Plant Physiol. (1996) 112: 141–148

Analysis of Cytokinin Metabolism in *ipt* Transgenic Tobacco by Liquid Chromatography-Tandem Mass Spectrometry[1]

Pascale Redig*, Thomas Schmülling, and Harry Van Onckelen

University of Antwerp, Department of Biology, Universiteitsplein 1, B-2610 Antwerpen, Belgium (P.R., H.V.O.); and Universität Tübingen, Lehrstuhl für Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany (T.S.)

The endogenous levels of the major, naturally occurring cytokinins in *Pisum sativum* ribulose-1,5-bisphosphate carboxylase small subunit promoter-isopentenyl transferase gene (Pssu-*ipt*)-transformed tobacco (*Nicotiana tabacum* L.) callus were quantified using electrospray-liquid chromatography-tandem mass spectrometry during a 6-week subcultivation period. An *ipt* gene was expressed under control of a tetracycline-inducible promoter for a more detailed study of cytokinin accumulation and metabolism. Activation of the *ipt* in both expression systems resulted in the production of mainly zeatin-type cytokinins. No accumulation of isopentenyladenine or isopentenyladenosine was observed. In Pssu-*ipt*-transformed calli, as well as in the tetracycline-inducible *ipt* leaves, metabolic inactivation occurred through *O*-glucoside conjugation. No significant elevation of cytokinin *N*-glucosides levels was observed. Side-chain reduction to dihydrozeatin-type cytokinins was observed in both systems. The levels of the endogenous cytokinins varied in time and were subject to homeostatic regulatory mechanisms. Feeding experiments of *ipt* transgenic callus with [^{3}H]isopentenyladenine and [^{3}H]isopentenyladenosine mainly led to labeled adenine-like compounds, which are degradation products from cytokinin-oxidase activity. Incorporation of radioactivity in zeatin riboside was observed, although to a much lesser extent.

Gene 4 of the plant pathogenic bacteria *Agrobacterium tumefaciens* (*ipt*) encodes the enzyme isopentenyl transferase that catalyzes the first step in the CK biosynthesis. It converts 5′-AMP and isopentenyl PPi into iPMP, which is rapidly converted to isopentenyl-types CKs (Akiyoshi et al., 1984; Barry et al., 1984). These can then be modified in a series of reactions to yield other types of CK ribotides, ribosides, and free bases. Introduction of the *ipt* into *Nicotiana tabacum* yields transgenic tissue that can grow phytohormone autotrophically (Beinsberger et al., 1987).

The actual pathway of CK production in plants is, in contrast to the biosynthetic pathway in *A. tumefaciens*, not yet well established. A partially purified enzyme with isopentenyl transferase activity has been reported in nontumorous tobacco cells (Chen and Melitz, 1979), but attempts to purify this enzyme to homogeneity have not yet been successful.

More information is available concerning CK metabolism. There are indications that the free base may be the most biologically active form (Laloue and Pethe, 1982). Formation of CK nucleosides and nucleotides from free bases, *N*-glucosylation, *N*-alanyl conjugation, *O*-glucosylation, *O*-acetylation, side-chain reduction, and side-chain oxidation have been identified as processes that play a role in CK metabolism (Letham and Palni, 1983). The reversibility of some of these metabolic reactions suggests a regulation of the level of biologically active CKs.

Increasing the rate of CK synthesis genetically can be useful in elucidating the pathway of hormone metabolism. How will the plant compensate if the concentration of the endogenous CKs is suddenly increased by 10- or 100-fold? Which CKs will be accumulated or broken down, and which metabolites will be formed in time?

We report here the kinetic analysis of the endogenous content of all major and naturally occurring types of CKs in transgenic tobacco callus tissue containing the *ipt* under the control of a light-inducible promoter. For a more detailed study, the *ipt* was expressed under the control of a tet promoter, and the time course of the formation of the different types of CKs was studied after gene induction. The nature and the accumulation of the endogenous CKs as

[1] This work was supported by a grant from the Belgian Program on Interuniversity Poles of Attraction (Prime Ministers Office, Science Programming, no. 38) to H.V.O. and a grant of the Deutsche Forschungsgemeinschaft to T.S. P.R. is a research assistant and H.V.O. is a research director of the Belgian National Fund for Scientific Research.

* Corresponding author; e-mail redig@uia.ac.be; fax 32–38202271.

Abbreviations: Ade, adenine; CK, cytokinin; DZ, dihydrozeatin; DZMP, dihydrozeatinriboside-5′-monophosphate; DZNG, dihydrozeatin-*N*-glucoside; DZOG, dihydrozeatin-*O*-glucoside; DZROG, dihydrozeatin riboside-*O*-glucoside; DZR, dihydrozeatin riboside; ES, electrospray; iPMP, isopentenyladenosine-5′-monophosphate, iPNG, isopentenyladenine-*N*-glucoside; 2iP, isopentenyladenine; 2iPA, isopentenyladenosine; 2iPMP, isopentenyl adenosine monophosphate; 2iPNG, isopentenyladenine-N- glucoside; *ipt*, isopentenyl transferase gene; LC, liquid chromatography; RP, reversed phase; tet, tetracycline inducible; Z, zeatin; ZMP, zeatin riboside-5′-monophosphate; ZNG, zeatin-*N*-glucoside; ZOG, zeatin-*O*-glucoside, ZR, zeatin riboside; ZROG, zeatin riboside-*O*-glucoside.

a result of the *ipt* activation and the subsequent different metabolic interconversions are the subjects of this report.

