles of complete nucleoli (Pn) and those enclosed within the nucleolar vacuoles (Pv). The relative volume density of the vacuoles was $Vv_{vac} = Pv/Pn$; this ratio is expressed as a percentage.

Determination of the nucleolar volume

For the determination of the nucleolar volumes (including vacuoles) photomicrographs of nucleoli of cortical cells were made using a Zeiss photomicroscope with a 40 × objective. The diameter of each nucleolus was measured directly on projections of the negatives on a screen at a final magnification of ×12 000.

At least 500 nucleoli were measured from five to eight radicles. The mean nucleolar volume at each experimental time was determined by the method of Gyger & Riedwill as described by Weibel (1979).

Determination of the numerical density of the pre-ribosomal particles of the granular component in the nucleolus

For each experimental period ultrathin sections were cut in cortical cells of five different radicles and stained preferentially for RNPs. Twenty five nucleoli were photographed with the electron microscope at a direct magnification of × 30 000. Only nucleoli with a diameter corresponding approximately to the mean diameter determined previously by the Gyger & Riedwill method were chosen. On prints at a final magnification of × 75 000 the central point of each nucleolus was determined and a sector of 45° was traced. Within this sector, which was a representative portion of the nucleolus, all the particles of the granular component were scored. The area of each sector was measured with a planimeter. The numerical density of the particles of the granular component (Nv) per unit area of nucleolar section is given by the formula:

$$Nv = \frac{N}{D + T - 2h}$$

(Weibel, 1979). D is the mean diameter of a granule of the granular component, N the mean number of pre-ribosomal particles of the granular component counted inside one 1 μm × 1 μm sector, T the section thickness (90 nm), h is the height of the minimum visible cap of a pre-ribosomal particle (9 nm) in ultrathin section.

The estimation of the numerical density of the granular component per mean nucleolar volume was calculated for each germination time.

Determination of the diameter of pre-ribosomal particles of the granular component in the nucleolus (S. alba)

For each experimental period, the diameters of particles of the granular component were measured in five nucleoli from five different radicles on the same prints as those used for the determination of numerical density. The diameters of 120 nucleolar particles chosen randomly in

Table 1. *Mean number of silver grains in autoradiographs of* Sinapis *root cells after 2 h of [^{3}H]uridine incorporation at different stages of germination*

Germination time (h)*	Mean number of silver grains on			Total
	Nucleolus	Chromatin	Cytoplasm	
Experiment 1				
0	0·7	1·2	7·1	9·0
1	2·7	5·0	7·3	15·0
3	4·1	9·1	4·9	18·1
6	6·6	8·6	3·0	18·2
16	6·3	7·7	6·5	20·5
48	33·9	40·1	43·9	117·9
Experiment 2				
0	0·1	0·8	3·5	4·4
6	4·8	9·1	7·3	21·2
24	8·3	14·5	14·7	37·5
48	12·7	20·8	35·6	69·1

Experiment 1: 5 μm thick sections, stripping film; experiment 2: 1 μm thick section, dipping.
* Incubation period not included.

each nucleolus were measured with a stereomicroscope fitted with a calibrated ocular micrometer (Wild Censor Heerbrugg M5) at a final magnification of × 900 000.

