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Dynamics of Endogenous Cytokinins during the Growth Cycle of a Hormone-Autotrophic Genetic Tumor Line of Tobacco

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ABSTRACT

The profile of endogenous cytokinins in a genetic tumor line of tobacco, namely, *Nicotiana glauca* (Grah.) × *Nicotiana langsdorffii* (Weinm.), following 1 to 10 weeks of growth on solid medium was determined by radioimmunoassay. ^{3}H-labeled cytokinins of high specific activity were added during tissue extraction to correct for the purification losses. Following subculture (of 4-week-old tissues when their cytokinin content is high) onto fresh medium the total cytokinin content continued to be high during the first week (1470 picomoles per gram fresh weight) when the tissue fresh weight remained essentially unchanged (lag phase). The cytokinin levels then declined by about half in 2- and 3-week-old tissues (626 and 675 picomoles per gram fresh weight, respectively), a period when rapid increase in tissue fresh weight was recorded. Increments of 840% and 2780% over initial fresh weight were obtained in 2- and 3-week-old cultures, respectively. The cytokinin content then increased to initial high levels in 4-week-old tissues (1384 picomoles per gram fresh weight) after which it gradually declined with tissue age. The lowest cytokinin levels (432 picomoles per gram fresh weight) were observed in 10-week-old tissues. Maximal tissue fresh weight (4030% increase over initial fresh weight) was recorded in 5-week-old cultures after which it decreased slowly to 77.5% of the highest tissue fresh weight in 10-week-old cultures. Zeatin appeared to be the dominant endogenous cytokinin in tissues of all ages. Other cytokinins quantified were dihydrozeatin, zeatin riboside, and dihydrozeatin riboside; the values may include contributions from aglucones derived from the hydrolysis of corresponding O-glucosides, since the entire basic fraction was treated with β-glucosidase before analysis. In addition the levels of isopentenyladenine, isopentenyladenosine, and the nucleotides of zeatin riboside, dihydrozeatin riboside, and isopentenyladenosine were also determined.

Plant tissues normally require the addition of auxin and cytokinin to the basal medium for growth *in vitro*. However, axenic tissues obtained from crown gall or genetic tumors can be cultured on phytohormone-free nutrient medium. While a portion of the bacterial transferred-DNA (from *Agrobacterium tumefaciens*) is responsible for the synthesis of enzymes for auxin and cytokinin production in crown gall tissues (12, 20), the genetic tumors arise spontaneously on certain interspecific hybrids, particularly in *Nicotiana* spp., without any apparent external cause (9).

Phytohormone imbalance has also been implicated in the induction and maintenance of these tumors (3). A recent study from this laboratory has indicated that endogenous cytokinins may play a role in tumorigenesis in *Nicotiana* hybrids (16). The cytokinin complex of 3.5-week-old genetic tumor tissues of *Nicotiana glauca* × *langsdorffii* and *Nicotiana suaveolens* × *langsdorffii* has previously been identified and the individual cytokinins quantified using deuterium-labeled internal standards and GC-MS (15) (GJM de Klerk, in preparation). Since the cytokinin content of tissues is known to change substantially during culture (12), it was felt important to estimate the cytokinin content of at least one line of *in vitro* cultured genetic tumors of tobacco. Thus, tissues derived from *Nicotiana glauca* × *langsdorffii* hybrids have been examined for their cytokinin content over a 10-week period following subculture, and an attempt has been made to relate this with callus growth.

MATERIALS AND METHODS

Plant Material

Genetic tumor callus line of tobacco was initiated from crown tumors formed on *Nicotiana glauca* (Grah.) × *Nicotiana langsdorffii* (Weinm.) hybrid plants as reported earlier (21). Cultures were maintained in 250 mL Erlenmeyer flasks containing 100 mL of auxin and cytokinin free medium (10) solidified with 0.8% (w/v) agar. The tissues were kept at 26°C in the dark and subcultured every 4 weeks.

