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OCCURRENCE AND BIOSYNTHESIS OF AUXIN IN PROTONEMA OF THE MOSS *FUNARIA HYGROMETRICA*

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Key Word Index—*Funaria hygrometrica*; Bryidae; moss; protonema; auxin; cell-density dependence.

Abstract—We have investigated the presence of auxin and the ability of chloronema cells to synthesize indole-3-acetic acid (IAA) in axenic protonema cell cultures of the moss *Funaria hygrometrica*. The endogenous level of auxin activity was 4 and 7 μg-IAA equivalents/kg in caulonema and chloronema cell types, respectively. Based on an indole-α-pyrone fluorometric assay, the level of putative IAA was observed to be 5.0 and 1.9 μg/kg in caulonema and chloronema cells, respectively. [^{3}H]Tryptophan was metabolized into IAA via the indole-pyruvate pathway by intact chloronema cells and also by the cell free homogenates. More [^{3}H]IAA accumulated when homogenates from cells pre-grown at low cell densities (< 0.5 mg/ml) as compared to those at high cell densities (> 0.5 mg/ml) were used. Since the activities of peroxidase and IAA-oxidase are known to be high at high cell densities, the lack of accumulation of radioactivity in IAA at high densities can be attributed to a high level of IAA-oxidizing enzymes. Our results suggest a possible relationship between IAA accumulation and caulonema differentiation.

INTRODUCTION

Protonema development in the moss *Funaria hygrometrica* shows two distinct developmental stages, the chloronema and the caulonema [1, 2]. On solid medium, a spore germinates, producing chloronema filaments, and later caulonema and secondary chloronema filaments are formed. Differentiation in suspension cultures depends on the inoculum density and on the relative levels of cAMP and IAA [3, 4]. At low cell densities (< 0.2 mg/ml), only 5–10% caulonema-type filaments are initiated, a level raised to 65–70% by biologically active auxins which inhibit secondary chloronema formation and enhance caulonema production [5–7]. Several observations indicate that endogenous auxins are involved in cell differentiation and protonema development in other mosses [8, 9].

The present experiments were carried out to examine the presence of auxin and the effect of cell density on IAA biosynthesis in protonema cultures of *F. hygrometrica*.

RESULTS

Free auxin levels in chloronema cells and pg-1 *mutant*

The level of endogenous auxin was determined in chloronema cells (wild type) and *pg*-1 mutant protonema collected at the mid-exponential phase of growth in suspension culture. The mutant *pg*-1 is a leaky, chloronema-repressed mutant, and produces predominantly caulonema filaments (> 70%), in the absence of any exogenous auxin [4]. We wanted to employ the wild-type caulonema cultures also for this experiment, but since caulonema differentiates in liquid cultures only in the presence of externally applied IAA or α-naphthaleneacetic acid (α-NAA), it is difficult to interpret the data on the level of endogenous auxin from such cultures. It was for this reason that the mutant *pg*-1 was used. The recovery of [^{14}C]IAA used as an internal standard varied from 4.2 to 28% in different experiments. Upon paper chromatography of the total acidic auxin fraction, a single zone of activity evoking the growth of coleoptile sections and also giving fluorescence in the fluorometric assay was noticed. This zone of auxin activity, with an R_f of 0.4–0.5 (solvent *i*-PrOH–NH_3–H_2O), co-chromatographed with authentic [^{14}C]IAA. Its level estimated by growth test and fluorometric assay is shown in Table 1.

Based on bioassay, the auxin activity of wild-type chloronema cells was *ca* 7 μg-IAA equivalent per kg fresh weight. The mutant *pg*-1 showed a slightly lower level, *ca* 4.3 μg-IAA equivalent. In chloronema cells, the level of auxin activity based on fluorometric assay was about one-third of that determined by bioassay. In the *pg*-1 mutant, however, the auxin level was similar whether determined by bioassay or by fluorometry (Table 1). Since the fluorometric method is specific for IAA, these results provide strong evidence for the presence of IAA in moss protonema but do not prove the chemical identity of the acidic auxin as IAA.

IAA biosynthesis by chloronema cell cultures

At the time of investigation, it was not established if bryophytes and mosses in particular can synthesize and produce IAA [10]. We therefore decided to investigate whether sterile cultures of chloronema can synthesize IAA from [^{3}H]tryptophan. First, the results show that the relative uptake of tryptophan at low cell density (0.025 mg/ml) is *ca* 10-fold greater (Table 2) than that at high cell density (2.5 mg/ml). Second, in the acidic auxin

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Table 1. Endogenous level of auxin in wild type and *pg*-1 mutant protonema of *Funaria hygrometrica*

Protonema	Chloronema (%)	Assay method	Wt of cells (g)	% Recovery of [^{14}C]IAA	μg IAA-equiv./ kg fr. wt
Wild type	100	B	171	9.3	6.6*
	100	B	70	14.7	6.9†
	100	F	374	4.2	1.9
Mutant	35	B	28	28.4	4.3
pg-1	35	F	25	22.0	5.0

*,†These are two separate experiments.
B: Oat coleoptile straight growth test; F: fluorometric assay.

