BIOLOGIA PLANTARUM 44 (3): 355-360, 2001

Effects of benzylaminopurine and abscisic acid on distribution of rRNA in the palisade cells of excised *Cucurbita pepo* cotyledons

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Abstract

rRNA was labelled with RNase-gold complexes applied to ultrathin *Lowicryl* sections of excised *Cucurbita pepo* L. (zucchini) cotyledons grown in darkness. Benzylaminopurine-caused stimulation of cotyledon growth decreases the density of ribosomes in palisade cells despite stimulating rRNA synthesis. Abscisic acid inhibits RNA synthesis and transport, but does not visibly affect the number of pre-existing ribosomes in retarded growth cells. The amount of rRNA in the cells of 24 h treated cotyledons is rather consequence of the growth rate than its leading factor.

Additional key words: nucleolus, rRNA synthesis and transport, cell ultrastructure, ribosomes, morphometry, cell growth.

Introduction

Cytokinins and abscisic acid (ABA), are the main hormones responsible for the growth, development and differentiation of excised cotyledons (Banerji and Laloraja 1965, Kulaeva 1973, Fofanova and Khokhlova 1983). Cytokinins enhance their growth, while exogenous ABA effectively inhibits it. Thus ABA behaves as a cytokinin antagonist. The control of protein synthesis in plants seems to be a common site of their action. Plant growth is related to protein and RNA metabolism, and cytokinins stimulate protein synthesis in plants. Exogenous ABA was found to decrease synthesis of RNA, the number of ribosomes, and the ratio of polysomes to monosomes in excised *Cucurbita* cotyledons grown on water (Klyachko *et al.* 1979). However, several other authors have reported that in some systems the decrease of RNA content results from RNase activation (Leshem and Schwarz 1972, Pilet 1972).

Numerous studies have been devoted to determining the role of cytokinins in transcriptional or post-transcriptional control of protein synthesis in plants. Roussaux *et al.* (1976) and Mikulovich *et al.* (1978) found that benzylaminopurine (BAP) increases cytoplasmic and etioplastic rRNA synthesis in isolated dark-grown cotyledons. But contrary to Roussaux *et al.* (1976), Mikulovich *et al.* (1978) showed that BAP was also able to stimulate rRNA synthesis in light-grown cotyledons. The results of Gordon and Letham (1975) suggest that growth in control cotyledons depend on pre-existing RNA, while BAP-induced growth is dependent on the continuing synthesis of RNA. They also report that BAP has a direct effect on protein synthesis, and that this effect precedes the effects on RNA synthesis. On the other hand, Selivankina *et al.* (1976) reports that BAP and kinetin stimulate RNA synthesis prior to the enhancement of protein activities. Naito *et al.* (1980) found BAP-stimulated synthesis of mRNA precedes initiation of DNA synthesis, and that synthesis of rRNA is not always necessary for cotyledon growth. Harvey *et al.* (1974) showed that BAP only weakly stimulates synthesis of proteins and lipids in dark-grown cucumber cotyledons.

The depletion of reserves limits the growth of excised cotyledons and the action of cytokinins in this system is related to temporal activation of cellular metabolism. However, it is unclear as to whether cytokinin-stimulated cotyledon growth is due to specific effects on RNA synthesis, or the result of increased anabolic activity (Murai 1994). Neither is it clear whether ABA exerts an inhibitory effect on growth because it delays RNA synthesis, or because it enhances catabolic activity in the cotyledon.

Received 7 August 2000, *accepted* 18 December 2000.
Abbreviations: ABA - abscisic acid, BAP - benzylaminopurine.
Acknowledgements: The authors wish to thank the anonymous reviewers for their constructive suggestions.
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Reports on the localisation of rRNA in plant cells, using ultrastructural techniques, have up to now been restricted to observations on meristems (Piche *et al.* 1984, Charest *et al.* 1985, Sato 1990, 1992, Sato *et al.* 1988, 1989) and green leaves (Cheniclet and Carde 1987). In general, these studies have not ascertained the changes in RNA content resulting from cell growth and the processes of RNA synthesis, transport and degradation.

The present investigation was undertaken to compare the action of cytokinin and abscisic acid on growth and on the redistribution of rRNA in cellular compartments. We aim to clarify by direct ultrastructural visualisation the location of rRNA, intracellular transport, and degradation during hormone-driven growth in isolated cotyledons.

