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Cytokinin Biosynthesis in Mutants of the Moss *Physcomitrella patens*[1]

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ABSTRACT

Three cytokinin-over-producing mutants of the moss, *Physcomitrella patens*, have been shown to convert [8-^{14}C]adenine to N^6-[^{14}C](Δ^2-isopentenyl)adenine, the presence of which was confirmed by thin layer chromatography, high performance liquid chromatography, and recrystallization to constant specific radioactivity. The labeled cytokinin was detected in the culture medium within 6 hours and the tissue itself appears to contain both labeled N^6-(Δ^2-isopentenyl)adenine and N^6-(Δ^2-isopentenyl)adenosine monophosphate.

The literature now contains several references to the biosynthesis of cytokinins from labeled precursors both *in vivo* (5, 10, 11) and *in vitro* (4, 12) and several of the systems employed, notably those based on tissue cultures (4, 10, 11), seem suitable for a detailed analysis of cytokinin production in plants. A central theme throughout these studies, apart from the elucidation of the basic pathways, is the question of whether cytokinins arise *de novo* or via the degradation of tRNA. This question has received much attention over the years but remains unresolved. Even though recent studies indicate that cytokinins can arise without tRNA degradation (3, 4), they have not invalidated the possibility that tRNA-dependent pathways exist in the intact plant.

Mutants of the moss *Physcomitrella patens* have recently been shown to produce i^{6}Ade[4] and zeatin (13, 14), both of which are exported into the culture medium. The levels occurring in the medium were relatively high and up to 100 times that estimated to be present in the WT culture medium. The presence of such levels of cytokinin suggested that this organism may be ideally suited to studies of cytokinin biosynthesis. This paper, therefore, outlines preliminary studies on this topic and demonstrates that cytokinin-over-producing mutants of *P. patens* rapidly convert adenine to i^{6}Ade which passes into the culture medium.

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[4] Abbreviations: i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; WT, wild-type; OVE, gametophore-over-producer; Ade, adenine; HPLC, high performance liquid chromatography; CV, column volumes; TEAB, trimethylammonium bicarbonate; Ado, adenosine; i^{6}Ado, N^6-(Δ^2-isopentenyl)-adenosine; io^{6}Ade, zeatin; io^{6}Ado, ribosylzeatin; i^{6}AMP, N^6(Δ^2-isopentenyl)adenosine monophosphate.

MATERIALS AND METHODS

Moss Culture Conditions. General procedures for the maintenance of cultures, preparation of homogenates, and the contents of media have been reported previously (6, 14); strains OVE 201, 200, and WT were grown under the conditions for OVE 201 and OVE 78 under those outlined for OVE 78.

[^{14}C]Ade Incorporation. Medium containing about 3 μCi [8-^{14}C]Ade (Radiochemical Centre, Amersham, 62 mCi/mmol) per incubation was filter sterilized and placed in 50-ml Ehrlenmeyer flasks with metal caps. Tissue for incubation was collected from 8-day-old cultures, initiated from homogenates, and placed in 10 ml [^{14}C]Ade-containing medium in flasks. The flasks were then placed on an orbital shaker (100 rpm) under fluorescent lighting (20 w/m^2) and at 25 C. Samples for the measurement of ^{14}C uptake (2 × 10 μl) and TLC (50 μl) were removed with a sterilized microsyringe under sterile conditions.

At the end of the incubation period, the moss tissue was filtered, washed, and frozen at −70 C prior to freeze-drying. Culture medium and washings were collected, frozen, and freeze-dried in the same way.

Analysis of Culture Medium. Fifty-μl samples of culture medium from each incubation were withdrawn and spotted directly onto thin layer plates. The plates were then developed in solvent A for 15 cm, dried, and autoradiographed. Further aliquots of medium were analyzed, without purification, by TLC in solvent B and by

