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Metabolism of adenine and hypoxanthine in a hormone autonomous genetic tumour line of tobacco

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Abstract

Genetic tumour tissues of *Nicotiana glauca* (Graham) × *N. langsdorffii* (Weinm.), which grow on auxin and cytokinin-free medium, were incubated with [^{14}C]-/[^{3}H]-adenine or [^{3}H]-hypoxanthine to investigate cytokinin biosynthesis. Adenine was supplied to tissues of two different ages (2- and 3.5-week-old) for 8, 24 or 30 h. The uptake was over 91.0 % (of "supplied radioactivity") by 2-week-old tissues as compared to around 50.0 % uptake by 3.5-week-old tissues. Incorporation into cytokinins could not be detected. While unmetabolized adenine accounted for only about 24.0 and 13.4 % of "extracted radioactivity" (following 8 and 30 h incubation, respectively) in 2-week-old tissues, relatively higher levels, *i.e.* 36.0 and 34.5 % (following 8 and 24 h incubation, respectively) were present in 3.5-week-old tissues. The metabolites formed were adenosine and its nucleotides (4.5 - 16.5 % and 37.4 - 60.2 % of the extracted radioactivity, respectively). Hypoxanthine was supplied to 3.5-week-old tissues for 8 and 24 h. While the uptake was low (< 28.0 % of supplied radioactivity), the major proportion of extracted radioactivity was due to unmetabolized hypoxanthine (79.8 % and 85.9 % after 8 and 24 h incubation periods, respectively); the minor metabolites were inosine and adenosine (both < 0.5 %) and their nucleotides (< 3.5 %). Radioactivity incorporation into cytokinins from hypoxanthine was not detected. Thus in the present investigations precursor incorporation from either adenine or hypoxanthine into cytokinins could not be demonstrated. It is possible that this may be due to slow rate of cytokinin turnover in these tissues.

Additional key words: biosynthesis, cytokinin, genetic tumour, *Nicotiana* hybrid.

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Abbreviations: Ade - adenine; Ado - adenosine; (diH)Z - dihydrozeatin; (diH) [9R]Z - dihydrozeatin riboside; HPLC - high performance liquid chromatography; Hyp - hypoxanthine; Ino - inosine or hypoxanthine riboside; iP - isopentenyladenine; [9R]iP - isopentenyladenosine; TLC - thin-layer chromatography; Z - zeatin; [9R]Z - zeatin riboside; [7G]Z - zeatin-7-glucoside; [9G]Z - zeatin-9-glucoside.

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Introduction

Genetic tumours arise spontaneously, without any apparent external cause, in certain interspecific hybrids, particularly in *Nicotiana* spp., and can be maintained indefinitely without the addition of auxin and cytokinin to the culture medium. Phytohormone imbalance has been implicated in the induction and maintenance of these tumours (Bayer 1982) and recent evidence suggests that cytokinins may be involved in tumour formation in *Nicotiana* hybrids (Kung 1989, 1991, Nandi *et al.* 1990). A number of cytokinins have been identified and quantified unambiguously in cultured genetic tumour tissues of *N. glauca* × *N. langsdorffii* (Nandi *et al.* 1990) but the levels are relatively low in comparison to crown gall tissues (Palni *et al.* 1983b). It is well known that crown gall tumour tissues can also be cultured on phytohormone-free nutrient medium but differ from genetic tumour tissues as the former contain a portion of the transferred-DNA (from *Agrobacterium tumefaciens*) which is responsible for the synthesis of enzymes for auxin and cytokinin production. The pathway of cytokinin biosynthesis has been elucidated in crown gall tissues by precursor (using both radioactive and heavy isotope labelled adenine) incorporation studies (Palni *et al.* 1983a, 1987). However, nothing is known about the biosynthesis of cytokinins in cultured genetic tumour tissues.

Therefore, it seemed desirable to investigate cytokinin biosynthesis by precursor incorporation studies using a cultured genetic tumour line of tobacco, namely *Nicotiana glauca* × *langsdorffii*.

