Plant Physiol. (1981) 68, 735–738
0032-0889/81/68/0735/04/$00.50/0

Cytokinins from the Moss *Physcomitrella patens*[1]

Received for publication September 19, 1980 and in revised form March 2, 1981

TREVOR L. WANG,[2] ROGER HORGAN,[3] AND DAVID COVE
Department of Genetics, University of Leeds, Leeds, LS2 9JT United Kingdom

ABSTRACT

Gametophore-over-producing mutants of the moss, *Physcomitrella patens*, when grown in liquid culture export high levels of cytokinin into their culture medium. The cytokinin produced by these mutants is postulated to account for their peculiar phenotype, that of mosses treated with exogenous cytokinin. N^6-(Δ^2-isopentenyl)adenine, the major cytokinin, has been identified previously in two of these mutants (Wang, Cove, Beutelmann, Hartmann 1980 Phytochemistry 19: 1103–1105) and now in additional representatives. A second cytokinin, zeatin, has been identified by its chromatographic behavior and mass spectrum including chemical ionization mass spectrometry of its permethyl derivative.

During its development, moss tissue passes through several distinct phases of growth each being characterized by specific cell types (3–5). One of the latter stages in moss morphogenesis is the production of leafy shoots or gametophores which eventually give rise sexually to the sporophyte generation and the production of spores. The production of gametophores can be influenced by plant growth regulator application; it is this influence that has led to the hypothesis that the naturally occurring compounds may be involved in gametophore development *in vivo* (12, 13). Consequently, moss tissue has been used for many biochemical and physiological studies of plant hormones (6, 7, 11, 17, 20), even though the presence of these substances in normal moss tissue has yet to be established.

The morphogenesis of the moss *Physcomitrella patens* and its response to plant growth regulators are similar to other moss species. However, its use as a system for genetic analysis has led to the isolation of several mutants altered in their response to and production of plant growth regulators (9). Several of these mutants (OVE mutants[4, 5]) phenotypically resemble the WT moss when it has been treated with a high concentration of cytokinin (2). Again, this raised the question of whether endogenous cytokinin could account for the abnormal development of OVE mosses and thus be indirectly implicated in the development of gametophores in WT tissue.

OVE mutants fall into three complementation groups (Featherstone and Cove, unpublished data; [14]); that is, when crossed parasexually via protoplast fusion, mutants from different complementation groups produce (diploid) hybrids which resemble the WT while fusions between members of the same group produce hybrids showing OVE characters. At least three genes, must be able to mutate to give rise to the OVE phenotype.

In a previous paper (23), the culture media of OVE 100 and 78 (each from different complementation groups) were analyzed for their cytokinin content and both found to contain i^6Ade while only OVE 100 contained another, more polar, cytokinin. In the present report, other OVE and WT mosses have been examined for the presence of cytokinin in their culture medium. In addition, the identity of the second cytokinin has been established.

MATERIALS AND METHODS

Moss Culture Conditions. The procedures for the maintenance of cultures, the preparation of homogenates for culture inocula, and the contents of media have been reported in previous studies (15, 23). Initially, cultures were grown as single batches in large reagent bottles. However, such vessels were superceded by 10 liters fermentor-type flasks equipped with sample outlet, air inlet, and air outlet (with an on-line condenser to prevent water loss) tubes of the type used for cell suspension cultures (21). The use of these vessels facilitated continuous monitoring of the growth of a culture. The protonemata were maintained in suspension by the flow of sterile air into the medium and no additional mechanical stirring was necessary.

Growth Analysis. Samples were withdrawn from the vessel under positive pressure and known volumes passed through dry Whatman GF/C filter papers. The retained protonemata were then dried at 60 C for 2 days before weighing.

