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Dynamics of Production of *trans*-Zeatin and *trans*-Zeatin Riboside by Immobilized Cytokinin-Autonomous and Cytokinin-Dependent Tobacco Cells

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Abstract. Two strains of cultured tobacco cells (*Nicotiana tabacum* L. cv. Wisconsin 38) differing in their requirement for exogenous cytokinins (cytokinin-dependent and cytokinin-autonomous) were immobilized on polyphenylenoxide (Sorfix) activated with glutaraldehyde. Columns packed with immobilized cells were continually eluted with diluted Murashige and Skoog's medium lacking or supplemented with synthetic cytokinin (6-benzylaminopurine; BA). Purified samples of column eluates were fractionated by HPLC, and *trans*-zeatin (*t*-Z) and *trans*-zeatin riboside (*t*-ZR) content was estimated by enzyme immunoassay. Both cytokinin-autonomous and cytokinin-dependent tobacco cells produced and excreted *t*-Z and its riboside, and there were significant quantitative differences between the strains. The steady-state excretion rate of *t*-Z was 19.8 ng · g^{-1} dw · h^{-1} and 4 ng · g^{-1} dw · h^{-1}, respectively, and that of *t*-ZR 4 ng · g^{-1} dw · h^{-1} and 1 ng · g^{-1} dw · h^{-1}, respectively. Exposure of cytokinin-dependent cells to BA after 72 h of starving for this synthetic cytokinin caused temporary increase in excretion of both zeatin and its riboside. After the application of 5 μM BA for 24 h, the excretion rate of *t*-ZR reached 5 ng · g^{-1} dw · h^{-1} (5-fold increase), and that of *t*-Z achieved 12 ng · g^{-1} dw · h^{-1} (3-fold increase). The elevation of *t*-Z excretion was delayed about 13 h compared with *t*-ZR excretion, which started increasing almost immedi-

Abbreviations: BA, 6-benzylaminopurine; *t*-Z, zeatin, (6-[4-hydroxy-3-methylbut-*trans*-2-enylamino]purine); *t*-ZR, zeatin riboside, (6-[4-hydroxy-3-methylbut-*trans*-2-enylamino]-9-β-D-ribofuranosylpurine); MS, Murashige and Skoog; NAA, α-naphthalene acetic acid; TTC, triphenyltetrazolium chloride; ELISA, enzyme-linked immunosorbent assay.

ately after BA application. A pulse of BA in lower concentration (1.5 μM for 30 h) provoked lower response.

Tobacco tissue cultures usually require an exogenous supply of auxin and cytokinin for their growth (for review, see Skoog and Armstrong 1970). This requirement may be lost following habituation (Meins et al. 1980, Meins and Hansen 1985) or specific bacterial infection (Braun 1958). Both auxins and cytokinins may be synthesized in habituated and transformed cells and tissues. The content of endogenous cytokinins is increased in habituated tobacco tissue (Einset and Skoog 1973), and recent studies have shown that many, but not all, tumor lines exhibit the same pattern (Einset 1980, Weiler and Spanier 1981).

Cytokinins supplied exogenously to cytokinin-dependent tissues are generally assumed to exert their biological activity in the same way as endogenously synthesized cytokinins. However, it is also possible that exogenous cytokinins may induce endogenous synthesis of naturally occurring cytokinins and in this indirect way stimulate cell division.

Our limited knowledge of this and related problems is due in part to the lack of methods for the continuous analysis of the production of plant hormones by living cells. The use of immobilized cells in column systems permits a continuous flow of nutrients and facilitates the analysis of excreted substances. A continuous system of this type allows one to follow the effects of changing exogenous factors on the production and excretion of specific metabolites by the immobilized cells. To date, immobilized cells have been used mainly for biotechnological purposes (for review see Brodelius 1984, Knor et al. 1985). The metabolism of a phytohormone by immobilized cells has been studied only in the case of microorganism *Aspergillus niger* (Baklashova et al. 1984).

Immobilized plant cells have been used to examine the production and excretion of cytokinins in the present study. The production and excretion of *trans*-zeatin (*t*-Z) and its riboside (*t*-ZR) by immobilized cytokinin-autonomous tobacco cells and the effect of exogenously applied 6-benzylaminopurine (BA) on endogenous cytokinin production in immobilized cytokinin-dependent tobacco cells have been investigated, and results are reported here.