Materials and methods

Plant material: Genetic tumour tissues of tobacco was initiated from crown tumours formed on *Nicotiana glauca* (Grah.) × *N. langsdorffii* (Weinm.) hybrid plants. Cultures were maintained in 250 cm^3 Erlenmeyer flasks containing 100 cm^3 of auxin and cytokinin-free Linsmaier and Skoog's (LS) medium (Linsmaier and Skoog 1965) solidified with 0.8 % (m/v) agar. The cultures were kept at 26 °C in the dark and subcultured every 4 weeks (Nandi *et al.* 1990). Two- and 3.5-week-old actively growing tissues were used.

Chemicals and enzymes: The cytokinin standards [iP, Z, (*cis*)Z, (diH)Z and their corresponding 9-β-D ribosides], adenine (Ade), adenosine (Ado), inosine (Ino) and hypoxanthine (Hyp) were obtained from *Sigma Chemical Co.*, St. Louis, USA. All other chemicals were of 'AnalaR' grade and unless otherwise stated were purchased from *Ajax Chemicals Pty. Ltd.*, Sydney, Australia. In addition, the following radioactive compounds (source: *Radiochemical Centre*, Amersham, UK) were used for incubation studies: [2-^{3}H]-Ade (1036 GBq $mmol^{-1}$), [U-^{14}C]-Ade (11 GBq $mmol^{-1}$) and [G-^{3}H]-Hyp (85.1 GBq $mmol^{-1}$). The source and the procedure of enzymatic hydrolysis by alkaline phosphatase (EC 3.1.3.1, Type III-S from *Escherichia coli*) have been reported earlier (Nandi *et al.* 1990).

Incubation of tissues with radiolabelled compounds:
1) Adenine as the precursor: Tissue (2-week-old; 2 g) was incubated in plastic Petri dishes (3 cm diameter) containing 2 cm^3 LS medium supplemented with [U-^{14}C]-Ade (200 kBq). In another experiment, 3.5-week-old tissue (10 g) was incubated in 50 cm^3 Erlenmeyer flasks containing 10 cm^3 LS medium supplemented with [2-^{3}H]-Ade (57 kBq).
2) Hypoxanthine as the precursor: Tissue (3.5-week-old; 20 g) was incubated for 8 and 24 h in 50 cm^3 Erlenmeyer flasks containing 20 cm^3 LS medium supplemented with [G-^{3}H]-Hyp (190 kBq).

The flasks or Petri dishes were placed on a reciprocating shaker (60 rpm) in darkness at 26 °C. Following incubation the tissues were washed with fresh medium, extracted and analyzed as described below. The metabolites remaining in the medium and washings were pooled and then analyzed.

Extraction and purification of metabolites: The detailed procedure has been described earlier (Nandi *et al.* 1990) and is summarised in Fig. 1. During tissue homogenization 100 μg each of the following internal standards (unlabelled) were added: Z, [9R]Z, iP, [9R]iP, Ade, Ado, AMP (and Hyp and Ino for ^{3}H-Hyp feeding studies) in order to correct for the purification losses following various chromatographic analyses.