Cytokinin Isolation from Culture Media. The extraction, isolation, and identification of i^6Ade from the culture media of the various moss tissues was performed as reported elsewhere for OVE 100 (23) by using 1-butanol partition, Sephadex LH20 chromatography, and MS. Biological activity was detected by using a modified soybean callus assay (23). Where sufficient activity, indicated by bioassay, eluted in the region of io^6Ade (22, Fraction I) from Sephadex chromatography, the relevant fractions were pooled and taken to dryness. Each sample was dissolved in 30 μl water (adjusted to pH 7 with TEAB) and subjected to HPLC on Hypersil ODS (Shandon Southern Ltd) with water (adjusted as above) and acetonitrile as solvents after the method of Horgan and Kramers (16). Peaks eluting in the region of io^6Ade were collected, dried, and analyzed either by MS as a direct probe sample or by GC-MS as the permethyl derivative.

Permethylation. Permethylation was performed by using dimethylsulfinyl carbanion and methyl iodide (18, 19). The use of the carbanion was simplified by following the procedure of Eagles *et al.* (10) and its preparation undertaken by warming potassium

[1] This work was supported by grant GR/A/3800.7 from the Science Research Council to D. J. C.

[2] To whom requests for reprints should be addressed at: Department of Applied Genetics, John Innes Institute, Colney Lane, Norwich, NR4 7UH, United Kingdom.

[3] Present address: Department of Botany and Microbiology, the University College of Wales, Penglais, Aberystwyth, Dyfed, SY23 3AN, United Kingdom.

[4] For details of the nomenclature used here for describing moss tissue, see ref. 2.

[5] Abbreviations: OVE, gametophore-over-producer; WT, wild type; i^6Ade, N^6-(Δ^2-isopentenyl)adenine; io^6Ade, 6-(4-hydroxy-3-methyl-2-butenylamino)purine, zeatin; TEAB, trimethylammonium bicarbonate; HPLC, high performance liquid chromatography; CI, chemical ionization; TMS, trimethylsilyl.

t-butoxide (112 mg/ml) in dimethylsulfoxide at 45 C for 1 h under an atmosphere of N_2. Samples for permethylation were dried in 1-ml Pierce Reactivials, sealed, and flushed with N_2. One hundred μl carbanion solution were added followed by 10 μl methyl iodide. The samples were then left at room temperature for a minimum of 30 min before halting the reaction by the addition of 200 μl water. The permethylated samples were partitioned into equal volumes of chloroform three times, the combined chloroform phases were reduced in volume and then backwashed with an equal volume of water before finally being dried for GC-MS.

MS and Combined GC-MS. Direct probe low resolution mass spectra were recorded on an AEI MS 30 single beam mass spectrometer operating at 70 ev. Scans were performed at 10 s/mass decade with a source temperature of 180 C and probe temperature of 150 C. Spectra were processed by a Kratos DS 50 data system.

GC-MS was carried out by using an AEI MS30 mass spectrometer linked via a jet separator to a Perkin Elmer Sigma 2 gas chromatograph equipped with a stream splitter. Scans were recorded at 10 s/mass decade and stored on a Kratos DS50 SM data system. CI conditions were produced by using ammonia gas introduced into the source (pressure, $2-5 \times 10^{-5}$ torr: temperature, 150 C) and with the ionization energy increased to 400 ev to give maximum sensitivity. Samples were run on a 4 × 1980 mm column of GasChrom Q (80–100 mesh) coated with 2% SE33 by using a helium flow rate of 40 ml/min, a column temperature of 210 C, and a separator temperature of 230 C.

Solvents and Reagents. All solvents were redistilled or HPLC grade (Jones Chromatography Ltd.). Authentic markers of zeatin and i^6Ade were obtained from Sigma Chemical Co. Ltd., and *cis*-zeatin was isolated by HPLC as a minor impurity of the zeatin.

RESULTS

Growth of Moss Tissue in Liquid Culture. The use of fermentor-type culture vessels for the growth of moss tissue allows continuous monitoring of the culture for any growth parameters and other features of interest. Small samples can be withdrawn easily under sterile conditions without disturbing the overall growth of the culture. OVE and WT tissues have very similar growth rates. Noticeable differences can arise, however, due to the condition of the original inoculum. Figure 1 shows the growth (as measured by dry weight) of three OVE cultures and a WT culture as batch cultures and illustrates this point.