Materials and Methods

Tissue Cultures

Cytokinin-dependent and cytokinin-autonomous tobacco callus cultures (*Nicotiana tabacum* L. cv. Wisconsin 38) were obtained from Dr. J. P. Jouanneau, Marseille, France. Cell suspension cultures derived from these tissues were grown on Murashige and Skoog's (1962) medium, modified according to Jouanneau and Tandeau de Marsac (1973). Media used for cultivation of cytokinin-autonomous and cytokinin-dependent strains were supplemented with auxin alone (NAA, 5 μM) and with auxin and cytokinin (5 μM NAA, 0.5 μM BA), respectively. Cultures were subcultured at 1-week intervals and used for immobilization when they were near the end of the logarithmic phase of growth (5 days after inoculation).

Cell Immobilization

Tobacco cells were immobilized on polyphenylenoxide (Sorfix) by the method described by Jirku et al. (1981). The porous polymer has been prepared according to Kubánek et al. (1978) and was kindly supplied by Dr. B. Veruovič, Institute of Chemical Technology, Prague, Czechoslovakia. To activate the carrier with glutaraldehyde, the Sorfix (10 g) was washed in 100 ml of ethanol and shaken for 24 h in 100 ml of 5% glutaraldehyde at room temperature. Free glutaraldehyde was removed by washing with 50 mM phosphate buffer, pH 5.7, until the reaction with 2,4-dinitrophenylhydrazine, indicating free aldehyde, was negative. Afterward the carrier was washed and equilibrated with 250 ml of potassium phosphate buffer (50 mM, pH 5.7) containing half-strength inorganic nutrients of MS medium. Tobacco cells from the cell suspension culture were suspended in the same buffer. The cell suspension (packed cell volume about 100 ml plus the same volume of buffer) was shaken with activated Sorfix (10 g) for 30 min at 20°C, allowed to stand for 30 min, then shaken again for the same period and left for 18 h at 4°C. The mixture was resuspended in 500 ml of the same buffer, and free cells were separated. Immobilized cells were packed into a column (5 × 11 cm) and eluted with the air-saturated culture medium (without vitamins and hormones and diluted 1:1, v/v, with water) at 26°C at a flow rate of 60 ml · h^{-1}, i.e. 52 μl · cm^{-2} · min^{-1}. Fractions of 240 ml of the effluent were immediately frozen and stored at −15°C until used for analysis of cytokinins.

Cytokinin Analysis

Cytokinins were extracted and purified from the cells, cultivation media, and column fractions by the modified method of Morris et al. (1982). Individual cytokinins were fractionated by HPLC and estimated by ELISA performed according to Weiler et al. (1981). The cells were frozen in liquid nitrogen, homogenized, and extracted with methanol (20 g of fresh weight and 50 ml of methanol of HPLC grade) in the presence of an antioxidative agent (sodium diethyldithiocarbamate, 200 μg · ml^{-1}). Extracts were concentrated under vacuum at 40°C. Methanol was replaced with ammonium acetate buffer (40 mM, pH 6.5). Ribotides were hydrolyzed by acid phosphatase (2 mg/20 g of cells, 27°C, 30 min). The samples were then applied to a column of DEAE cellulose (20 ml). In the case of cultivation media and column fractions, the methanol extraction was omitted. Cytokinins were adsorbed on reverse-phased column cartridges of Sep-Pak Si C_{18} (Waters), washed with water, and eluted with 8 ml of 80% methanol. Eluates were concentrated under vacuum and stored at −15°C. Samples were fractionated by HPLC (Spherisorb 5 ODS, 4 × 250 mm column) using a gradient of methanol in water (10–80%, v/v) as a mobile phase. Content of cytokinins in HPLC fractions was estimated by ELISA. Immunoassays were carried out as described by Weiler et al. (1981) with the following modifications: $IgG_{t\text{-}ZR}$ (2 μg · ml^{-1}) was used for coating the wells of microtiter plates; alkaline phosphatase conjugated to *t*-ZR (2 μg · ml^{-1}) was used to detect the remaining free $IgG_{t\text{-}ZR}$ binding sites. Be-

cause of the cross reactions of antibodies prepared against *t*-ZR with *t*-Z (100%) and BA (20%), it was possible to evaluate all three cytokinins in separate HPLC fractions using one antiserum. There was very low cross reaction with N^6-(Δ^2-isopentenyladenine) and its riboside (<4%). Therefore, these cytokinins would not have been detected even if present in the HPLC fractions. For detection of cytokinins reacting with $IgG_{t\text{-}ZR}$, the whole HPLC profile was analyzed. In addition to *t*-Z, *t*-ZR, and BA, an additional cytokinin corresponding to the BA riboside standard was detected in effluents from column containing cytokinin-dependent cells that were supplied with medium containing BA. The concentration of this cytokinin was about 1 order of magnitude lower than that of BA, and it was not analyzed in the present experiments. The identity of the analyzed compounds with *t*-Z, *t*-ZR, and BA was confirmed using several solvent systems for HPLC.