Table 2. Synthesis of IAA by chloronema cells grown at low and high cell densities

Initial cell density (mg/ml)	Time (hr)	Caulonema (%)	[^{3}H]Tryptophan uptake (nmol/g fr. wt)	Incorporation (cpm/mg protein) IPA	IAA	Incorporation % of uptake IPA	IAA
0.025	19	0	1599	228	1280	3.45	10.52
	42.5	20	1724	118	3327	1.18	33.48
2.5	19	0	159	96	340	7.49	26.62
	42.5	0	136	33	136	3.64	15.05

fraction, peaks of ^{3}H-radioactivity co-chromatographing with indole-3-pyruvic acid (IPA) and IAA were observed. Some ^{3}H-radioactivity was also present (especially the 19 hr, low density sample) behind the solvent front, but the identity of this compound is unknown. In both low and high density cultures, no radioactivity was found in tryptamine in any of the experiments. Since a peak corresponding to [^{3}H]IPA and [^{3}H]IAA could be detected, we conclude that sterile cultures of this moss can synthesize auxin, and that tryptophan is metabolized into IAA via the transamination pathway. Cells grown at low cell density (0.025 mg/ml), accumulated more radioactivity (per mg protein) in IPA and IAA than cells at high density (2.5 mg/ml) after 19 and 42.5 hr of incubation (Table 2). This effect is clearly due to a higher uptake of tryptophan by the low density cultures. In the latter cultures, however, the amount of [^{3}H]IAA increased with time, while in the high cell density cultures it decreased with time. Thus both types of cultures can synthesize IAA, and the cell density does not seem to affect the synthesis of IAA, but a later step. Since we have shown earlier that the activities of IAA-oxidase and peroxidase increase with increasing density of cells in culture [11], the observed decrease in the accumulation of newly synthesized IAA with time could be due to its degradation. After 42 hr, cultures inoculated at low cell density had started to differentiate and showed *ca* 20% caulonema filaments, while those at the high cell density showed no differentiation (Table 2).

IAA synthesis in a cell-free system and effect of cell density

The results presented so far show that intact chloronema cells can synthesize IAA from [^{3}H]tryptophan via the transamination pathway. In order to confirm these results, the biosynthesis of IAA was studied in a cell-free system, as described in the Experimental. In a cell-free system as well, [^{3}H]tryptophan was metabolized into [^{3}H]IAA, which co-chromatographed with the authentic [^{14}C]IAA. Among other acidic indole compounds, ^{3}H-radioactivity was detected in IPA and in an unidentified compound moving slower than IPA. IPA seems to be converted into IAA via 3-indoleacetaldehyde (IAAld) as radioactivity was detected at an R_f identical to that of authentic IAAld. In the cell-free system, [^{3}H]IAA was oxidized further to IAld as radioactivity was also detected in this compound.

The inoculum cell density affected the relative distribution of ^{3}H-radioactivity in the various indole derivatives accumulated in the cell-free system. At low cell densities ($\leqslant$ 0.5 mg/ml), a substantive amount of radioactivity in the acidic fraction was found in IPA and IAA peaks. At high cell densities ($>$ 0.5 mg/ml), no free IAA accumulated and most of the radioactivity co-migrated

Table 3. Effect of cell density on *in vitro* synthesis of IAA

Final cell density (mg/ml)	Cpm incorporated/mg protein IPA	IAA	IAld
0.24	28913	29780	20153
0.50	4080	11027	47422
3.45	3304	0	67305
9.50	0	0	51295
16.00	0	0	120916

with IAld. Thus, at higher cell densities, no [^{3}H]IAA accumulated and it was oxidized to IAld. The radioactivity in IPA, IAA and IAld normalized to unit protein basis is shown in Table 3. These data show that as the cell density increased, the amount of radioactivity in IPA and IAA decreased, whereas it increased in IAld. This result is consistent with the high levels of IAA-oxidase and peroxidase at high cell densities observed earlier [11], and it could account for a decrease of IAA accumulation with increasing cell density. Thus one of the ways by which cell density can regulate cell differentiation in moss protonema is by its effect on the enzymes of IAA catabolism.