RESULTS

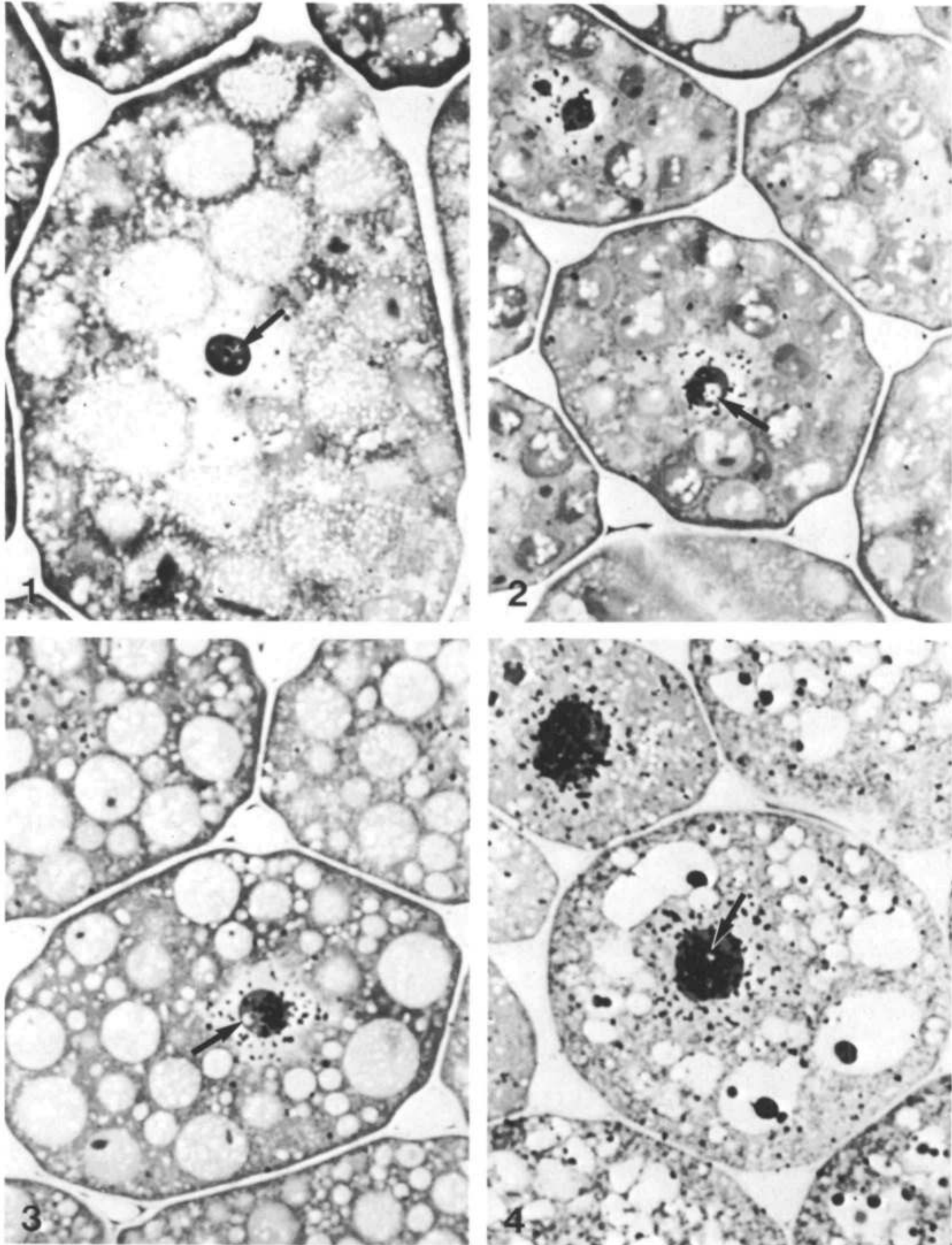
Histoautoradiography

In *Sinapis*, as previously observed in maize (Van de Walle *et al.* 1976), a continuous increase in the mean number of silver grains over the nucleolus was observed from 0 to 48 h (Table 1; Figs 1–4). This indicates that the rate of pre-rRNA synthesis resumed progressively and speeded up during the first 2 days of germination. During the same period, the radioactivity of both the cytoplasm and the chromatin strongly increased also. It is likely that the increase in radioactivity in the cytoplasm was not only due to resumption of cytoplasmic organelle transcription but also to translocation of newly synthesized nuclear RNAs. Thus it is likely that the nuclear export of RNPs had started between 24 and 48 h.

Nucleolar ultrastructure of Sinapis *during early germination*

The nucleolus in quiescent nuclei appeared like a homogeneous fibrillar spherule in which the granular component was scattered and sometimes formed a very thin layer at the periphery of the organelle. Nucleolar vacuoles were rarely seen (Fig. 5).

In quiescent nuclei as well as in nuclei of germinating embryos, one or two knobs of dense nucleolus-associated chromatin were observed lying at peripheral positions (Figs 6, 7), their size diminishing during germination. These knobs were connected with a chromocentre that was attached to the nuclear envelope (Fig 6). In addition, fibrillar centres (FCs) were seen at all germination stages (Figs 7, 8). They were



Figs 1–4

frequently associated with the nucleolar vacuoles (Fig. 7). On 25 ultrathin sections observed, the association frequency was: 18·7% (0h), 13·6% (6h), 26·4% (24h) and 48·8% (48h).

