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Changes in Nucleus, Nucleolus and Cell Size Accompanying Somatic Embryogenesis of *Theobroma cacao L.* II. Relation Between Basic Protein Content and Size of Nucleus, Nucleolus and Cell

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ABSTRACT

Embryo formation from callus of *Theobroma cacao L.* was associated with the changes in relationship between nuclear, nucleolar and cell sizes and the content of basic proteins (FG-FCF-stained). Together with the increase in nuclear size of callus and proembryo cells the increase in the amount of nuclear basic proteins was found. In the callus cells the increase in nucleolar protein content exceeded that in nucleolus size, which led to the rise in basic protein concentration in the nucleolus. However, in the early stage of embryogenesis the increase in protein content was not so marked as that in callus, which indicated that embryogenesis involved a decrease in concentration of nucleolar basic proteins. Differences between callus and proembryo cells were also observed in the concentration of cytoplasmic proteins. The increase in size of callus cells was the same as the increasing amount of cytoplasmic proteins. In proembryos a significant increase in cell size was accompanied by only slight changes in cytoplasmic proteins.

The stimulation of embryogenesis by 2,4-D resulted in an increase of nuclear concentration of basic proteins in proembryos. The intensification of embryogenesis involved the decrease of the concentration of nucleolar proteins together with the increase in concentration of basic cytoplasmic proteins.

H. KONONOWICZ, J. JANICK: *Zmiany rozmiarów jąder, jąderek i komórek towarzyszące somatycznej embriogenezie u Theobroma cacao L. II. Zależność pomiędzy zawartością białek zasadowych i rozmiarami jąder, jąderek i komórek.*

Powstawanie embrionów z kalusa *Theobroma cacao L.* było związane ze zmianami zależności pomiędzy rozmiarami jąder, jąderek i komórek i zawartością białek zasadowych (bawiących się FG-FCF). Zwiększeniu rozmiarów jąder w kalusie i proembrionach towarzyszyło zwiększenie ilości zasadowych białek jądrowych. W komórkach kalusa zwiększenie zawartości zasadowych białek jąderkowych było większe

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niż zwiększenie rozmiarów jąderek, co prowadziło do zwiększenia stężenia białek zasadowych w jąderku. Jednakże we wczesnych stadiach rozwoju embrionów zwiększenie zawartości białek nie było tak znaczne jak w kalusie, co sugeruje, ze embiogeneza związana jest z obniżeniem stężenia zasadowych białek jąderkowych. Obserwowano także różnice stężenia białek cytoplazmatycznych pomiędzy komórkami kalusa i proembrionów. Rozmiary komórek kalusa zwiększały się w jednakowym stopniu jak ilość białek cytoplazmatycznych. Natomiast w proembrionach zwiększeniu rozmiarów komórek towarzyszyła jedynie nieznaczna zmiana ilości białek cytoplazmatycznych.

Stymulacja embriogenezy pod wpływem 2,4-D powodowała zwiększenie stężenia jądrowych białek zasadowych w proembrionach. Intensyfikacja embriogenezy związana była także z obniżeniem stężenia zasadowych białek jąderkowych oraz zwiększeniem stężenia zasadowych białek cytoplazmatycznych.

Following abbreviations have been used: 2,4-D = dichlorophenoxyacetic acid; CW = coconut water; FG-FCF = FG = Fast Green; TCA = trichloroacetic acid; AU = arbitrary units.

INTRODUCTION

It was suggested that developmental processes were highly sensitive to cell and nucleus size, and different sizes were optimal for different tissue [4]. In eukaryota DNA content is strongly correlated with the cell and nuclear volume [2]. These correlations were explained by postulating that DNA had two functions unrelated to its protein-coding capacity, eg. determination of nuclear volume by the overal bulk of DNA and control of cell volume by the number of replicon origins [4]. However, relationship between nuclear protein content and nucleus size was observed [8] which suggested that increased nuclear size, with constant DNA content may be due to increased nuclear protein content. This raises the question about the nature of these proteins.

