

# Changes in Endogenous Cytokinin Levels in Partially Synchronized Cultured Tobacco Cells<sup>1</sup>

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## ABSTRACT

During the course of partially synchronized cell divisions in cultured tobacco (Xanthi) cells the amount of endogenous cytokinins in the butanol-soluble fraction increased 5 to 10 times in 3 hours and paralleled the increase in frequency of mitosis. Among three cytokinins detected in tobacco cells, the activity corresponding to the R<sub>F</sub> of authentic zeatin in thin layer chromatography changed in parallel with the mitotic index.

The relationship between exogenous cytokinin and cell division has been studied extensively (2). Tobacco pith and soybean callus require exogenous cytokinin for cell proliferation *in vitro* in the presence of auxin. However, the callus of many other species grown in culture does not require exogenous cytokinin. This implies that the tissue produces enough cytokinin for cell division (4, 10). Although there are a few reports relating endogenous cytokinin and mitotic activity (20, 21, 23), no information on the role of endogenous cytokinins in cell cycle regulation has been published. To clarify this role, we examined changes in the endogenous cytokinins during the cell cycle using partially synchronized tobacco cell suspension cultures (14).

## MATERIALS AND METHODS

**Cell Strain and Culture Conditions.** The cell strain XD6S (a single cell clone) was originally isolated by Filner (1) in 1961 from the stem of *Nicotiana tabacum* cv. "Xanthi." The cells were cultured in Murashige and Skoog medium (11) in which IAA and kinetin were replaced by 1 mg/liter 2,4-D. The conditions for suspension culture and the methods for synchronization of cell division by starvation, alternating light (15,000 lux, 12 h)-dark (12 h) treatment and anaerobic conditioning (40 h) were described previously (14). For another synchronization, the cells in the early stationary phase were inoculated with initial density of  $2.0 \times 10^4$  cells/ml into medium without 2,4-D for 48 h and then 1  $\mu$ g/ml IAA was added. These cultures were kept at  $26 \pm 1$  C in the dark.

**Determination of MI<sup>2</sup>.** MI, the percentage of cells in mitosis, was determined by counting the nuclei in late prophase to telophase under the microscope after fixing the cells with formalin (15%) and staining by acetoorcein. At least five samples of 1,000-1,500 cells each were measured from different culture batches.

**Extraction of Cytokinins.** The cells were collected by filtration and homogenized thoroughly with 80% ethanol in a chilled mortar

and centrifuged at 10,000g for 20 min at 4 C. The precipitate was extracted twice with 80% ethanol for 1 h in a freezer, and centrifuged at 10,000g for 20 min at 4 C. The pooled 80% ethanol extracts were concentrated *in vacuo* at 40 C, dissolved in water, adjusted to pH 3.0 with 1 N HCl, and extracted three times with *n*-hexane. The residual aqueous phase was adjusted to pH 8.0 with 1 N NaOH and extracted three times with water saturated 1-butanol. The 1-butanol extracts were combined and are designated as butanol-soluble fraction. The butanol-soluble fraction is concentrated *in vacuo* at 40 C. The dry residue was dissolved in 80% methanol and streaked on TLC.

**TLC of Cytokinins.** Each fraction was applied as a thin streak on aluminum TLC plates (20  $\times$  20 cm) coated with Silica Gel GF<sub>254</sub> or cellulose powder GF<sub>254</sub> (Merck). The silica gel plates were developed in chloroform-methanol (4:1, v/v) and the cellulose powder plates in water. After development (about 15 cm), each chromatogram was air-dried and divided into 10 equal fractions. Each R<sub>F</sub> zone was scraped off and eluted with 80% methanol. The eluate was evaporated to dryness and tested for cytokinin activity using the soybean callus bioassay. The positions of the authentic cytokinins on the chromatogram were determined by UV absorption.