Between 0 and 6 h of germination numerous nucleoli showed one or several clearly distinct vacuoles each containing RNP granules (about 18 nm in diameter) and fibrils (Figs 6, 9, 10). Some thicker RNP granules varying from 35 to 40 nm in diameter were also present in many vacuoles (Fig. 10).

These unusual RNP granules, observed previously in maize embryo (Deltour *et al.* 1979), probably represent a storage form of unprocessed pre-rRNA molecules associated with proteins (see Faken & Deltour, 1981; Deltour, 1985). The majority of the nucleolar vacuoles occurred inside the nucleolus; however, in some cases they appeared to be connected to the nucleoplasm (Fig. 9).

After 1 day of germination the nucleolus was larger, with the granular component scattered throughout the whole organelle; however, its structure was still predominantly fibrillar (Fig 7). From 48 h it became more heterogeneous, showing intermingled areas on the inside constituted of either granular or fibrillar component and on the outside a thick layer of granular component. Nucleolar vacuoles were seen inside the fibrillar and granular areas (Fig. 8).

Percentage of vacuolated nucleoli and relative volumes of nucleolar vacuoles (Vv_{vac}) *in* Sinapis

To determine the timing and the extent of the nucleolar vacuolation, we have recorded the percentage of vacuolated nucleoli and estimated the relative volume of the nucleolar vacuoles (as a percentage of the nucleolar volume) during the first 3 days of germination. Results (Table 2) show that during the first 6 h of germination the percentage of vacuolated nucleoli increased significantly; 18 h later it dropped significantly and remained constant from 48 h.

The mean number of vacuoles per vacuolated nucleolus increased significantly between 0 and 4 h, remained unchanged until 24 h and dropped significantly at later times (Table 3).

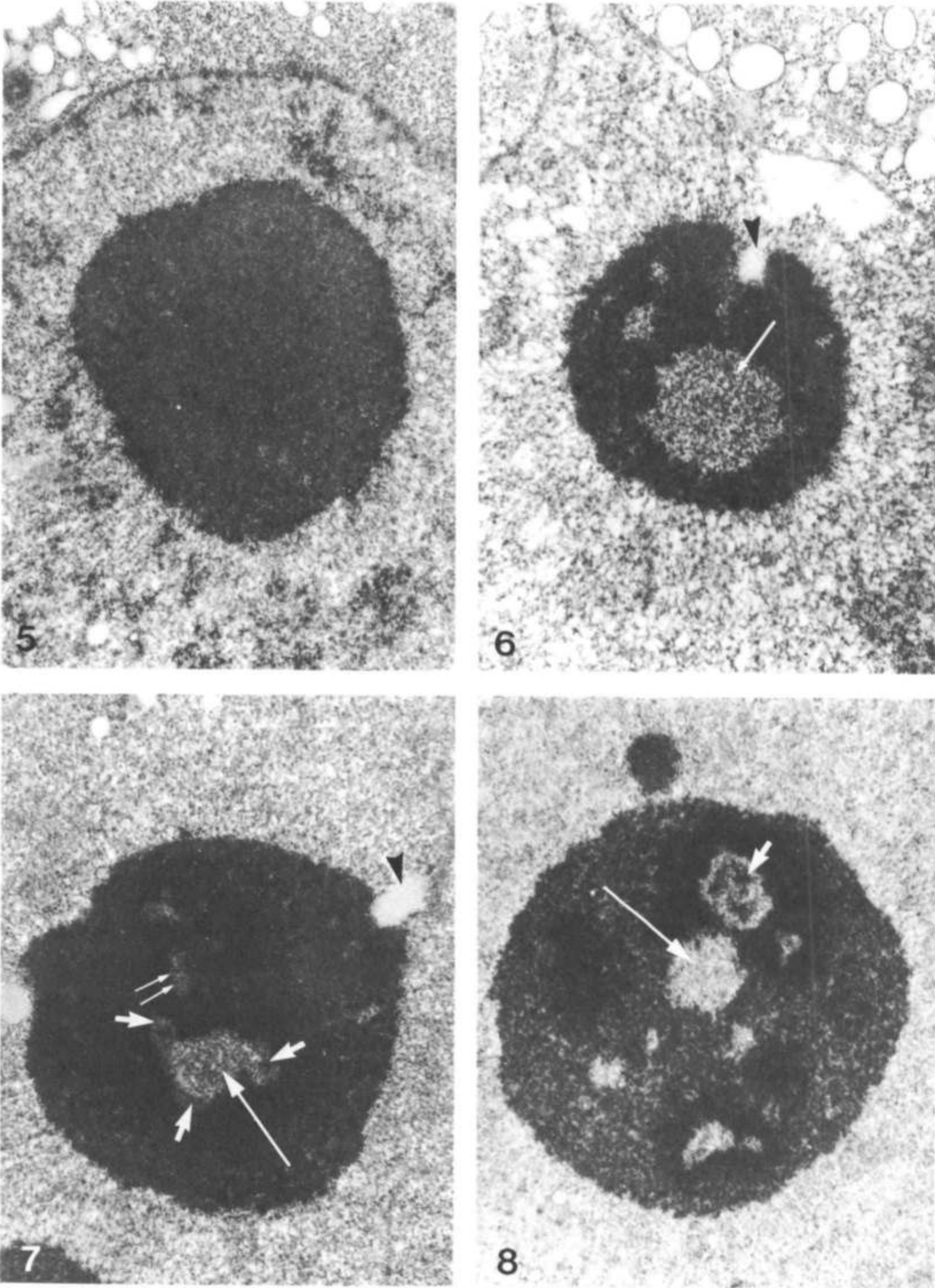
Figs. 1–4 Autoradiographs of *S. alba* radicle cells after [^{3}H]uridine incorporation at different moments of germination.

