Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 510-515

CYTOKININS IN TOBACCO CROWN GALL TUMORS

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Received February 4,1980

SUMMARY: Bacteria-free tobacco (Nicotiana tabacum cv. Wisconsin #38) crown gall strains incited by Agrobacterium tumefaciens C58, 27, B6, CGIC, and AT4 have been analyzed for cytokinin content with the tobacco callus bioassay. All tumor strains contained high total levels of cytokinins ranging from 4-810 kinetin equivalents per kg fresh weight compared to 0.5 kinetin equivalents per kg for normal callus growing on medium with 0.1 μM N6-benzyladenine. Fractionation on a column of Sephadex LH-20 separated cytokinin activity from B6 tumors into a number of components among which ribosyl-trans-zeatin has been purified and characterized based on uv spectrum, biological activity and mass spectrum.

INTRODUCTION

Crown gall is a bacterial disease of dicotyledons caused by the pathogen Agrobacterium tumefaciens (Smith and Townsend) Conn. and characterized by tumor-like overgrowths on infected plants. The unique properties of crown gall tumors such as production of high levels of opines and the ability of bacteria-free tissue cultures to grow on medium lacking auxin and cytokinin apparently result from the expression of Ti plasmid DNA (T-DNA) by transformed plant cells (3).

Growth of bacteria-free crown gall tissues in the absence of phytohormones contrasts with the requirement of normal tissues for exogenous supplies of auxin and cytokinin. Miller and associates have reported definitive evidence based on chemical methods for a high cytokinin content in an A6 line of Vinca rosea L. tumor tissue. They found unusually high concentrations of ribosyl-trans-zeatin in this tissue and also identified three other cytokinins; i.e. zeatin and the glucosyl derivatives of zeatin and ribosyl-trans-zeatin (7,8,9).

0006-291X/80/060510-06$01.00/0

In experiments to determine whether high cytokinin content is found in tobacco crown gall strains incited by a number of different strains of A. tumefaciens, we have analyzed callus tissue of normal tobacco and of five bacteria-free crown galls for cytokinins. This paper reports on the high relative cytokinin activity in these crown gall tissues as measured with the tobacco bioassay and on the purification and characterization of ribosyl-trans-zeatin from a B6-incited crown gall strain.

MATERIALS AND METHODS

Crown galls were induced on young tobacco (Nicotiana tabacum cv. Wisconsin #38) plants and obtained as bacteria-free tissue cultures as described (5). The five different tumor strains analyzed for cytokinin contents are listed in Table 1.

To prepare extracts for cytokinin analysis, tissue from 5-week-old cultures was homogenized with 95% ethanol (2 ml per g of tissue) to give a final ethanol concentration of 70%, and insoluble material was removed by filtration. After the pH was adjusted to 2.5, the ethanol-soluble material was applied to an AG50W-X4 (H^+) cation exchange column (0.5 ml of resin per g of tissue) which was eluted with 2 bed volumes of water and then 8 bed volumes of 1N NH_4OH. The ammonia eluate was evaporated to dryness and the residue was extracted 10 times with 20 ml volumes of water-saturated ethyl acetate. After evaporation of the ethyl acetate, the extract was analyzed for cytokinin activity in the tobacco bioassay (10) either directly or after further fractionation on a Sephadex LH-20 column eluted with 35% ethanol (1). Cytokinin content is expressed as kinetin equivalents (KE) where 1 KE is the growth response obtained on medium containing 1 µg per liter of kinetin.

Ribosyl-trans-zeatin was purified from B6-incited crown gall tissue by the extraction methods described above followed by fractionation on a Sephadex LH-20 column (150 ml bed volume). Fractions corresponding to the elution volume of ribosyl-trans-zeatin were pooled, evaporated to dryness and the residue was further fractionated on a column of Porapak Q (15 ml bed volume) equilibrated in 15.2% ethanol (2). The sample (2 ml) was dissolved in the same solvent and 3 ml fractions were collected. Fractions corresponding to the elution volume of ribosyl-trans-zeatin, between 45 ml and 58 ml, were analyzed for their uv spectrum, pooled and concentrated by evaporation to dryness. Mass spectral

Table 1. Characteristics of Agrobacterium tumefaciens strains and tobacco crown gall strains analyzed for cytokinin contents.