MATERIALS AND METHODS

Chemicals

All CK-deuterated tracers ([$^2H_5$]Z, [$^2H_5$]-ZR, [$^2H_5$]zeatin-9-glucoside, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]2iP, [$^2H_6$]2iPA, [$^2H_6$]isopentenyl-9-glucoside, [$^2H_3$]DZMP) were purchased from Apex (Honiton, UK). Radioactive tracers that were obtained from the Institute of Experimental Botany (isotope laboratory, Prague Czech Republic) included 1.65 TBq/μmol [2-^{3}H]N^3-2iPA and 1.65 TBq/μmol [2-^{3}H]-N^6-2iP. Alkaline phosphatase (10 mg/mL, 2500 units/mg, enzyme immunoassay grade) was purchased from Boeh- ringer Mannheim. RP C_{18} columns (1 mL, 0.5g) were purchased from Varian (Harbor City, CA) and DEAE- Sephadex (A-25) was purchased from Pharmacia Biotech (Sweden).

ipt Tobacco Callus Tissue

Nicotiana tabacum L. cv Petit Havana SR1 callus tissue transformed with the *ipt* of *Agrobacterium tumefaciens* under the control of the *Pisum sativum* ribulose-1,5-bisphosphate carboxylase small subunit promoter sequence (Pssu-*ipt*) (strain pGV2488) (Beinsberger et al., 1991) was cultivated (25°C, 16 h of light, 8 h of darkness) on Murashige-Skoog medium (Murashige and Skoog, 1962) containing 3% Suc, 200 mg/L *myo*-inositol, 10 mg/L thiamine dichloride, 1 mg/L pyridoxine hydrochloride, 1 mg/L nicotinic acid, and 0.8% agar.

N. tabacum L. cv Petit Havana SR1 untransformed callus tissue (SR1) and callus tissue transformed with the vector lacking the *ipt* (pGV831) (Table I) were grown on the Murashige-Skoog medium mentioned above, supplemented with 1 mg/L NAA and 0.1 mg/L BA. Every 6 weeks the tissues were transferred to new culture medium.

tet-*ipt* Plants

Nicotiana tabacum L. cv Wisconsin 38 was transformed with an *ipt* under control of a tet promoter (Gatz et al., 1992). Clone 35So IPT-5/TETR used for the present study will be described in detail elsewhere (M. Faiss and T. Schmülling, unpublished data). The plants were grown in the greenhouse (20–24°C, 15 h of light, 9 h of darkness).

Table I. *Description of the callus tissue*

Tissue Line	Description
pGV2488 Pssu-*ipt*	*N. tabacum* cv Petit Havana SR1 containing the octopine *ipt* coupled to the light-inducible promoter of the gene encoding the small subunit of Rubisco from *P. sativum*
pGV831	*N. tabacum* cv Petit Havana SR1 tissue transformed with the vector lacking *ipt*
SR1	*N. tabacum* cv Petit Havana SR1 untransformed tissue

From 16-week-old plants (approximately 70–80 cm tall), leaves 9 to 15 (starting from the top) were harvested. The *ipt* expression was induced by vacuum infiltration of the leaves with 1 mg/L chlortetracycline in 50 mM sodium citrate (pH 5.6). Control tissue was untransformed *N. tabacum* L. cv Wisconsin 38.

Extraction and Purification of CKs

The frozen tissue (0.5–1 g) was ground under liquid nitrogen and extracted overnight at −20°C in $CHCl_3$:CH_3OH:H_2O:HCOOH (5:12:2:1, v/v) (Bieleski, 1964). Deuterated CKs were added as internal standards. After the sample was centrifuged (24,000*g*, 4°C, 20 min) the supernatant was concentrated by rotary film evaporation until the water phase, and the pH was adjusted to 7.0. This extract was then purified on a combination of a DEAE-Sephadex column (2 mL, HCO_3^- form) and an RP C_{18} column. After the columns were washed with water, the fraction containing the CK free bases, ribosides, and glucosides were eluted from the RP C_{18} column with 10 mL of 80% methanol. The eluate was concentrated in vacuo until the water phase and applied to a combination of a nonimmune column (1 mL), an immunoaffinity column (0.5 mL), and an RP C_{18} column. The immunoaffinity columns were prepared with monoclonal anti-ZR antibodies, which were able to bind a broad spectrum of CKs, including all major isoprenic and aromatic CK free bases, ribosides, and *N*-glucosides (Ulvskov et al., 1992; P. Redig, unpublished results). They had a binding capacity of more than 7000 pmol ZR/mL gel. The nonimmune columns were prepared with bovine γ-globulins as described by Ulvskov et al. (1992). After washing with 10 mL of water the immunoaffinity column was eluted with 4 mL of ice-cold methanol and immediately reconditioned with water. The eluate from the immunoaffinity column contained the CK free bases, ribosides, and *N*-glucosides and was dried and stored at −70°C until further analysis by LC-tandem MS. The CK-*O*-glucosides, which were not retained on the immunoaffinity column, were eluted from the RP C_{18} column with 4 mL of 80% methanol, dried, and stored at −70°C until further analysis.