Fig. 1 Quiescent embryo cell, 2 h of incubation. The radioactivity of the whole cell is weak. Vacuoles are seen inside the nucleolus (arrow), since the process of nucleolar vacuolation started during the 2 h of incubation in the radioactive precursor.

Fig. 2. After 6 h of germination, 2 h of incubation. The whole nucleus is weakly labelled. A large vacuole is seen in the nucleolus (arrow).

Fig. 3. After 24 h of germination, 2 h of incubation. The nucleoplasm and the nucleolus are more labelled than earlier during germination (Figs 1, 2). A small nucleolar vacuole is seen (arrow).

Fig. 4. After 48 h of germination, 2 h of incubation. The nucleus is strongly labelled. The silver grains are numerous over the nucleolus and have masked the nucleolar vacuole (arrow). Compared to earlier germination periods, the cytoplasmic radioactivity is strongly increased.



Figs 5–8

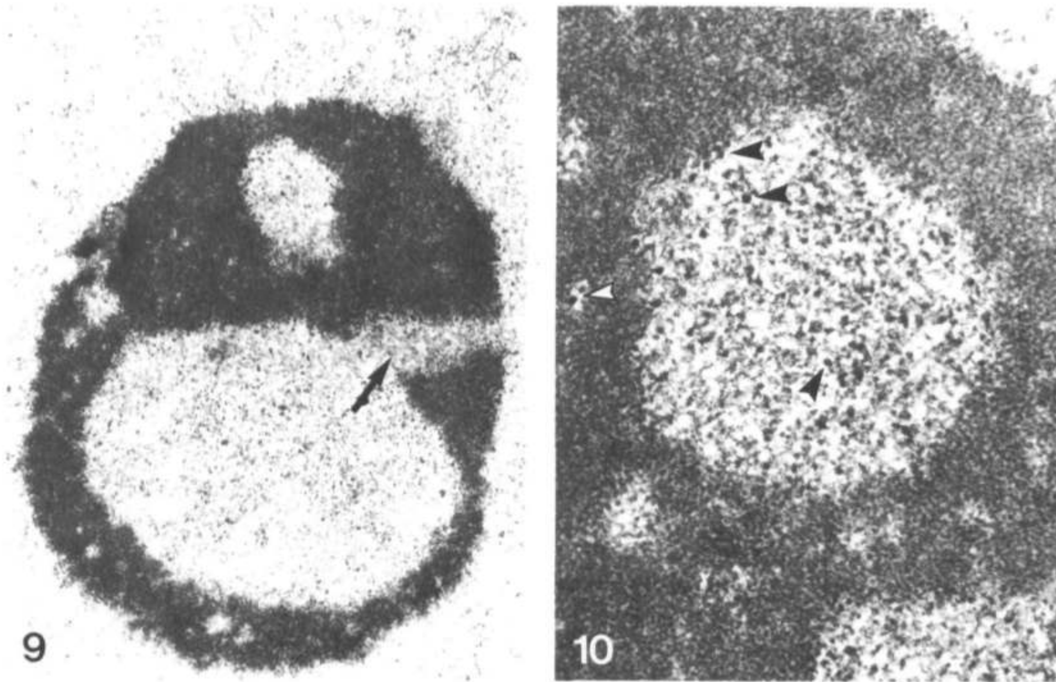


Fig. 9–10. Glutaraldehyde fixation, EDTA staining, *S. alba*.