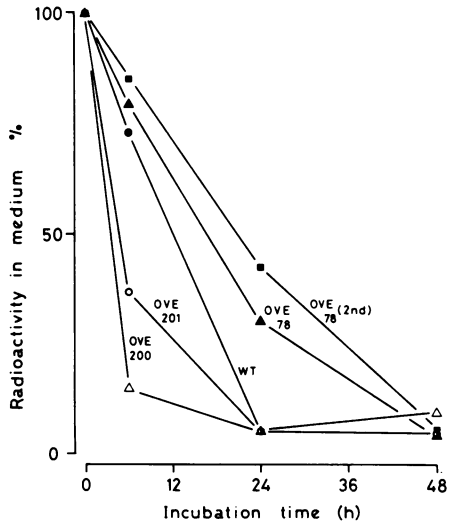

FIG. 1. Uptake of radioactivity by moss tissue in liquid culture. Moss tissue was incubated with [^{14}C]Ade according to the text. Duplicate 10-μl samples were withdrawn and measured by liquid scintillation counting. Values are expressed as a percentage of the [^{14}C]Ade supplied. Weights (mg) of tissue used: OVE 201, 13.2 mg; WT, 10.7; OVE 200, 10.2; OVE 78, 2.3; OVE 78 second incubation, 3.1.

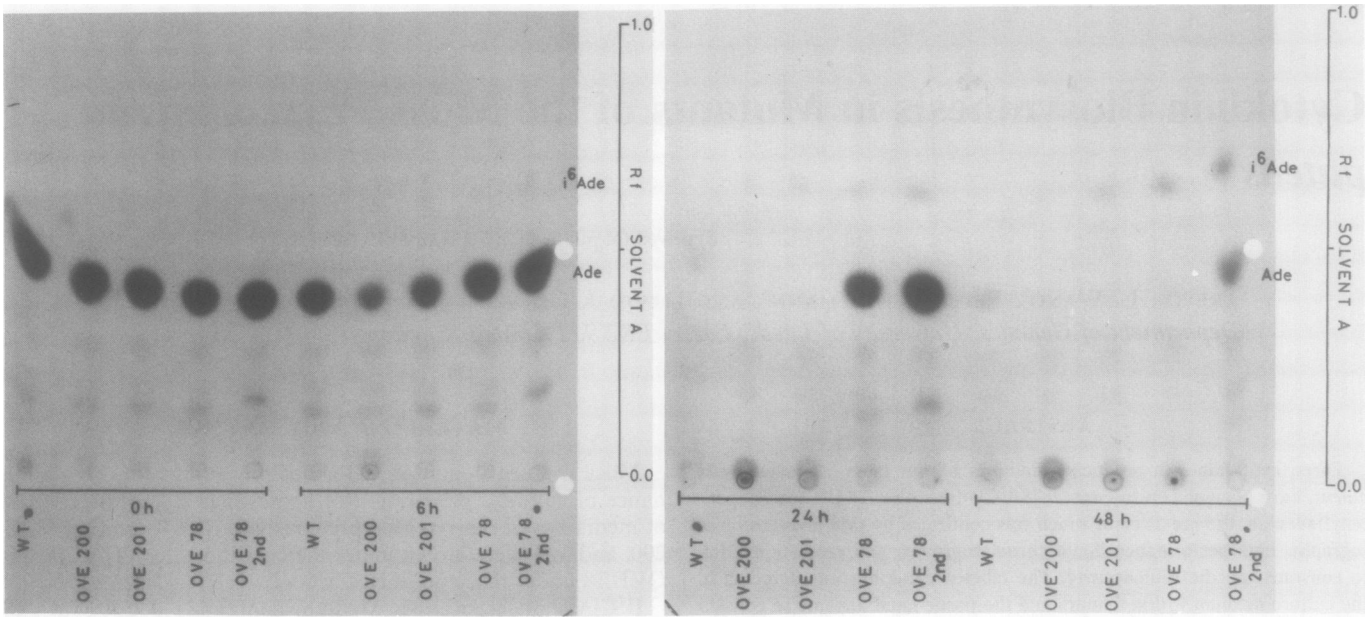

FIG. 2. Metabolism of [^{14}C]Ade by moss tissue. Moss tissue was incubated with [^{14}C]Ade as described in the text. Fifty-μl samples were withdrawn after 0, 6, 24, and 48 h and spotted directly onto silica gel plates. The TLC plates were developed in solvent A, dried, and autoradiographed for 3 weeks.

HPLC. Final cytokinin purification was achieved by separation on TLC in solvent A followed by recrystallization of the relevant radioactive compounds as outlined below.

