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Induction of Indoleacetic Acid Synthetases in Tobacco Pith Explants[1]

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ABSTRACT

Formation of indoleacetic acid synthetases in tobacco pith explants was determined by following the growth of tissue cultures under conditions of indole-3-acetic acid (IAA) deprivation and by measuring the enzymatic conversion of tryptophan to IAA in the cultures. The pith explants obtained from the parent plant (*Nicotiana glauca*) and from basal regions of the tumor-prone hybrid (*N. glauca* × *N. langsdorffii*) both show a requirement for exogenous IAA for growth initiation in culture. The parent pith requires the constant presence of added IAA for continued growth, but hybrid pith, after initial treatment with IAA, will grow without further additions. IAA synthetases are detected in the cell homogenates of hybrid pith explants cultured with either continuous or initial IAA addition. These observations indicate that IAA may induce its own production. In contrast, IAA synthetases are not found in the parent pith under comparable culture conditions. Besides IAA, nonhormonal compounds such as indole and tryptophan are also capable of stimulating growth of hybrid pith, possibly through the induction of IAA synthetases needed for IAA formation. Indole and tryptophan are, however, inactive in growth promotion of the parent pith. These results suggest that the genomic expression of IAA synthetase formation is more stringently controlled in *N. glauca* than in the tumor-prone hybrid.

Since the development of the tumor-prone tobacco hybrid of *Nicotiana glauca* × *N. langsdorffii* by Kostoff (9), the results of numerous studies have suggested that spontaneous tumor formation may be related to an elevated auxin level in the hybrid (1–3, 8). White (16) and Skoog (13) demonstrated that callus tissue derived from this tumor-prone hybrid plant could grow in the absence of exogenous auxins. These observations consistently indicate that the proliferative growth of hybrid cells, both *in vivo* and *in vitro*, is probably regulated at the level of genomic expression, as a result of production of auxin at greater than physiological levels.

Recently, Hagen (6, 7) showed that pith explants from both *N. glauca* and the hybrid require an external supply of auxins for growth in culture but do not require any other plant growth-promoting substances such as kinins or gibberellins. This finding suggested that this culture system offers a unique opportunity to study the cellular mechanisms regulating the formation of the enzymes involved in the synthesis of the phytohormone IAA. The advantage of using primary pith cultures in such research is that these cells could become capable of growth in culture in the absence of exogenous IAA if IAA synthetases are induced by exposure to an appropriate chemical stimulus. Thus, the growth response of pith explants can be examined on a medium free of added IAA. Also, the production of IAA synthetases can be measured directly by the enzymatic conversion of tryptophan, an IAA precursor, into IAA.

The experimental results presented here demonstrate that IAA synthetases are inducible in the tumor-prone tobacco hybrid pith explant by IAA and also by the nonhormonal compounds indole and tryptophan. The induction phenomenon may be closely related to the genetic constitution of these tobacco cells.

MATERIALS AND METHODS

Plants and Growth Conditions. The tumor-prone tobacco hybrid of *Nicotiana glauca* × *N. langsdorffii* and one parent, *N. glauca,* were used as the source of material for all the experiments. *N. langsdorffii* was not included because it is very difficult to obtain plants in the vegetative state.

The medium used to culture pith tissue was similar to that used by Murashige and Skoog (11) but with the omission of glycine, nicotinic acid, and pyridoxine, as previously described (4). The agar was omitted in the preparation of liquid medium. The concentrations of various compounds added to the medium to determine their effect in promoting growth of the pith explant are detailed in the captions to the figures and tables. To avoid decomposition of these compounds, they were sterilized separately from the culture medium by Millipore filtration.