Materials and methods

Plant material and cotyledon treatment: Seeds of zucchini (*Cucurbita pepo* L. cv. Cocozelle) were soaked for 4 h in tap water and germinated on moistened filter paper for 96 h in darkness at 28 °C. After excision of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water and kept in darkness for further 24 h in order to decrease endogenous cytokinin and abscisic acid content. The cotyledons were then transferred and kept another 24 h on either distilled water, or on an aqueous solution of either 10 μM BAP or 10 μM ABA. The experiments were carried out in darkness in order to eliminate the stimulatory effect of light on the growth and development of the excised cotyledons, which can interfere with the cytokinin action. Cellular growth and localisation of rRNA were observed using paradermal sections of the palisade tissue from the middle part of the cotyledons.

Determination of the cellular areas: The growth of epigeal cotyledons is entirely lateral (Gordon and Letham 1975). Thus during growth of the cotyledon, changes in cell area, as observed in transversal cuts of the first palisade layer, represent changes in cell enlargement.

Samples (5 cotyledons per variant) were taken after the 24-h treatments with phytohormones and at the end of cotyledon growth. The samples were fixed in 3 % glutaraldehyde in 0.2 M phosphate buffer, pH 7.2, and embedded in Spurr's epoxy resin (Spurr 1969). Semi-thin sections (2 - 3 μm thick) were cut longitudinal to the direction of the cotyledon blade by ultramicrotome and dried on glass plate. The sections were stained with 0.01 % (m/v) toluidine blue, and the palisade cells were observed using a light microscope (*Carl Zeiss*, Jena, Germany). The microscope images were saved on a digital image processor (*International Micro-Visio*, Redwood City, USA). Cell area of 20 cells per cotyledon was measured with software *3D-Doctor* (*Able Software Corp.*, Lexington, USA).

Preparation for electron microscopic cytochemistry: The cotyledon samples were fixed in 1.6 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 2 h, washed in the same buffer, and dehydrated through graded ethanol solutions, and embedded in *Lowicryl K4M* (*Chemische Werke Lowi*, Waldraiburg, Germany) under UV light at -18 °C for 24 h. *Epon* (Cheniclet and Carde 1987, Sato *et al.* 1989) or Spurr's (Piche *et al.* 1984) resins were successfully used in experiments on rRNA content in meristematic and leaf cells. Our experiments failed when we used Spurr's or *Epon* resin and were successful only when we used *Lowicryl K4M*. The ultrathin sections were mounted on gold grids.

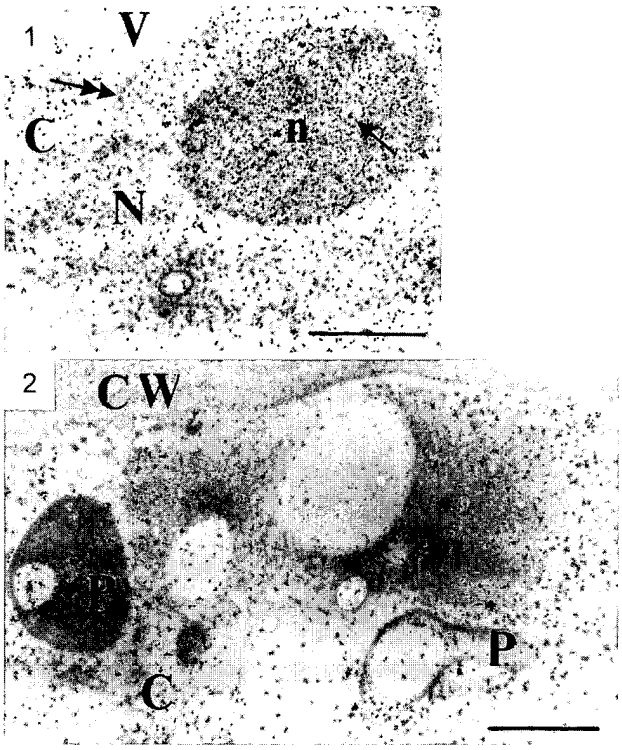
The colloidal-gold suspension was prepared according to Frens (1973), the enzyme-gold complex prepared according to Bendayan (1982). The RNase-gold complex was prepared by adjusting a gold suspension to pH 9 with 0.2 M K_2CO_3, to which RNase III-A (*Sigma*, Diesenhofen, Germany) sufficient to bring the solution to a final concentration of 25 μg cm^{-3} was added. RNase-gold complex suspended in phosphate-buffered saline (PBS), containing 0.2 μg cm^{-3} polyethylene glycol, was applied to the specimens. The gold grids bearing thin sections on *Pioloform* (Structure Probe Inc., Westchester, USA) membranes were floated for 10 min at room temperature on a drop of PBS containing 0.2 μg cm^{-3} of PEG and immediately transferred on to a large drop of RNase-gold suspension, and kept there for 30 min at 37 °C in the dark. After labelling, the grids were rinsed twice for 1 min in de-ionized distilled water. At each transfer step special care was taken not to let the grids dry out. Some grids, serving as controls, were incubated at 37 °C with the enzyme-gold complex, which was previously heated to 100 °C for 120 min.