DISCUSSION

Endogenous auxin has been reported from liverworts [12, 13], but very little is known about its occurrence in mosses. The auxin from *Marchantia polymorpha* is active in oat coleoptile bioassay and appears to be IAA [13]. Thomas *et al.* [14] have recently demonstrated IAA in rapidly elongating setae of the liverwort *Pellia epiphylla*. Auxin of microbial origin is present in substrata supporting the growth of bryophytes and it seems important in the induction of rhizoids [10]. As mentioned earlier, in the moss *Physcomitrella patens*, a factor, most likely auxin, leaches out when fresh medium is dripped onto the growing protonema [9]. Several factors are known to diffuse into the medium from the protonema, but their chemical identity is unknown [15–17]. A substance resembling an oxidation product of IAA has been detected in the vicinity of old protonema in the moss *Tortella caespitosa* [18].

The results described here show that axenic and actively proliferating protonema cultures of the moss *F. hygrometrica* contain IAA and also have the ability to synthesize it from exogenously supplied tryptophan. The level of auxin activity in the *pg*-1 mutant, which grows predominantly as caulonema, was slightly lower than that of chloronema cells (oat coleoptile bioassay). However, the level of putative IAA based on fluorometric assay was *ca* 2.5-fold more in *pg*-1 than that in the chloronema cells. The elevated level of putative IAA could be important in changing the ratio of cAMP:IAA such that caulonema formation is favoured in *pg*-1. At a high concentration, auxin inhibits the production of buds and gametophores [18, 19]. An increase in the level of putative IAA in *pg*-1 could also explain the inability of bud initials to develop into gametophores in this mutant. As reported by Handa and Johri [4], the bud initials in *pg*-1 dedifferentiate into chloronema, which in turn differentiate into caulonema. These results suggest a role of endogenous auxin in cell differentiation in moss protonema.

The chloronema cell cultures metabolized [^{3}H]tryptophan into IPA, IAAld, IAA and IAld. In higher plants, tryptophan can be metabolized into IAA via several different pathways but predominantly by transamination [20]. A similar situation has also been found for IAA synthesis in *F. hygrometrica*. The inoculum size seems to affect the accumulation of IAA in moss cultures. The cells pre-grown at a low cell density accumulated more IAA than those pre-grown at a high cell density. In a cell-free system, homogenates from cells pre-grown at high cell density completely oxidized IAA into IAld and no accumulation of free IAA was observed. Since the activities of peroxidase and IAA-oxidase are highest at high cell densities [11], the disappearance of IAA can be explained.

The emerging trend strongly suggests that IAA is the predominant form of native auxin in bryophytes as well. In chloronema cells, since the auxin activity based on oat coleoptile bioassay was *ca* 3- to 4-fold more than that determined by the fluorometric assay, the presence of another acidic auxin in addition to IAA is indicated. The solvent system, *i*-PrOH–NH_3–H_2O, did not separate this unidentified auxin from IAA. Alternatively, it is conceivable that a substance moving along with IAA quenches the fluorescence in the fluorometric assay. There was no indication of the presence of such substances in caulonema cells from *pg*-1.

In comparison to the previous studies on bryophytes, the protonema cultures of the moss *F. hygrometrica* showed a much lower level of endogenous free auxin (4–7 μg/kg fr. wt) and putative IAA (2–4 μg/kg fr. wt). These values are also low when compared to the free IAA content reported from dry seeds [21]. The levels in *Funaria* are, however, consistent with the IAA content of vegetative tissues from higher plants, where most values fall into the range of 0.5–15 μg/kg fr. wt [22]. We do not know the content of bound auxin in the moss protonema. The absolute level of free auxin seems to be very different in the actively dividing and non-dividing tissues. The chloronema and caulonema cultures with a low auxin level were actively proliferating and showed a dry matter content of 16–20%. On the other hand, the setae of *Pellia* showing several hundred-fold more IAA than the *Funaria* cultures represent a system where non-dividing cells are rapidly elongating and all the auxin is present in the free form [14].

EXPERIMENTAL

Cell line and media. The experiments described here were carried out using the J-2 cell line of *Funaria hygrometrica* Hedw. (wild type) and the *pg*-1 mutant grown in axenic cell suspension cultures. The method of isolating the cell line, its maintenance and media have been described earlier [11, 23].