Cell Viability

During the whole experiment, the cell viability was followed according to the sugar consumption (concentration of sucrose and its hydrolysis products). Sugar concentration in column fractions was measured by means of HPLC using a refractometric detector and Lichrosorb NH_2 column (4 × 250 mm). The mobile phase was a mixture of acetonitrile and water (75:25 v/v).

At the beginning and end of each experiment, reductase activity of the cells was tested with triphenyltetrazolium chloride (Towill and Mazur 1975). The cells were incubated in 0.8% TTC in phosphate buffer (50 mM, pH 7.5) 18 h in darkness at room temperature. The formed red insoluble formazan was extracted with ethanol for 30 min at room temperature. The amount of the dye was estimated by its absorbance at 485 nm.

Results

Immobilized cytokinin-autonomous tobacco cells were continually eluted with half-strength MS medium without vitamins and hormones. The cells produced and excreted the two major natural cytokinins (*t*-Z and its riboside) into the effluent (Fig. 1). At the very beginning of the experiment, high excretion rate of both cytokinins was recorded (129 ng *t*-Z · g^{-1} dw · h^{-1} and 125 ng *t*-ZR · g^{-1} dw · h^{-1}). The cytokinin level decreased rapidly, and after 1.5 days a steady state was reached with the excretion rate of *t*-Z at 19.8 ng · g^{-1} dw · h^{-1} and that of *t*-ZR at 4 ng · g^{-1} dw · h^{-1}.

A higher amount of *t*-Z, compared to *t*-ZR, was also found in cultivation medium analyzed 5 days after inoculation with cell suspension. Opposite ratio of the two cytokinins was determined in cultured cells (Table 1). The values for *t*-ZR include also the amount of its mono-, di-, and triphosphates.

Excretion of *t*-Z and *t*-ZR by immobilized cytokinin-dependent tobacco cells was investigated using the same experimental arrangement as for cytokinin-autonomous cells. In the first experiment, the medium applied on the column contained 0.25 μM BA. The elution profiles of *t*-Z and *t*-ZR were approxi-

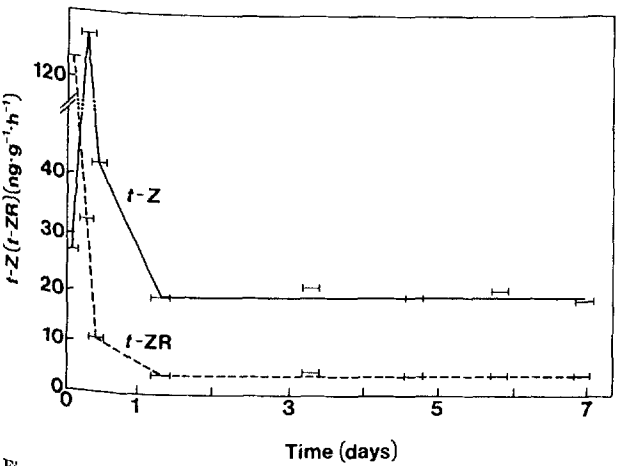

Fig. 1. Excretion of *trans*-zeatin (*t*-Z) and *trans*-zeatin riboside (*t*-ZR) by immobilized cytokinin-autonomous tobacco cells. Elution medium: Half-strength MS without growth regulators. The length of horizontal bars corresponds to the time interval the sample was collected.

Table 1. Cytokinin concentration in the cultured cells and cultivation media of cytokinin-autonomous (J-13) and cytokinin-dependent (J-193) tobacco strains (5 days after inoculation).