The sections were first stained with uranyl acetate using conventional techniques, and then, for less than 1 min, with lead citrate before examination with a *JEM 100B* (*JEOL*, Kyoto, Japan) electron microscope.

Estimation of RNase-gold labelling on cell structures: Ultrastructural photographs were taken at magnification ×10 000 and printed. Photographic slides were scanned and digitized using a *ScanJet* (*Hewlett Packard*, USA). The density of gold particles in each cell compartment was counted using a software product *3D-Doctor*. The labelling was evaluated by subtracting from intensity values the background levels of RNA recorded in vacuoles and resin free of plant material.

Results and discussion

Evaluation of the specificity of the cytochemical labelling: About 85 % of total RNA are ribosomal RNA. The observations showed that gold particles are indeed present at cell locations known to contain rRNA. In the nucleus (Figs. 1,3,5), the condensed chromatin and interchromatic space extending between clumps of condensed chromatin were also found to contain rRNA. In the nucleolus, the fibrillar component showed less gold particle density than the granular component, and the gold particles were primarily located at the periphery of dense fibrils situated around the fibrillar centres. Generally the fibrillar centres (when visible) appeared devoid of gold particles; a few particles were occasionally detected on the dense fibrils in fibrillar centres (Figs. 1,3). The



Figs. 1 and 2. Transverse sections of palisade cells in *Cucurbita* cotyledons embedded in *Lowicryl K4M* and incubated with the RNase-gold complex. Gold particles are present on the rRNA-containing compartments of the cells. Parts of palisade cells of *Cucurbita* cotyledon grown on water (control). N - nucleus, n - nucleolus, C - cytoplasm, V - vacuole, CW - cell wall, P - leucoplast, *arrow* - fibrillar centre, *double arrow* - place of the nuclear envelope; *bar* denotes 0.5 μm (Fig. 1) or 1 μm (Fig. 2).

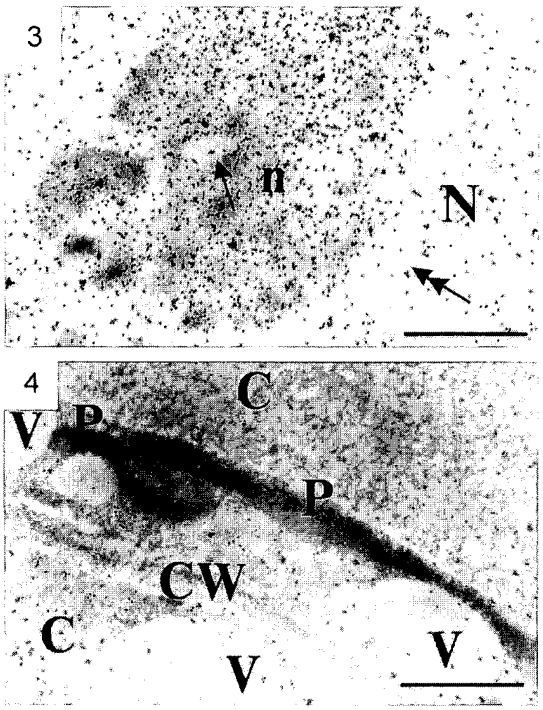
abundant presence of RNA in the dense fibrillar component around the fibrillar centres clearly marks the place of rRNA synthesis. Although the biochemical processes related to the formation of rRNA are known (for a review see Hadjiolov 1985), the corresponding *in situ* description has not been so well developed. Though some autoradiographic data have identified the site of rRNA transcription to be the dense fibrillar component of the nucleolus, RNA polymerase I has been found immunocytochemically in fibrillar centres. The present results showed very little label in fibrillar centres, but its abundant presence around the centres. This conforms to the view that fibrillar centres are composed of proteins, a little DNA, but no RNA, and this is in agreement with other authors using the RNase-gold method (Charest *et al.* 1985, Sato 1992).

In cytoplasm-located structures (Figs. 1,2,4,6), the gold particles were located mainly in the ribosomes of the leucoplasts and in the cytoplasm, with hyaloplasm matrix of the cytoplasm only faintly labelled. Some of the particles in the cytoplasm form chain-like structures, presumably polysomes. Mitochondria contain little rRNA and retain few gold particles. Vacuoles are not thought to contain RNA and represent background labelling in our experiments. A few gold particles were found in the lipid droplets. Cell walls and intercellular spaces were entirely absent of gold particles.