Chemicals and Enzymes

All chemicals were of 'AnalaR' grade and unless otherwise stated were purchased from Ajax Chemicals Pty. Ltd., Sydney, Australia. The following radioactive compounds were used to estimate recovery and/or to facilitate detection of appropriate fractions during TLC and HPLC: [8- ^{3}H](diH)Z[2] (1110 GBq/mmol), [2- ^{3}H]AMP (48 GBq/mmol); both from the Radiochemical Centre, Amersham, UK, and [8- ^{3}H](diH)[9R]Z

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[2] Abbreviations: (diH)Z, dihydrozeatin; RIA, radioimmunoassay; Z, zeatin; (diH OG)Z, *O*-glucoside of dihydrozeatin; iP, isopentenyladenine (their respective 9-β-D-ribosides are denoted by the prefix [9R]).

(189 GBq/mmol); synthesized by Dr. D. S. Letham. β-D-Glucosidase (EC 3.2.1.21, from sweet almonds) was purchased from Boehringer, Mannheim, FRG.

Determination of Growth Curve

For establishing a growth curve, three pieces (about 2 g fresh weight) of 4-week-old tissues were transferred into 250 mL Erlenmeyer flasks containing 100 mL of medium. The flasks were maintained in darkness at 26°C for up to 10 weeks. At the end of each week tissue was harvested from a minimum of five flasks and total callus yield from each flask determined. All manipulations were carried out under aseptic conditions, and the tissue from various flasks was gently mixed following harvest and an appropriate amount removed for subsequent estimation of cytokinin levels by RIA and for some other studies (15).

Estimation of Endogenous Cytokinins by RIA

For this study, tissue samples (10 g fresh weight; 1–10-week-old) were obtained as described above. Tissue extraction was carried out sequentially with chloroform: methanol: formic acid: water (5:12:1:2, v/v; 10 mL/g fresh weight, −20°C), methanol: formic acid: water (6:1:4, v/v; 10 mL/g, −20°C) and methanol: water (8:2, v/v; 10 mL/g fresh weight, 4°C) as described by Palni *et al.* (23). The extracts (1–10 weeks; prepared immediately after harvest) were stored at −20°C and all were subjected to further purification at the same time. The purification was carried out as detailed in Figure 1. The cytokinins were finally fractionated by HPLC and their levels determined by RIA using antibodies raised against [9R]Z, (diH)[9R]Z, and [9R]iP (1, 2). The assay was performed essentially as in the cited references; further details of assay conditions, and the method of recovery estimation of ^{3}H-labeled compounds and subsequent correction for purification losses of cytokinins, have been described by Nandi *et al.* (16).

The assays were done in triplicate. In the present investigation anti-[9R]Z-serum, anti-(diH)[9R]Z-serum, and anti-[9R]iP-serum were used to estimate the levels of [9R]Z and Z, (diH)[9R]Z and (diH)Z, and [9R]iP and iP, respectively. The determination of Z and (diH)Z in Z/(diH)Z fraction and that of [9R]Z and (diH)[9R]Z in [9R]Z/(diH)[9R]Z fraction (Fig. 1) was carried out by taking suitable aliquots (in triplicate) from these fractions obtained after purification on HPLC and/or C-18 Baker columns. Separate aliquots were assayed using antisera raised against [9R]Z or (diH)[9R]Z. It should be noted that (diH)Z and (diH)[9R]Z exhibit very low cross-reactivity (<2%) with anti-[9R]Z-serum used in this study. Similarly the cross-reactivity of Z and [9R]Z was also very low (<3.7%) with anti-(diH)[9R]Z-serum. The details of per cent cross-reactivity of naturally occurring cytokinins and other purines with the three antisera used have been reported previously (1, 2).

Chromatographic Procedures

TLC

Normal phase TLC was performed on layers of silica gel (0.25 or 0.5 mm thickness; $60PF_{254}$ Keiselgel; E. Merck, Darmstadt, FRG) developed in butan-1-ol: acetic acid: water (12:3:5, v/v).