**Bioassay of Cytokinins.** The methanol extracts from each R<sub>F</sub> zone were incorporated into the SCF medium of Fosket and Torrey (3). Cytokinin activity was assayed by measuring soybean cv. "Acme" callus growth. Four pieces of soybean callus, each 10 mg in fresh weight, were inoculated on 10 ml of SCF medium in 50-ml Erlenmeyer flasks. Five flasks were used for each assay. The cultures were maintained at 26 C under dim red light. After 25 days, the average fresh weight per flask was determined and the concentration of cytokinins was calculated from the growth curve for authentic zeatin and expressed as ng zeatin equivalent/g fresh material.

**Determination of DNA Contents.** The cells were fixed in formalin (15%), washed and stained by Feulgen-staining procedure, including hydrolysis for 1 min in 1 N HCl at 60 C (13, 22), and permanently mounted on a microscope slide with Canada balsam. The relative amount of DNA/nucleus was estimated with the microspectrophotometer (Model MMSP-Tu, Olympus), attached to a minicomputer (NOVA 01 8KW, Nihon Minicon) at 530 nm (12, 19). At least 50 nuclei were measured for each sample. The observed values were grouped into classes. The relative amount of DNA/nucleus in 2 C and 4 C were determined as the standard by using the cell in mitosis.

## RESULTS

**Content of DNA in Synchronously Dividing Cells.** The observed values of DNA content of synchronously dividing cells were grouped into classes (Fig. 1). The amounts of DNA in 2 C and 4 C nuclei were 15-25 and 35-45 arbitrary units, respectively. The frequency of 2 C and 4 C nuclei at each period is summarized in

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<sup>2</sup> Abbreviation: MI: mitotic index.

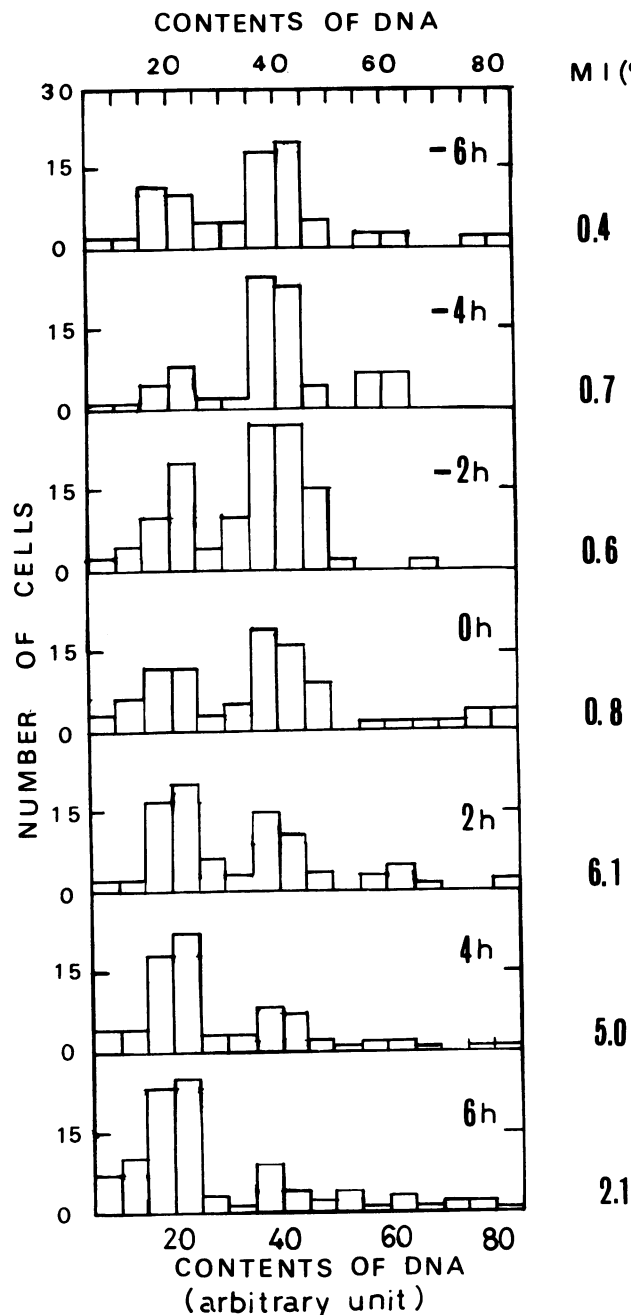


FIG. 1. Distribution of relative amount of DNA/nucleus during synchronous cell division. Synchronization was obtained by inoculating a low initial density of cells at an early stationary phase, culturing for 24 h at  $26 \pm 1$  C in the dark and then subjecting the cultures to two light (12,000-lux, 12 h)-dark (12 h) cycles supplemented by anaerobic conditioning for 40 h. Time zero indicates the beginning of the third irradiation and aerobic conditioning. MI is the mitotic index at each time.

