OCCURRENCE AND LEVELS OF *cis*- AND *trans*-ZEATIN RIBOSIDES IN THE CULTURE MEDIUM OF A VIRULENT STRAIN OF *AGROBACTERIUM TUMEFACIENS*

James A. McCLOSKEY, Takeshi HASHIZUME+, Brenda BASILE, Yoko OHNO+ and Shigenori SONOKI*

Departments of Medicinal Chemistry and Biochemistry, University of Utah, Salt Lake City, UT 84112, USA and *+Laboratory of Bioorganic Chemistry, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan*

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1. Introduction

Agrobacterium tumefaciens is a widely distributed plant pathogen which induces crown gall tumors in a variety of plants, most of which are dicotyledonous. Large tumor-inducing (Ti) plasmids of the bacterial cells code for functions essential to tumorigenesis in dicotyledonous plants [1–4]. Although active investigations have been undertaken pertaining to the plasmids [5–8], little has been known about the cytokinins which may possibly be excreted into the culture medium by the bacterium, except [9], published during this study. The bases iPAde and *trans*-zeatin were found at 5.4–8.8 μg iPAde/l culture medium, and 2–3 μg *trans*-zeatin/l, relative to kinetin added as internal standard. *cis*-Zeatin was also shown present [10]; the virulent strain produced higher levels of *trans*- and *cis*-zeatin and iPAde than the avirulent strain.

We report here the presence and levels of *cis*- and *trans*-zR and, possibly, *trans*-mszR, in the culture medium of the virulent (plasmid-containing) strain (C58) of *Agrobacterium tumefaciens*, while none of the 4 nucleoside cytokinins, iPA, msiPA, zR, and mszR, were found in the culture of an avirulent (plasmidless) strain (*A. tumefaciens* NT1).

Abbreviations: iPA, N^6-(Δ^2-isopentenyl)adenosine; iPAde, N^6-(Δ^2-isopentenyl)adenine; msiPA, N^6-(Δ^2-isopentenyl)-2-methylthioadenosine; zR, zeatin-9-β-D-ribofuranoside; *m/z*, mass-to-charge ratio; M, molecular ion

Address correspondence to T. Hashizume

*Present address: Department of Environment and Health Sciences, Azabu College of Veterinary Science, Sagamihara, Kanagawa, Japan

2. Materials and methods

2.1. *Microorganisms*

A plasmid-containing strain, *A. tumefaciens* C58, and a plasmidless strain, *A. tumefaciens* NT1, were used. The bacteria were grown at 30°C for 60 h in 1 liter vol. in 3 liter flasks on the medium below. Continuous agitation was maintained in the dark by a horizontal shaker.

2.2. *Composition of the culture medium*

A mixture of sucrose (10 g), casein hydrolysate (8 g), yeast extract (4 g), K_2HPO_4 (2 g), and $MgSO_4$ (0.3 g), was dissolved in 1 liter distilled water and sterilized.

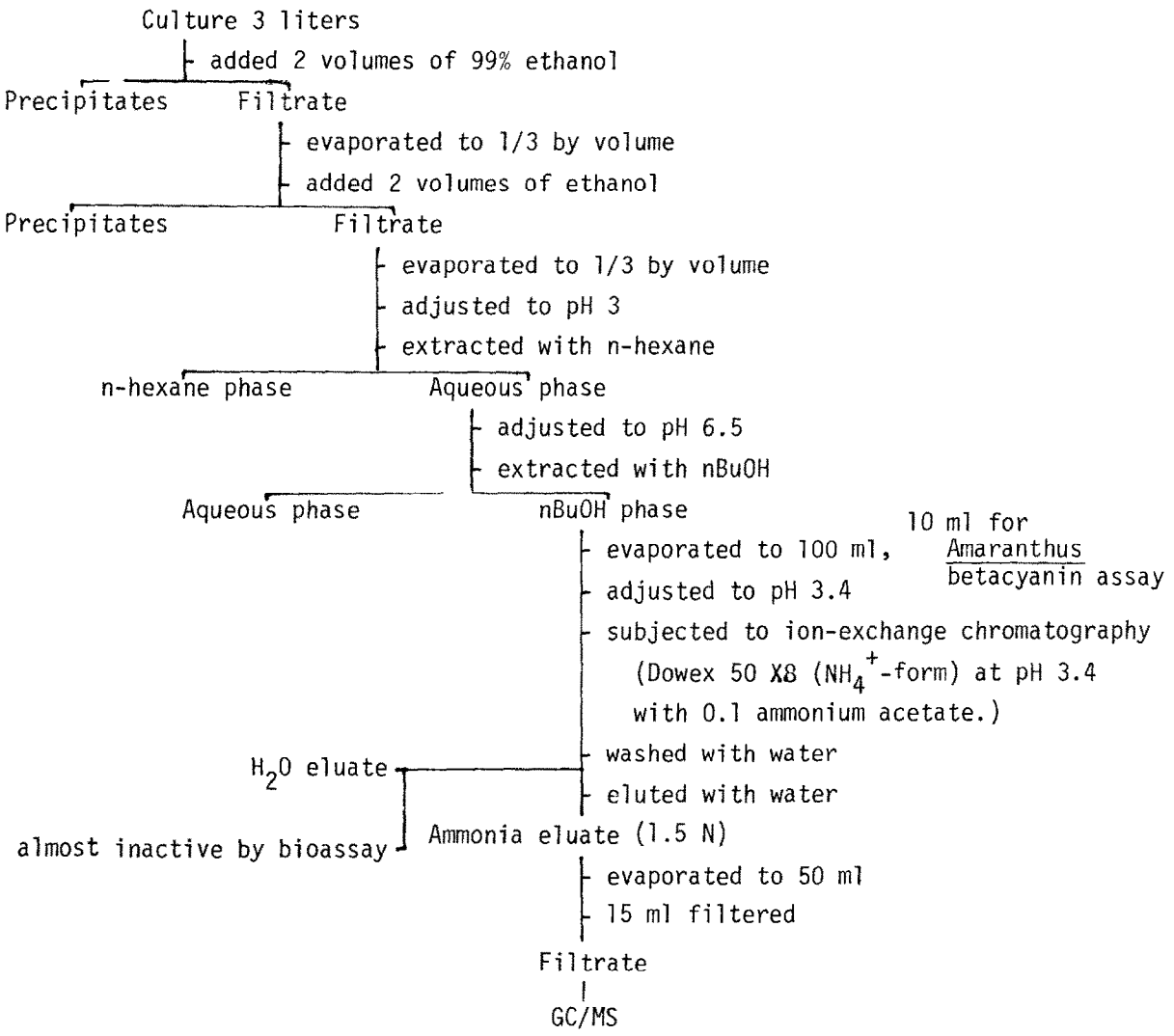
2.3. *Procedures for purification of cytokinins*