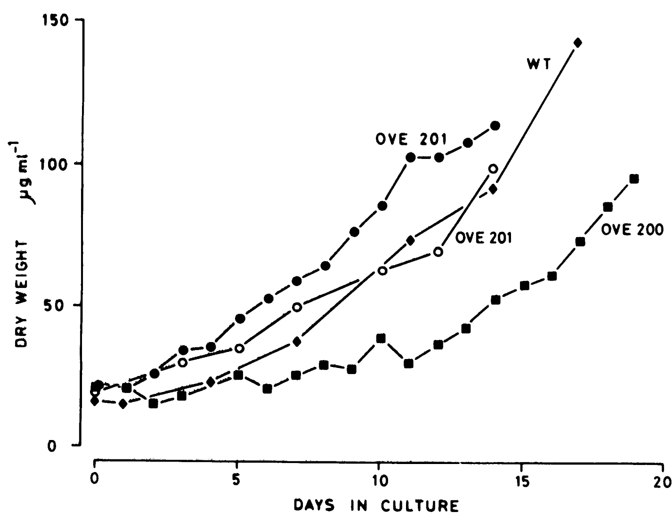

FIG. 1. Growth of moss tissue in liquid culture. Ten liters liquid cultures were inoculated with homogenates of moss protonemata and samples withdrawn under sterile conditions. For dry weight analysis, 40 ml suspension was collected on glass fiber filters and dried at 60 C.

Cultures are inoculated from young agar-grown colonies after homogenization (which initiates regeneration and chloronemal growth). It is this homogenization which accounts for the lag, often present, and most noticeable in the OVE 200 culture. When viewed under the microscope 24 h after inoculation, such a culture shows extensive cell lysis due to over-homogenization, although this is only reflected in the dry weight analysis as a small decline. The extensive lag that follows is not due to a lack of growth but to the recovery, via regeneration of viable cells, that ensues. Moss tissue does not exhibit cell density-dependent growth as do many cell suspension cultures of higher plants (21). Once established the growth is similar to the other cultures. Both OVE 201 cultures included illustrate the difference that can occur in the growth of the same moss.

i^6Ade in Moss Culture Medium. Culture medium partitioned against butanol and subjected to chromatography on Sephadex LH20 requires no further purification for the isolation of i^6Ade. After bioassay, fractions containing biological activity from the elution region of i^6Ade were pooled, reduced in volume, their UV spectra measured, and they were then taken to dryness for MS. Spectra were measured via direct probe insertion and all were identical with the authentic compound. Examples of both have been published previously (23). Table I summarizes the data obtained to date.

Zeatin from Culture Medium of Moss Tissue. Unlike putative i^6Ade samples from moss culture medium, fractions eluting in the region of io^6Ade from Sephadex chromatography frequently contain impurities which show on the UV spectrum as increased absorption (over a standard solution) in the region below 270 nm. Such samples were, therefore, purified before mass spectral analysis by reverse-phase HPLC. Figure 2A shows the HPLC trace of an aliquot of fraction 21 from Sephadex chromatography of 12 liters OVE 201 culture medium. Peak A was collected and further analyzed by CI-MS as the permethyl derivative. Figure 2B shows the GC trace obtained for peak A and Figure 3A shows its CI spectrum. This spectrum contained the same ions as that obtained from authentic zeatin (Fig. 3B). From their chromatographic positions on Sephadex LH20, GC, and reverse-phase HPLC, their UV and mass spectra, the minor peaks of biological activity from OVE culture media (fractions I, 23) were identified as zeatin. Table I includes data on io^6Ade isolations from moss culture media. Using the recovery of [^{14}C]benzylaminopurine as a standard, we estimated that on average 65% of the cytokinin was recovered during the extraction procedure.