Cells	*t*-ZR		*t*-Z		BA	
	ng · g^{-1} fw	μg · g^{-1} dw	ng · g^{-1} fw	μg · g^{-1} dw	ng · g^{-1} fw	μg · g^{-1} dw
J-13	127	2.54	29	0.58	—	—
J-193	38	0.76	19	0.38	89	1.78

Cultivation media	*t*-ZR		*t*-Z		BA	
	ng · ml^{-1}	ng · g^{-1} dw	ng · ml^{-1}	ng · g^{-1} dw	ng · ml^{-1} (original)	ng · ml^{-1} (final)
J-13	1.60	32.0	2.9	58	—	—
J-193	0.76	15.2	1.4	28	113	3.7

mately of the same shape as in the case of immobilized cytokinin-autonomous cells, only the amount was 4–5 times lower. The maximal excretion rates were 34.1 ng *t*-Z · g^{-1} dw · h^{-1} and 37.3 ng *t*-ZR · g^{-1} dw · h^{-1}. Steady-state values were 1.6 ng *t*-ZR · g^{-1} dw · h^{-1} and 5 ng *t*-Z · g^{-1} dw · h^{-1}.

Comparing the cytokinin content in cytokinin-dependent and cytokinin-autonomous cells, relatively high values were found for the dependent strain (only 2–3 times lower than for the autonomous one). A similar ratio was estimated in cultivation medium (Table 1).

Another column with cytokinin-dependent cells was eluted with medium lacking BA. The cells were in contact with this synthetic cytokinin only during their previous cultivation period. Excretion of BA, *t*-ZR, and *t*-Z was monitored (Fig. 2). At the beginning, the elution of all three cytokinins followed a

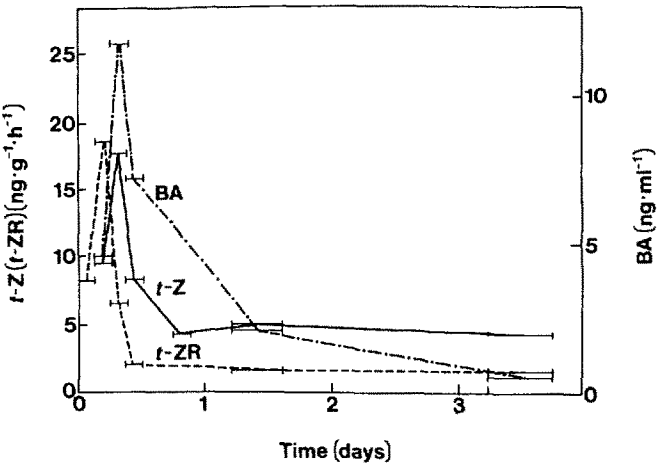

Fig. 2. Excretion of 6-benzylaminopurine (BA), *trans*-zeatin (*t*-Z), and its riboside (*t*-ZR) by immobilized cytokinin-dependent tobacco cells. Elution medium lacked BA.

similar trend. This fact indicates that the high excretion rate of cytokinins by the cells that appeared soon after their immobilization represented a gradual depletion of cytokinins which accumulated in the cells during their cultivation on medium supplemented with BA. The possibility that it was a consequence of elevated synthesis—e.g., as a response to immobilization stress—could be excluded. Later on, the amount of BA gradually decreased while the excretion rate of *t*-Z and *t*-ZR reached constant level (4 ng · g^{-1} dw · h^{-1}, 1 ng · g^{-1} dw · h^{-1}, respectively). Stability of *t*-Z and *t*-ZR levels indicated that the steady state was caused by synthesis and excretion of these natural cytokinins by immobilized cells.

Immobilized cytokinin-dependent cells were also used for testing the inductive effect of BA on synthesis and excretion of natural cytokinins. In the first experiment, immobilized cells were supplied for 96 h with the medium containing 0.5 μM BA. Then the BA supply was interrupted for 50 h, and BA was applied again at concentration 1.5 μM (Fig. 3). All cytokinins (BA, *t*-ZR, *t*-Z) were excreted at a high rate for the first 24 h. When the BA supply was stopped, its concentration in the effluent dropped by approximately 75%. The excretion of *t*-Z and its riboside was not significantly influenced. Supplying the column with a second wave of BA caused a gradual increase of *t*-Z and *t*-ZR excretion. It is interesting that the rise in zeatin riboside content in the column effluent was recorded almost immediately after BA application and about 15 h sooner than that of BA. A temporary increase in *t*-Z excretion was delayed at least 13 h.