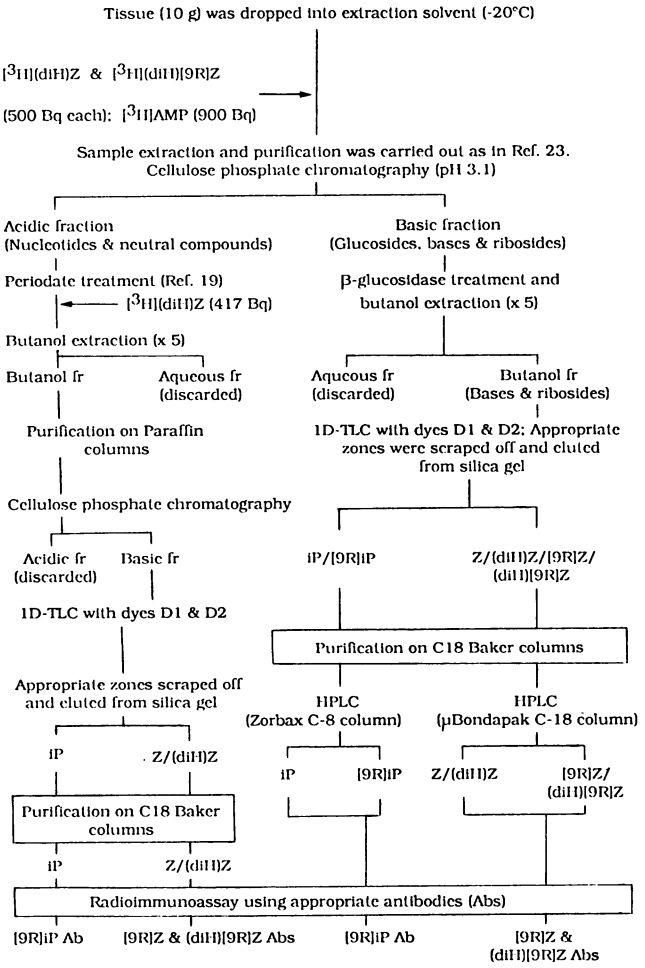

Figure 1. Summary of the extraction and purification procedure used for the identification and quantification of endogenous cytokinins in cultured genetic tumor tissue by RIA.

Two dyes, namely Rhodamine B (Hopkins and Williams, Chadwell Heath, Essex, UK; designated D1) and Drimarine brilliant blue-KBL (Polysciences, Washington, PA; designated D2) were used as markers to locate zones of interest during purification of cytokinins by TLC. One-cm zone above D1 would contain iP and [9R]iP while the zone between dyes D1 and D2 would contain [9R]Z, (diH)[9R]Z, Z, and (diH)Z. The cytokinins were eluted from silica gel with methanol: water: acetic acid (8:8:1, v/v).

HPLC

HPLC was performed with equipment supplied by Waters Associates (Milford, MA) and details have been described elsewhere (22). The samples were purified either on a Zorbax C-8 semipreparative column (9.4 × 250 mm; Du Pont) eluted isocratically with 70% methanol (v/v) at the flow rate of 4 mL/min, or on a μBondapak C-18 column (7.8 × 300 mm; Waters) eluted isocratically with 20% methanol (v/v) at the flow rate of 3.0 mL/min. All solvents (containing 1% acetic acid, v/v) were HPLC grade and were filtered before use.

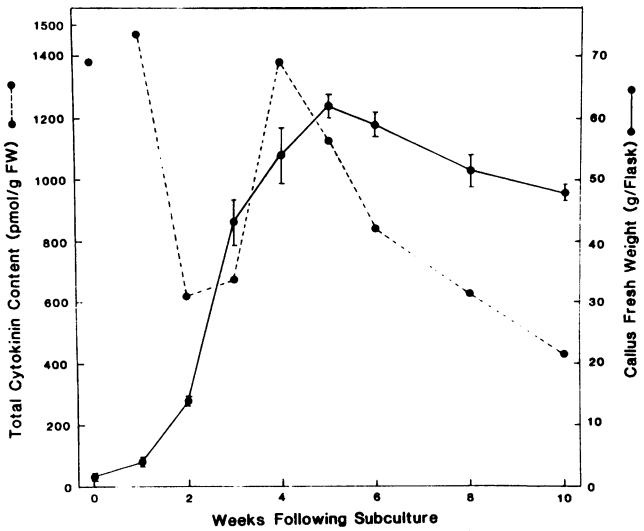

Figure 2. Changes in the total cytokinin content in relation to growth of cultured genetic tumor tissue of tobacco *N. glauca* × *langsdorffii*. Fresh weight data were obtained as described in the text and values for total cytokinin content have been taken from Table I.

Other Chromatographic Procedures

Details of chromatographic procedures for purification of cytokinins using paraffin-coated, silica gel columns and Baker columns (Fig. 1) have been described earlier (6, 8).