In our previous paper [7] we found that somatic embryogenesis of *Theobroma cacao L.* was associated with changes in the relationship between an increase in DNA content and sizes of nuclei, nucleoli and cells. The process of embryo formation form callus was also associated with changes in the relationship between nuclear, nucleolar and cell sizes and total (DNFB-steined) protein content.

The aim of this study was to ascertein if there is a relationship between sizes of nucleus, nucleolus and cell and basic protein content (FG-stained). Amount of basic proteins was estimated cytophotometrically after FG staining procedure [3].

MATERIAL AND METHODS

Methods for maintenance of stock callus culture were described in previous paper [6]. Embryogenic competent callus of cacao may be shifted toward homogenous callus production without any evidence of embryogenesis with 1.0 mg/l 2,4-D+10% coconut water (CW) or to high frequency of embryogenesis with 10^{-2} mg/l 2.4-D (6). Basal media (control) were supplemented as described with 2,4-D at concentration 10^{-2} mg/l or 1.0 mg/l+10% CW.

Pieces of white yellow callus 10—15 um³ were used to initiate experiments. After three weeks, two stages were distinquished under the stereoscopic microcsope: white-yellow callus- stage I callus, and nodular bodies -stage II callus. All experiments were performed on these two developmental stages.

Staining procedure.

Protein content in nucleus, nucleolus and cytoplasm were estimated cytophotometrically- with Zeiss (Jena) microspectrophotometer. Preparations were made according to Feulgen-FG method [3]. Material was hydrolized with 1N TCA at 60°C for 15 min. After rinsing 3 times in H_2O it was stained by Feulgen procedure at room temperature for 60 min. The material was washed 3 times with 1N TCA and stained with FG, pH 8.1 to 8.2 for 30 min.

Measurements of relative amount of FG-stained proteins per nucleus, nucleolus and cytoplasm were made at 589 nm. Nuclear, nucleolar and cell dimensions were determined from the cytophotometrical procedure. In each experimental series 100 cells were analized.

RESULTS

Relationship between FG-stained protein content and size of nucleus

There is the linear relation between the amount of FG-stained nuclear proteins and nuclear size in callus and proembryo cells (Fig. 1: 1A, 1B). The increase in nuclear proteins content was associated with an increase in nuclear size. The increase in sizes of nuclei, however, was not so significant as the increase in protein content, which resulted in the

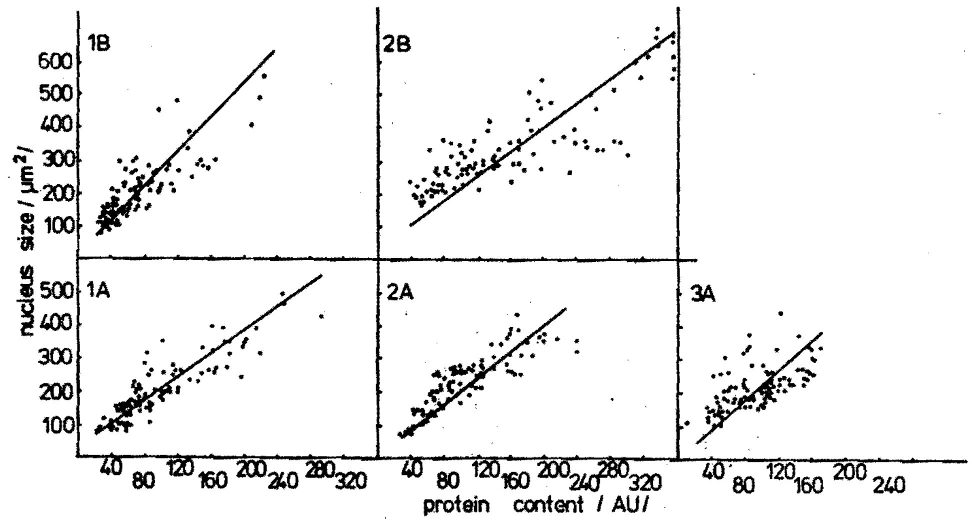

Fig. 1. The relationship between FG-stained nuclear protein content and nucleus size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 14.34+0.57\times(r^2 = 0.77)$; 2,4-D: $y = 0.52+0.5\times(r^2 = 0.17)$; 2,4-D+CW: $y = 2.49+0.45\times(r^2 = 0.46)$. B) control: $y = 0.49+0.5\times(r^2 = 0.59)$; 2,4-D: $y = 15.49+0.54\times(r^2 = 0.34)$