The CK nucleotides that were bound to the DEAE-Sephadex column were eluted with 10 mL of 1 M NH_4HCO_3. The CK nucleotides in the eluate were bound on another RP C_{18} column, which was then eluted with 10 mL of 80% methanol. The eluate was dried by rotary film evaporation and redissolved in 0.01 M Tris (pH 9.0). The CK nucleotides were treated with alkaline phosphatase (30 min, 37°C), and the resulting nucleosides were further purified by immunoaffinity chromatography as described above.

Quantitative Analysis of CKs by ES Tandem MS

CK fractions were quantified by HPLC that was linked to a mass spectrometer (Quatro II, Fisons, Beverly, MA) equipped with an ES interface ((+)ES LC-tandem MS). Samples (10 μL) were injected onto an RP C_8 column (LiChrosphere 60 RP Select B, 5 μm, 125 × 4 mm; Merck, Darmstadt, Germany) and eluted with 0.01 M methanol:ammonium acetate (70:30,

v/v) at 800 μL/min. The effluent was introduced into the ES source (80°C source, +3.5-V capillary, and 20-V cone) using a post-column split of 1/20. Quantitation was obtained by multiple reaction monitoring of $(MH)^+$ and the appropriate product ion (Prinsen et al., 1995b).

Metabolic Studies

Callus tissue (0.5 g) was transferred aseptically to a Petri dish (3.5 cm in diameter) containing 3 mL of Murashige-Skoog medium (described above) supplemented with 0.2 mg/L 2iP and 0.2 mg/L 2iPA and containing 1.0×10^4 Bq of [^{3}H]2iP and [^{3}H]2iPA, respectively, and incubated at 25°C (16 h of light, 8 h of darkness). After 8 h of incubation samples were taken, washed with distilled water, and immediately frozen in liquid nitrogen.

The callus tissue was extracted overnight at −20°C in 2 mL of $CHCl_3$:CH_3OH:H_2O:HCOOH (5:12:2:1, v/v) (Bieleski, 1964), and the extract was dried under nitrogen. The samples were redissolved in 20 μL of 70% methanol and 80 μL of water and analyzed by HPLC.

HPLC

CKs were separated on a Microsorb C_{18} column (150 mm, 4.6 mm i.d, 5-μm particle size; Rainin, Woburn, MA) at a flow rate of 0.5 mL/min using a gradient of solvent A (10% methanol in 40 mM triethylammonium acetate, pH 3.35) and solvent B (80% methanol in 30 mM acetic acid). The following gradient profile was used: 0 min, 90% solvent A plus 10% solvent B; 20 min, 50% solvent A plus 50% solvent B; 39 min, 45% solvent A plus 55% solvent B; and 40 min, 100% solvent B (Prinsen et al., 1995a). Fractions (0.25 mL) were collected, and 3 mL of liquid scintillation cocktail (Ultimo Gold, Packard, Meriden, CT) were added for ^{3}H counting (Tricarb 1500, Packard). Corrections for quenching of the radioactivity were made.

RESULTS

CK Levels in *ipt* Transgenic Tobacco Callus Tissues

The levels of 16 CKs were monitored during a 6-week subcultivation period of the callus tissue. By sampling the callus tissue throughout the entire period of subcultivation, the interconversions among the nucleotide, riboside, and base forms of the CKs could be studied. Eventual changes in CK contents due to physiological changes during the culture could be taken into account. During the first 4 d of subculture, samples were taken daily. Afterward, the callus tissues were sampled at 2-d intervals. The tissue samples were taken after 7 h of light induction and were immediately frozen in liquid nitrogen. The morphological changes of the callus tissue during the subcultivation period have been described by Beinsberger et al. (1991).

The major CKs accumulating in the Pssu-*ipt* transgenic callus tissue (pGV2488) are ZMP, ZR, and Z (Fig. 1). Both in the pGV831 callus tissue (lacking the *ipt*) and in pGV2488 calli the levels of the 2iP-type CKs were close to the detection limit (0.5 pmol/g fresh weight for 2iPA and 2iPNG, 1 pmol/g fresh weight for 2iP, and 2 pmol/g fresh weight for 2iPMP). The CK content of the *ipt* calli varied with time after subculture (Fig. 1). The concentration of ZMP reached a peak value of 10,800 pmol/g fresh weight approximately 2 d after subculture, after which it declined to a steady-state level of approximately 3,000 pmol/g fresh weight (Fig. 1C). A transient increase in Z levels (peak value of 12,000 pmol/g fresh weight) on approximately the 3rd d of subculture was followed by an increase in ZR (peak value of 7,500 pmol/g fresh weight) (Fig. 1, A and B). Concentrations of Z-type CKs in the pGV831 callus tissue were approximately steady-state concentrations of 30 pmol/g fresh weight for Z, 10 pmol/g fresh weight for ZR, and 10 pmol/g fresh weight for ZMP throughout the entire cultivation period (Fig. 1, E–G). The levels of DZ-type CKs in the *ipt* calli (pGV2488) were also elevated (steady-state concentrations of approximately 200 pmol/g fresh weight) in comparison with the tissues that lacked the *ipt* (steady-state concentrations of approximately 4 pmol/g fresh weight) but not to the extent of the Z-type CKs. A similar kinetic pattern was observed for the DZ-type CKs as well as for the Z-type CKs (Fig. 1, A–C): DZMP reached a peak value of 2,700 pmol/g fresh weight after 2 d of culture, DZ reached its peak value (1,300 pmol/g fresh weight) on approximately the 3rd d of culture, followed by an increase in DZR (peak value 970 pmol/g fresh weight).