Enzymatic Hydrolysis and Chemical Treatment

The entire basic fraction obtained after cellulose phosphate chromatography, which would include *O*-glucosides of Z, (diH)Z, [9R]Z, and (diH)[9R]Z (Fig. 1) was treated with β-glucosidase to hydrolyze these cytokinin *O*-glucosides to their respective aglucones as described earlier by Palni and Horgan (18). The 5′-nucleotides of purines, including those of cytokinins, were degraded chemically to the corresponding bases using sodium periodate and cyclohexylamine (19).

RESULTS

Growth Curve

Figure 2 shows the growth of *N. glauca* × *langsdorffii* genetic tumor tissue following subculture onto fresh medium. The callus growth, measured in terms of increase in fresh weight, showed little increase in fresh weight during the first week (lag phase), after which rapid growth occurred. Increments of 840% and 2780% over initial fresh weight were recorded in 2- and 3-week-old cultures. Maximum fresh weight (4030% increase over initial fresh weight) was achieved 5 weeks following subculture, after which it decreased gradually to about 77.5% of the highest callus fresh weight in 10-week-old cultures. This decline appears to be associated with tissue drying and depletion of nutrients in the medium.

Endogenous Cytokinins

Table I shows RIA results indicating the levels of various cytokinins in tobacco genetic tumor tissue at different times after subculture; Figure 2 shows total cytokinin content in relation to tissue growth. The cytokinin content of starting tissue (0-week-old) was not estimated; 4-week-old tissue was used for subculture and therefore the values obtained for 4-week-old tissues should reflect the cytokinin levels at the time of subculture. Zeatin appears to be the dominant endogenous cytokinin in tissues of all ages. Other cytokinins quantified were (diH)Z, [9R]Z, (diH)[9R]Z, iP, [9R]iP, and the nucleotides of [9R]Z, (diH)[9R]Z, and [9R]iP. In this study the cytokinin-*O*-glucosides were not quantified separately; the entire basic fraction obtained after cellulose phosphate chromatography was treated with β-glucosidase (Fig. 1). Therefore, the values quoted in Table I for Z, (diH)Z, [9R]Z, and (diH)[9R]Z may include contributions from the aglucones derived from the hydrolysis of respective *O*-glucosides. In keeping with previous reports only small amounts of [9R]iP were found. The nucleotide levels were low at all times, with the possible exception of [9R]iP nucleotide. It is interesting to note that (diH)[9R]Z and [9R]iP were detected in *N. glauca*

Table I. *Cytokinin Content of Cultured Genetic Tumor Tissues of Tobacco (N. glauca × langsdorffii) at Various Times after Subculture*
The determinations were carried out by RIA. The assays were done in triplicate. Results are an average of the two closest values.

Tissue Age	Cytokinin Content									Total Cytokinin Content	Recovery of Labeled Compounds			
	Bases and ribosides (including *O*-glucosides)						Nucleotides[a]				Bases	Ribosides	Nucleotides	
	Z[b]	(diH)Z[b]	[9R]Z[b]	(diH) [9R]Z[b]	iP	[9R] iP	Z	(diH)Z	iP		(diH)Z	(diH) [9R] Z	AMP	(diH)Z
wk	*pmol/g fresh wt*										%			
1	473.2	173.9	92.8	119.7	141.9	7.5	150.9	37.0	272.9	1469.8	11.5	31.0	59.7	40.8
2	168.0	64.5	51.1	73.6	110.8	5.2	37.0	10.2	105.1	625.5	24.0	44.1	81.9	41.5
3	189.3	56.5	72.4	86.1	88.3	16.4	13.8	18.0	133.9	674.7	32.3	50.4	89.0	45.8
4	717.4	162.9	65.9	78.6	199.8	21.3	16.3	19.0	102.4	1383.6	19.0	37.5	85.8	51.3
5	640.0	78.3	24.6	42.2	234.4	3.8	18.5	15.5	73.4	1130.7	17.1	47.2	90.2	66.2
6	340.0	87.0	14.3	34.1	204.5	5.1	13.4	17.4	123.7	839.5	22.5	56.4	82.7	52.7
8	216.4	86.6	17.9	36.4	112.3	5.5	10.8	21.2	122.7	629.8	21.7	58.0	83.1	47.9
10	143.4	59.1	37.0	30.0	25.8	3.9	10.6	21.7	100.6	432.1	31.6	46.3	74.9	50.0

[a] Nucleotides were analyzed after chemical degradation to respective bases. [b] Values include aglucones derived from the hydrolysis of corresponding *O*-glucosyl derivatives.