Figure 2. Seven h before maximum mitosis (at -4 h), about 70% of all nuclei had a DNA content equal to 4 C, and 8% were at the 2 C level. DNA duplication was complete by this time. Thereafter, the frequency of 4 C nuclei decreased, while that of 2 C increased. After mitosis reached a peak at 3 h the DNA became equal to 2 C in 60% of cell nuclei. In this synchronized system for cell division, the amount of DNA/nucleus also changed synchronously.

**Endogenous Cytokinins.** The main cytokinin activities of late log phase of tobacco suspension cultures were found at  $R_f$  0.2-0.4 and 0.5-0.6 on silica gel TLC and at  $R_f$  0.1-0.2, 0.2-0.4, and 0.6-

0.7 on cellulose TLC (Fig. 3). The activity at  $R_f$  0.5-0.6 on silica gel corresponded to those of zeatin and its riboside. On cellulose TLC zeatin and its riboside migrated to  $R_f$  0.2-0.4 and 0.6-0.7, respectively. Almost all activity at  $R_f$  0.2-0.3 on silica gel TLC and 0.1-0.2 on cellulose TLC disappeared after treatment with  $\beta$ -glucosidase. Two glucosides were identified at this region of cytokinin activity (unpublished data).

The endogenous cytokinin levels, as reflected in the butanol-soluble fractions, were measured at different stages of growth (Fig. 4). High cytokinin activity at  $R_f$  0.5-0.6 on silica gel TLC, corresponding to zeatin and its riboside, occurred in the middle of the log phase of growth. This activity declined as the growth rate decreased. On the other hand, the activity of cytokinin at  $R_f$  0.2-0.3, corresponding to cytokinin glucosides, increased at the beginning of the growth phase and decreased gradually. However, the

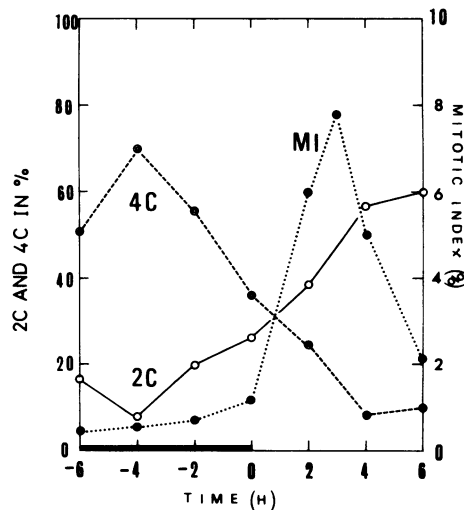


FIG. 2. Frequency of 2 C and 4 C nuclei as per cent of all nuclei counted at each period. This was summarized from Figure 1. Relative amount of DNA in 2 C and 4 C nuclei was determined as cited under "Materials and Methods."

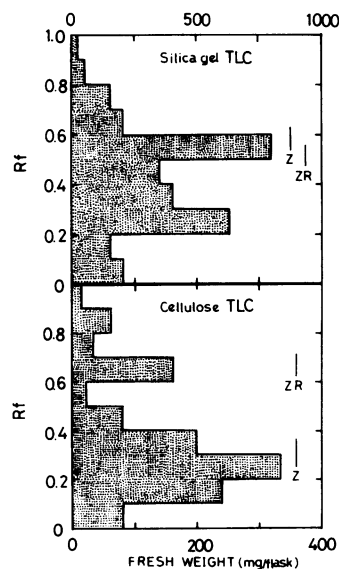


FIG. 3. Cytokinin activity of the butanol-soluble fraction. Silica gel TLC in a solvent system of  $\text{CHCl}_3$ - $\text{CH}_3\text{OH}$  (4:1, v/v). Cellulose TLC in a solvent system of water. Extracts applied were made from 15 g fresh weight of cells which were subcultured for 6 days. Bars indicate  $R_f$  values for authentic zeatin (Z) and zeatin riboside (ZR).

amount of increase was much less than that of the cytokinins at  $R_f$  0.5–0.6.