Extraction of Moss Tissue. Freeze-dried material was extracted in 10 ml methanol:chloroform:formic acid:water (60:25:5:10 v/v) and the extracts taken to dryness. The residues were suspended in 5 ml water, adjusted to pH 3.1, and applied to cellulose phosphate columns (Whatman P1, NH_4^+ form [pH 3.1], 10 ml bed volume). The columns were washed with 5 CV water (adjusted to pH 3.1 with acetic acid) producing fractions A and then eluted with 5 CV 0.3 N ammonia to give fractions B.

Fractions B were taken to dryness, dissolved in 5 ml water, adjusted to pH 8, and partitioned five times into equal volumes of water-saturated 1-butanol. The combined butanol phases were taken to dryness, dissolved in 100 μl 80% ethanol (v/v), and one-half spotted onto TLC plates with cold markers. The plates were then developed for 15 cm in two dimensions with solvent C followed by solvent A. TLC plates were dried and autoradiographed.

Fractions A from cellulose phosphate chromatography were adjusted to pH 6.5 and applied *in toto* to DEAE-cellulose columns (Whatman DE 22) at pH 6.3. These columns were washed with 5 CV distilled H_2O and then eluted with 5 CV 0.4 M TEAB (fractions C). An aliquot from each fraction C was chromatographed ascendingly on paper in solvent D, the chromatogram dried and then autoradiographed. The remainders of fractions C were taken to dryness, dissolved in 1 ml 50 mM ethanolamine (adjusted to pH 9 with HCl) containing 10 mM $MgCl_2$ and incubated with 30 μg alkaline phosphatase overnight. The incubation mixture was partitioned directly into equal volumes of water-saturated 1-butanol three times and the combined butanol fractions were dried under N_2. This butanol fraction was finally analyzed by TLC and HPLC in the same way as fraction B.

Paper and TLC. Paper chromatography was performed on 20-cm squares of Whatman No. 3MM; TLC on 20 × 20 cm Merck SG 60 F_{254} plates 0.25 mm thick. The following solvents were used: A: 1-butanol, 14 N ammonia, water (6:1:2; v/v; upper phase); B: chloroform:methanol (9:1; v/v); C: 1-butanol:acetic acid:water (12:3:5; v/v); D: *t*-amyl alcohol:formic acid:water (3:1:2; v/v).

HPLC. HPLC was performed with either a Pye LC3X liquid chromatograph or a LDC dual pump (Constametric I and III) chromatograph linked via a Gradient Master solvent programmer; UV flow cells were 8 and 20 μl, respectively. Columns of Hypersil ODS (150 × 10 mm or 250 × 4.5 mm, Shandon Southern Ltd., United Kingdom) were employed for the separation on gradients between water (adjusted to pH 7 with TEAB) and acetonitrile (9). All solvents were redistilled or HPLC grade (Jones Chromatography, Glamorgan, United Kingdom, and Fisons Scientific Apparatus, Loughborough, United Kingdom). One-min samples were collected under a flow rate of 5 (10-mm column) or 2 ml/min (4.5-mm column), dried, and counted.

Crystallizations. Putative [^{14}C]i^{6}Ade samples for crystallization were eluted with 3 ml 80% ethanol from TLC plates developed in solvent A, taken to dryness, and about 1 mg authentic i^{6}Ade added to each. Each sample was then recrystallized three times from minimum volumes of acetonitrile:ethanol (3:1; v/v). The specific activity of the i^{6}Ade was calculated after each crystallization by measuring the UV absorption of a known volume of sample at λ_{max} together with its radioactivity.

Autoradiography and Scintillation Counting. Autoradiography was carried out with either Kodak Kodirex or Fuji RX X-ray film layed directly onto TLC plates and aligned via radioactive ink. Radioactive samples were counted in toluene:Triton X-100:2,5-diphenyloxazole:1,4-bis(2[5-phenyloxazolyl])benzene (1 liter:0.5 liter:4 g:250 mg) on either a LKB Rackbeta or Phillips PW4540 counter. HPLC samples were thoroughly dried before counting to avoid any possible solvent effects.

Solvents and Reagents. All solvents were redistilled unless otherwise stated. Authentic markers, AMP, ADP, ATP, Ado, Ade, i^{6}Ade, i^{6}Ado, io^{6}Ade, and io^{6}Ado were obtained from Sigma Chemical Co. and i^{6}AMP from P-L Biochemicals Inc. Alkaline phosphatase (type VII from calf intestinal mucosa, 880 units/mg protein) was also obtained from Sigma.