The CK-*O*-glucoside levels also were elevated in comparison with the pGV831 tissue (Fig. 1, D and H). The initial high levels of ZOG and DZOG, which correspond with the buildup of the concentrations of these compounds at the end of the cultivation period, were immediately hydrolyzed starting from d 1 of the culture (Fig. 1D). Afterward, we again observed an elevation of ZOG and DZOG concentrations, and this buildup coincided with the decrease in the levels of the Z and DZ free bases (Fig. 1A). From the middle of the subculture period on, levels decreased to a steady-state level of approximately 3000 pmol/g fresh weight and increased again toward the end of the subculture period. The levels of ZROG and DZROG remained stable throughout the entire cultivation period, with steady-state levels of approximately 300 pmol/g fresh weight (Fig. 1D). In the pGV831 tissue, the levels of the CK-*O*-glucosides and CK riboside-*O*-glucosides were approximately steady-state concentrations of 50 pmol/g fresh weight for ZOG, 30 pmol/g fresh weight for DZOG, 3 pmol/g fresh weight for ZROG, and 20 pmol/g fresh weight for DZROG (Fig. 1H). The levels of ZOG and DZOG in the pGV831 tissue were higher than the concentrations of the Z and DZ free bases and ribosides (Fig. 1, E and F).

Only very low levels of ZNG could be detected in the *ipt*-transformed tissue, ranging from 2 to 70 pmol (Fig. 1D), whereas no ZNG was found in the pGV831 tissue (Fig. 1H). Neither DZNG nor 2iPNG could be detected in pGV2488 or pGV831 tissue.

tet-*ipt* Tobacco Plants

Leaves of *N. tabacum* cv Wisconsin plants, transformed with the *ipt* under control of a tet promoter (35So IPT-5/TETR) and the untransformed control, were induced by vacuum infiltrating chlortetracycline. Samples were harvested after different times. The leaves were subsequently