Table I. *Cytokinin Content of Moss Culture Medium*

Concentrated culture media from all cultures other than OVE 78 were partitioned against butanol and chromatographed on Sephadex LH20. Biological activity in the region of i^6Ade detected by the soybean callus assay was further analyzed directly by MS. Activity in the region of io^6Ade was purified by HPLC and then analyzed by GC-MS. For further details see text and ref. 23. Measurements are based on UV absorption of the isolated compounds at λ_{max}. Those marked with an asterisk were estimated from data obtained from the soybean callus bioassay. Values in μg/g fresh wt are based on the yield of cytokinin obtained from the tissue obtained at the end of the growth period. Nd = not detected.

Strain	Cytokinin Content in Culture Medium			
	i^6Ade		Zeatin	
	nM	*μg/g fresh wt*	*nM*	*μg/g fresh wt*
WT	<1*	<0.04*	<0.2*	<0.004*
OVEA200	60	7.20	5.0	0.300
OVEA78	100	5.70	nd	nd
*ove*B100	25	5.30	1.1	0.270
OVEC200	23	4.60	<0.5*	<0.100*

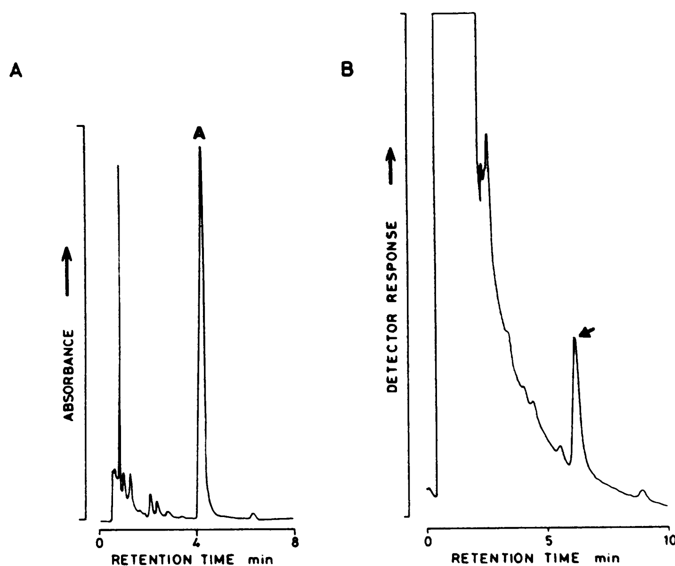

FIG. 2. A, HPLC profile of fraction I from the culture medium of OVE 201. Medium from an OVE 201 culture was partitioned against butanol and subjected to Sephadex LH-20 chromatography. An aliquot from fraction I was chromatographed on Hypersil ODS with 12% acetonitrile in water. Peak A eluting in the region of zeatin was collected for further analysis. *cis*-zeatin would have eluted about 1 min later. B, GLC profile of Peak A from HPLC (Fig. 2A). Peak A from HPLC was collected, permethylated, and chromatographed on 2% SE33 at 210 C. The major peak (arrowed) eluted at the retention time of zeatin (*cis*-zeatin would have eluted about 0.5 min earlier).

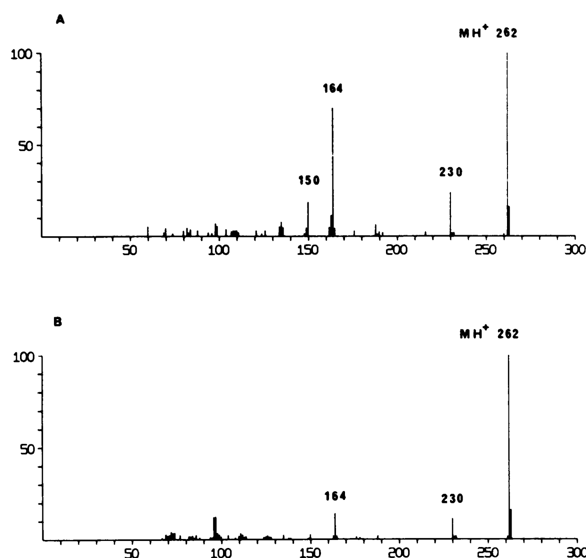

FIG. 3. A, CI mass spectrum of the minor cytokinin from OVE 201 culture medium. Fraction I from Sephadex chromatography, purified as described in the text, was permethylated and analyzed by GC-MS. Spectra were recorded on an AEI MS30 with ammonia as the ionizing gas. B, CI mass spectrum of zeatin. Ten μg of authentic zeatin was permethylated and analyzed by GC-MS as in Fig. 3A. For the nature of the fragment ions see ref. 24.

