EXAMINATION OF 2, 4-D INDUCED ENLARGEMENT OF NUCLEOLI AND CHANGES IN RNA SYNTHETIC ACTIVITY IN PEA EPICOTYL[3]

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Abstract: Four-day old etiolated seedlings were sprayed with 2.5×10^{-3}M 2, 4-D (pH 6.0) for 24 hr (treated seedlings) and the morphological, anatomical and biochemical changes were examined. The elongation of cells in the young meristematic region and in the early stages of differentiation were inhibited. The radial enlargement of the mature epicotyl was due to the increase in diameter of cortical and pith cells. The 2, 4-D treatment not only altered the size of the cells in the cortex and pith, but also increased the size of the nucleolus, especially in the cortex and vascular strands. The diameter of nucleolus was increased by 40% to 60% (1.5-2.2 μm to 2.2-3.6 μm), but the size of nuclei was only slighly increased by the auxin treatment. The increase in diameter of nucleoli up to 6-8 μm observed previously by the auxin treatment in isolated nuclei was not found in situ in these cross sections. From these studies, the enlargement of nucleoli by auxin treatment was not found to be localized in any particular region and the nuclei with enlarged nucleoli isolated from the treated seedlings for in vitro studies were not preferentially obtained from any particular region of the tissue.

INTRODUCTION

Although the molecular action of auxin is obscure, from several lines of evidence it is seen that one of the roles of auxin is in the regulation of nucleic acid metabolism[7,8]. When the synthetic auxin, 2, 4-dichlorophenoxyacetic acid (2, 4-D), at sublethal and lethal dosages was applied to young etiolated soybean seedlings, cells of the mature hypocotyl enlarged radially and ultimately proliferation took place, while the apical meristem became quiescent[6,7]. This abnormal proliferation of the mature hypocotyl was preceded by a large accumulation of RNA, especially ribosomal RNA[6,7]. This large accumulation of RNA in the mature tissue was associated with dramatic increases in the size of the isolated nuclei and RNA polymerase I activity[1,2]

Isolated nuclei and nucleoli have been used to great advantage in the study of many important problems, such as the study of the transcription of defined RNA species synthesis[3,5,11,12]; the modification of chromatin proteins and their role in gene expression[9,14,15]; the study of RNA polymerases[1,10] and the hormonal regulation of RNA synthesis[7,7]. Using isolated nuclei to study the auxin regulation of RNA synthesis in higher plants, one needs to emphasis that the isolated nuclei are from all parts of epicotyl. By using anatomical methods in combination with biochemical methods, in this report the authors have tried to resolve the above problems.

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MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L.) which had been treated with 10% sodium hypocotyl were germinated in moist vermiculite at 26°C. After 96 hr of germination those seedlings designated as "treated" were sprayed to run-off with a 2.5×10^{-3} M (pH 6.0) solution of 2, 4-D. Mature epicotyl tissue from control and treated seedlings were harvested after an additional 24 hr.

1. **Anatomical studies:**

Tissues used in the anatomical studies were fixed in a mixture of 5% acetic acid, 5% formalin, and 90% ethanol. The fixed material was imbedded in paraffin and cut at 8 μ in thickness with a rotatory microtome. Hematoxylin was used to stain the tissues for anatomical and nuclear figure studies.

2. **Preparation of nuclei:**

The nuclei were isolated by a method described by Chen *et al.*[1]. For RNA polymerase activity assay, the nuclear pellet was resuspended in 1 M sucrose containing 25 mM MES-NaOH buffer (pH 6.0), 20 mM KCl, 30% glycerol, and 10 mM 2-mercaptoethanol.

3. **RNA polymerase assay:**

The RNA polymerase activities of the nuclear preparation were assayed at 28°C in a 0.25 ml reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 5 mM $MgCl_2$, 20% glycerol, 0.4 mM each of ATP, GTP, and CTP, and 0.02 mM ^{3}H-UTP (1 μCi). Other additions are described in the taqle legends. The reaction was terminated after 20 min of incubation by addition of 2 ml of 10% trichloroacetic acid containing 8 mM sodium pyrophosphate. The precipitate was collected on GF/C glass fiber disks, washed four times with 3 ml of 5% trichloroacetic acid and twice with 4 ml of 95% ethanol. The filters were dried under heat lamps and counted in a liquid scitillation spectrometer (Beckman LS-100C).

RESULTS

The treatment of intact pea seedlings with 2, 4-D at the concentration of 2.5×10^{-3} M leads to very marked aberrations in the growth pattern. The elongation of young meristematic cells and cells in the early stages of differentiation were inhibited. The radial enlargement of fully elongated and differentiated zones were promoted (Fig. 1). Transverse sections of mature epicotyl zone from untreated and treated plants are shown in Fig. 2 to Fig. 5. When the intact seedlings were sprayed with 2, 4-D for 24 hr, the most pronounced radial enlargement of the epicotyl was in the fully elongated and differentiated zone just below the young meristematic zone. This radial enlargement was due to the increase in diameter of cortical and pith cells (Fig. 3 and Fig. 5).