RESULTS

Uptake and Metabolism of [^{14}C]Ade in Moss Tissue. Moss tissue grown in liquid culture rapidly takes up exogenous [8-^{14}C]Ade. Samples removed from the culture medium show that radioactivity present in the medium falls over the 48-h incubation period to reach equilibrium at approximately 5% of the original value (Fig. 1) in all cases. The time taken to reach this point varies

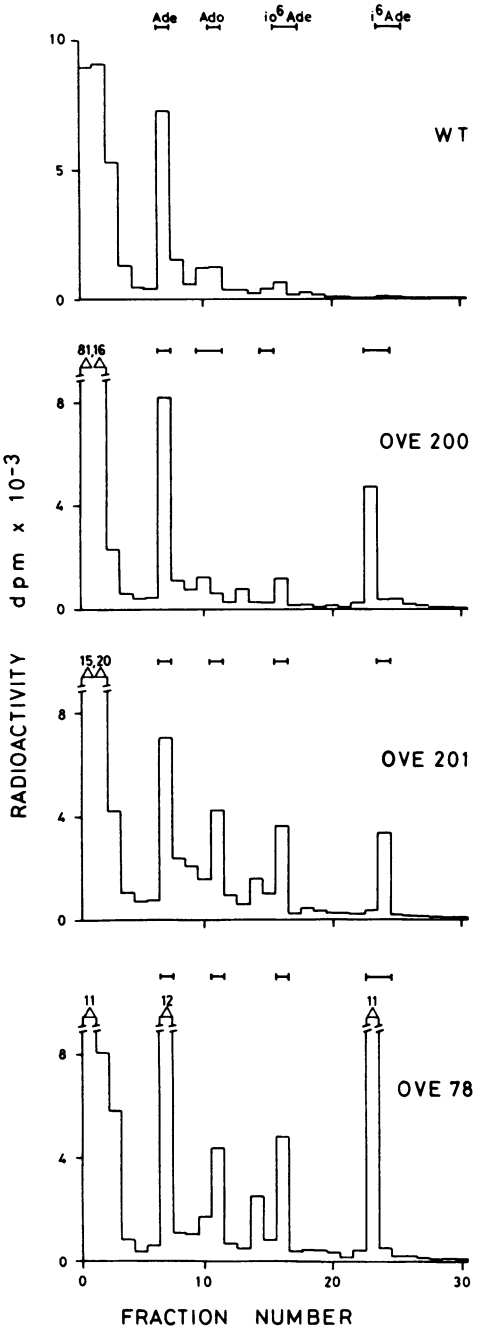

FIG. 3. HPLC profiles of [^{14}C]Ade metabolites from moss culture medium. Aliquots of culture medium from moss tissue incubated with [^{14}C]Ade for 48 h were analyzed directly by HPLC on Hypersil ODS by using a gradient of water to 30% acetonitrile in 30 min. One-min samples were collected, dried, and measured by liquid scintillation counting. Horizontal bars indicate the elution positions of authentic compounds.

with each incubation although the differences between different strains may be accounted for by the differences in the weights of tissue employed. Within the incubation period, however, Ade had passed into the tissue and its metabolites passed out into the medium. Hence, the uptake of Ade is likely to be an underestimate if the radioactivity present in the culture medium is used as a measure. Fewer Ade metabolites appear in WT culture medium over the experimental period (Fig. 2).

The uptake of [^{14}C]Ade and incorporation into cytokinin appears to depend on the amount of Ade available. This is demonstrated by the second OVE 78 incubation shown in Figure 1. In this instance, in absolute terms, almost twice the amount of Ade was supplied, twice the amount taken up, and twice the amount of labeled i^{6}Ade formed as analyzed by HPLC (data not shown). It is also of note that OVE 78 incorporated more label into i^{6}Ade than the other OVE.

Isolation and Identification of Cytokinins in Culture Medium and Derived from [^{14}C]Ade. Samples of culture medium were analyzed directly by TLC and HPLC without prior purification. Figure 2 indicates the change in radioactive compounds in the culture media over the 48-h incubation period which were separated in TLC in solvent A. Ade (R_F 0.43) disappears rapidly from the culture medium although several impurities present in the [^{14}C]Ade stock persist. Two new radioactive spots appear at R_F values 0.05 and 0.62. These latter compounds are detectable after only 6 h in all OVE culture media and have increased in the two samples taken at 24 and 48 h. At all sampling times only the polar Ade metabolite occurs in WT culture medium and not to any great extent. The compound at R_F 0.62 co-chromatographed with i^{6}Ade.