Fig. 9. A nucleolus showing a nucleolar vacuole in connection with the nucleoplasm (arrow). × 18 000.

Fig. 10. High magnification of a vacuole from a nucleolus of a 6 h germinating embryo. Unusual RNP granules about 35–40 nm in diameter (arrowheads) and fibrillar material are clearly visible inside. × 27 500.

The relative volume of nucleolar vacuoles (Vv_{vac}) increased in parallel with the percentage of vacuolated nucleoli and reached 16·4 % at 6 h. Then it decreased rapidly and reached a value of about 3 % after 24 h (Table 2). The decrease in the Vv value indicated that the size of vacuoles dropped rapidly after 6 h of germination.

Figs 5–8. Glutaraldehyde fixation, EDTA staining, *S. alba*.

Fig. 5. A nucleolus from a quiescent embryo cell. The granular component is scattered throughout the whole organelle and forms a very thin peripheral layer. × 13 700.

Fig. 6. A nucleolus from a 6 h germinating embryo. A large nucleolar vacuole is apparent (arrow) as well as destained nucleolus-associated chromatin (arrowhead). × 15 700.

Fig. 7. A nucleolus from a 24 h germinating embryo. It is predominantly fibrillar. A vacuole (long arrow) associated with three fibrillar centres (short arrows) and nucleolus-associated chromatin (arrowhead) are visible. Small homogeneous fibrillar centres are also visible (two thin arrows). × 12 220.

Fig. 8. A nucleolus from a 72 h germinating embryo. Intermingled fibrillar and granular zones are easily visible. One large vacuole is visible (long arrow) and one heterogeneous fibrillar centre (short arrow). × 9000.

Table 2. *Percentage of vacuolated nucleoli and relative volume of nucleolar vacuoles* (Vv_{vac}) *of cortical cells during germination of* S. alba

Germination time (h)	% Vacuolated nucleoli	χ^2 comparison with next time	Vv_{vac} (% of the nucleolar volume)
0	6·1	+	0·2
2	19·5	+	2·0
6	43·6	−	16·4
8	48·2	+	9·1
24	21·4	+	3·7
48	15·2	−	3·1
72	15·3		3·3

The % of vacuolated nucleoli were compared by the χ^2 test: + = $P < 0{\cdot}01$ (significant); − = $P > 0{\cdot}01$ (not significant).

Volumes of nucleoli and numerical densities of the pre-ribosomal particles per nucleolus in Sinapis *and maize*

Modifications of the nucleolar volumes and of the mean number of pre-ribosomal particles per nucleolus were estimated during the first 3 days of germination in *Sinapis* and maize root cells. Results in Tables 4 and 5 show that in both species a significant decrease in the nucleolar volume occurred during the first 6–8 h of germination. The decrease in volume was more than 50 % for *Sinapis* and more than 40 % for maize. After this early decrease, the nucleolar volume increased rapidly with the progression of germination. In *Sinapis* and maize the volume increased more than 5 and 4·3 times, respectively, at 72 h of germination.

These changes in the nucleolar volumes were paralleled by quantitative changes in the density of the granular component. Between 0 and 6 h the nucleolus of *Sinapis* had lost $1{\cdot}5 \times 10^6$ pre-ribosomal particles whereas the nucleolus of maize had lost $2{\cdot}0 \times 10^6$ (Tables 4 and 6). After 6–8 h the nucleolus of both species rapidly gained new granular component and at 72 h there were $8{\cdot}3 \times 10^6$ and $15{\cdot}2 \times 10^6$ pre-ribosomal particles per nucleolus in *Sinapis* and maize, respectively. It thus appears that the nucleolar vacuolation was paralleled by a significant decrease in the nucleolar volume

Table 3. *Mean number of vacuoles per vacuolated nucleolus in* S. alba

Germination time (h)	Mean no. of vacuoles per vacuolated nucleolus (n = 100)*
0	1·46
4	2·37
6	2·38
24	2·34
48	1·35

* L.S.D. ($P = 0{\cdot}01$) = 0·57.