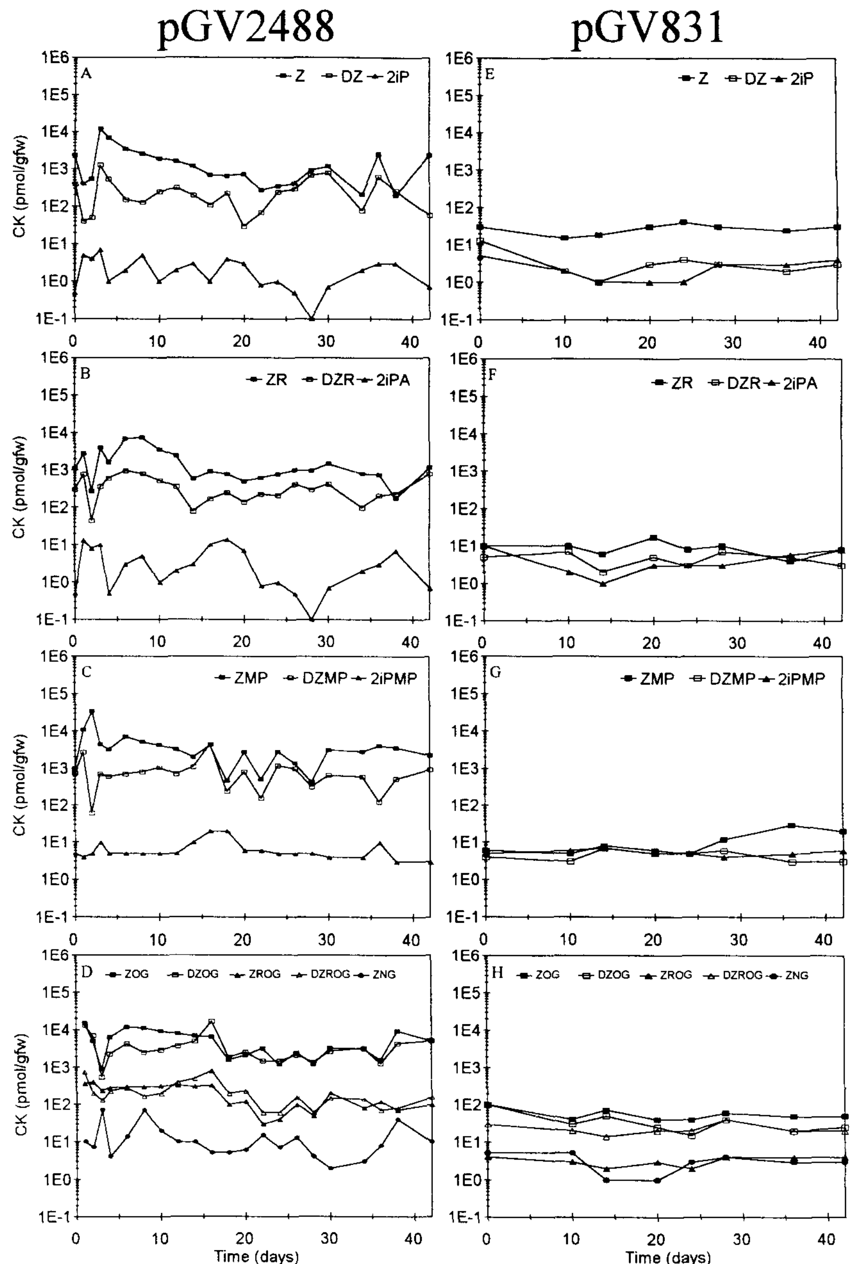

Figure 1. Endogenous CK levels in *ipt*-transformed callus tissue (pGV2488) and callus tissue lacking the *ipt* (pGV831) during a 6-week subcultivation period. Concentrations are expressed as pmol g^{-1} fresh weight (fw). A, Levels of Z, DZ, and 2iP in pGV2488 callus tissue; B, levels of ZR, DZR, and 2iPA in pGV2488 callus tissue; C, levels of ZMP, DZMP, and 2iPMP in pGV2488 callus tissue, the curve of 2iPMP represents the detection limit; D, levels of ZOG, DZOG, ZROG, DZROG, and ZNG in pGV2488 callus tissue; E, levels of Z, DZ, and 2iP in pGV831 callus tissue; F, levels of ZR, DZR, and 2iPA in pGV381 callus tissue; G, levels of ZMP, DZMP, and 2iPMP in pGV381 callus tissue, the curve of 2iPMP represents the detection limit; H, levels of ZOG, DZOG, ZROG, DZROG, and ZNG in pGV381 callus tissue, the curve of ZNG represents the detection limit.

analyzed for the accumulation of the different CKs and their metabolites (Tables II and III). The CK contents of the tet-*ipt* tobacco leaves at 0 h (no *ipt* induction) were similar to those of untransformed control tobacco leaves. No change in endogenous CK content could be observed up to 4 h after tetracycline application. In particular, accumulation of ZMP, ZR, and ZROG and to a lesser extent of Z and ZOG was observed after 8 h of gene induction. After 24 h, ZMP was the main CK present together with ZR and ZROG. From this time on conversion to DZ started and conjugation into DZOG CKs took place. DZR was already present after 8 h of induction, and after 48 h of gene induction all Z-type CKs reached high steady-state levels. In our experiments, accumulation of DZ-type CKs started later than the Z-type CKs and never reached the high levels of the Z-type CKs after 48 h of gene induction. No conjugation into N-type CK glucosides (ZNG, DZNG, and 2iPNG) was observed (Table II). Throughout the entire time (up to 48 h of gene induction) examined, no increase in 2iP-type CKs was found. No change in endogenous CK content in the untransformed control leaves was observed after tetracycline treatment (Table III).

Metabolic Studies

Table IV shows the uptake and metabolism of [^{3}H]2iP and [^{3}H]2iPA (0.2 mg/L, 1.0 × 10^4 Bq) by the *ipt*-transformed callus tissue and SR1 callus tissue (2 weeks

Table II. *CK contents of N. tabacum cv Wisconsin 38 leaves transformed with ipt under control of a tet promoter after induction of the gene with chlortetracycline (1 mg/L) for different periods*

Type	CK Content					
	0 h	2 h	4 h	8 h	24 h	48 h
	pmol/g fresh wt					
Z	13	9	5	30	120	600
DZ	1.5	2	3	5	4	20
ZR	17	15	12	100	820	920
DZR	4	3	2	17	20	75
ZNG	1	1.5	0.5	<0.3	0.6	1.5
DZNG	<0.6	<0.3	<0.5	<0.3	<0.3	<0.3
ZOG	50	40	30	140	90	430
DZOG	13	30	30	6	10	20
ZROG	22	15	10	100	830	1270
DZROG	2	3	3	7	20	35
ZMP	30	13	2	100	1040	930
DZMP	5	1	2	10	20	40
2iP	8	5	5	10	13	14
2iPA	0.4	1	2	4	6.5	3
iPNG	1.4	1	2	10	1	1
iPMP	<1	<1.5	<2	<1.5	<2	<2

after subculture). The identity of the CKs shown in Table IV was based on co-chromatography of CK standards on a Rainin column eluted with the gradient described in "Materials and Methods."

The uptake of [^{3}H]2iP and [^{3}H]2iPA by the callus tissue, *ipt*-transformed (pGV2488) and control tissue (SR1), after 8 h of the supplied radioactivity was 47 and 45%, respectively. In the case of addition of [^{3}H]2iP to pGV2488, 80% of the total radioactivity extracted was associated with Ade-type compounds (Ade, adenosine, and Ade nucleotides). About 12% was associated with 2iP-type CKs, and 5% of the radioactivity co-chromatographed with ZR. After 8 h of incubation of pGV2488 with [^{3}H]2iPA, 60% of the radioactivity extracted was associated with the Ade-type fractions, 30% was associated with the 2iP-type CKs, and 7% was associated with ZR. Uptake and metabolism of the radioactivity by the SR1 tissue was comparable (Table IV). No incorporation of radioactivity in Z, ZMP, DZ, DZR, and DZMP could be observed.