Direct HPLC analysis of moss culture medium by reverse-phase chromatography by using a water-acetonitrile gradient showed several peaks of radioactivity. Of note are the large polar component in the first three fractions and the radioactivity co-chromatographing with Ade and i^{6}Ade (Fig. 3). Again, the radioactivity co-chromatographing with i^{6}Ade was absent from WT culture medium. (Although not very apparent due to the scale of Figure 3, WT culture medium shows approximately 50 dpm above background in the region that i^{6}Ade elutes.) Chromatography on TLC in solvent B again showed the same differences, a radioactive spot co-chromatographing with i^{6}Ade appeared in the culture medium of all OVE but not of WT tissue. It seemed very likely, therefore, that the nonpolar metabolite of Ade in the culture medium of the moss mutants was i^{6}Ade.

To confirm this, the remains of the culture media were separated on TLC in solvent A, the bands co-chromatographing with i^{6}Ade eluted and the radioactivity co-crystallized with cold i^{6}Ade to constant specific activity. The specific activities obtained were as follows (dpm/μg): OVE 78, 12.3 ± 3.3%; OVE 200, 2.4 ± 5.9%; OVE 201, 2.0 ± 6.6%. Hence, the compound at R_F 0.62 (solvent A) in OVE culture medium was identified as [^{14}C]i^{6}Ade.

The position of the compound at R_F 0.05 on TLC in solvent A suggested that it may be a more polar purine such as inosine or guanosine or even a purine nucleotide. After elution from the silica gel, this band was treated with alkaline phosphatase and subjected to HPLC as before. The major peak of radioactivity appeared in the second fraction for each sample analyzed from the column and coincided with the only significant endogenous UV absorbing peak (data not shown). A small radioactive peak coeluting with Ado was also noted. This fraction, therefore, probably contains Ade nucleotides as well as unknown polar compounds but no cytokinin-active nucleotides.

Isolation Cytokinins Derived from [^{14}C]Ade in Moss Tissue. Moss tissue was extracted under conditions inhibiting phosphatase activity (2) and the extract subjected to cation exchange chromatography producing a fraction (A) containing nucleotides and neutral compounds and a fraction (B) containing bases and ribosides. The bases and ribosides were extracted into 1-butanol and subjected to two-dimensional TLC.

The autoradiograms indicated several radioactive compounds in each extract. However, in all OVE tissue extracts but not the WT, a spot was present which co-chromatographed with i^{6}Ade in both solvents (data not shown). Figure 4 shows autoradiograms from OVE 201 and WT tissue extracts. Ade and Ado also appeared to be present together with a major unknown compound running slightly ahead of Ado and io^{6}Ado in solvent A but between the two in solvent C. Radioactive material was not available for a more detailed analysis.