By the examining transverse sections of the epicotyl using a light microscope, the 2, 4-D treatment caused the enlargement of nucleoli in the cells of the cortex, vascular strands, and pith, especially in the cortex and in the vascular strands (Table 1). The size of nucleoli in the cortex had increased the most after 2, 4-D treatment (the diameter of nucleoli was 2.2 μm for untreated and 3.6 μm for treated, but the size of nuclei had only slightly increased).

The nuclei were isolated from the mature zone of epicotyl, and the RNA synthetic activity was measured in vitro. The relative activities of RNA polymerase I and II were assessed by varying the ammonium sulfate and by an addition of α-amanitin in the assay; RNA polymerase I is optimally active at 50 mM ammonium sulfate and insensitive to α-amanitin while RNA polymerase II is optimally active at 200 mM ammonium sulfate and is inhibited by a low concentration of α-amanitin. The ionic strength optima and α-amanitin sensitivities for RNA polymerase I and II in pea nuclei are similar to those reported previously with soybeans[1],

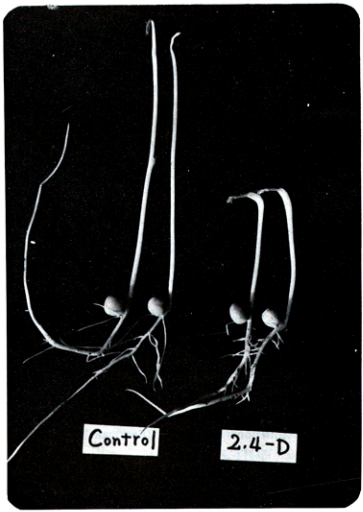

Fig. 1. The external features of control (untreated) and 2, 4-treated 5-day old pea seedlings.

Table 1. Effects of 2, 4-D on the size of nuclei and nucleoli in the mature zone of pea epicotyl

Tissue	The diameter of nucleoli or nucleoli (μm)	
	Control	2, 4-D treated
Cortex		
Nuclei	6.90±0.62	8.70±0.70
Nucleoli	2.20±0.10	3.60±0.41
Vaseular strand		
Nuclei	5.50±0.51	6.70±0.62
Nucleoli	2.00±0.15	2.81±0.21
Pith		
Nuclei	3.72±0.29	5.22±0.49
Nucleoli	1.50±0.13	2.22±0.21

The data was obtained by direct measurement from the cross section of the mature zone of the epicotyl with an ocular micrometer.

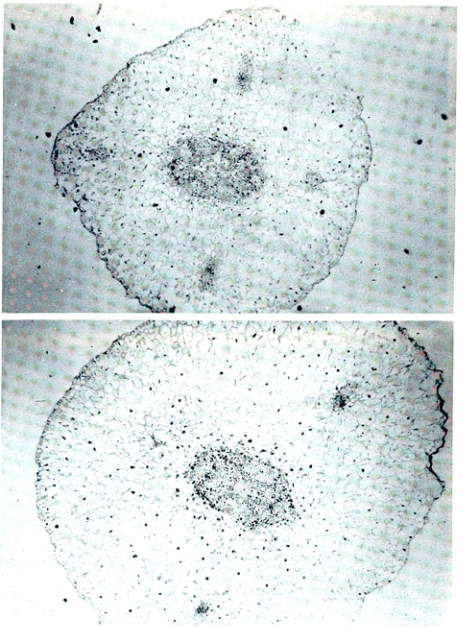

Fig. 2. Transverse section of a mature epicotyl of pea from untreated (A) and from 2,4-D treated epicotyl (B). 4×

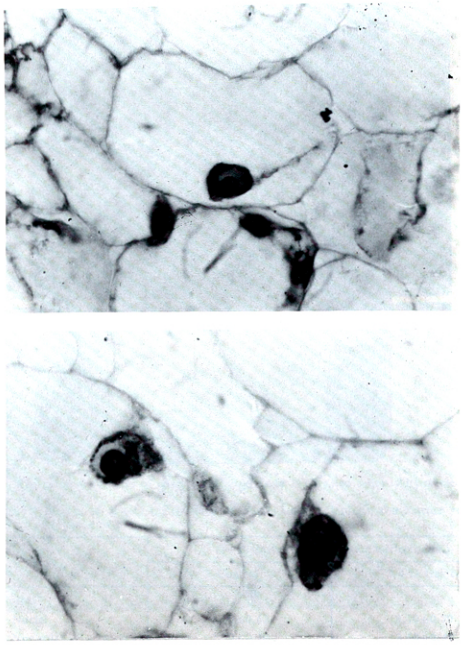

Fig. 3. Portion of cortex of epicotyl from untreated (A) and from 2, 4-D treated (B) epicotyl. 100×
The nucleoli were significantly enlarged in the nuclei from 2, 4-D treated cortex. This was also reflected by the pronounced enlargement of cortex cells.