Fresh pith explants were routinely cultured for assessment of the activity of various compounds in promoting cell growth. Tobacco stems obtained from rapidly growing vegetative plants were sterilized in 5% Clorox (5.25% sodium hypochlorite, The Clorox Co., Oakland, Calif.) plus 0.2% Alconox (glass detergent, Scientific Glass Apparatus, Bloomfield, N.J.) for 5 min. Pith explants were obtained by cross-sectionally slicing the pith from the centers of stems that had been trimmed to remove the outer tissue layers. Nine to 16 pieces of pith weighing approximately 5 to 10 mg were then cultured in 100- × 20-mm Petri dishes (Falcon Plastics, Oxnard, Calif.) containing 50 ml of solid medium variously supplemented. The pith cultures were incubated at 28 C in the dark for the stated times. Under these culture conditions the hybrid and the *N. glauca* pith explants grow as a friable, undifferentiated callus mass. For studying

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Table I. *Comparison of the Increases in Fresh and Dry Weight of Hybrid and Parent Pith Cultures from Subapical and Basal Regions of the Plants*

In cultures a, b, d, and e, the tissue was incubated at 28 C for 30 days. In cultures c and f, the tissue was harvested after 22 days on a medium containing 2.5 μM IAA. The fresh weight was obtained after removal of the excess liquid around the tissue. The dry weight was obtained after drying the tissue in a 60 C oven for 48 hr. Each mean weight and its standard error was calculated from at least 10 samples.

Position of Pith (Internode)	IAA	Fresh Weight		Final/Initial	Dry Weight		Final/Initial
		Initial	Final		Initial	Final	
		mg/piece			*mg/piece*		
1. *N. glauca* × *N. langsdorffii* (hybrid)							
a. 3rd-5th	−	2.9 ± 0.6	739.0 ± 98.0	255	0.2 ± 0.1	35.0 ± 2.9	250
b. below 7th	−	9.4 ± 1.6	9.5 ± 0.7	1	0.4 ± 0.1	0.7 ± 0.2	2
c. below 7th	+	9.4 ± 1.6	1168.4 ± 216.0	125	0.4 ± 0.1	47.6 ± 6.4	136
2. *N. glauca* (parent)							
d. 3rd-5th	−	2.2 ± 0.9	5.6 ± 1.5	3	0.2 ± 0.1	0.5 ± 0.1	3
e. below 7th	−	4.9 ± 0.9	9.2 ± 1.0	2	0.3 ± 0.1	0.7 ± 0.2	2
f. below 7th	+	4.9 ± 0.9	1179.3 ± 283.0	235	0.3 ± 0.1	56.1 ± 15.4	170

the effect of IAA deprivation on cell growth, pith explants were removed at given intervals from the solid culture medium containing IAA and washed with an excess of liquid medium free of IAA. The same washing procedure was used for all changes of medium. Growth of the cultures was estimated from increases in fresh and dry weight.

Preparation of Crude Extract for Enzyme Assay. The procedure for the preparation of crude extract from tobacco pith explants was similar to that of Phelps and Sequeira (12). After blotting with filter paper, 2.0 g of tissue were ground with 0.2 ml of 0.5 M potassium phosphate buffer, pH 6.0, in a mortar with a small amount of purified sand (J. T. Baker Chemical Co., Phillipsburg, N.J.) for 5 min at 4 C. The cell debris was removed by centrifugation in a Lourde 9RA rotor at 10,000*g* for 30 min. The supernatant was adjusted to a volume of 4.0 ml and to a final concentration of potassium phosphate buffer of 25 mM. The 2.0-ml reaction mixtures used for measuring the enzymatic synthesis of IAA from tryptophan contained 5 μmoles of ∂-ketoglutaric acid, 0.05 μmole of pyridoxal phosphate, 2.5 μmoles of DL-tryptophan, 0.5 μc of ^{14}C-DL-tryptophan (17.1 mc/mmole, New England Nuclear, Boston, Mass.), and 1.0 ml of crude extract. They were incubated for various times at 28 C with shaking. The reaction was stopped by chilling the sample to 4 C, and acidic ether-soluble compounds were immediately extracted as described below. The protein concentration of the crude extract was measured by the biuret method with bovine albumin used as a standard (5).