The isolation and purification procedures used are summarized in scheme 1. Following filtration of a 15 ml aliquot in the final step, the precipitates were washed with 80% hot ethanol. The washings and the filtrate were combined and evaporated to 2 ml. An aliquot (50 μl) was withdrawn, and 2 μl of each of the 4 internal standards (see section 2.4) in DMF solution were added. The solution was dried, and silylated in a mixture of DMF (10 μl) and hexamethyldisilazane (90 μl) at 90°C for 1 h. The silylated product (8 μl) was injected into the gas chromatograph–mass spectrometer (GC/MS). The culture medium of avirulent strain, NT1, was treated similarly.

2.4. *Deuterium-labeled internal standards*

Internal standards used for these experiments were iPA-d_6, msiPA-d_6, *cis*- and *trans*-zR-d_3, and *cis*- and *trans*-mszR-d_3. Their syntheses were reported in [11].

Scheme 1
Extraction procedures of cytokinin containing fraction from the culture of *Agrobacterium tumefaciens* C58



2.5. *GC/MS using selected ion monitoring*

Mass spectrometric measurements were obtained using an LKB 9000S GC/MS interfaced to a PDP-11/40 computer. Operating conditions were: carrier gas separator and ion source temperatures, 270°C; ionizing energy, 70 eV; 3 ft. 1% OV-17 liquid phase, temperature programmed from 190–270°C at 6°C/min. Procedural details for mass spectrometry were described earlier, including construction of a standard calibration curve [11]. The following ions were monitored: zR, *m/z* 624 (M-15, d_0), 627 (M-15, d_3); mszR, *m/z* 670 (M-15, d_0), 673 (M-15, d_3).

3. Results and discussion

The presence of *cis*- and *trans*-zR, and *trans*-mszR was established at the levels shown in table 1. Discussion of the method of measurement of these cytokinins and the differentiation of the *cis*- and *trans*-isomers of zR and mszR were presented in [11]. A similar method was developed in [12].

Although iPAde, *trans*-zeatin and *cis*-zeatin were reported at maximal levels of 8.8 μg/l–5.46 μg/l and 0.43 μg/l, respectively, using kinetin as internal standard, the report dealt with bases [9]. We report

Table 1
Cytokinins found in the culture medium of *Agrobacterium tumefaciens* C58

Cytokinin	Found (μg/l)
cis-zR	1.1
trans-zR	0.31
cis-mszR	–
trans-mszR	0.19[a]
iPA	--
msiPA	–

[a] Identification tentative; see text

here the presence of the cytokinin nucleosides as shown in table 1. By comparison, our measurements showed that the level of *cis*-zR was substantially higher than that of *trans*-zR, the level of *trans*-zeatin was higher than that of the *cis*-isomer in [9,10]. This notable difference raises the question as to whether this is related to the particular growth stage of the bacteria. The growth stage involved in the earlier work was not reported [9,10], therefore, this factor cannot be further considered at present. The signal obtained for *trans*-mszR was distinct but sufficiently close to the limit of detection corresponding to ~0.20 μg/l that positive identification based on the present data must remain tentative. Identical experiments were also carried out on similar extracts obtained from a culture of *A. tumefaciens* NT1, but none of the cytokinin nucleosides were found at the minimum detection limit.

The fact that tumor cells are able to grow in vitro in the absence of viable bacteria on agar lacking auxin and cytokinin, a medium on which normal plant tissue is unable to grow, may possibly be explained by their capability to produce cytokinins [2]. Whether or not a certain region of the plasmid (Ti) codes for the production of cytokinin remains to be studied.

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