Table 4. *Nucleolar volume and mean number of pre-ribosomal particles of granular component per nucleolus during early germination of* S. alba *and* Z. mays

Germination time (h)	Nucleolar volume (μm^3)		Pre-ribosomal particles ($\times 10^6$)/nucleolus
S. alba			
0	49·9 ± 3·5*		2·1
		−4·8†	
6	21·1 ± 3·5		0·6
		+3·2	
24	79·3 ± 3·3		4·4
		+0·3	
48	87·1 ± 6·1		6·2
		+1·2	
72	115·4 ± 4·8		8·3
Z. mays			
0	41·7 ± 4·2		4·6
		−2·3†	
8	23·2 ± 3·2		2·6
		+0·7	
24	35·2 ± 4·7		3·2
		+1·1	
48	61·9 ± 6·9		9·9
		+0·6	
72	76·8 ± 7·9		15·2

* S.D., standard deviation.
† Variation in the nucleolar volume (μm^3 per h).

and by a loss of part of the granular component. These data are in good agreement with previous qualitative observations on maize (de Barsy *et al.* 1974).

Size evolution of the pre-ribosomal particles of the granular component of the nucleolus in Sinapis

Results show (Table 5) that there was a significant increase in the diameter of the nucleolar granules of the *S. alba* nucleolus, which began between 24 and 48 h of germination and persisted until 72 h.

Table 5. *Mean diameter of the pre-ribosomal particles during early germination of* Sinapis

Germination time (h)	Mean diameter of the nucleolar pre-ribosomal particle (nm) ($n = 600$)*
0	16·26
6	16·13
24	15·86
48	17·86
72	21·59

* L.S.D. ($P = 0·01$) = 1·68.

Table 6. *Estimation of the participation of the granular component in the nucleolar volume changes observed during four successive periods of germination in* Sinapis

Periods of germination (in h)	Changes of the mean nucleolar volume (μm^3)	Changes in the mean no. of pre-ribosomal particles per nucleolus ($\times 10^6$)	Part of the nucleolar volume change imputable to granular component (μm^3)
0–6	−28·8	−1·5	− 3·3 (−11·4%)
6–24	+58·2	+3·8	+ 7·9 (+13·5%)
24–48	+ 7·8	+1·8	+ 5·3 (+67·9%)
48–72	+28·3	+2·1	+11·1 (+39·2%)

Calculations from data of Tables 4 and 5.

Relationship between changes in the nucleolar volumes and changes in the granular component during early germination of Sinapis

As large changes in the nucleolar volume (decrease and increase) occurred during early germination (Table 4) the question arises as to what extent these changes are due to changes in granular component. As we have estimated the mean diameter (Table 5) as well as the mean number of the pre-ribosomal particles per nucleolus (Table 4) at different germination times, it is possible to calculate the part of the change in nucleolar volume that is due to modification of the granular component (Table 6). During the period of decrease in nucleolar volume extending over the first 6 h of germination the loss of granular component is responsible for only about 11·4% of

Table 7. *Evolution of the surface of the nucleolus and of the nucleolar vacuoles (considered as one sphere) during four successive periods of germination of* Sinapis *and maize*

Germination time (h)	Surface of the nucleolus (μm^2)		Surface of the nucleolar vacuoles (μm^2)	
S. alba				
0	65·3		1·0	
6	37·0	−28·3	11·1	+10·1
24	88·9	+51·9	9·8	− 1·3
48	95·0	+ 6·1	9·4	− 0·4
72	114·6	−19·6	11·8	+ 2·4
Z. mays				
0	58·2		0·0	
8	39·3	−18·9	18·4	+18·4
24	52·0	+12·7	14·7	− 3·7
48	75·7	+23·7	21·6	− 6·9
72	87·4	+11·7	24·9	+ 3·3

Data calculated from Tables 2 and 4.

the size diminution. This means that the nucleolus decreased by first exporting nucleolar components other than the granular, i.e. most certainly, the fibrillar RNP component. During the nucleolar growth period extending from 6 to 24 h the nucleolus increased by first accumulating fibrillar component principally constituted of still unprocessed pre-rRNA (see Discussion). From 24 to 48 h the growth of the nucleolus was mainly linked to the accumulation of granular component. From 48 h the output of newly synthesized and processed nucleolar RNPs had begun and the pre-ribosomal subunits were probably exported at a higher rate.