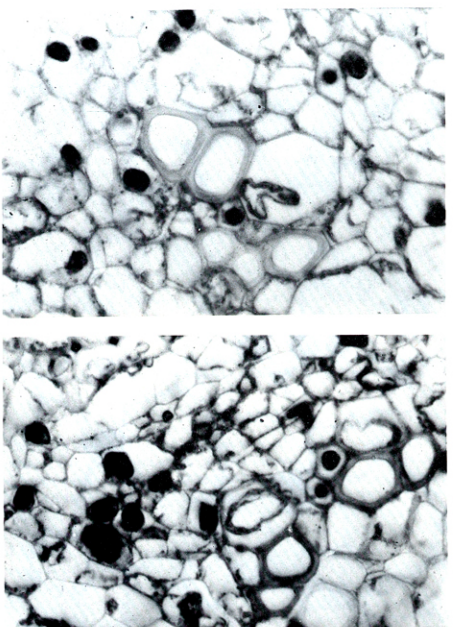

Fig. 4. Portion of vascular strand from nutreated (A) and 2, 4-D treated (B) epicotyl. 100×

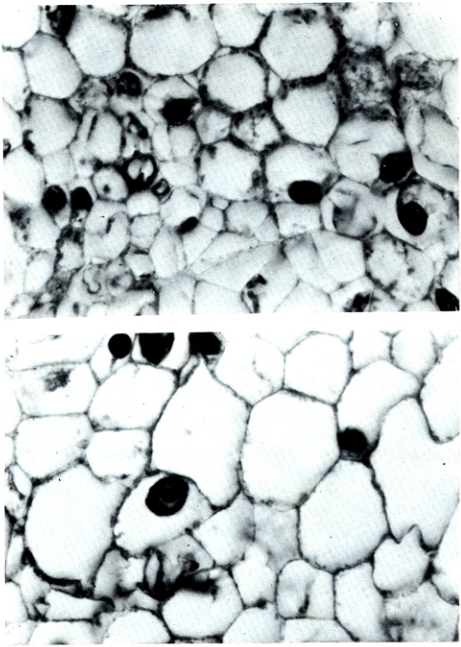

Fig. 5. Portion of pith from untreated (A) and from 2,4-D treated (B) epicotyl. The nucleoli were slightly enlarged in nuclei from 2,4-D treated epicotyl. This is also reflected in a pronounced enlargement of pith cells after the treatment with 2,4-D.

and animal nuclei[6,13,18]. Based on this differential assay, the level of RNA polymerase I activity expressed in nuclei from untreated tissue was 40% higher than RNA polymerase II activity. Nuclei from auxin-treated tissue contained about 5 times as much RNA polymerase I activity as control nuclei (Table 2); in contrast, RNA polymerase II activity was increased by auxin treatment by an average of only about 1.9 times over a series of experiments.

Table 2. RNA polymerase activities of nuclei isolated from the untreated and 2, 4-D treated pea epicotyl

Nuclei source	$(NH_4)_2SO_4$ (mM)	cpm		RNA polymerase (cpm)	
		− α-amanitin	+ α-amanitin	I (*)	II (**)
Untreated	50	735	638		
	200	730	357	638	373
Treated	50	3,303	3,131		
	200	1,435	746	3,131	689

The RNA polymerase activities were assayed at 28 C for 20 min in a 0.25 ml reaction mixture (as described in Material and Methods). The data is expressed as cpm per 4 μg of DNA in isolated nuclei.

(*) RNA polymerase I activity was based on 4 μg/ml α-amanitin insensitivity at 50 mM ammonium sulfate.

(**) RNA polymerase II activity was determined as the difference of incorporation in the absence and in the presence of 4 μg/ml α-amanitin at 200 mM ammonium sulfate.

DISCUSSION

Following the treatment of pea seedlings with sublethal and lethal concentrations of synthetic auxin, 2, 4-D, the elongation in the meristematic zone of the epicotyl was severely inhibited because of an impairment of cell division. But the radial enlargement in the mature zone of epicotyl is enhanced. This radial enlargement is due to the increase in diameter of cortical and pith cells. (Figs. 2, 3, 5). The growth pattern of this marked aberration is very similar to that of etiolated soybean seedlings when they were sprayed with 2, 4-D at sublethal or lethal concentrations. Key *et al.* had reported that herbicidal action of 2, 4-D was associated with renewal of RNA and protein synthesis leading to massive tissue proliferation, disorganized growth, and final death of the plant[13].