× *langsdorffii* by RIA, whereas these compounds were not identified in extracts of 3.5-week-old tissue by GC-MS analysis (15). The level of [9R]iP quantified by RIA was quite low, but (diH)[9R]Z was detected in substantial amounts (Table I). It is possible that (diH)[9R]Z was derived from the hydrolysis of corresponding *O*-glucoside since (diH OG) [9R]Z was found as an endogenous compound in this tissue by GC-MS (15).

It can be observed from Table I that following subculture onto fresh medium the total cytokinin content remained unchanged during the lag phase (note similar levels in 0- and 1-week-old tissues; Fig. 2). However, after 2nd and 3rd week of culture, a period of rapid increase in fresh weight, the cytokinin levels declined by about half. After this the endogenous cytokinin content increased rapidly reaching a high value at week 4 (1384 picomoles per gram fresh weight), 1 week before maximum fresh weight was recorded. By week 5 the cytokinin levels had already started to decline gradually (1131 picomoles per gram fresh weight), and reached about half maximum values by week 6. The cytokinin content continued to decrease in essentially ageing tissues, and lowest endogenous levels (432 picomoles per gram fresh weight; less than one third of maximum value) were observed for 10-week-old tissues. The results of this study show that the endogenous cytokinin levels reached high values immediately after the period of most rapid increase in fresh weight; the tissue fresh weight, however, continued to show a gradual increase up to week 5 when the cytokinin levels had already started to decrease (Fig. 2). The most rapid increase in total cell number per flask occurred between week 2 and 3 (coinciding with the time of most rapid increase in fresh weight) after which the total cell number remained nearly constant up to week 6 and then gradually declined (15). However, the total fresh weight continued to increase up to week 5 (Fig. 2); the increase in fresh weight after week 3 is due largely to cell enlargement rather than cell division. This is supported by the data on cell size; initially in 1- to 3-week-old tissues there were more smaller cells whereas later in the culture period (4-week and older tissues) the percentage of larger cells increased (15).

During tissue extraction high specific activity [^{3}H](diH)Z and [^{3}H](diH)[9R]Z were added as recovery markers. Since radiolabelled cytokinin nucleotide was not available, [^{3}H] AMP was used for the recovery estimation of nucleotides up to the point of periodate treatment. [^{3}H]Dihydrozeatin was then added to the periodate-treated fraction before butanol extraction (Fig. 1). The per cent recovery of all ^{3}H-labeled compounds added during purification is shown in Table I. The recovery of [^{3}H](diH)Z and [^{3}H](diH)[9R]Z was used to correct for the purification losses of all bases and ribosides, respectively, while the recovery of [^{3}H]AMP and [^{3}H](diH)Z was used to correct for the purification losses of all nucleotides. As expected the recovery of cytokinin bases was lower than that of ribosides.

DISCUSSION

The RIA data indicate that *in vitro* cultured genetic tumor tissues of *N. glauca* × *langsdorffii* produce a number of endogenous cytokinins. These include iP, Z, (diH)Z, and their respective ribosides and nucleotides; Z would appear to be the dominant endogenous cytokinin in this tissue. Substantial amounts of (diH)[9R]Z were also detected by RIA in tissues of all ages; these may have possibly resulted from (diH OG)[9R]Z, since the complete basic fraction was treated with β-glucosidase before analysis. It should be noted that (diH)[9R]Z was not identified in extracts of 3.5-week-old tissue by GC-MS in a separate study (15). High levels of Z and (diH)Z cited in Table I may also include contributions from the hydrolysis of corresponding *O*-glucosides. *O*-Glucosides of Z, (diH)Z, [9R]Z, and (diH)[9R]Z have previously been detected in this tissue by GC-MS (15). RIA is known to be a very sensitive method; however, proper sample purification has to be done for reliable identification and quantification. Keeping this in mind it is relevant to discuss some problems commonly associated with such studies. First, [^{3}H](diH)Z and [^{3}H](diH)[9R]Z were used as recovery markers to correct for purification losses of all cytokinin bases and ribosides, respectively (see Table I), and the same approach was also taken for nucleotides (see Materials and Methods). It is well known that substantial and varied losses of individual cytokinins occur during purification. Thus the values quoted for individual cytokinins in Table I would be subject to slight error. There are, however, a number of reports in the literature where recovery markers have not been used (*e.g.* refs. 26 and 28). As purification losses could not be calculated, the reported values must be considerable underestimates in the cited studies. Second, fractional separation of closely related cytokinins by chromatographic techniques may also add to error in estimating levels. In this study final sample purification was carried out by HPLC, and fractions containing 'coeluting' [9R]Z and (diH)[9R]Z were collected for RIA. These fractions were identified by radioactivity (and not by UV) due to [^{3}H](diH)[9R]Z added initially. To avoid the possibility of fractional separation of [9R]Z from (diH)[9R]Z during HPLC, which otherwise may result in partial loss and thus underestimation of [9R]Z, a relatively broad fraction was collected during HPLC. The same also holds true for 'coeluting' Z and (diH)Z fractions. It should be noted that during HPLC fractional separation of plant growth substances from their labelled analogues has been reported (4, 5).