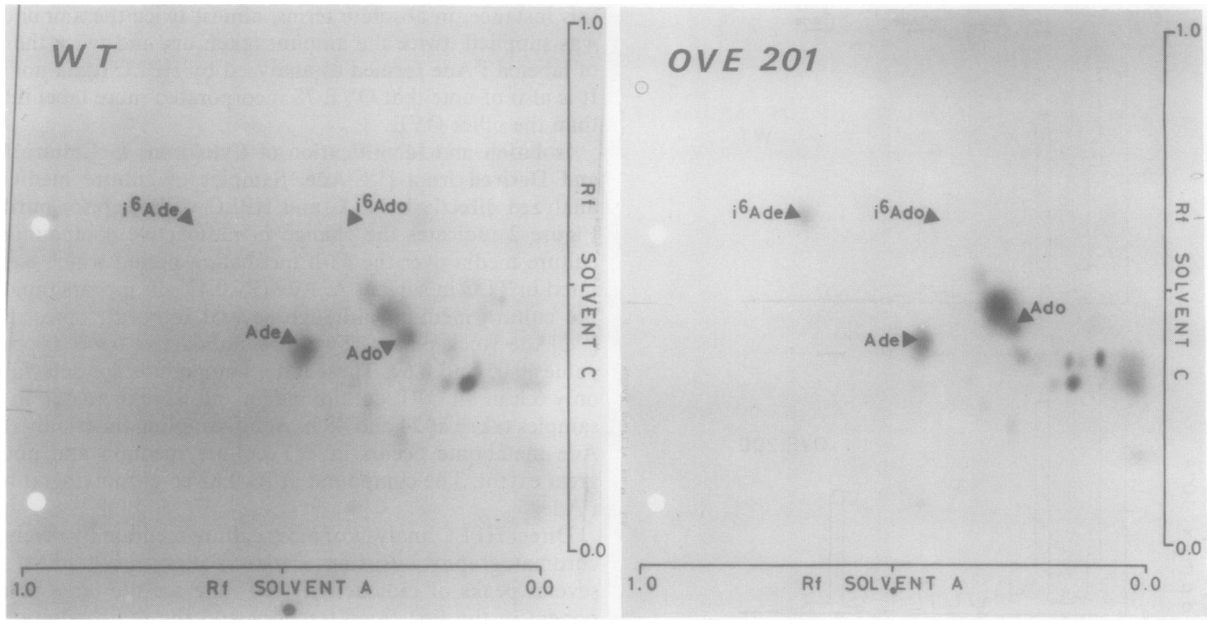

FIG. 4. [^{14}C]Ade basic metabolites from moss tissue. Moss tissue, after incubation with [^{14}C]Ade for 48 h, was extracted and the extract subjected to ion exchange chromatography. The base wash was partitioned into butanol and analyzed by two-dimensional TLC as described in the text. After TLC the plates were dried and autoradiographed for 2 weeks. Arrowheads indicate the positions of authentic markers.

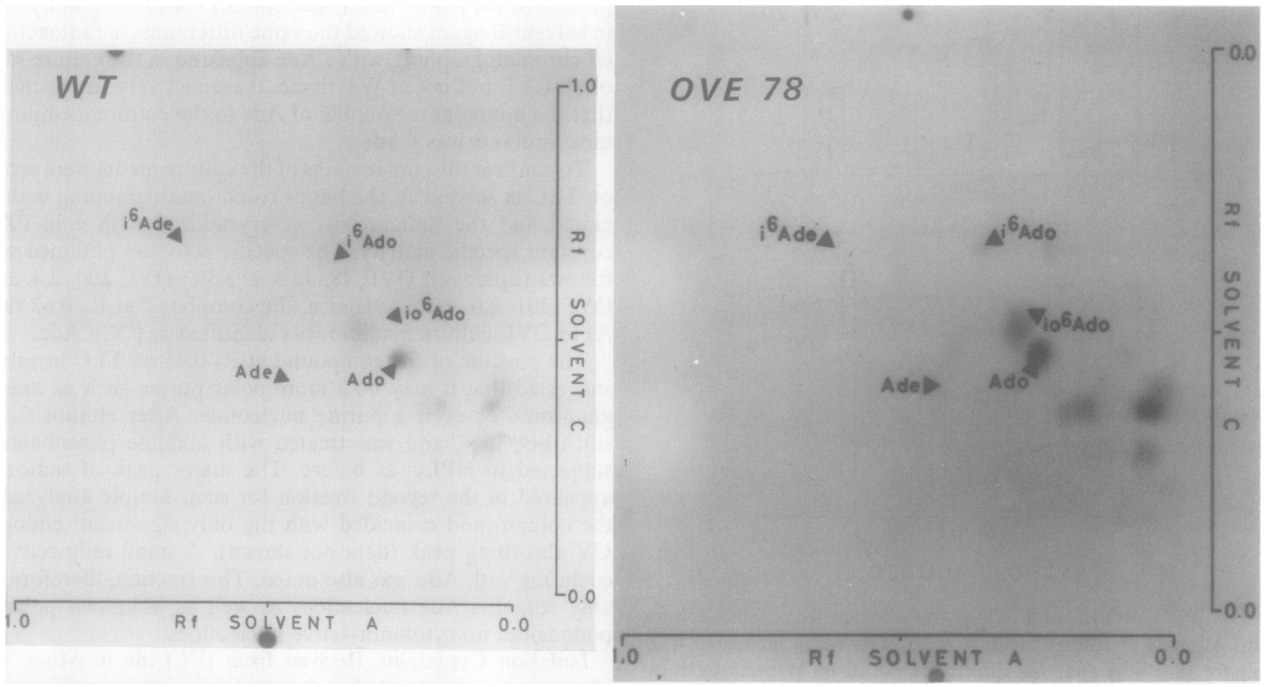

FIG. 5. [^{14}C]Ade acidic metabolites from moss tissue. Moss tissue, after incubation with [^{14}C]Ade, was extracted, and the extract was subjected to ion exchange chromatography. The acidic wash was treated with alkaline phosphatase and then partitioned into butanol. The butanol phase was finally analyzed by two-dimensional TLC according to the text. After TLC plates were dried and autoradiographed for 2 weeks. Arrowheads indicate the positions of authentic markers.

