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ISOPENTENYLADENINE FROM MUTANTS OF THE MOSS, *PHYSCOMITRELLA PATENS*

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Abstract—The culture media from gametophore over-producing mutants of the moss *Physcomitrella patens* have been examined for their cytokinin content. Two cytokinins have been detected, one of which has been identified as N^6-(Δ^2-isopentenyl) adenine (2iP).

INTRODUCTION

Although normally defined by their action on plant tissue cultures [1], the cytokinins are recognized to have diverse effects on the growth and metabolism of plants. One such effect—that on bud formation in mosses—has been known for many years [2] and has frequently been used as a bioassay for this particular group of plant growth regulators, e.g. [3].

Even though there have been several investigations of the phenomenon of bud formation and its associated events, e.g. [4–6], there have been no isolations or identifications of endogenous cytokinins in moss. Callus tissue from the sporophyte of the moss hybrid *Funaria hygrometrica* (L.) Sibth × *Physcomitrium piriforme* Brid., however, has been found to contain large quantities of N^6-(Δ^2-isopentenyl) adenine [7] and cytokinin activity has also been detected in other bryophytes [8].

The moss *Physcomitrella patens* has been developed as a system for genetical analysis by Cove and co-workers ([9] and refs. cited therein.). Chemically-induced mutants have been isolated, several of which over-produce gametophores (category 9 – OVE – mutants; 10) and, hence, phenotypically, resemble the WT strain treated with cytokinin.

Although gametophore over-producers, it was not certain whether category 9 mutants in fact also over-produced cytokinin [10]. This report, therefore, presents preliminary findings on the cytokinin content of the culture medium from OVE mutants of *P. patens*. Two mutants, each from different complementation groups [11], were employed in the investigation, and different procedures used in the isolation of the cytokinins.

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RESULTS

Cytokinins in ove*B*100 pab*A*3 (*OVE* 100)

Fifteen 1. of medium from a culture of OVE 100 grown for 12 days in aerated culture was partitioned into butanol and then separated on a Sephadex LH20 column eluted with 35% EtOH. Fig. 1 shows the bioassay profile for the equivalent of 1.3 1. of culture medium or 1.25 g fr. wt of protonema using the soybean cotyledonary callus assay. It is evident that two peaks of activity are present—Fr I and Fr II (Fig. 1). The UV spectrum of Fr II (which co-eluted with 2iP) gave a $\lambda_{max}^{80\%\ EtOH}$ of 269 nm and a shift to 275 nm with a shoulder at 284 nm at pH 9. This indicated that Fr II contained a N^6-substituted purine. An aliquot of Fr II was analysed, without further purification, by MS as a probe sample and gave the following ions: *m/e* 203 (59.8%; M^+), 188 (62.3), 160 (97.4), 149 (20.3), 148 (16.1), 135 (100), 121 (7.1), 120 (14.8), 119 (30.2), 108 (36.1), which are characteristic of 2iP. Thus Fr II was identified as 2iP on the basis of its UV absorption, chromatographic properties and MS. About 76 μg of 2iP was isolated from the culture medium giving a concentration of 25 nM. Fr I which co-eluted with zeatin on Sephadex LH20 is at present under investigation and, as indicated by the bioassay, represents a small component of the cytokinin activity in the culture medium.

*Cytokinins in OVEA*78 pab*A*3 (*OVE* 78)

After purification of 8 l. of medium from a 10-day-old suspension culture of OVE 78 using ion-exchange chromatography followed by column chromatography in ethyl acetate, the cytokinin-containing fraction was applied to a Sephadex LH20 column and eluted with 35% methanol. Assay of the column eluate indicated that the only detectable activity eluted in the same fractions as 2iP. These fractions were combined and