From anatomical studies we have shown that 2, 4-D treatment caused the enlargement of nucleolar size in the cells of the cortex, vascular strands, and pith. However the enlargement of the nucleoli was most obvious in the cortex and least noticeable in the pith. This change was found in all types of the cells in the mature epicotyl, rather than in any particular region.

The size of nucleoli in untreated seedlings by anatomical observation was identical to the nucleoli in the nuclei isolated. The diameters of nucleoli in situ and in the isolated nuclei both ranged in size from 1.5 to 2.2 μm. However the diameter of nucleoli from treated seedlings was significantly changed after cell disintergration and purification of nuclei by sucrose gradient (2.2-3.6 μm to 4-6 μm) during preparation. Nevertheless the enlargement of nucleoli by auxin treatment took place in the cells of the cortex, vascular strands and pith and those isolated nuclei with enlarged nucleoli which were used for in-vitro studies were not obtained from any particular localized region of the tissue.

REFERENCES

1. CHEN, YIH-MING, CHU-YUNG LIN, H. CHANG, T. J. GUILFOYLE, and J. L. KEY. 1975. Isolation and properties of nuclei from control and auxin treated soybean hypocotyl. plant Physiol. **56**: 78-82.
2. GUILFOYLE, T. J., C. Y. LIN, Y. M. CHEN, R. T. NAGAO, and J. L. KEY. 1975. Enhancement of soybean RNA polymerase I by auxin. Proc. Natl. Acad. Sci. U. S. A. **72**(1): 69-72.
3. GURLEY, W. B., C. Y. LIN, T. J. GUILFOYLE, R. T. NAGAO, and J. L. KEY. 1976. Analysis of plant RNA polymerase I transcript in chromatin and nuclei. Biochim. Biophys. Acta. **425**: 168-174.
4. JACOB, S. T. 1973. Mammalian RNA polymerases. Prog. Nucleic acid Res. Mol. Biol. **13**: 93-126.
5. JACOBSON, A., R. A. FIRTEL, and H. F. LODISH. 1974. Synthesis of messenger and ribosomal RNA precursor in isolated nuclei of the cellular slime mold *Dictyostelium discoideum*. J. Mol. Biol. **82**: 213-230.
6. KEY, J. L., C. Y. LIN, E. M. GIFFORD JR., and R. DENGLER. 1966. Relation of 2, 4-D induced growth aberration to changes in nucleic acid metabolism in soybean seedlings. Bot. Gaz. **127**(2-3): 87-94.
7. KEY, J. L. 1969. Hormones and nucleic acid metabolism. Ann. Rev. Plant Physiol. **20**: 449-474.
8. KEY, J. L., and L. N. VANDERHOEF. 1973. Plant hormones and developmental regulation: role of transcription and translation. In: S. J. Coward, ed. Aspects of cell differentiation. Academic Press, New York. pp. 49-83.
9. KLEIMSMITH, L. J., and V. G. ALLFREY. 1969. Nuclear phosphoproteins I. Isolation and characterization of a phosphoprotein fraction from calf thymus nuclei. Biochemistry **13**: 3689-3896.
10. LIN, C. Y., T. J. GUILFOYLE, Y. M. CHEN, and J. L. KEY. 1975. Isolation of nucleoli and localization of ribonucleic acid polymerase I from soybean hypocotyl. Plant physiol. **56**: 850-852.
11. MARZLUFF, W. F. JR., E. C. MURPHY JR., and R. C. C. HUANG. 1974. Transcription of the gene for 5 S ribosomal RNA and transfer RNA in isolated mouse myeloma cell nuclei. Biochemistry **13**: 3689-3896.
12. PRICE, R., and S. PENMAN. 1972. A distinct RNA polymerase activity, synthesizing 5.5 S, 5 S and 4 S RNA in nuclei from adenovirus 2-infected Hela cells. J. Mol. Biol. **70**: 435-450.
13. RAYNAUD-JAMMET, C., F. BIERI, and E. E. BAULIEU. 1971. Effects of estradiol, α-amanitin, and ionic strength on the in vitro synthesis of RNA by uterus nuclei. Biochim. Biophys. Acta **243**: 355-360.
14. RICHWOOD, D., P. G. RICHES, and A. J. MACGILIVRAY. 1973. Studies of the in vitro phosphorylation of chromatin non-histone proteins in isolated nuclei. Biochim. Biophys. Acta **299**: 162-171.
15. STEIN, G. C., T. C. SPELSBERG, L. J. KLEISMITH. 1974. Non-histone chromosomal proteins and gene regulation. Science **183**: 812-824.
16. WIDNELL, C. C., and J. R. TATA. 1964. Evidence for two DNA-dependent RNA polymerase activities in isolated rat liver nuclei. Biochim. Biophys. Acta **87**: 531-533.