Strain of Agrobacterium	Octopine or Nopaline Utilization by Bacteria	Octopine or Nopaline Production by Tumor
C58	Nopaline	Nopaline
27	Nopaline and octopine	Nopaline
B6	Octopine	Octopine
CG1C	Octopine	Octopine
AT4	Neither	Neither

data on the purified material was obtained by direct inlet introduction of the sample and ionization by electron impact at 70 ev with a Finnigan 1015 S/L mass spectrometer.

RESULTS AND DISCUSSION

Table 2 shows the results of analyses of crown gall tissues for their total cytokinin activity compared to normal tobacco tissue growing on a medium giving rapid growth of friable calus and optimal fresh weight yield. Although the levels of cytokinin varied considerably between the different tumor strains (4-810 kinetin equivalents per kg fresh weight), all crown gall strains tested had high relative cytokinin activities.

When an extract from the normal tobacco callus strain grown on medium supplemented with N6-benzyladenine was fractionated on a Sephadex LH-20 column, the major part of the cytokinin activity eluted between 1.2 and 1.5 bed volumes which corresponds to the elution volume of zeatin and between 2.3 and 3.0 bed volumes which corresponds to the elution volume of N6-benzyladenine (Figure 1). Cytokinin activity from the B6 crown gall strain had a different elution pattern with the majority of the activity eluting between 0.7 and 1.2 bed volumes and the remainder eluting between 1.2 and 2.3 bed volumes. On the basis of the Sephadex LH-20 fractionation, B6 tobacco crown gall tissues have cytokinins with elution volumes corresponding to the glucose esters of zeatin and ribosylzeatin (I), ribosylzeatin (II), zeatin (III), N6-isopentenyladenosine (IV) and N6-isopentenyladenine (IV).

Table 2. Cytokinin contents of normal and crown gall tissues.

	Cytokinin concentration	
Tissue	KE/kg	Relative
Wis #38 callus[a]	0.5	1X
C58 tumor	4	8X
27 tumor	110	220X
B6 tumor	810	1620X
CG1C tumor	20	40X
AT4 tumor	225	450X

[a]Callus grown on medium supplemented with 11.4 μM indole-3-acetic acid and 0.1 μM N6-benzyladenine.

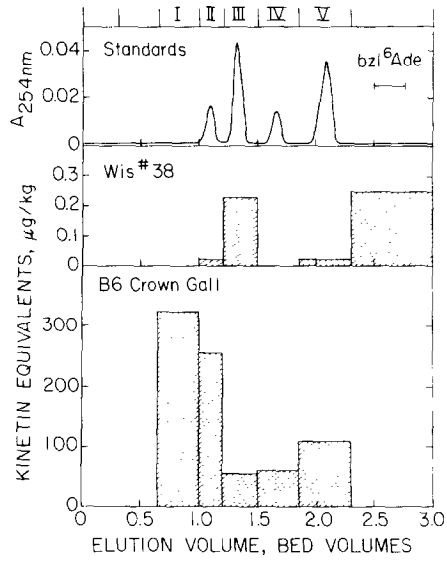

Figure 1. Fractionation of cytokinins from normal tobacco callus and B6 crown gall tissues on a Sephadex LH-20 column (150 ml bed volume) equilibrated and eluted with 35% ethanol. The sample volume in each case was 2 ml. Peaks of absorbance in the top graph indicate 50 µg each of ribosylzeatin (II), zeatin (III), N6-isopentenyl-adenosine (IV) and N6-isopentenyladenine (V). The I indicates the expected elution volume of glucose esters of ribosylzeatin and zeatin and bzl^{6}Ade indicates the expected elution volume of N6-benzyladenine. Bars indicate cytokinin activity determined with the tobacco callus bioassay.