increase in protein concentration (Table 1). While in callus nuclei of 50 µm² protein concentration was 0.20 AU/µm², in nuclei of 400 um² it was 0.52 AU/µm². The increase in nuclear protein concentration in proembryos was not so remarkable as in callus (Table 1).

The stimulation of embryogenesis with 10^{-2} mg/l 2,4-D did not alter the relationship observed in the control callus cells (Fig. 1: 2A). In proembryos, however, 2,4-D increased the amount of nuclear proteins as compared to the same size nuclei of the control (Fig. 1 2B). For example, in nuclei of 400 µm² the content of basic proteins in control proembryos was 148 AU and increased to 200 AU in proembryos growing in the presence of 2,4-D.

TABLE 1

Effect of the conditions stimulating (2.4-D) or inhibiting (2,4-D+CW) embryogenesis on the concentration (AU/μm²) of FG-stained nuclear proteins in callus and early stage of embryogenesis of *Theobroma cacao L.*

Treatment	Stage	Size of nucleus (μm²)			
		50	100	200	400
control	I	0.24	0.40	0.48	0.52
	II	0.28	0.32	0.36	0.37
10^{-2} mg/l	I	0.28	0.50	0.50	0.50
2,4-D	II	—	0.40	0.47	0.50
1.0 mg/l 2,4-D +10% CW	I	0.4	0.44	0.45	0.44

Relationship between FG-stained nucleolar protein content and size of nucleolus

Changes in FG-stained nucleolar protein content were accompanied by parallel changes of nucleolar sizes (Fig. 2). In callus cells growing on basal medium the increase in nucleolar size was not equivalent to the increasing protein content (Fig. 2: 1A), for example, doubling nucleolus size from 40 to 80 μm² was accompanied by 2.7 times increase in pros tein content. It led to an significant increase in nucleolar protein concentration in callu-

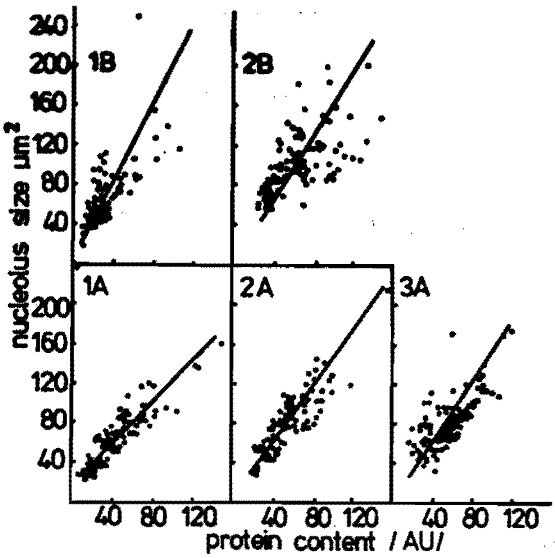

Fig. 2. The relationship between FG-stained nucleolar protein content and nucleolus size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 15.17+0.96\times(r^2 = 0.76)$; 2,4-D $y = 1.62+0.71\times(r^2 = 0.79)$; 2,4-D+CW: $y = 1.31+0.14\times(r^2 = 0.59)$. B) control: $y = 0.49+0.5\times(r^2 = 0.58)$; 2,4-D: $y = 2.86+0.81\times(r^2 = 0.40)$

TABLE 2

Effect of the conditions stimulating (2,4-D) or inhibiting (2,4-D+CW) embryogenesis on the concentration (AU/μm²) of FG-stained nucleolar proteins in callus and early stage of embryogenesis of *Theobroma cacao* L.