DISCUSSION

Present and previous observations (Deltour & Bronchart, 1971; de Barsy *et al.* 1974; Deltour *et al.* 1979) show that during early germination of *Sinapis* and maize embryos, both cytological and functional changes of the radicle cell nucleolus are very similar when the germination of the two species occurs at the same rate but at a different temperature.

In both species the progressive increase in nucleolar radioactivity after [^{3}H]uridine incorporation (Table 1; Van de Walle *et al.* 1976) as well as the increase in the granular component of the nucleolus (which results from pre-rRNA processing) (Table 4) show that pre-rRNA synthesis and processing are activated within the first 2 days of germination. Electron microscopic observations (Figs 5–8; and Deltour *et al.* 1979) have also shown a good parallel between the ultrastructural changes in the nucleolus of both species. Morphometrical studies have established that after an initial decrease in volume, which takes place within the first 6–8 h of germination, the nucleolus starts a spectacular increase in volume lasting until at least the third day of germination (Table 4). Accompanying the initial decrease in volume a strong output of granular and fibrillar components has been described as occurring (Tables 4 and 6). In addition, a vacuolation of the nucleolus has been observed in *Sinapis* culminating at 6–8 h of germination and slowing down during the following 14–16 h. This nucleolar vacuolation pattern is similar to that observed in germinating maize embryos (de Barsy *et al.* 1974). Nucleolar vacuolation thus occurs in a very similar way during germination of embryos of two species taxonomically and morphologically very different.

If we take into account that in maize (Deltour & Jacqmard, 1974) and in *Sinapis* (Deltour & Jacqmard, unpublished) the majority of the cells of quiescent embryos are in G_1 and that DNA replication for both species takes place after about 2 days of germination, our present and previous observations essentially concern changes in the nucleolus during the G_1 phase of the first mitotic cycle of germination. Taken together, these observations indicate a strong activation of ribosomal gene transcription as well as profound structural changes in the nucleolus during very early germination before the resumption of both DNA replication and mitotic activity in embryonic cells. There are indications in the literature that a similar nucleolar activation occurs for other species studied (for a review, see Deltour, 1985). This suggests that the activation of the nucleolus may be essential for germination, several aspects of which are worthwhile considering.

Possible significance of the nucleolar RNP output during early germination

Among changes affecting the nucleolus during very early germination one of the most impressive is the massive loss of pre-existing RNPs that have been synthesized during embryo maturation (Table 4). Thus the question of the possible significance of this process for embryo germination arises. We will consider three possibilities: (1) The nucleolar RNPs of the quiescent nucleolus are strongly desiccated during the dehydration at seed maturation. Some of them could be physically altered and eliminated. This supposition appears unlikely because other RNP structures, i.e. cytoplasmic ribosomes, are still functional in dry embryos as shown by the very rapid start of translation following inhibition of maize kernels (Deltour, 1977) or seeds of other species (Bewley & Black, 1978). (2) The pre-ribosomal RNP granules and fibrils exported from the nucleolus form an appreciable batch of new cytoplasmic ribosomes directly available for use in translation. (3) The nucleolar capability of building up RNP granular and fibrillar components could be abnormal at the end of embryo maturation. Consequently, the granular and fibrillar RNPs present in the quiescent nucleolus would be non-functional and actively eliminated during early germination before the nucleolus starts the synthesis of new functional RNPs. This possibility is supported by the fact that up to 24 h in *Sinapis* the granular component is constituted of smaller particles than later during activation of the nucleolus (Table 5). Such an observation can hardly be attributed to a shrinkage caused by low hydration of quiescent cells, since the smaller size of pre-ribosomal particles lasts until 24 h when the rehydration of embryonic tissues is already taking place. It can rather be attributed to an unusual protein/rRNA ratio as suggested by the biochemical study of Weidner & Zalewski (1982), which indicated both qualitative and quantitative changes in embryo ribosomal proteins during ripening of cereal kernels. In particular, the synthesis of low molecular weight proteins was enhanced during the last phases of ripening. More information will be required to establish which of these possibilities is correct.