**Endogenous Cytokinin Levels in Synchronously Dividing Cells.** The activities for different periods at  $R_f$  0.5–0.6 on silica gel TLC (Fig. 5) agreed with that of  $R_f$  0.5–0.6 on silica gel TLC (Fig. 3) and corresponded to authentic zeatin and its riboside. The activities at  $R_f$  0.2–0.3 corresponded to the cytokinin glucosides. The maximum mitotic index of about 7.5% was achieved about 2.5 h after the start of the irradiation and aerobic conditioning. The amount of cytokinins at  $R_f$  0.5–0.6 on silica gel TLC increased sharply to a maximum of 5.2 ng zeatin equivalents/g fresh weight of cells at 2.5 h and then decreased. The peak in the amount of

cytokinin coincided with that of the MI. On the other hand, the amount of cytokinin at  $R_f$  0.2–0.3 was much less than at  $R_f$  0.5–0.6 and remained almost constant. As already reported, synchronized cell division was also observed in the next cell cycle (14). In the second cell cycle, the amount of cytokinins in the butanol-soluble fraction increased in accordance with mitotic activity (Fig. 6).

To confirm the relationship between cytokinin content and MI, changes in the cytokinin content were examined in another synchronous culture system. IAA (1  $\mu\text{g}/\text{ml}$ ) supplied to tobacco suspension cultures precultured for 48 h in the dark without auxin induced synchronous cell division after 8 h. This suggested a relationship between IAA and cell division (15). The cytokinins in the butanol-soluble fraction of this synchronized system also increased considerably with the increase in the MI, and decreased as the MI declined (Fig. 7).

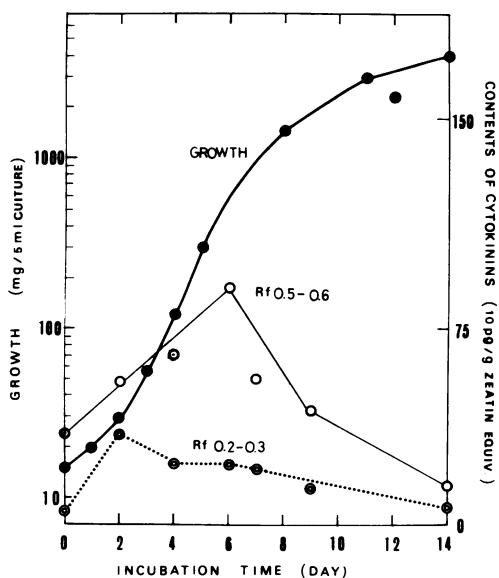


FIG. 4. Growth curve and changes in the amount of endogenous cytokinins in synchronously dividing tobacco cells. Amount of cytokinins in the butanol-soluble fraction was measured by silica gel TLC in a solvent system of  $\text{CHCl}_3\text{-CH}_3\text{OH}$  (4:1, v/v) followed by soybean callus bioassay.