Nucleotides were removed from fraction A by anion exchange chromatography and analyzed directly by paper chromatography in solvent D against authentic i^{6}AMP and adenine nucleotides. The main radioactivity co-chromatographed with the latter compounds but small spots were noted against i^{6}AMP in OVE extracts but not in WT extracts. However, these spots were not clearly resolved from an unknown compound running marginally slower. The remainder of each sample was degraded with alkaline phosphatase, extracted into butanol and analyzed by two-dimensional TLC and HPLC. Again, in all OVE extracts but not WT, faint spots of radioactivity on the autoradiograms co-chromatographed with i^{6}Ado in both solvents; the most pronounced was from the OVE 78 extract (see Fig. 5). Analysis by HPLC confirmed this finding (Fig. 6). The unknown metabolite noted in fractions B was also present in these enzyme treated extracts; this compound appears to be present in the tissues as both base or nucleoside and nucleotide. Significant radioactivity co-chromatographed with Ado on both TLC and HPLC and, hence, it is likely that labeled Ade nucleotides were present in moss tissue.

On the basis of these data, OVE moss tissue was found to

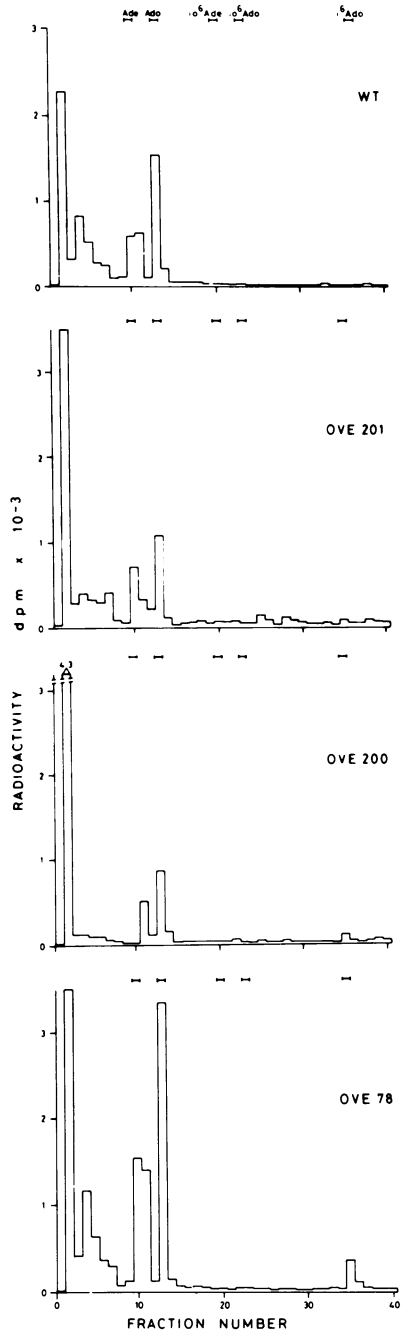

FIG. 6. HPLC profiles of [^{14}C]Ade acidic metabolites from moss tissue. Samples from extracts of moss tissue treated as in Figure 5 were analyzed by HPLC on Hypersil ODS with a gradient of water to 25% acetonitrile in 40 min. One-min samples were collected, dried, and measured by liquid scintillation counting. Horizontal bars indicate the elution positions of authentic compounds.

incorporate rapidly [^{14}C]Ade into [^{14}C]i^{6}Ade which appears in the culture medium as well as in the tissue. In addition, the tissue also appears to contain [^{14}C]AMP. A summary of the radioactivity recovered as cytokinin from each incubation is given in Table I.