Since the RNP output of pre-existing nucleolar and nucleolar vacuolation are simultaneous processes, both occurring during early germination when other nucleolar functions are still at very low rates, the question arises as to whether they are related.

Nucleolar vacuolation

Our results show that for both species nucleolar vacuolation takes place with seed imbibition and culminates 6–8 h later. During this very early period of germination, the size of the nucleolus decreases rapidly and greatly, the rates of variation of nucleolar volume are the greatest we have measured during germination: they are $-4{\cdot}8\,\mu m^3\,h^{-1}$ and $-2{\cdot}3\,\mu m^3\,h^{-1}$ for *Sinapis* and maize, respectively (Table 4). This decrease in nucleolar size is due largely to a loss of both granular and fibrillar components (Table 6). Since the network of nucleolar vacuoles is always connected to the nucleoplasm by a small channel as shown in Fig. 9 and by a serial sectioning study (Deltour *et al.* unpublished data), this strongly supports the idea that they could

participate actively in this output, as suggested already by observations on living cells (Johnson & Jones, 1967; Kohlenbach, 1967; Rose *et al.* 1972; Erdelská, 1973).

We thus suggest that the strong nucleolar vacuolation occurring during early germination could be due to a transient inability of the external surface area of the small quiescent nucleolus to accommodate the strong output of both granular and fibrillar components observed at the start of germination.

These views on a possible RNP export function for the nucleolar vacuoles are in good agreement with those of authors who suggest that these structures are involved in the transport of some nucleolar material (Soudek, 1960; Esper, 1965; Rose *et al.* 1972; Moreno-Díaz de la Espina *et al.* 1980), and contradict the idea of a storage function (Chouinard, 1964; Rose *et al.* 1972; Moreno-Diáz de la Espina *et al.* 1980).

In apparent contradiction to our above hypothesis are the observations that we have made after 6–8 h of germination. After 24 h the percentage of vacuolated nucleoli remains constant (Table 2; de Barsy *et al.* 1974) while the export of newly synthesized nucleolar RNPs increases (as suggested by data in Table 1). Consequently, an increase in nucleolar vacuolation is expected, but is not actually observed. It is likely, however, that the increasing surface area of the growing nucleolus is, at that time after the start of germination, almost sufficient on its own to accommodate nucleolar RNPs export (Table 7).

In this connection it must be emphasized that, from observations made on our material, the intensity of vacuolation of the nucleolus appears related to nucleolar RNP export rather than to the rates of pre-rRNA transcription and/or processing. This could explain why a strong vacuolation could occur in either actively transcribing nucleoli like those in cotton fibre (Delanghe *et al.* 1978) or in non-transcribing nucleoli (the present study).

To establish with certainty whether, as we suppose, there is a causal relationship between nucleolar vacuolation and the rate of release of RNPs additional quantitative studies are still needed.

Nucleolar growth during early germination

After an initial decrease in volume we have observed in both species a rapid growth in the nucleolus between 6 h and 72 h of germination. One can distinguish two successive patterns of growth during this period. From 6 h to 24 h the nucleolus has little granular component (Table 4). Growth is most probably due to the accumulation of slowly processing RNP fibrils. The biochemical data reported on maize are in good agreement with this supposition (Van de Walle *et al.* 1976). After 24 h the nucleolar volume first increases by the accumulation of the granular component. This results from the acceleration of both pre-rRNA synthesis and processing (Table 1; Van de Walle *et al.* 1976). One can compare the growth pattern of the small nucleolus of quiescent embryos during the G_1 and S phases of the first mitotic cycle of germination with the post-mitotic nucleologenesis in synchronized cells. In synchronized cells the nucleolar size increases greatly in G_1 and S, especially by the accumulation of granular component (Noël, Dewey, Abel & Thompson, 1971; Sacristán-Gárate, Navarrete &

De la Torre, 1974), and pre-rRNA synthesis accelerates at the end of G_1 and at the beginning of S (Yeoman & Aitchison, 1976).

These changes are very similar to those we have observed in *Sinapis* and maize embryos root cells during early germination. However, the high growth rate of the nucleolus during germination is preceded by a strong vacuolation and a concomitant massive loss of RNPs. These late processes may reflect at the structural level the particular qualitative and quantitative changes accompanying the conversion of the nucleolus from the quiescent to the active state.

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