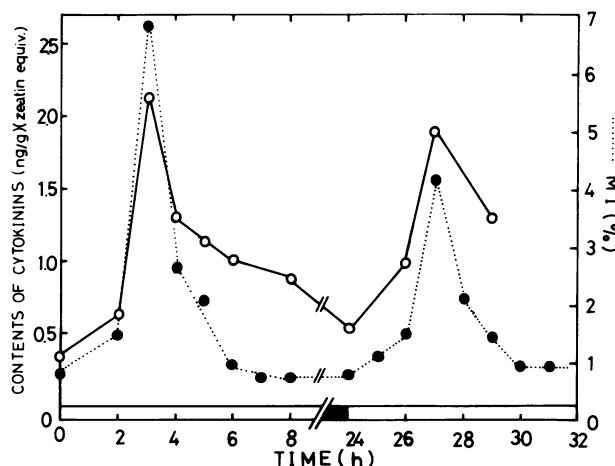


FIG. 6. Changes in amount of endogenous cytokinins and MI in synchronously dividing tobacco cells. Amount of cytokinins in butanol-soluble fraction was measured by silica gel TLC in  $\text{CHCl}_3\text{-CH}_3\text{OH}$  (4:1, v/v) followed by soybean callus bioassay. Synchronization method as in Figure 1. Cultures were irradiated from 0 to 12 h and 24 to 32 h and were kept in the dark.

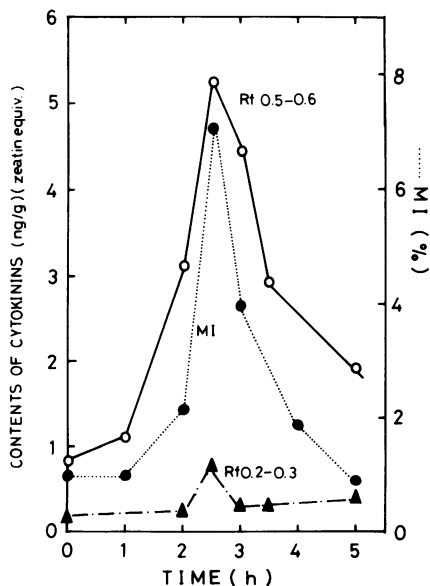


FIG. 5. Changes in amount of endogenous cytokinins and MI in synchronously dividing tobacco cells. Amount of cytokinins in butanol-soluble fraction was measured by silica gel TLC in  $\text{CHCl}_3\text{-CH}_3\text{OH}$  (4:1, v/v) followed by soybean callus bioassay. Synchronization of cell division was obtained as in Figure 1. Time zero indicates beginning of irradiation and of aerobic conditions.

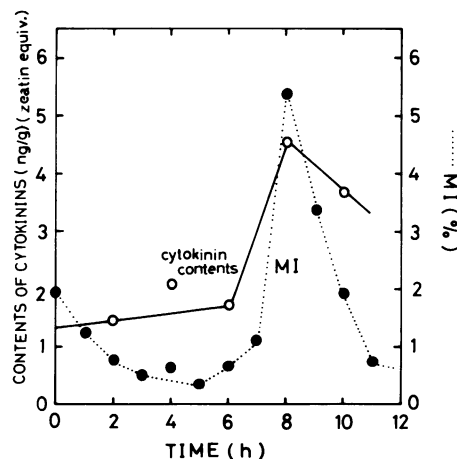


FIG. 7. Changes in the amount of endogenous cytokinins and MI in synchronously dividing tobacco cells. Amount of cytokinins in the butanol-soluble fraction was measured by silica gel TLC in  $\text{CHCl}_3\text{-CH}_3\text{OH}$  (4:1, v/v) followed by soybean callus bioassay. For synchronization of cell division cells in the early stationary phase were inoculated with initial density of  $2.0 \times 10^4$  cells/ml into medium without 2,4-D and cultured in the dark for 48 h at  $26 \pm 1$  C. At zero time, 1  $\mu\text{g}/\text{ml}$  of IAA was added and cultures were kept in the dark.

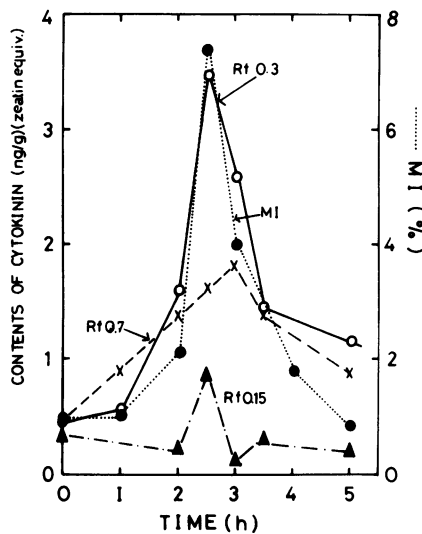


FIG. 8. Changes in amount of endogenous cytokinins and MI in synchronously dividing tobacco cells. Amount of cytokinins in the butanol-soluble fraction was measured by cellulose TLC in water followed by soybean callus bioassay. Synchronization of cell division as in Figure 1. Time zero indicates beginning of irradiation and of aerobic conditions.