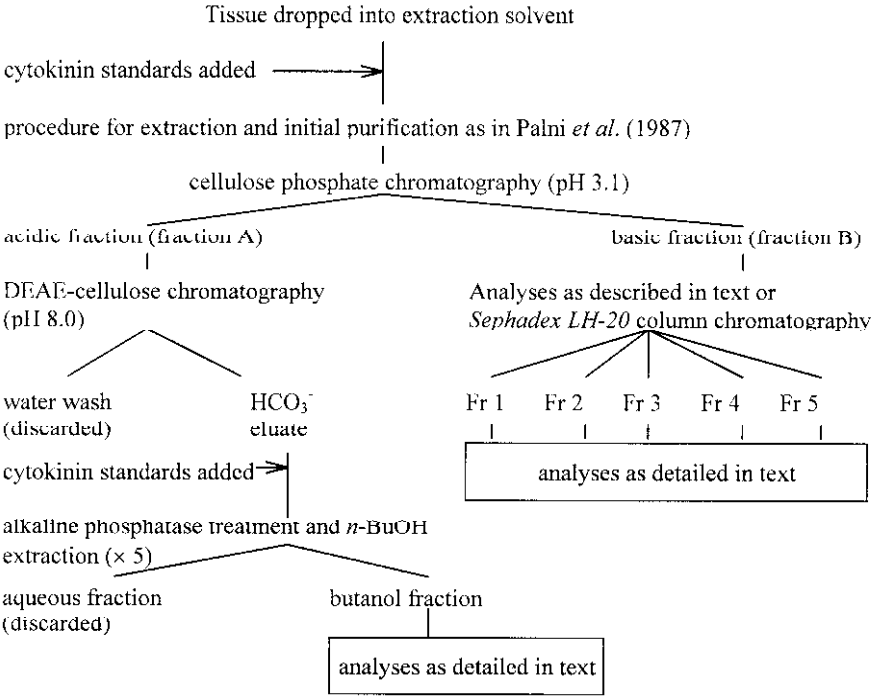

Fig. 1. Summary of the purification steps in the analysis of various metabolites.

Chromatographic procedures: *Sephadex LH-20* chromatography was done on a column (80 × 2.5 cm) of *Sephadex LH-20* (bead size 25 - 100 μm; *Pharmacia,* Uppsala, Sweden) eluted continually with 35 % ethanol at 30 cm^3 h^{-1} (Stuchbury *et al.* 1979). Normal phase thin layer chromatography (TLC) was carried out either on layers of silica gel (0.25 or 0.5 mm thickness, *60 PF_{254} Keiselgel, E. Merck,* Darmstadt, Germany) using methanol:chloroform (1:9, v/v) plus a trace of ammonia - system A, or on layers of cellulose (0.25 or 0.5 mm; *Serva, Feinbiochemica,* Heidelberg, Germany with a fluorescent indicator PF_{254}) using *n*-butanol:acetic acid:water (12:3:5, by volume) - system B. Two-dimensional TLC analysis was carried out on silica gel (0.25 or 0.5 mm) plates first developed in the 1st dimension with *n*-butanol:acetic acid:water (12:3:5), allowed to air dry and then developed in the 2nd dimension with *n*-butanol:14 M ammonia:water (6:2:1, by volume, upper phase) - system C. High Performance Liquid Chromatography (HPLC) was carried out with equipment supplied by *Waters Associates* (Milford, USA). The samples were analysed using a *Zorbax C-8* semipreparative column (9.4 × 250 mm; *Du Pont*) and eluted isocratically with 25 % methanol (flow rate 4 cm^3 min^{-1}). All solvents (containing 1 % acetic acid, v/v) were HPLC grade and were filtered before use.

Results

Adenine as the precursor: The uptake of Ade was higher (91.0 and 96.9 % of supplied) by 2-week-old tissues compared to 50.5 and 54.4 % by 3.5-week-old tissues, but nearly the same amount of radioactivity was extracted (as % of radioactivity taken up) in both cases (Table 1). After incubation, the tissues were extracted and purified (Fig. 1). The basic fraction (Fr B; containing bases, ribosides and glucosides) and the acidic fraction (Fr A; containing nucleotides and neutral compounds) were analyzed as follows.

Analysis of fraction B: A small portion of this fraction was analyzed by 2D-TLC

Table 1. Uptake and distribution of radioactivity during the initial purification steps and in various metabolites following incubation of cultured genetic tumour tissues of tobacco with [U-^{14}C]-Ade (2-week-old tissue) or [2-^{3}H]-Ade (3.5-week-old tissue). The metabolites were identified by D-TLC.