The specificity of the method used is also supported by previous work on various other plant cells (Charest *et al.* 1985, Cheniclet *et al.* 1987, Piche *et al.* 1984, Sato 1990, Sato *et al.* 1988, 1989).

The effect of phytohormones on RNA localisation in the cell: Changes in the RNA content of cell compartments could be a result of RNA synthesis, transport, or degradation. The total density of gold markers in the cytoplasm, nucleus, and especially, in the nucleolus of BAP-treated cells (Fig. 3, Table 1) is lower than that in control cells (Figs. 1,2, Table 1) after 24 h growth in darkness. On the other hand, treatment with cytokinin promotes growth of the cotyledon and the treated cells have more active nucleoli, showing well mixed granular and fibrillar components, and a loose outline (Fig. 3). These characteristics are normally found in cells undergoing intensive transcription. Low levels of rRNA in the nucleus might be found in conjunction with intensive transcription if a more rapid export of ribosomes is supposed. In the opposite case, rRNA in the nucleus is found to increase during periods of low or absent transcription of rDNA (Hadjiolov 1985). In the cytoplasm, the lower density of gold-marked ribosomes found in BAP-treated cells could simply be the result of more intensive growth (Table 1). Cotyledon cells treated with BAP for 24 h were 78 % larger (in comparison to controls). This enlargement effect decreased during cotyledon development, with BAP-treated cotyledons 168 h after posting about 50 % larger than controls. The exact relationship of the biochemical changes involved in

the expansion of detached cotyledons is not clear. Letham (1971) indicates that cytokinin-induced expansion of detached radish cotyledons is due to, at least in part,



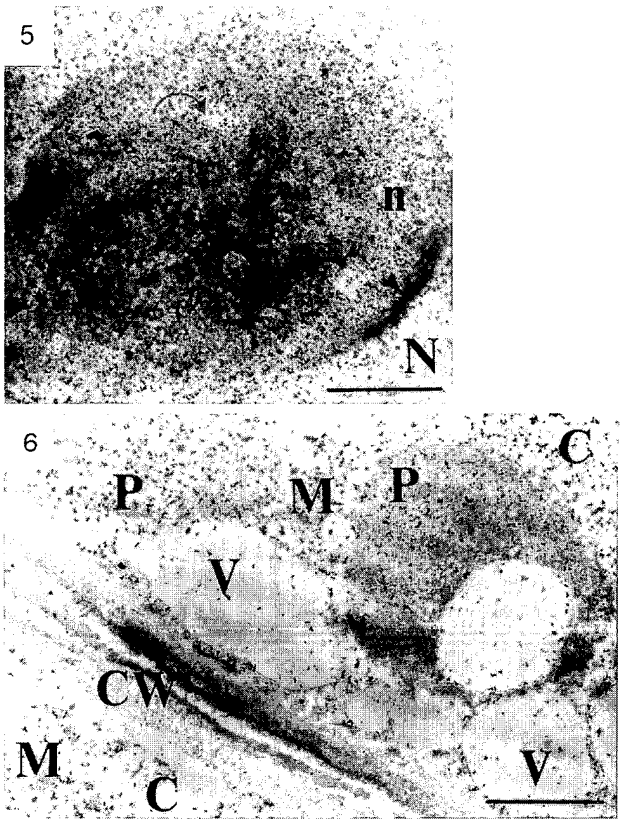
Figs. 3 and 4. Parts of palisade cells of BAP-treated cotyledon. N, n, C, V, CW, P, *arrow* and *double arrow* as in Fig. 1, 2. *Bar* denotes 0.5 μm (Fig. 3) and 1 μm (Fig. 4).

stimulated water uptake. Bewli and Witham (1976) were the first to point out that kinetin-stimulated mass gain in such cotyledons is dependent upon RNA and protein synthesis. The experiments of Trewavas (1980) are, in addition, indicative that during BAP-increased growth the rate of both rRNA synthesis and degradation simultaneously increased. The larger rate of cell growth registered in the present experiments could be suggestive, on the one hand, of more intensive translation and of a higher ribosome turnover rate during translation. Enhancement of protein synthesis by BAP can also be argued on the basis that, despite the lower total density of ribosomes, aggregates of polysomes in the cells of cytokinin-treated cotyledons can be seen. Some of the ribosomes stored during seed maturation, or produced *de novo* during the growth in Petri dishes, are used in the intensive translation, and possibly these ribosomes have already been degraded in BAP-treated cells. In contrary, the effect of ribosome dilution in the larger cells of BAP-treated cotyledons could also be responsible for the lower density of labelling.