In another experiment, cytokinin-dependent cells were eluted with the medium without BA for 72 h, then a pulse of BA (5 μM) was applied for 24 h. For the next 8 days, the column was supplied with medium lacking BA (Fig. 4). The BA pulse caused, as in the previous experiment, an almost immediate increase in *t*-ZR excretion rate. Comparing the two experiments, it appears

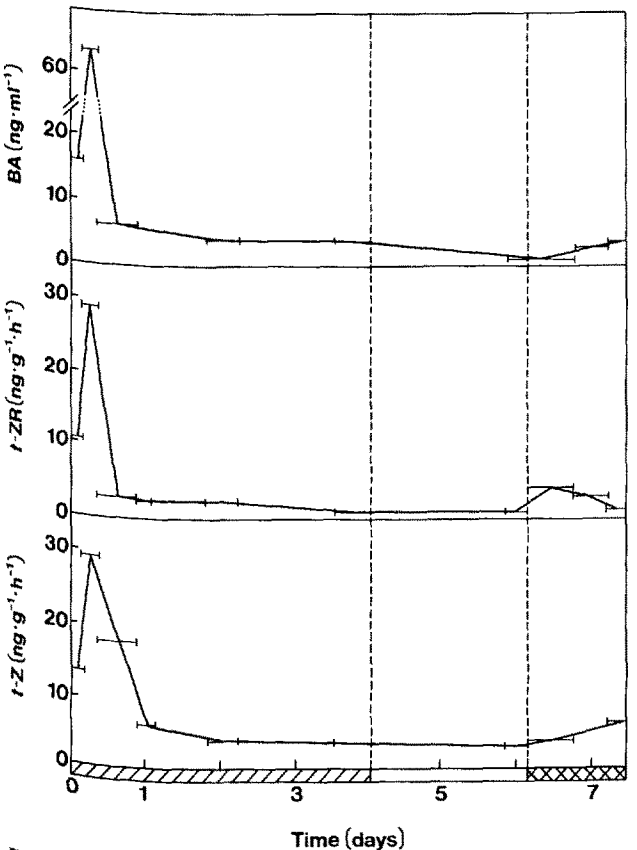

Fig. 3. Excretion of 6-benzylaminopurine (BA), *trans*-zeatin (*t*-Z), and its riboside (*t*-ZR) by immobilized cytokinin-dependent tobacco cells. Concentration of BA in the elution medium: 0–96 h: 0.5 μM; 96–146h: 0 μM; 146–176 h: 1.5 μM.

that a higher amount of BA in the elution medium induces a higher rate of *t*-ZR excretion; i.e., 1.5 μM BA increased *t*-ZR excretion from 1 ng · g^{-1} dw · h^{-1} to 3.4 ng · g^{-1} dw · h^{-1} (Fig. 3), whereas 5 μM BA increased the excretion rate to 5 ng · g^{-1} dw · h^{-1} (Fig. 4). The excretion rate of *t*-Z grew more slowly than that of *t*-ZR (from 4 ng · g^{-1} dw · h^{-1} to 12 ng · g^{-1} dw · h^{-1}) but still increased to a higher level than at the 1.5 μM BA application (7 ng · g^{-1} dw · h^{-1}). At 5 μM, BA seemed to exceed the cell capacity for its uptake even after their starving for this exogenous cytokinin. In this case, the BA level in the effluent increased immediately.

The viability of cells was not significantly influenced by the immobilization procedure (as evaluated by TTC reduction). Sugar consumption of immobilized cells in the column increased slightly during the first 12 h; then it remained constant for approximately 5–7 days, after which it started decreasing slowly (results not shown). For this reason the experiments did not usually extend more than 7–10 days.