Tissue age [weeks]	2-week-old		3.5-week-old	
Time of incubation [h]	8	30	8	30
Radioactivity supplied [kBq]	200.00	200.00	57.00	57.00
Uptake[a]	90.98	96.90	54.40	50.50
Extracted radioactivity[b]	50.15	38.30	50.00	46.25
Acidic fraction[c]	64.40	67.10	61.70	42.00
Basic fraction[c]	35.20	21.50	38.20	57.50
Ade[c]	24.00	13.35	36.00	34.53
Ado[c]	9.00	4.54	5.61	16.45
Ado ntd[c]	60.20	59.81	54.86	37.37

[a] - [% of radioactivity supplied]; [b] - [% of radioactivity taken up]; [c] - [% of extracted radioactivity]; Z, [9R]Z and [9R]Z were not detected (ntd).

(system C). Only about 1 % of radioactivity applied to the TLC plate, co-chromatographed with Z and [9R]Z standards in samples from 2-week-old tissues only, while all the remaining radioactivity was associated with unmetabolized Ade and Ado (Table 1). The remaining portions of 8 and 30 h samples (2-week-old tissue) were purified by 1D-TLC (system B) after the addition of (*cis*)Z and (*cis*) [9R]Z standards. Areas of the plate co-chromatographing with Z and [9R]Z standards were eluted and further analyzed by 1D-TLC (system A) which resolved *cis* and *trans* isomers of Z and [9R]Z: Rf values: (*cis*)Z = 0.42, Z = 0.34, (*cis*) [9R]Z = 0.30 and [9R]Z = 0.26. However, radioactivity was not associated with any of these compounds.

Analysis of fraction A: This fraction was further purified on a column of DEAE-cellulose and 50 μg of [9R]Z was added as an internal standard; then it was treated with alkaline phosphatase and extracted with *n*-butanol. Upon analysis of the butanol fraction by 2D-TLC (as in fraction B), the major portion of the radioactivity, derived from the corresponding nucleotide(s), was found to co-chromatograph with Ado (Table 1).

It was observed that labelled Ade was not incorporated into cytokinins (Table 1). The major proportion of Ade remained unmetabolized, accounting for about 24.0 and 13.4 % of extracted radioactivity (8 and 30 h incubation, respectively) in 2-week-old tissues but higher amounts, *i.e.* 36.0 and 34.5 % was found in 3.5-week-old tissues. The predominant metabolite formed was Ado nucleotide(s) representing 60.2 % of extracted radioactivity in 2-week-old tissues but a lower amount (54.9 and 37.4 % following 8 and 24 h incubation, respectively) was detected from 3.5-week-old tissues. The other metabolite, Ado was formed in levels between 4.5 - 16.5 % of extracted radioactivity, in both the tissues (Table 1).

Hypoxanthine as the precursor: The uptake was only 23.2 % and 27.9 % of supplied radioactivity following 8 and 24 h incubation of tissues with [^{3}H]-Hyp (Table 2). The tissues were purified and the basic fraction (Fr B) and the acidic fraction (Fr A) were analyzed as follows.

Analysis of fraction B: This fraction was subjected to *Sephadex LH-20* column chromatography (Stuchbury *et al.* 1979) and fractions containing radioactivity were appropriately combined as follows: Fr 1 - 270 - 390 cm^3, elution volume of Hyp/Ino/cytokinin glucosides; Fr 2 - 420 - 510 cm^3, elution volume of [9R]Z/(diH)[9R]Z/Ado; Fr 3 - 540 - 630 cm^3, elution volume of Z/(diH)Z/Ade; Fr 4 - 660 - 750 cm^3, elution volume of [9R]iP; Fr 5 - 840 - 930 cm^3, elution volume of iP.

Fr 1: A small portion of this fraction was analyzed by *2D-TLC* (system C) after the addition of Z, [9R]Z, Ade and Ado standards. Almost all the radioactivity co-chromatographed with Hyp standard and no radioactivity was associated with cytokinin glucosides. The remaining portion was analyzed by *2D-TLC* (system C) after the addition of Z and [9R]Z standards. Areas of the plate corresponding to Z, [9R]Z, Ino and Hyp standards were scraped off and directly counted for radioactivity. No radioactivity was associated with Z and [9R]Z, however, the major proportion of radioactivity was associated with Hyp standard and less than 0.3 % with Ino (Table 2).