Treatment	Stage	Size of nucleolus (μm²)			
		20	40	80	160
control	I	0.20	0.55	0.75	0.86
	II	0.50	0.50	0.50	0.50
10^{-2} mg/l 2,4-D	I	0.50	0.65	0.68	0.69
	II	0.70	0.67	0.64	0.65
1.0 mg/l 2,4-D +10% CW	I	0.60	0.70	0.68	0.65

cells (Table 2). However, the process of embryos formation did not involve any changes in the concentration of basic nucleolar proteins (Table 2).

2,4-D slightly increase the concentration of nucleolar protein in proembryo cells as compared to control.

Relationship between FG-FCF-stained cytoplasmic protein content and size of cells

Changes in cytoplasmic proteins content were accompanied by changes in cell sizes (Fig. 3). The increase in the size of callus cells growing on basal medium was the same as the increasing amount of cytoplasmic proteins (Fig. 3: 1A). In proembryos, however, significant increase in cell size was accompanied by only slight changes in cytoplasmic proteins (Fig. 3: 1B), which caused a marked decrease in protein concentration in cells of greater size. In cells of 500 μm² protein concentration was 0.48 AU/μm² and only 0.2 AU/μm² in cells of 2000 μm² (Table 3). Differences in cytoplasmic proteins content between callus and proembryo cells were evident in larger cells. Cells of callus contained twice as many FG-stained proteins as the equivalently sized cells of proembryos. For example, in cells of 2400 μm², the content of cytoplasmic proteins in callus was 940 AU but only 450 AU in proembryos.

2,4-D did not cause any essential changes in the relationship between protein content and cell size in callus (Fig. 3: 2A). However, in proembryos, there was a remarkable increase in cytoplasmic protein content when compared to the control (Fig. 3: 2B). For example, in cells of 2000 μm² protein content in control proembryos was 400 AU and in proembryos growing in the presence of 2,4-D — 690 AU, but nevertheless the increase in cell sizes led to the increase in protein concentration (Table 3). In contrast, 2,4-D+CW, which eliminated proembryos, significantly decreased cell protein content as compared to the control (Fig. 3: 3A). Protein content in control cells of 2000 μm² was 780 AU and in the same size cells growing on media supplemented with 2,4-D+CW — 600 AU. These changes suggest that the transition from nonorganized growth of callus to embryogenesis is associated with an increase in cytoplasmic protein concentration.