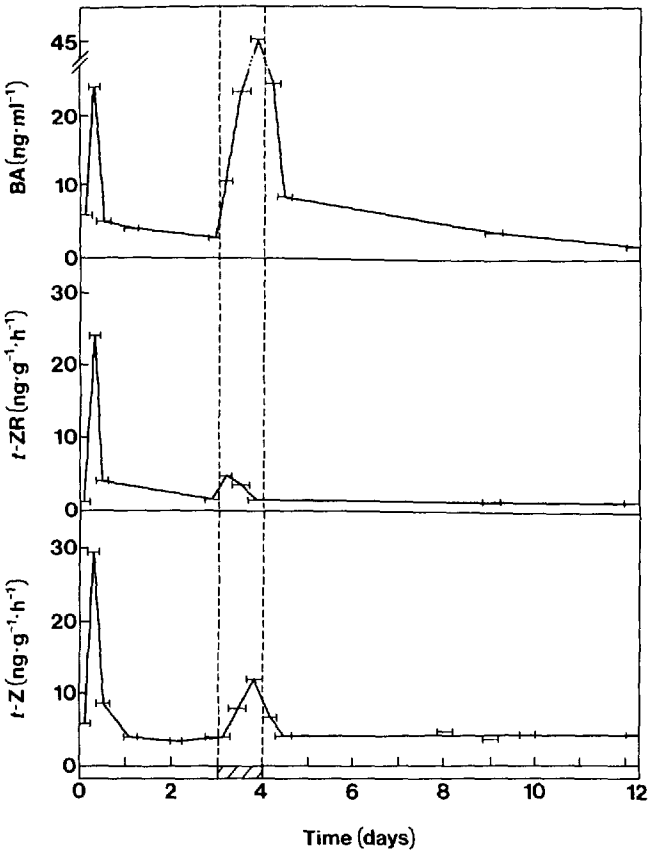

Fig. 4. Excretion of 6-benzylaminopurine (BA), *trans*-zeatin (*t*-Z), and its riboside (*t*-ZR) by immobilized cytokinin-dependent tobacco cells. Concentration of BA in the elution medium: 0–72h: 0 μM; 72–96h: 5 μM; 96–288h: 0 μM.

Discussion

Previous results (Kamínek and Luštinec 1974 a,b, Einset 1980) indicated that cytokinin-dependent cells are able to synthesize natural cytokinins. In our experiments we found relatively small differences in cytokinin content between cytokinin-autonomous and cytokinin-dependent tobacco cells. Cytokinin autotrophy, however, need not correlate with high cytokinin level (Inoue et al. 1979, Weiler and Spanier 1981). This feature cannot be explained just on the basis of differences in cytokinin concentration. Comparing our results with those reported by Einset (1980) and Morris et al. (1982), our tobacco strains contain relatively high levels of cytokinin. A possible explanation could be that the above-mentioned authors used tobacco callus tissue as a source whereas our biological material were rapidly growing cell suspensions. It is generally accepted that the level of cytokinins changes with growth activity (Wyndaele

et al. 1985). This explanation cannot, of course, be used for immobilized cells, the growth of which is suppressed. In this case high synthetic activity induced by the exponential growth during previous cultivation may be maintained by continual elution of cytokinins from the cell environment. Removal of the products could positively shift the equilibrium.

In accordance with results reported on maize roots (Van Staden 1976), we found more *t*-ZR than *t*-Z in tobacco cells (value for *t*-ZR also includes its mono-, di-, and triphosphates). The situation was opposite in the medium (more *t*-Z). Similar proportion *t*-Z and *t*-ZR were also found in cultivation medium of crown gall *Catharanthus roseus* (Palni 1984). The difference between cells and medium was probably caused by higher permeability of the cell membrane for the free base than for the riboside. This difference seems to be more evident in the case of immobilized cells, where continual elution with fresh medium supports cytokinin excretion.

Continuous elution enabled us to follow the effect of synthetic BA on excretion of the natural cytokinins *t*-Z and its riboside in cytokinin-dependent cells. Exogenous growth regulators can influence metabolism of cells in two basic ways: (1) to affect cell metabolism in the same way as the endogenous hormones, or (2) to effect the synthesis, translocation, or metabolic conversion of natural hormones (see Bruinsma 1980). An example of the latter mechanism may be the influence of 2,4-D on the cytokinin content in rice callus (Inoue et al. 1979) or on the IAA level in auxin-dependent callus of sugar beet (Kevers 1981). In this respect it is an interesting finding of Thomas and Katerman (1986) that thidiazuron, a powerful synthetic cytokinin (Mok et al. 1982), significantly increases content of natural purine cytokinins in soybean callus. This increase is probably related to the effect of urea-type cytokinins on cytokinin oxidase as reported by Laloue and Fox (1985) for wheat germ and by Chatfield and Armstrong (1986) for *Phaseolus vulgaris* tissue culture.

A similar mode of action can also be at least partly adopted for BA. Application of this synthetic regulator caused an increase in cytokinin levels in a cytokinin-dependent tobacco strain. A positive correlation between the amount of BA added and natural cytokinin concentration was found. So it seems probable that biological activity of BA at least partially depends on the stimulation of endogenous cytokinin synthesis. In more general terms, application of any exogenous cytokinin to cytokinin-dependent cells might switch on an autoinductive mechanism which increases synthesis and/or limits degradation of endogeneous cytokinins.

Our results show that immobilization techniques offer a unique advantage for the study of plant hormones. This method allows continuous monitoring of the production and excretion of metabolites and facilitates the following of the changes in their levels as a consequence of various chemical or physical factors. The latter possibility has been demonstrated using the synthetic cytokinin BA.

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