To obtain definitive evidence for the presence of ribosyl-trans-zeatin in B6 tobacco crown gall tissue, this cytokinin was purified and chemically characterized. Using the procedure described, 34 µg of ribosyl-trans-zeatin was obtained from 225 g fresh weight of B6 crown gall tissue. This amount which was calculated based on the ultraviolet absorption spectrum of the purified material corresponds to approximately 150 µg per kg fresh weight and is close to the concentration of ribosyl-trans-zeatin determined from Figure 1 which is 250 µg per kg assuming biological activity comparable to that of kinetin. The purified cytokinin had a maximum uv absorption at 269 nm indicative of ribosyl-trans-zeatin and cochromatographed with synthetic standards on the Sephadex LH-20 column and the Porapak Q column which separates the cis and trans isomers of ribosylzeatin. The mass spectrum obtained at 210 C had prominent peaks at m/e 351 (molecular weight of ribosylzeatin), 334, 320, 278, 219 (molecular weight of zeatin), 202, 188, 186, 174, 160, 136, 135, 119 and

108. The relative intensities of these fragment ions corresponded closely to those obtained with a synthetic sample of ribosyl-trans-zeatin (Table 3). Collectively these data identify the purified cytokinin from the B6-incited tobacco crown gall as ribosyl-trans-zeatin.

These results demonstrate high relative cytokinin activity in bacteria-free tobacco crown galls incited by 5 different strains of A. tumefaciens and representing the three major types of tumors; namely, nopaline, octopine and null type crown galls. The experiments also show that the total cytokinin activity of tumors incited by A. tumefaciens B6 is due to a number of components among which ribosyl-trans-zeatin has been purified and chemically characterized. When the results are considered together with similar findings by Miller with a Vinca rosea crown gall strain, it appears that cytokinin overproduction may be a common feature of crown gall tumor physiology.

The mechanism by which T-DNA causes cytokinin overproduction in transformed plant cells is unknown but two alternative hypotheses seem plausible. One hypothesis, the direct hypothesis, states that the T-DNA has a structural gene(s) coding for an enzyme of the cytokinin biosynthetic pathway in tumors. The second hypothesis, the indirect hypothesis, states that T-DNA has a gene(s) that

Table 3. Mass spectra of synthetic ribosyl-trans-zeatin and purified cytokinin from B6 tobacco crown gall tumor tissue.

	Relative intensity	
m/e	Ribosyl-trans-zeatin	B6 cytokinin
351	0.19	0.15
334	0.60	0.40
320	0.41	0.40
278	0.10	0.30
219	0.22	0.20
202	1.56	1.50
188	1.43	1.35
186	0.82	0.60
174	0.29	0.39
160	1.00	1.00
136	2.00	1.73
135	1.53	2.10
119	0.94	1.50
108	0.79	1.20

regulates plant gene expression of cytokinin biosynthesis. While information to date does not distinguish between these two hypotheses, there is evidence on cytokinins in both crown galls and culture filtrates of A. tumefaciens (6).

Normal, non-transformed tobacco tissues require exogenous sources of both auxin and cytokinin for sustained growth on nutrient media. As the concentration of auxin in the medium is increased, the concentration of cytokinin required for growth is decreased (4). Because of this, the finding of high relative cytokinin levels in crown galls is not sufficient evidence, in itself, to account for the capability of crown galls to grow on nutrient medium devoid of cytokinin. An explanation for the phytohormone autotrophy of tumor tissues will require data on auxin contents, in addition to the information on cytokinin contents reported here.

ACKNOWLEDGEMENTS

I thank E. W. Nester, University of Wasington, Seattle, for the strains of Agrobacterium tumefaciens and N. T. Keen, University of California, Riverside, for assistance with the mass spectrometric analyses. This work was supported by National Science Foundation Grant PCM-7911229 to J. W. Einset.

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