DISCUSSION

In a previous report (23) it was demonstrated that the gametophore over-producing mutants OVE 100 and OVE 78 of *P. patens* produced i^6Ade which was present in significant quantities in the culture media. A second peak of biological activity was also detected by the soybean callus assay in OVE 100 culture medium. In the current investigation, further OVE mutants and WT tissue have been tested for their cytokinin production. The second peak of activity has been identified as zeatin from its chromatographic behavior and mass spectrum. In all cases, zeatin occurs at much lower levels than i^6Ade in the culture medium.

It has not yet been possible to identify any cytokinins in WT culture medium or tissue inasmuch as they occur in the former at least 100-fold less a concentration than that in OVE and their presence is intimated only by bioassay.

Zeatin was not detected in OVE 78 culture medium perhaps due to the bioassay employed. OVE 201 which falls into the same complementation group as OVE 78 has been demonstrated here to contain significant quantities of zeatin in its culture medium, and hence, it is likely that OVE 78 produces some but too little zeatin to detect. OVE 200 is a similar instance. In this case, zeatin was only detected by bioassay and not enough available for further analysis. Nevertheless, it is clear that OVE mutants which over-produce gametophores are, in addition, indeed cytokinin over-producers. This latter characteristic is highly likely to be responsible for the former inasmuch as a high concentration of cytokinin applied to WT tissue will induce a response which is an imitation of the OVE phenotype (1). In this study, representatives of each of the three OVE complementation groups have been examined for cytokinin production and all export elevated levels, with regard to the WT, of the same cytokinins. We have not yet been able to distinguish between the roles of the three gene products which must be involved in determining normal cytokinin levels in the WT.

One of the techniques examined here, CIMS of permethylated cytokinins, has been reported recently by Claeys and co-workers (8) for the identification of *Agrobacterium tumefaciens* cytokinins. Permethylation has been employed before for cytokinin analysis (19, 24) and its advantages are 2-fold: only one derivative per cytokinin is formed and the intensities of the molecular ions are increased relative to TMS derivatives. When coupled with CIMS the advantages are increased. The quasi-molecular ion (MH^+) is the most abundant ion in the spectrum and its m/e value for complex cytokinins (such as glucosides and ribosyl glucosides) is far lower than the respective TMS derivatives (22). Hence, it is hoped that such a technique will help in the identification of very small quantities of cytokinin that are likely to be present in WT mosses.

Acknowledgments—We thank J. Eagles, Food Research Institute, Norwich, for his help in the running of the CI mass spectra and J. K. Heald, University College of Wales, for the running of probe samples.

LITERATURE CITED

1. ASHTON NW, DJ COVE, DR FEATHERSTONE 1979 The isolation and physiological analysis of mutants of the moss, *Physcomitrella patens*, which over-produce gametophores. Planta 144: 437–442
2. ASHTON NW, NH GRIMSLEY, DJ COVE 1979 Analysis of gametophytic development of the moss, *Physcomitrella patens*, using auxin or cytokinin resistant mutants. Planta 144: 427–435
3. BOPP M 1954 Ein Beitrag zur differenzierung in Moosprotonema. Ber Deut Bot Ges 67: 177–184
4. BOPP M 1961 Die Morphogenese der Laubmoose. Biol Rev 36: 237–280
5. BOPP M 1968 Control of differentiation in fern-allies and bryophytes. Annu Rev Plant Physiol 19: 361–380
6. BOPP M 1973 Action mechanism of cytokinins in mosses (a model for the effects of cytokinins). *In* Plant Growth Substances 1973, Part IV. Hirokawa Publishing Co Inc, Tokyo. pp 934–944
7. BOPP M, U ERICHSEN, M NESSEL, B KNOOP 1978 Connection between the synthesis of differentiation specific proteins and the capacity of cells to respond to cytokinin in the moss *Funaria*. Physiol Plant 42: 73–78
8. CLAEYS M, E MESSENS, M VAN MONTAGU, J SCHELL 1978 Quantitative determination of cytokinins in culture media of *Agrobacterium tumefaciens*. *In* AP De Leenheer, RR Roncucci, C Van Peteghem, Quantitative Mass Spectrometry in Life Sciences Vol II. Elsevier, Amsterdam. pp 409–418
9. COVE DJ, NW ASHTON, DR FEATHERSTONE, TL WANG 1980 The use of mutant strains in the study of hormone action and metabolism in the moss *Physcomitrella patens*. *In* DR Davies, DA Hopwood, The Plant Genome. Proceedings of