Treatment of cotyledons with ABA resulted in retarded cellular growth. ABA-treated for 24 h cotyledon cells were about 15 % smaller than that of the controls. This inhibition markedly increases during further cotyledon development: the area of 168 h ABA-treated cotyledons was about 35 % lower than that of the control. Although ABA-suppressible genes have been reported, the molecular mechanism underlying ABA suppression is completely unknown (Shen and Ho 1997). ABA is considered a general inhibitor of RNA synthesis (Wareing *et al.* 1968, Pilet 1972, Milborrow 1974, Sussex *et al.* 1975, Klyachko *et al.* 1979, Zeevaart and Cleelman 1988). The more compact structure of the nucleolus in ABA-treated cotyledon cells indicates a delay in the

Table 1. Effects of 24-h treatment with phytohormones on palisade cell area and the density of RNase-gold labeling over cell compartments (* - the nucleus without the nucleolus, ** - the term "cytoplasm" here includes the organelles).

Cotyledon treatment	Cell area [μm^2]	Number of gold particles [μm^{-2}] nucleolus	nucleus*	cytoplasm**
water	147 ± 10	108 ± 8.0	38 ± 3.0	39 ± 3.0
BAP	262 ± 12	63 ± 4.0	34 ± 2.0	24 ± 2.0
ABA	125 ± 8	142 ± 7.8	63 ± 5.5	64 ± 6.2



Figs. 5 and 6. Parts of palisade cells of ABA-treated cotyledon. M - mitochondrion, *arrowhead* - local segregation of fibrillar from granular material. N, n, C, V, CW, P, *arrow* and *double arrow* as in Fig. 1, 2. *Bar* denotes 1 μm.

synthesis and transport of rRNA. Strong inhibition of rDNA transcription by ABA could be manifested morphologically as a spatial redistribution of nucleolar components, known as a nucleolar segregation. Small regions of partial segregation of nucleolar material from granular material are indeed seen in the nucleoli of ABA-treated palisade cells (Fig. 5). However, structural damage caused by ABA is not severe. Concomitant with inhibition on growth is abundant presence of rRNA in the cytoplasm and nucleus - the density of labelling in cells treated with ABA is higher than in control cells (Figs. 5, 6, Table 1).

The biosynthesis of rRNA and protein in isolated cotyledons gradually decreases. The zucchini cotyledon contains half the amount of protein after 72-h growth on water (Ananieva and Ananiev 1999). The state of cellular reserves of rRNA in ABA-treated cells mostly reflects reserves inherited during seed formation. We were not able to confirm that ABA-caused reduction of the number of ribosome and polysome, which was reported by Klyachko *et al.* (1979).

Cytokinins (Kulaeva 1973, Klyachko *et al.* 1979, Naito *et al.* 1980, Ananiev *et al.* 1987) and ABA (Wareing *et al.* 1968, Pilet 1972, Sussex *et al.* 1975, Klyachko *et al.* 1979, Zeevaart and Cleelman 1988) might control translation *via* an effect on transcription. Cytokinin/ABA antagonism at this level was first reported by Sussex *et al.* (1975). Phytohormone-dependent changes in the functional organisation of the nucleoli in the present experiments are in support to this opinion, but confirm only that the level of transcription corresponds to the state of growth. The number of ribosomes in the cytoplasm is not the limiting factor for the rate of growth and protein synthesis in the present experimental system. That is why the effects on the synthesis of tRNA and mRNA could also be taken in consideration (Murai 1994). Despite ABA inhibiting rRNA synthesis and cotyledon growth, ABA does not have an effect on ribosome number. One might suppose that ABA acts mainly on translation, but without provoking visible structural degradation of cytoplasmic ribosomes. Some authors have showed the state the pre-formed mRNAs as a reason for ABA-caused preventing of translation (Ho and Varner 1976, Ihle and Dure 1970). Post-transcriptional control in the mechanism of action of cytokinins has also been supposed (Kulaeva 1973, Gordon and Letham 1975, Klyachko *et al.* 1979). The present results also show that rRNA accumulation in the cell is rather consequence than leading factor in ABA-cytokinin interplay during cotyledon growth.

In conclusion, the used RNase-gold protocol enables ultrastructural localisation of rRNA in cotyledon cells. The functional meaning of the changes in rRNA abundance and location at a given stage of development are best evaluated by taking in consideration both the changes in cell enlargement and the concomitant changes in the functional organisation of the cellular organelles.

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