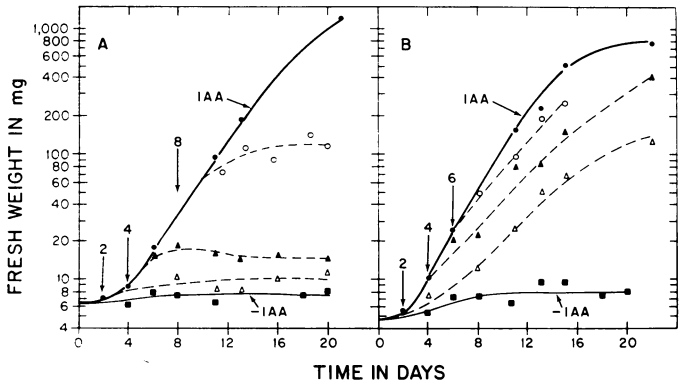

FIG. 1. Effect of IAA deprivation on the growth of (A) parent (*N. glauca*) and (B) hybrid (*N. glauca* × *N. langsdorffii*) pith explants cultured on a defined medium. The solid curves show growth of pith tissue cultured at 28 C in the dark in the presence of 2.5 μM IAA from zero time (●) and in its absence (■). The dashed curves show the growth of pith tissue exposed to IAA for 2 days (△), 4 days (▲), and (A) 8 days or (B) 6 days (○) and then transferred to a medium free of IAA. Each point is the average weight of eight samples.

Extraction and Identification of ^{14}C-IAA. At the end of the incubation period, the reaction mixture was adjusted to pH 3.0 with 0.5 N HCl. The acidic ether-soluble compounds were extracted from the sample by vigorous shaking with 3.0 ml of diethyl ether twice for 2 min at room temperature. The ether phase was separated from the aqueous phase by centrifugation and evaporated to dryness. The compounds were then redissolved in about 50 μl of diethyl ether. Radioactive IAA was identified by thin layer chromatography. The samples obtained from the acidic ether-soluble fraction were spotted on a 5- × 10-cm Silica Gel F 254 thin layer plate (E. Merck, Darmstadt, Germany), which was developed in chloroform-acetic acid (95:5) to 8.5 cm from the origin. The spots corresponding to the R_F value of the IAA reference (R_F = 0.46) were excised from the thin layer plates into scintillation vials. Ten milliliters of toluene-based counting fluid were added to each sample, and the radioactivity was assayed (4) in a model 2420 Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

IAA Requirement for Growth of Pith Explants. The growth capabilities of pith explants obtained from various parts of both the tumor-prone tobacco hybrid and one parent, *N. glauca*, were examined in culture in the presence and absence of IAA (Table I). In the hybrid system, an auxin-autotrophic proliferation was observed for pith explants dissected from near the apical region (3rd to 5th internode) (Table Ia), but pith explants from the basal section of the plant (below the 7th internode) were incapable of growth unless exogenous IAA was added (Table I, b and c). With pith explants from *N. glauca*, regardless of the location of the tissue in the plant, an absolute growth requirement for exogenous IAA was found for all cells (Table I, d, e, and f). Therefore, in the subsequent experiments primary cultures of pith explants having an absolute growth requirement for IAA were used, namely, those from *N. glauca* and from the basal region of the hybrid plant.

Effect of IAA on the Growth of Pith Explants. When pith explants from both parental and hybrid plants were cultured in the continuous presence of 2.5 μM IAA (Fig. 1), the growths

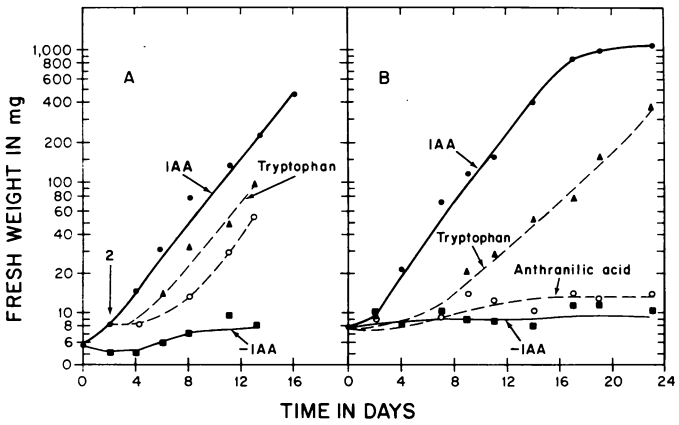

FIG. 2. Effect of DL-tryptophan and anthranilic acid on the growth of hybrid (*N. glauca* × *N. langsdorffii*) pith explants cultured on a defined medium. Solid curves show growth with 0.25 μM IAA (●) and without it (■), as in Figure 1. Dashed curves (A) show growth of pith tissue removed from medium containing IAA after 2 days and cultured on a medium free of IAA, with (▲) and without (○) DL-tryptophan (5.0 μM). Dashed curves (B) show growth of pith tissue cultured in the presence of DL-tryptophan (▲) or of anthranilic acid (○), each 5.0 μM from zero time.

of both types of explants, as expressed by the fresh weights of the samples, were comparable; both showed an initial lag period of 2 days followed by an exponential phase of about 15 days, and the fresh weight increased by approximately two orders of magnitude.