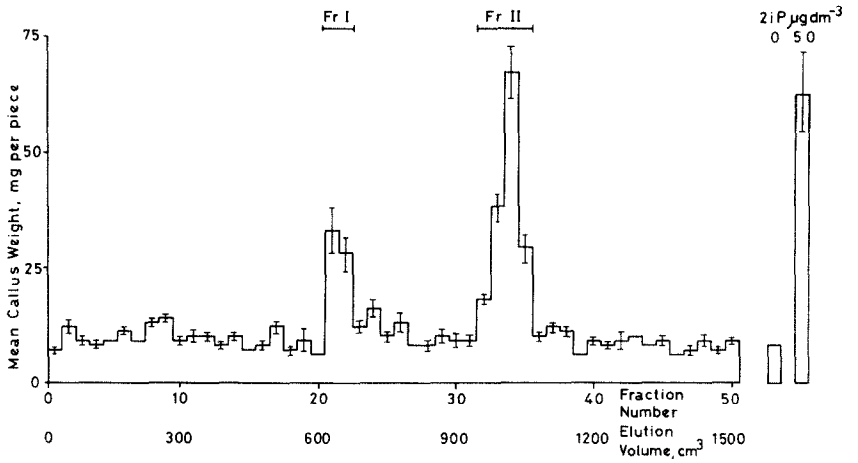

Fig. 1. Soybean bioassay profile of an extract of OVE 100 culture medium separated on Sephadex LH20. Vertical bars represent 2×s.e.m. Where bars have been omitted s.e.m. was less than 0.6 mg.

further purified by picrate precipitation and TLC. Final analysis was carried out by MS using a direct probe sample and gave the following ions: *m/e* 203 (21.2%; M^+), 188 (38), 160 (100), 149 (3.1), 148 (15.4), 135 (68.7), 121 (6.7), 120 (8.2), 119 (22.9), 108 (51.7), indicating that the sample was 2iP. Hence, on the basis of its chromatographic properties and MS, the active fraction in OVE 78 culture medium was identified as 2iP. In this instance, 160 μg of 2iP was isolated giving a concentration of 100 nM in the culture medium.

DISCUSSION

The availability of mutants of an organism altered in their response to and production of cytokinins should aid in the understanding of the mode of action of these plant-growth regulators. The isolation of a group of mutants from *P. patens* which over-produce gametophores has previously been reported [10]. Since these mutants (category 9 – OVE – mutants) when grown on minimal medium appeared the same as WT cultures on medium to which a relatively high level of benzyladenine had been added, and, since OVE mutants had the ability to cross-feed with WT cultures, increasing gametophore production in the latter, it was proposed that these gametophore over-producers were also cytokinin over-producers [10].

The isolation and identification of relatively high levels (when compared to other plant tissues) of 2iP from the culture medium of two of the OVE mutants, reported here, signify that the original hypothesis is likely to be correct. Although the procedure employed for the isolation of 2iP from OVE 78 was one which would normally be required for more complex plant extracts, it has been shown (in the method used with OVE 100) that moss culture medium is a relatively clean source of cytokinins and very little purification is necessary for physical determination of the cytokinins present. Thus the moss should be invaluable for further research into cytokinin production.

EXPERIMENTAL

Growth and culture conditions for OVE 100. The maintenance of stock cultures and prepn of homogenates have been described in ref. [12]. For liquid culture, a homogenate prepared from ten 14-day-old agar cultures of *ove*B100 *pab*A3 inoculated into a 15 l. reagent bottle containing the following medium (pH 6.5) sterilized at 120° and 1.1 kg/cm² for 55 min (quantities per l.): $Ca(NO_3)_2$—59 mg; KNO_3—1.04 g; KH_2PO_4—250 mg; $FeSO_4 \cdot 7H_2O$—10 mg; $MgSO_4 \cdot 7H_2O$—250 mg; 1 ml Hoagland's trace element soln [13]; *p*-aminobenzoic acid—0.25 mg; nicotinic acid—1 mg; thiamine hydrochloride—0.1 mg. The culture was grown under sterile conditions at 25°, under continuous fluorescent lighting (20 W/m²). Although forced aeration is not required for growth, sufficient was used to mix the culture and maintain it as a suspension. After 12 days, the medium was filtered from the protonema and evapd to a small vol. *in vacuo* at 40°.