the IV John Innes Symposium. John Innes Charity, Norwich. pp 231–241
10. EAGLES J, WM LAIRD, R SELF, RLM SYNGE 1974 Permethylation for mass spectrometry: rearrangements of ester linkages and use of potassium *t*-butoxide. Biomed Mass Spec 1: 43–48
11. GARDNER G, MR SUSSMAN, H KENDE 1978 *In vitro* cytokinin binding to a particular cell fraction from protonema of *Funaria hygrometrica*. Planta 143: 67–73
12. GORTON BS, RE EAKIN 1957 Development of the gametophyte in the moss *Tortella caespitosa*. Bot Gaz 119: 31–38
13. GORTON BS, CS SKINNER, RE EAKIN 1957 Activity of some 6-substituted purines on the development of the moss *Tortella caespitosa*. Arch Biochem Biophys 66: 493–496
14. GRIMSLEY NH, DR FEATHERSTONE, GRM COURTICE, NW ASHTON, DJ COVE 1980 Somatic hybridisation following protoplast fusion as a tool for genetic analysis of the moss *Physcomitrella patens*. *In* Advances in Protoplast Research. Hungarian Academy of Sciences, Budapest. pp 363–376
15. GRIMSLEY NH, NW ASHTON, DJ COVE 1977 The production of somatic hybrids in the moss, *Physcomitrella patens*. Mol Gen Genet 154: 97–100
16. HORGAN R, MR KRAMERS 1979 High performance liquid chromatography of cytokinins. J Chromatogr 173: 263–270
17. JOHRI MM 1974 Differentiation of caulonema cells by auxins in suspension cultures of *Funaria hygrometrica*. *In* Plant Growth Substances 1973 Part IV. Hirokawa Publishing Co Inc, Tokyo. pp 925–933
18. LECLERQ PA, DM DESIDERIO JR 1971 A laboratory procedure for the acetylation and permethylation of oligopeptides on the microgram scale. Anal Lett 4: 305–316
19. MORRIS RO 1977 Mass spectroscopic identification of cytokinins glucosyl zeatin and glucosyl ribosylzeatin from *Vinca rosea* crown gall. Plant Physiol 59: 1029–1033
20. SPEISS LM 1979 Antagonism of cytokinin induced callus in *Pylaisiella selwynii* by nucleosides and cyclic nucleotides. Bryologist 82: 47–53
21. STREET HE 1977 Plant, Tissue and Cell Culture, Ed 2. Blackwell, London
22. SUMMONS RE, B ENTSCH, DS LETHAM, BI GOLLNOW, JK MACLEOD 1980 Metabolites of zeatin in sweet-corn kernels: purifications and identifications using high-performance liquid chromatography and chemical-ionization mass spectrometry. Planta 147: 423–434
23. WANG TL, DJ COVE, P BEUTELMANN, E HARTMANN 1980 Isopentenyladenine from mutants of the moss, *Physcomitrella patens*. Phytochemistry 19: 1103–1105
24. YOUNG H 1977 Identification of cytokinins from natural sources by gas-liquid chromatography/mass spectrometry. Anal Biochem 79: 226–233