Comparison of the growth responses of parental and hybrid explants to IAA deprivation revealed distinct differences in the growth requirement for auxin between the two systems. In *N. glauca* pith explants (Fig. 1A), regardless of whether the transfer was during the early exponential phase (2 and 4 days) or during the actively proliferating mid log phase (8 days), the tissues all ceased to grow when transferred from a medium containing IAA to a medium free of IAA. The slight increases in fresh weight in these cultures immediately after removal from the IAA medium were probably due to residual intracellular IAA supporting growth for a short time. Thus, an external supply of IAA is essential for continued growth of pith cells from *N. glauca*. In sharp contrast, when hybrid cells (Fig. 1B) from various stages of the growth period (2, 4, and 6 days) in culture with IAA were transferred to a medium devoid of IAA, they were capable of proliferative growth. The results indicate that, for continued growth, hybrid pith cells require only a brief exposure to IAA during the intial culture period. Furthermore, the slopes of the growth curves of hybrid pith cells deprived of IAA were all similar although the cells had been transferred at different points on their initial growth curves. These observations suggest that rather than storing IAA the cells have a mechanism for production of (additional) IAA after initial treatment with IAA itself.

Since the continued growth of hybrid pith explants after brief treatment with exogenous IAA suggested that IAA synthetases may have been induced, the effect of tryptophan, a precursor of IAA, on cell growth was examined. Hybrid explants were removed from a medium containing IAA (0.25 μM) after 2 days and cultured on a medium free of IAA either with or without tryptophan (Fig. 2A). Since tryptophan probably does not act directly as a plant hormone, the growth enhancement of the culture may be due to the stimulatory effect of newly synthesized IAA enzymatically converted from tryptophan. When hybrid pith was cultured from zero time on medium supplemented with tryptophan or with anthranilic acid (Fig. 2B), growth of pith cells was stimulated by the IAA precursor, tryptophan, but not by the tryptophan precursor, anthranilic acid. Anthranilic acid has been shown to be ineffective in promoting growth of pea stem sections (10).

Growth Promotion by Nonhormonal Compounds. The findings with tryptophan and anthranilic acid suggested that other compounds containing an indole moiety might be active in the promotion of cell growth. Therefore, the growth response of parent and hybrid explants to various indole compounds (1–10 μM) was tested (Table II). In *N. glauca*, the growth-regulating substance IAA was the only compound capable of stimulating cell growth, but in hybrid cells growth was promoted by IAA, D- and DL-tryptophan, and indole. It is interesting that indole, although it is not a phytohormone, effectively initiated the growth of hybrid cells. Furthrmore, exposure of the hybrid cells to indole for just 3 days was more than sufficient to stimulate cell proliferation, as shown by the approximately 80-fold increase in fresh weight of the tissue during a subsequent 23-day culture period without indole; this increase is directly comparable to that of the tissue cultured with continuous presence

Table II. *Effect of Nonhormonal Compounds on the Growth of Parent and Hybrid Pith Explants Cultured on a Defined Medium*

The pith tissue was cultured at 28 C in the dark on a defined medium containing various compounds as indicated: a, The compound was present in the medium throughout; b, tissue was exposed to the compound for various periods and then transferred to medium free of the compound. At the end of the culture period, the tissue was harvested for fresh weight determination. Each mean weight and its standard error was calculated from at least eight samples.