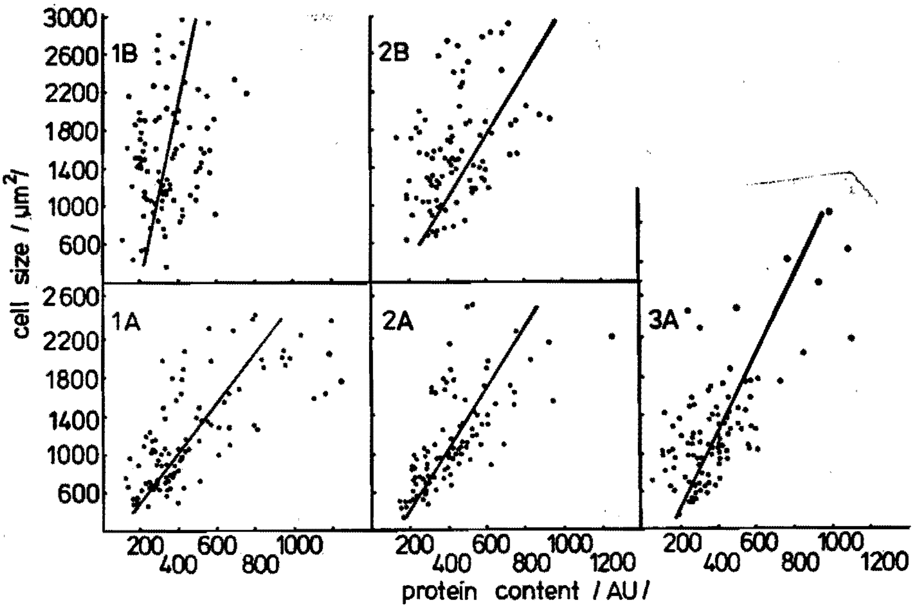

Fig. 3. The relationship between FG-stained cytoplasmic protein content and cell size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 value as follows: A) control: $y = 25.56+0.37\times(r^2 = 0.51)$; 2,4-D: $y = 81.54+0.34\times(r^2 = 0.53)$; 2,4-D+CW: $y = 105.15+ +0.25\times(r^2 = 0.49)$. B) control: $y = 183.99+0.11\times(r^2 = 0.26)$; 2,4-D: $y = 110.61+0.25\times(r^2 = 0.3)$

TABLE 3

Effect of the conditions stimulating (2,4-D) or inhibiting (2,4-D+CW) embryogenesis on the concentration (AU/μm²) of cytoplasmic FG-stained proteins in callus and early stage of embryogenesis of *Theobroma cacao L.*

Treatment	Stage	Size of cell (μm²)			
		500	1000	1500	2000
control	I	0.40	0.40	0.39	0.39
	II	0.48	0.29	0,23	0.20
10^{-2} mg/l	I	0.46	0.39	0.36	0.34
2,4-D	II	0.46	0.38	0.35	0.34
1.0 mg/l	I	0.46	0.35	0.31	0.30
2,4-D+10% CW					

DISCUSSION

A relationship between sizes of nuclei, nucleoli and cell and basic protein content was observed in cells of callus and proembryos of *Theobroma cacao L.*

The increase in sizes of nuclei in callus and proembryo cells was accompanied by the increase in the amount of nuclear basic proteins. This effect was especially evident in callus cells (Table 1). Similar relation found for nucleoli in callus cells, which resulted in increase in nucleolar protein concentration (Table 2). The significant differences also appeared in the concentration of cytoplasmic proteins between cells of callus and proembryos (Table 3). Callus cells contained about twice as many basic proteins as proembryogenic cells of corresponding sizes (Table 3).

The induction of embryo formation by 2,4-D from callus involve remarkable increase in the concentration of nuclear and cytoplasmic protein. The intensification of embryogenesis did not involve significant changes in concentration of basic nucleolar proteins.

Similar correlation between protein content and the sizes of nuclei were detected by MITCHELL and VAN DER PLOEG [8]. The increase in nuclear sizes without any changes in DNA content ascribed to an increase in nuclear protein content. It was confirmed by analysis of protein content.

The increase of protein content along with the increase of the nucleus size was observed in *Vicia faba* [1]. However, not all differences in nuclear sizes could be related to differences in protein content, because some large nuclei contained less proteins than small ones.

In our previous paper [7] we reported correlation between nucleus size and DNA content. CAVALIER-SMITH [4] suggested, that the general correlation between the size of actively dividing cells and their DNA content found in bacteria [5] as well as in eucaryotes, which supported the idea that all cells have common mechanism of the size determination. She suggested [4] that DNA acted as a nucleoskeleton, determining the volume of the nucleus, and that larger cells requited larger nuclei and therefore correspondingly more DNA.

Significant changes in the concentration of nuclear, nucleolar, and cytoplasmic proteins accompanying the stimulation of embryogenesis in cacao suggest that these proteins play an important role in differentiation processes leading to embryo formation. The quantitative changes in FG-stained proteins and DNFB-stained proteins [7] may be the result of changes in particular groups or kinds of proteins. The histochemical methods however, do not permit precise determination of the kinds of proteins involved.

In conclusion the results presented in these papers indicated that DNA as well as protein were responsible for the increase in nuclear size. However, the fact that proteins content may increase although DNA content remains constant, indicates that the proteins play an important role in this process.

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