Table I. *[^{14}C]Adenine Recovered as Cytokinin from OVE Moss Tissue*

Samples of moss culture medium after incubation of moss tissue with [^{14}C]Ade were chromatographed on TLC and HPLC and crystallized to constant specific activity against authentic i^{6}Ade. Samples from tissue were co-chromatographed on TLC and HPLC against authentic markers of i^{6}Ade or i^{6}AMP, eluted, and counted. All ^{14}C values are total radioactivity values for each compound in dpm × 10^{-3}. Molarities are calculated on the following bases: dry weight of moss tissue equals 5% fresh wt and 1 mg fresh wt moss tissue has been taken to be equal to 1 μl volume.

	OVE 200	OVE 78	OVE 201
[^{14}C]Ade supplied	6900	6600	6200
[^{14}C]i^{6}Ade in medium	9.8	30.1	6.9
[^{14}C]i^{6}Ade in tissue	1.3	0.2	0.7
[^{14}C]i^{6}AMP in tissue	0.2	0.6	0.05
Total [^{14}C]cytokinin	11.3	30.9	7.7
[^{14}C]Ade incorporation, %	0.16	0.47	0.12
Cytokinin in culture medium (nM)	4.8	14.9	3.4
Cytokinin in tissue (nM)	37	86	14

DISCUSSION AND CONCLUSIONS

Cytokinin-over-producing mutants of *P. patens* have been shown to produce both i^{6}Ade and io^{6}Ade (13, 14). To date, genetic analysis of OVE mutants using somatic hybridization via protoplast fusion, has indicated that three complementation groups exist (Featherstone and Cove, unpublished data; see ref. 7 for summary). It follows that there are at least three genes in which a mutation can lead to the OVE phenotype. However, there is no reason to suppose that these genetic changes directly influence cytokinin production by, *e.g.* preventing cytokinin catabolism. It is possible that a gross disturbance in purine metabolism could occur which would be reflected by an increase in purine levels including the cytokinins, although the only feature manifested in the phenotype of the mutant, by virtue of their unique biological activity, is the increase in the cytokinins.

In recent papers on cytokinin biosynthesis (4, 12) purified enzyme extracts have been shown to catalyze the formation of i^{6}AMP directly from 5′-AMP. Only in crude enzyme preparations was the presence of either i^{6}Ade or i^{6}Ado detected. Furthermore, in crown-gall tissues of *Vinca rosea* the initial products from [^{14}C]Ade incorporation similarly appear to be cytokinin nucleotides (11). Because the results presented here indicate the occurrence of labeled i^{6}AMP in OVE moss tissue but not its culture medium, it seems possible that synthesis of the cytokinin may have occurred via the nucleotide but that i^{6}Ade was the major product in the reaction and it was this product that passed into the culture medium. No i^{6}Ado or io^{6}Ade were detected in either culture medium or tissue, although OVE mosses are known to contain the latter. Further studies will be necessary before their absence as labeled products of [^{14}C]Ade incorporation into OVE moss tissue can be confirmed or denied.

The likelihood of cytokinin nucleotides being the initial products in cytokinin biosynthesis is high since purine metabolism itself proceeds at the level of the nucleotide rather than the base or riboside (8). The identification of cytokinin nucleotides in plant extracts are few, perhaps due to their more difficult extraction and their lability. However, as demonstrated in recent studies and as recently emphasized (11), they can no longer be neglected if a total picture of cytokinin production in plants is to be attained.

In a previous paper, several proposals were made to explain the OVE phenotype (1). The results obtained for the mutants analyzed in this present work and the accompanying paper strongly support the first hypothesis outlined, that the OVE phenotype is due solely to a high endogenous level of cytokinin brought about by an increase in biosynthesis. Preliminary work on cytokinin metabolism has not revealed any significant differences between OVE and WT tissue, (both mainly degrade i^{6}Ade to Ade) and the high cytokinin production of OVE tissue, therefore, is unlikely to be due to a lack of hormone catabolism (McGeary, Wang, Cove, unpublished data). Up to 0.5% [^{14}C]Ade fed to OVE moss tissue

was recovered as [^{14}C]cytokinin (Table I).

Hence, moss tissue has several important features which make it particularly suitable for studies on cytokinin biosynthesis, namely (a) it is a green plant; (b) it can be cultured easily and intact; (c) mutants are available for comparative studies; (d) cytokinin isolation is relatively easy especially from the culture medium; and (e) OVE mutants rapidly incorporate adenine into cytokinin.

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