Cytokinin isolation from OVE 100. After reduction of its vol., the culture medium was adjusted to pH 8 and partitioned×5 against H_2O-satd *n*-BuOH. The combined BuOH fractions were taken to dryness and then dissolved in 2.5 ml 35% EtOH. Precipitated salts were removed by centrifugation and the supernatant injected on to a Sephadex LH20 column and eluted with 35% EtOH at a flow rate of 30 ml/hr. Hourly fractions were collected and aliquots of each fraction taken to dryness for assay using the soybean callus assay as described below. The remainder of the main active fraction (Fr II) from Sephadex chromatography was taken to dryness and dissolved in 80% EtOH. After measurement of its UV absorption, the sample was again taken to dryness and used for MS analysis.

MS of OVE 100, *Fr* II. Low resolution MS were recorded at 70 eV. Scans were taken at 10 sec/mass decade from a direct probe sample at 100° and with a source temp of 180°.

Bioassay. The soybean callus assay used was that of Miller [14] with the following modification: 10 pieces of callus were placed on 50 ml of medium in 7 cm crystallizing dishes. After 3 weeks the fr. wt of each piece of callus was recorded and the mean and s.e.m. calculated for each sample.

Growth and culture conditions for OVE 78. A homogenate from 14-day-old cultures of OVEA78 *pab*A3 was inoculated into 3 l. inverted conical flask culture vessels (*ca* 0.5 g fr. wt of tissue per flask) containing the following medium sterilized at 120° and 1.1 kg/cm² for 30 min (quantities per l.): KNO_3—100 mg; $CaCl_2$—10 mg; KH_2PO_4—136 mg; $MgSO_4 \cdot 7H_2O$—10 mg; Fe-citrate—2.7 mg; thiamine hydrochloride—0.5 mg; *p*-aminobenzoic acid—2.5 mg. The cultures were maintained

at 20°, under continuous fluorescent lighting (15 W/m^2) and aerated as described for OVE 100.

Cytokinin isolation from OVE 78. A total of 8 l. of medium was filtered from 10-day-old suspension cultures and its vol. reduced *in vacuo*. The medium was then adjusted to pH 2 and passed down a 15×2.5 cm Amberlite CG 120 cation exchange column equilibrated in 10 mM HCl. The column was washed with 200 ml H_2O (pH 5) and finally the cytokinin-containing fraction eluted with 200 ml 35% MeOH adjusted to pH 11.5 with 15 M NH_4OH. The eluate from this column was reduced to 20 ml, adjusted to pH 8 and loaded onto a Extrelut column (Merck) followed by elution with 60 ml EtOAc. The EtOAc fraction was then taken to dryness and redissolved in 85% MeOH. For separation of cytokinins, the sample was divided into 3 and each part passed down a Sephadex LH20 column (0.5×60 cm) eluted with 35% MeOH at a flow rate of 4 ml/hr; 30 min fractions were collected. Cytokinin activity was detected by assaying aliquots of each fraction with the moss bud induction bioassay [15] using protonema of *Bryum caespititium*. A single region of activity was detected (8.2–9.6 column vols) co-eluting with 2iP. The fractions from this region were taken to dryness, redissolved in 0.5 ml 50% MeOH and 0.5 ml of a satd soln of picric acid added. After 1 hr at −15°, the ppt. was collected by centrifuged, washed with 0.5 ml ice-cold H_2O and dried. Finally, the picrate was separated from the cytokinin by chromatography on TLC in $CHCl_3$–MeOH (9:1). The band co-chromatographing with 2iP was eluted with EtOAc, the eluate dried and used for MS.

MS of OVE 78 *cytokinin*. Low resolution MS were recorded at 70 eV. Scans were taken at 10.8 sec/mass decade from a direct probe sample at 150° with a source temp. of 250°.

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