Table 2. The distribution of radioactivity in various metabolites following incubation of 3.5-week-old cultured genetic tumour tissues of tobacco with 190 kBq [G-^{3}H]-Hyp. The results are based on 2D-TLC analyses.

Time of incubation [h]	8	24
Uptake[a]	23.15	27.89
Extracted radioactivity[b]	98.50	99.30
Acidic fraction[c]	11.23	19.60
Basic fraction[c]	88.77	80.40
Hyp[c]	85.93	79.75
Ino[c]	0.19	0.25
Ado[c]	0.27	0.40
Ado ntd[c]	0.63	0.76
Ino ntd[c]	2.80	2.16

a, b, c - as described in Table 1; Ade, Z, [9R]Z and [9R]Z ntd were not detected.

Fr 1 was also subjected to HPLC and 72 % of applied radioactivity eluted at the retention time (Rt) of Hyp (Rt = 3.75 min) while the remaining (28 %) was associated with Ado (Rt = 5.4 min; results not shown). But, upon further HPLC analysis of the latter fraction, all the radioactivity was found to co-chromatograph with Hyp standard.

Fr 2 and Fr 3: These fractions were analyzed separately by *2D-TLC* (system C) after the addition of standards which they lacked. Only a small amount of radioactivity was associated with Ado standard in Fr 2, and radioactivity was not associated with either [9R]Z/(diH)[9R]Z or Z/(diH)Z (Table 2). A small amount of radioactivity was present in Fr 2 and 3 and was due to [^{3}H]-Hyp which was carried over from Fr 1 during *Sephadex LH-20* column chromatography.

Fr 4 and Fr 5: Since radioactivity was not associated with compounds eluting in this region, no further analysis was attempted.

Analysis of fraction A. After purification of this fraction through a DEAE-cellulose column, some internal standards (Ino and [9R]Z, 50 μg each) were added. Following alkaline phosphatase treatment and butanol extraction, a small portion of the butanol fraction (containing ribosides) was analyzed by 2D-TLC (as in fraction B) after the addition of standards (Z, [9R]Z, Ade, Ado, Hyp and Ino; 10 μg each). Radioactivity was associated only with Ado and Ino standards indicating formation of the corresponding nucleotides.

The major proportion of extracted Hyp (79.8 % and above) remained unmetabolized (Table 2). Only a small fraction of radioactivity (< 1 % of extracted) was found associated with Ado or Ino, while 3.4 and 2.9 % (following 8 and 24 h incubation, respectively) of the extracted radioactivity was associated with their nucleotides. Radioactivity was not incorporated into any of the cytokinins.

Discussion

In this study with cultured genetic tumour tissues of two different ages incorporation of Ade or Hyp into cytokinins could not be demonstrated. Although cytokinin biosynthesis via tRNA has been reported (Gray *et al.* 1996 and the references cited therein), many *in vitro* and *in vivo* experiments indicate that plant tissues contain enzyme(s) for the direct synthesis of free cytokinins, independent of tRNA turnover (Hommes *et al.* 1985, Taya *et al.* 1978). Ade incorporation into free cytokinins occurs in a few cultured plant tissues, particularly crown gall (Palni *et al.* 1983a, 1987, Stuchbury *et al.* 1979) and cytokinin nucleotides appear to be the primary products of biosynthesis. More recently Blackwell and Horgan (1994) reported synthesis of isopentenyladenosine-5-monophosphate and isopentenyladenosine in cell free extracts of immature *Zea mays* kernels incubated with [^{3}H]-AMP and dimethylallyl pyrophosphate. Although uptake of radioactive Ade was found to be higher and rapid in younger tissues (Table 1), incorporation into cytokinins could not be detected. The formation of Ado nucleotide(s) as the dominant metabolite of Ade indicates that this metabolite presumably results from the action of adenine phosphoribosyltransferase which catalyzes the first step in utilization of free purines by tissues (Hirose and Ashihara 1983b). Some of the interconversion reactions may also be analogous for other cytokinins, have been discussed in a recent review (Van Staden and Crouch 1996). The lack of Ade incorporation into cytokinins has been frequently reported in many plant tissues (Van Staden and Vos 1989 and references therein), including cultured tissues (Forsyth and Van Staden 1986, Palni *et al.* 1983a). While [^{14}C]-Ade incorporation, at very low levels, into cytokinins has been reported by young leaves of tobacco, fully expanded green leaves and senescing leaves exhibited no such incorporation (Singh *et al.* 1992). A relatively short incubation period was chosen in this study of cytokinin biosynthesis using Ade as precursor because of reported rapid cytokinin turnover in tobacco cells (Nishinari and Syono 1980) and numerous problems associated with long incubation periods in biosynthesis experiments, as Ade plays a central role in cellular metabolism (Stuchbury *et al.* 1979). The different specific activities of radiolabelled Ade used in the incubation experiments might have slightly influenced uptake and/or incorporation.