DISCUSSION

Elevated CK levels have been reported previously in tissues transformed with an *ipt* (Medford et al., 1989; Beinsberger et al., 1991; Smigocki, 1991; Zhang et al., 1995). In most of the studies only a limited number of CKs were quantitated in different tobacco varieties. Moreover, tissues were mostly assayed at only one time during culture. In this way contributions of physiological changes during the culture period were not taken into account, nor was the continuous interconversion of the nucleotide, riboside, and base forms of the CKs (Laloue and Pethe, 1982). Our

Table III. CK *contents of N. tabacum cv Wisconsin 38 leaves (control) after treatment with chlortetracycline (1 mg/L) for different periods*

Type	CK Content					
	0 h	2 h	4 h	8 h	24 h	48 h
	pmol/g fresh wt					
Z	10	8	3	4	6	8
DZ	2	3	3	3	2	4
ZR	10	16	10	12	17	16
DZR	2	6	6	4	3.5	3
ZNG	0.5	0.7	3	2	2	1.5
DZNG	<0.2	<0.7	<0.6	<0.5	<0.8	<0.3
ZOG	30	30	25	20	22	32
DZOG	10	7	5	4	6	5
ZROG	12	14	19	12	15	15
DZROG	3	1	2	2	2	2
ZMP	3	5	2	3	4	6
DZMP	7	9	7	6	11	5
2iP	7	3	2	4	3	5
2iPA	0.7	1.4	3	3	2	2
iPNG	0.8	1	1	1.1	1.5	1
iPMP	<0.6	<1.5	<2	<0.8	<1	<1.6

Table IV. *Uptake and metabolism of [^{3}H]iP and [^{3}H]iPR by ipt transformed callus tissue (pGV2488) and untransformed callus tissue (SR1)*

Isolation and analysis of the CKs and metabolites by HPLC were performed 8 h after the start of incubation. The results are expressed as percentages of the total radioactivity taken up by the callus tissue.

[^{3}H]	Tissue	^{3}H Incorporation (%)								
		Ade nucleotide	Ade	Adenosine	2iP nucleotide	2iP	2iPA	Z nucleotide	Z	ZR
2iP	pGV2488	6	28	50	11	1	–	–	–	5
	SR1	7	27	37	16	–[a]	3	–	–	10
2iPA	pGV2488	7	12	40	9	15	10	–	–	7
	SR1	7	14	33	17	–	7			

[a] –, No incorporation of radioactivity detected.

aim was to investigate how the plant tissue would cope with the high endogenous CK concentrations present as a result of *ipt* expression.

In our present study all major, naturally occurring CKs in *ipt*-transformed callus tissues have been assayed by LC-tandem MS. To our knowledge, this is the first report of the kinetics of 16 different unequivocally identified CKs during a 6-week subcultivation period. The use of the Bieleski solvent prevented the hydrolysis of nucleotides by phosphatase action. CKs were purified by a combination of solid-phase extraction and immunoaffinity chromatography. During the purification procedure, the endogenous CKs were separated into three fractions: one fraction contained the CK free bases, ribosides, and *N*-glucosides, a second fraction contained the CK-*O*-glucosides, and a third fraction contained the CK nucleotides. After treatment with alkaline phosphatase, this last fraction was further purified as the CK nucleosides. The different CK fractions were quantified by HPLC coupled to a Quatro II mass spectrometer equipped with an ES interface, using multiple reaction monitoring of the (MH^+) ion and the corresponding product ion (Prinsen et al., 1995b).

We used two *ipt* expression systems to study the production of the different CKs upon *ipt* activation and the subsequent interconversions among the different CKs and metabolites. In the first system the *ipt* was positioned under control of a light-inducible promoter, the Pssu-*ipt* construct (Beinsberger et al., 1991). The pGV831 callus tissue (lacking the *ipt*, Table I) was used to take into account eventual changes in CK contents due to particular culture conditions. Comparing its endogenous CK contents with that of the *ipt*-expressing pGV2488 callus tissue is not entirely valid since the pGV831 callus tissue has to be cultivated on medium supplemented with auxin and CK to obtain a comparable growth rate. BA and NAA are known to influence the endogenous CK metabolism in this callus tissue (Beinsberger et al., 1991). Initial results (Beinsberger et al., 1991) showed that the addition of NAA to the *ipt* transgenic SR1 calli resulted in a maximal 40% decrease of ZR-type CKs, whereas BA had little or no influence on the ZR-type CKs. These results indicate that the pronounced difference (100-fold) in CK contents can only marginally be attributed to the difference in culture conditions.

The second system that was studied contained an *ipt* expressed under the control of a tet promoter, 35So IPT-5/TETR (M. Faiss and T. Schmülling, unpublished data). The tet-*ipt* expression system is almost not leaky. At time 0 the endogenous CK contents of the tet-*ipt*-transformed leaves were comparable to that of the untransformed control leaves. Incubation of the untransformed control leaves with tetracycline did not influence the endogenous CK contents. The major endogenous CKs present in the Pssu-*ipt*-transformed callus tissue and in the tet-*ipt* tobacco leaves are CKs of the Z-type. The accumulation of the same type of CKs could be observed also in *N. tabacum* cv Petit Havana as well as in cv Wisconsin 38 . The main CKs present in the Pssu-*ipt* callus tissue and in the tet-*ipt*-induced tobacco leaves are ZMP, together with ZR, Z, ZOG, and DZOG. In the Pssu-*ipt* callus tissue the CK contents depend on the time after subculture. Because we are looking at a circadian expression of the *ipt*, a certain degree of fluctuation is not unexpected. The pool of the 2iP compounds, namely 2iP and 2iPA, shows the most fluctuations (Fig. 1, A and B), which indicates that these compounds are under the strictest metabolic control. A buildup of 2iP and 2iPA was observed over a period of approximately 4 d, and at the end this period, a critical level was reached and a breakdown of these compounds could be observed. 2iP and 2iPA are under a tight metabolic control, most probably provided by the CK oxidase.