Zeatin and its riboside were separated when developed with water on cellulose TLC (Fig. 3). Accordingly, changes in the amount of zeatin ( $R_f$  0.3) and zeatin riboside ( $R_f$  0.7) were investigated by cellulose TLC (Fig. 8). The amount of cytokinin at  $R_f$  0.3 (zeatin) increased sharply in parallel with the increase in MI. The amount of cytokinin at  $R_f$  0.7 (zeatin riboside) increased more gradually and reached a maximum after the MI had reached its highest value. The maximum amount of zeatin riboside was 1.7 ng/g fresh weight of cells, half of that of zeatin. On the other hand, the amount of cytokinin at  $R_f$  0.15 (cytokinin glucosides) increased at 2.5 h and then decreased. But the amount was much less than that of zeatin and its riboside.

## DISCUSSION

To study the mechanisms of cell cycle regulation by endogenous plant growth regulators, two different methods were used to synchronize cell divisions in suspension cultures of tobacco cells. In one method starvation, alternating light-dark treatment and anaerobic conditioning were combined, and in the other method, exogenous IAA was supplied to 2,4-D-starved cultures. In the former case we confirmed that not only the cell number and MI, but also, the content of DNA/nucleus changed synchronously. In this synchronous system, the DNA duplication was completed 7 h before mitosis (Figs. 1 and 2).

In these two different culture systems, we found a dramatic 5- to 10-fold increase in endogenous cytokinin levels in the butanol-soluble fraction during the cell cycle. This increase in endogenous cytokinins coincided with an increase in the MI in both synchronous culture systems (Figs. 4-6). In detached etiolated cotyledons of squash cytokinin activity rapidly increased after 2 h of illumination and sharply decreased after 3 h of illumination (25). The increased cytokinin levels in the butanol-soluble fraction from the tobacco cell cultures do not seem to be the result of illumination because the cultures were kept in the dark when IAA was used.

In general, actively dividing tissues, such as found in root and shoot apices, have much greater cytokinin activity than mature tissues (9, 21). Also, high endogenous cytokinin levels break dormancy in potato tubers (26). Short and Torrey (20) found high cytokinin activity associated with high frequency of mitoses in pea root callus cultures but gave no information about changes in

endogenous cytokinins during the cell cycle. This report is the first one concerning the relationship between the cell cycle and the amount of endogenous cytokinins.

It is known that exogenously supplied cytokinin induces cell division (2, 24) and partial synchronization of tobacco cells (8). Using a synchronous culture system, Jouanneau (8) suggested that cytokinin regulated events necessary for the continued progress of cells through their division cycle. These events seem to be localized in  $G_2$ , or in transition from  $G_2$  to mitosis. Similar conclusions have been reached for soybean cell cultures by Fosket (2). Although the relationship between effects of exogenously supplied cytokinins and increase in endogenous cytokinins associated with mitosis is not yet clear, it is reasonable to conclude that endogenous cytokinin plays an important role in cell cycle regulation.

At least three kinds of cytokinins were found in the butanol-soluble fraction.  $R_f$  values of two were identical with those of authentic zeatin and zeatin riboside in two different solvent systems in TLC (Fig. 3). The third  $R_f$  region contains two cytokinin glucosides judging both from published  $R_f$  values (5-7) and the effect of  $\beta$ -glucosidase (unpublished data). Zeatin glucoside is one of the principal metabolites of exogenously supplied zeatin and may have a storage function in plants (16-18).

Identification of the cytokinins by high performance liquid chromatography combined with MS which increased during mitosis will be published in a subsequent paper. Experiments are also in progress to elucidate the roles of endogenous cytokinin in cell division, especially in mitosis or cytokinesis.

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