Although there are a few kinetic studies of endogenous cytokinins in cultured tissues (11, 14, 17, 25, 27, 28), they can not be directly compared with the present investigation for a number of reasons. (a) Most of the studies have been carried out with suspension cultured cells (11, 14, 27, 28) including partially synchronized cultures (17). (b) The cytokinin estimation in a number of these studies was confined to only a few days after subculture, usually less than 18 days. (c) In none of these studies (11, 14, 17, 25, 27, 28) was the entire cytokinin complex determined, usually the estimations have been confined only to one or two individual cytokinins. (d) Many of the reported studies are based on bioassay estimations (11, 17, 25). (e) No attention has been given to purification losses which can be varied and substantial; with one exception (14) all other studies have not used recovery markers. (f) The identity of cytokinins was not established by unequivocal methods. The present study would appear to be different from other investigations because an attempt has

been made to estimate all endogenous cytokinins (their identity having been established previously by GC-MS [15]) during 1–10 weeks' growth.

The genetic tumor tissues derived from *N. glauca* × *langsdorffii* do not appear to contain particularly high endogenous cytokinin levels. Cytokinin-independent tissues on the other hand contain even lower amounts of cytokinin. For example, using bioassay, [9R]Z content (kinetin equivalents) of tobacco crown gall cells was estimated to be 40 to 80 ng/g fresh weight and that of habituated cells to be less than 0.1 ng/g fresh weight (13). More recently, Scott and Horgan (24) found that the total cytokinin content of 3-week-old tobacco crown gall callus tissue was about 1520 picomoles per gram fresh weight (675 picomoles per gram fresh weight in 3-week-old genetic tumor tissue; Table I), whereas in autonomous, normal tissue the reported values appeared to be at least 100-fold lower (and barely detectable). Furthermore, Hansen *et al.* (7), using HPLC and immunoassay, were unable to detect the presence of any cytokinins in a cytokinin-independent, normal tobacco cell line.

It can be seen from Figure 2 that high cytokinin levels were found immediately following the period of most rapid increase in fresh weight. This, however, is at slight variance with reports on growing cultures of sycamore (11), pea (25), tobacco (17), and some crown gall cultures (7, 14, 26, 28) which reported high levels during the early part of exponential growth; the levels had declined considerably well before maximal fresh weight was recorded, and continued to decline through the stationary phase. The observed differences could be attributed to a number of reasons as described previously. A somewhat different profile of cytokinin accumulation was observed for crown gall cells of *Beta vulgaris* in which the cytokinin levels peaked toward the end of growth (27) as in the present study. The endogenous cytokinin content of a tissue at any given time will depend on the rates of biosynthesis and utilization (degradation). In this context relatively high cytokinin levels observed at week 4 may have resulted from higher rates of cytokinin biosynthesis. This is in accord with the observations of Palni *et al.* (23) who found that in *Datura innoxia* crown gall tissue maximal [^{14}C]adenine incorporation into cytokinins coincided with the time of rapid increase in callus fresh weight after which the rate of cytokinin biosynthesis declined rapidly. This may explain the decline in total endogenous cytokinin levels observed in this tissue after week 4 (Fig. 2). Relatively low levels observed in 2- and 3-week-old tissues may reflect lower rates of biosynthesis and much higher rates of utilization.

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