The possibility of cytokinin biosynthesis via the purine salvage pathway was investigated. Since hypoxanthine riboside 5'-phosphoric acid (IMP) is the precursor of AMP in the purine biosynthesis pathway, several studies of Hyp metabolism in plant tissues suggest that Hyp is salvaged for IMP (Ashihara 1983, Hirose and Ashihara 1983a). Hyp has been shown to be a precursor of [9R]Z in *Rhizopogon roseolus* cultures (Miura and Miller 1969). Yet following incubation of genetic tumour tissues with [^{3}H]-Hyp, radioactivity was not found associated with any of the cytokinins; a lack of incorporation of Hyp into cytokinins was also found in germinating corn seeds (Hocart 1985).

In addition to the purine salvage pathway, a degradative pathway operates within plant tissues where purine bases are degraded to allantoin, allantoic acid and carbon dioxide. These degradative metabolites of Ade or Hyp were not identified, and it is

possible that some of the radioactivity associated with the unknown metabolites could be due to these compounds. While a considerable proportion (> 20 %) of labelled Hyp was found to be salvaged for the nucleotides (Hirose and Ashihara 1983a), a major proportion of supplied Hyp is generally degraded to ureides which are the major organic nitrogen compounds present in a wide variety of plants (Ashihara 1983, Umesh Kumar and Montalbini 1994). Extremely low levels of radioactivity associated with Ino or Ado nucleotides, and high levels of unmetabolized Hyp observed in this study indicate that Ade salvage pathway is more active than the Hyp salvage pathway as also reported for several other plant systems (Hirose and Ashihara 1983a,b, 1984). It would also appear that the Hyp degradative pathway is not fully functional in cultured genetic tumour tissues of *N. glauca* × *N. langsdorffii*. It is interesting to note that the rate of carbon dioxide released from Hyp by tobacco leaf tissue was found to be 11 times higher in comparison to the callus tissue (Ashihara and Nobusawa 1981).

The endogenous cytokinin levels in this genetic tumour line are quite low [675 pmol g^{-1}(f.m.); Nandi *et al.* 1990] and it appears that the capacity of cytokinin independent growth by these tissues may not necessarily be related to an increase in the extractable cytokinin pool size. The cytokinin levels are probably determined by the rates of synthesis and/or metabolism, and it is possible that due to the slower rate of cytokinin turnover in these tissues, incorporation of adenine into cytokinins could not be detected. The results of [9R]Z metabolism which indicate enhanced cytokinin stability in this tissue (Nandi *et al.* 1990, Nandi and Palni 1997) are consistent with this suggestion. The regulation of cytokinin levels by cytokinin oxidase has been discussed (Motyka and Kamínek 1994, Nandi and Palni 1997, Redig *et al.* 1997). Cultured genetic tumour tissues, although similar to crown gall tissues in their capacity for cytokinin (and auxin)-independent growth, appear to maintain cytokinins at physiologically effective levels, sufficient for *in vitro* growth, by reduced rate of cytokinin turnover.

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