Extraction and purification of free acidic auxins. Exponentially growing chloronema cells were harvested, homogenized in 80% MeOH in the presence of a known amount of [^{14}C]IAA as an internal standard, and the homogenate was kept overnight at 4°. It was then centrifuged at 27 000 *g* for 20 min and the supernatant was concd under red. pres. at 45°. The aq. phase was partitioned against *n*-hexane to remove lipids. Chlorophyll was removed by addition of 2% $NaHCO_3$ and storing at −20° for 15 hr. The supernatant was adjusted to pH 8.0 and neutral compounds were extracted 5 × with freshly distilled Et_2O. The aq. fraction was acidified to pH 3.0 with HCl and partitioned against Et_2O to isolate acidic indole compounds. The Et_2O was evapd and the residue was dissolved in 50% MeOH and spotted on 20 × 27 cm Whatman No. 1 paper. The paper was developed by ascending PC in the dark using either *i*-PrOH–NH_3–H_2O (10:1:1) as solvent, or the upper, organic phase of the solvent, C_6H_6–HOAc–H_2O (2:2:1). The paper was dried at 37° and cut into 10 equal strips of 2 cm each. When acidic auxins were determined by bioassay, each strip was eluted with 2 ml 0.01 M NaPi buffer, pH 5.9. A sample of the eluate (0.2 ml) was used for determining the recovery of IAA while the remainder was used for assay. For fluorometric assay, each strip was eluted in 2 ml of distilled MeOH. The eluate was dried by flushing N_2 and the residue was used for assay.

Estimation of auxin activity. The level of acidic auxins was determined using oat coleoptile straight growth test [24]. *Avena*

sativa (var. Kent, EC 13594) was obtained from I.A.R.I., New Delhi. The auxin levels were also determined using the fluorometric assay as described by Stoessl and Venis [25].

Synthesis of IAA by chloronema cell cultures from [^3H]tryptophan. The experiments on the synthesis of IAA were carried out using the methods described by Gibson *et al.* [26, 27]. The chloronema cells were subcultured at various cell densities in minimal glucose medium supplemented with 0.1 mM [^{3}H]tryptophan (4×10^7 cpm, sp. act. 5.6 Ci/mmol). After 18 and 42.5 hr, respectively, cell were harvested, washed with 50 ml medium containing 0.1 mM tryptophan, and homogenized in 50% MeOH after the addition of [^{14}C]IAA as an internal standard. The homogenate was centrifuged at 27 000 *g* for 20 min and the supernatant concd under red. pres. at 45°. Ten μg of each of IAA, indole-3-pyruvic acid (IPA), indole-3-lactic acid (ILA), indole-3-ethanol (IE), indole-3-aldehyde (IAld), 3-indoleacetaldehyde (IAAld) and tryptamine (TNH_2) were added as carriers to the concentrate. The mixture was adjusted to pH 8.0, and neutral indole compounds were extracted 5 × with freshly distilled Et_2O. The aq. fraction was acidified to pH 3.0 with HCl, and acidic indole compounds were partitioned into Et_2O. The aq. phase was finally adjusted to pH 11.0 and basic compounds were separated by 5 × extraction with Et_2O. The various indole compounds were separated by ascending PC. After chromatography, the paper was dried at 37° and cut into 1 cm wide 20 strips. The strips were suspended in Bray's scintillation fluid [28] and the radioactivity was determined. The indole compounds were identified on the basis of R_f and co-chromatography with authentic non-radioactive indole compounds spotted on the same chromatogram. The indole compounds were detected by treating the chromatograms with *p*-dimethylaminocinnamaldehyde reagent [17].

Synthesis of IAA in cell-free system. The chloronema cells were subcultured at various cell densities (0.05–5 mg/ml); after 48 hr, cells at each density were harvested, weighed and homogenized in 0.1 M KPi buffer (pH 8.0) containing 0.01 M EDTA, 0.01 M β-mercaptoethanol and 0.1 mM pyridoxal phosphate. The homogenate was centrifuged at 27 000 *g* for 20 min, and 0.5 ml of supernatant was incubated with 0.5 ml of reaction mixture containing 0.1 M KPi buffer (pH 8.0), 0.01 M EDTA, 0.01 M β-mercaptoethanol, 0.8 mM pyridoxal phosphate, 100 μg thiamine pyrophosphate, 1 mg nicotinamide adenine dinucleotide, 6–7 $\times 10^6$ cpm [^{3}H]tryptophan, 0.04 M *l*-tryptophan, and 0.01 M α-ketoglutarate. One set of reaction mixture was stopped immediately while the other set was incubated in darkness for 2 hr at 30°, and then the acidic and neutral indole compounds were extracted. These compounds were separated by ascending PC as already described.

Estimation of protein. Protein from cells or cell homogenates was precipitated with ice-cold 10% TCA. The ppt. was washed repeatedly with 95% EtOH and dissolved in 0.1 M NaOH. Insoluble material was removed by centrifugation, and protein in the supernatant was determined by the method of Lowry *et al.* [29].

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