Only very low levels of CK-*N*-glucosides were observed. This is in contrast to the results of Scott and Horgan (1984), who found elevated levels of Z-7-glucoside and only minor quantities of CK-*O*-glucosides in tobacco crown gall tissue. Laloue et al. (1977) demonstrated that Z-7-glucoside is biologically inactive, and its accumulation is considered a terminal inactivation pathway (Laloue et al., 1981). *O*-glucosylation, on the other hand, is considered a reversible process and may represent only a temporary inactivation (Sembdner et al., 1994). In both systems that we studied, *N. tabacum* cv Petit Havana *Pssu-ipt* calli and *N. tabacum* cv Wisconsin tet-*ipt* leaves, no conjugation of the CKs into *N*-glucosides was observed.

Accumulation of DZ-type CKs in the *ipt*-transformed tissue and in the tet-*ipt*-induced leaves was also observed. DZ-type CKs are formed by reduction of the Z side chain. The significance of this reaction is still a little unclear. CKs bearing saturated N^6 chains (DZ-type CKs) are resistant to attack by the CK oxidase (Motyka and Kaminek, 1994) and might constitute in this way a pool of stable CKs.

Conversion of Z to DZ-type CKs could occur at the level of the nucleotides. From the experiments with the tet-*ipt* tobacco leaves, levels of DZ nucleotides and DZR start to increase already after 8 h of induction of the *ipt*. Reduction

of the side chain at the level of the free base, however, as demonstrated by Martin et al. (1989), cannot be excluded.

We expected an increase in 2iP-type CKs because these are the products of the isopentenyl transferase enzyme activity. However, only an increase in Z- and DZ-type CKs was observed after *ipt* activation. No accumulation of 2iP, 2iPA, 2iPNG, or 2iPMP was detected in Pssu-*ipt*-transformed callus tissue and tet-*ipt* transformed tobacco leaves. 2iPMP, the product of isopentenyl transferase, is thought to be rapidly converted to 2iPA and 2iP (Akiyoshi et al., 1984; Barry et al., 1984). The absence of the 2iP-type compounds could indicate that the hydroxylation to Z metabolites happens rapidly.

To test the hypothesis that 2iP and 2iP riboside are converted rapidly to Z-type CKs, feeding experiments of transgenic and control callus tissues with [^{3}H]2iP and [^{3}H]2iPA were carried out. 2iP and 2iPA can undergo three basic reactions: (a) cleavage of the isopentenyl side chain, leading to complete inactivation of the CK (Terrine and Laloue, 1980); (b) ring substitution, resulting in a lower activity of the CK (Laloue et al., 1977); and (c) hydroxylation of the terminal methyl group in the side chain, leading to an increase in CK activity (Miura and Hall, 1973). Our results show that externally applied 2iP and 2iPA undergo a rapid oxidative cleavage of the side chain. Between 70 and 80% of all radioactivity taken up by the callus tissue was found to be present in the form of Ade-type compounds. For the SR1 tissue this was between 50 and 70%.

2iP and 2iPA also seem to undergo metabolic conversion to the phosphate ester, which was also observed by Laloue and co-workers (1977, 1982) in cell suspensions of tobacco and *Acer*. Endogenous levels of 2iPMP, however, were not detected during the growth kinetic in the Pssu-*ipt* callus tissue or in the tet-*ipt* leaves after induction of the *ipt*. Incorporation of radioactivity in ZR was observed but, in comparison with the side-chain cleavage reaction, to a much lesser extent; this could be the result of a hydroxylation of the side chain. From our results it cannot be concluded at which level the *trans*-hydroxylation takes place. Similar results were obtained by Palni and Horgan (1983) with crown gall tissue of *Vinca rosea*. Feeding experiments of tobacco crown gall tissue with [^{3}H]Ade yielded radioactive 2iPMP, which was rapidly hydroxylated to yield ZMP, which was then metabolized to the free bases and ribosides (Zhang et al., 1995). These results suggest that 2iPMP is immediately converted to ZMP and that 2iP and 2iPA are not produced as a result of expression of the *ipt*. This is also confirmed by our results. We found no elevated levels of 2iP and 2iPA in comparison with the control tissue upon activation of the *ipt*. The results of our feeding experiments suggest that 2iP could be an intermediate in the synthesis of Z-type CKs, since some incorporation of radioactivity in ZR could be detected. It cannot be excluded, however, that the radioactive ZR measured is formed directly from Ade or from an Ade derivative. The results from our feeding experiments also indicate that the enzyme CK-oxidase, which is responsible for the side-chain cleavage and subsequent inactivation of the CK, is very active in the callus tissue. Data concerning the CK-oxidase in Pssu-*ipt*-transformed callus tissue will be presented elsewhere.

In summary, similar conclusions can be drawn from the two different expression systems, the Pssu-*ipt* and the tet-*ipt*, concerning CK accumulation and metabolism. No accumulation of 2iP-type CKs could be detected upon *ipt* activation, which raises questions about the endogenous substrate of the isopentenyl transferase. The main CKs initially accumulating in *ipt* transgenic tissue are Z-type CKs, which are then converted to DZ-type CKs. Metabolic inactivation was achieved through *O*-glucoside conjugation, and no accumulation of CK-*N*-glucosides was observed.

ACKNOWLEDGMENTS

The authors wish to thank W. Van Dongen for LC-MS analyses and N. Schrijvers and S. El Kamili for technical assistance.

Received April 1, 1996; accepted May 17, 1996.
Copyright Clearance Center: 0032–0889/96/112/0141/08.

LITERATURE CITED

Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP (1984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc Natl Acad Sci USA **81:** 5994–5998

Barry GF, Rogers SG, Fraley RT, Brand L (1984) Identification of a cloned cytokinin biosynthetic gene. Proc Natl Acad Sci USA **81:** 4776–4780

Beinsberger SE, Rudelsheim P, Inzé D, Van Lysebettens M, De Greef J, Van Onckelen H (1987) Full expression of chimeric T-DNA gene 4 constructions in tobacco tissues. Arch Int Physiol Biochim **95:** 2

Beinsberger SEI, Valcke RLM, Deblaere RY, Clijsters HMM, De Greef JA, Van Onckelen HA (1991) Effects of the introduction of *Agrobacterium tumefaciens* T-DNA *ipt* gene in *Nicotiana tabacum* L. cv. Petit Havana SR1 plant cells. Plant Cell Physiol **32:** 489–496

Bieleski RL (1964) The problem of halting enzyme action when extracting plant tissues. Anal Biochem **9:** 431–442

Chen CM, Melitz DK (1979) Cytokinin biosynthesis in a cell free system from cytokinin autotrophic tobacco tissue culture. FEBS Lett **107:** 15–20

Gatz C, Frohberg C, Wendenburg R (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. Plant J **2:** 397–404

Laloue M (1977) Cytokinins: 7-glucosylation is not a prerequisite of the expression of their biological activity. Planta **134:** 273–275

Laloue M, Pethe C (1982) Dynamics of cytokinin metabolism in tobacco cells. *In* J Guern J, C Peaud-Lenoel, eds, Plant Growth Substances 1982. Springer, New York, pp 80–96

Laloue M, Pethe-Terrine C, Guern J (1981) Uptake and metabolism of cytokinins in tobacco cells: studies in relation to the expression of their biological activities. *In* J Guern, C Peaud-Lenoel, eds, Metabolism and Molecular Activities of Cytokinins. Springer, New York, pp 80–96

Laloue M, Terrine C, Guern J (1977) Cytokinins: metabolism and biological activity of N^6-(Δ^2-isopentenyl)adenosine and N^6-(Δ^2-isopentenyl)adenine in tobacco cells and callus. Plant Physiol **59:** 478–483

Letham DS, Palni LMS (1983) The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol **34:** 163–197

Martin RO, Mok MC, Shaw G, Mok DWS (1989) An enzyme mediating the conversion of zeatin to dihydrozeatin in *Phaseolus* embryos. Plant Physiol **90:** 1630–1635

Medford JI, Horgan R, El-Sawi Z, Klee HJ (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell **1:** 403–413

Miura G, Hall RH (1973) *Trans*-ribosylzeatin. Its biosynthesis in *Zea mays* endosperm and the mycorrhizal fungus, *Rhizopogon roseolus*. Plant Physiol **51:** 563–569

Motyka V, Kaminek M (1994) Cytokinin oxidase from auxin- and cytokinin-dependent callus cultures of tobacco (*Nicotiana tabacum* L.). J Plant Growth Regul **13:** 1–9

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant **15:** 473–496

Palni LMS, Horgan R (1983) Cytokinin biosynthesis in crown gall tissue of *Vinca rosea*: metabolism of isopentenyladenine. Phytochemistry **22:** 1597–1601

Prinsen E, Redig P, Strnad M, Galis I, Van Dongen W, Van Onckelen H (1995a) Quantifying phytohormones in transformed plants. *In* KMA Gartland, MR Davey, eds, Methods in Molecular Biology, Vol 44: Agrobacterium Protocols. Humana Press, Totowa, NJ, pp 245–262

Prinsen E, Redig P, Van Dongen W, Esmans E, Van Onckelen H (1995b) Quantitative analysis of cytokinins by electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom **9:** 948–953

Scott IM, Horgan R (1984) Mass-spectrometric quantitation of cytokinin nucleotides and glycosides in tobacco crown-gall tissue. Planta **161:** 345–354

Sembdner G, Atzorn R, Schneider G (1994) Plant hormone conjugation. Plant Mol Biol **26:** 1459–1481

Smigocki AC (1991) Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. Plant Mol Biol **16:** 105–115

Terrine C, Laloue M (1980) Kinetics of N^6-(Δ^2-isopentenyl)adenosine degradation in tobacco cells. Plant Physiol **65:** 1090–1095

Ulvskov P, Nielsen T, Seiden P, Marcussen J (1992) Cytokinins and leaf development in sweet pepper (*Capsicum annuum* L.). Planta **188:** 70–77

Zhang R, Zhang X, Wang J, Letham DS, McKinney SA, Higgins TJV (1995) The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expressing an *ipt* gene. Planta **196:** 84–94