Compound	Concentration	Days of Culture	Final Fresh Weight	
			Parent	Hybrid
	μM		mg/piece	
1. No addition	...	(a) 22	7.5 ± 1.5	9.4 ± 1.6
2. IAA	2.5	(a) 22	1179.3 ± 283.0	1168.0 ± 216.0
		(b) 3 with; 19 without	10.0 ± 1.3	318.0 ± 47.1
3. DL-Tryptophan	10	(a) 26	9.2 ± 1.0	208.0 ± 49.9
		35	...	822.2 ± 264.0
4. DL-Tryptophan	10	(a) 26	11.0 ± 2.4	161.3 ± 48.6
		35	...	1164.0 ± 240.0
5. Indole	17	(a) 26	10.5 ± 1.7	720.0 ± 128.0
		(b) 3 with; 23 without	...	822.0 ± 160.0

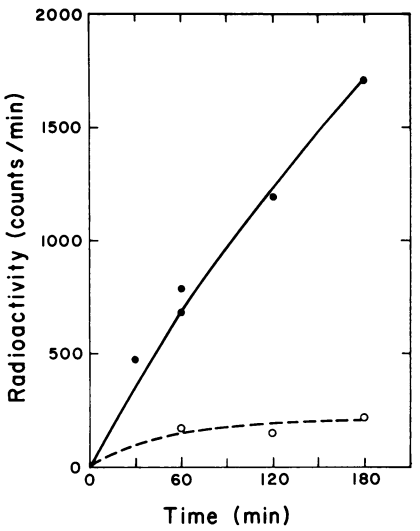

FIG. 3. Rate of IAA synthesis by crude extracts from hybrid pith explants. The solid line shows data for extracts from transferred cells, and the dashed line shows data for extracts from cells cultured without exogenous IAA (see text). Preparation of crude extracts, conditions for conversion of ^{14}C-tryptophan to ^{14}C-IAA, and identification of ^{14}C-IAA are described under "Materials and Methods."

of indole for the same length of time. Thus, indole was more effective than tryptophan in promoting cell growth.

Enzymatic Conversion of Tryptophan to IAA. The enzymatic activity in synthesizing IAA from tryptophan was determined for cell extracts prepared from parental and hybrid pith explants cultured under various conditions. The rate of conversion of ^{14}C-tryptophan to ^{14}C-IAA by a crude extract prepared from transferred hybrid cells (cells treated with IAA for 3 days and then transferred to IAA-free medium and cultured for 5 days) was approximately linear for 3 hr, whereas the rate for a crude extract from comparable tissue cultured from zero time without exogenous IAA remained near zero (Fig. 3). This lack of conversion was not caused by cell inactivation since the cells retained their viability over an extended period of time without auxin (unpublished results).

The above data provide direct evidence for the appearance of IAA synthetase activity in transferred cells and suggest that IAA synthetases had been induced in the hybrid system. In *N. glauca,* activity of IAA synthetases, as measured by synthesis of ^{14}C-IAA, was not detected in crude extracts whether they were prepared from cells cultured with IAA for 14 days, from transferred cells (10 days with IAA followed by 4 days without), or from fresh pith explants (Table III, a, b, and c). In the hybrid system, the crude extract from the transferred cells was more active in IAA synthesis than that from cells cultured in the continuous presence of exogenous IAA (Table III, e and d). The enzymatic activity of the extract from hybrid cells was not markedly reduced by the addition of an equal volume of extract from *N. glauca* (Table IIIi). This finding tends to rule out the possibility that extract from *N. glauca* contains inhibitors that suppress the enzymatic activity converting ^{14}C-tryptophan to ^{14}C-IAA.

DISCUSSION

Pith tissue, an essentially homogenous mass of highly differentiated parenchymal cells, was used to study the mechanism of IAA synthetase induction. These cells are suitable for such a study because they are not a site for IAA production in intact plants. IAA synthetases either are not pith cell constituents or exist in an inactive conformation. Consequently, for growth in culture, pith explants from the tumor-prone hybrid of *N. glauca* × *N. langsdorffii* and from the parent, *N. glauca,* have an absolute requirement for an exogenous supply of IAA or an equivalent amount of a similar plant growth-regulating substance (6, 7). However, an exception exists in that hybrid explants obtained from near the stem apex are capable of growth and proliferation in the absence of external auxin. The previously reported auxin gradient from the apical toward the basal region of various species of plants (14, 15, 17) and the high auxin content in the tumor-prone *Nicotiana* hybrids (1–3, 8) may contribute directly to an elevated auxin level in the apical region. Hence the endogenous auxin in the apical region may be more than sufficient to trigger cell growth in culture. But it is very unlikely that these cells would initially contain amounts of auxin high enough to support continuous growth. Thus, the growth behavior of pith explants from subapical regions seems to be analogous to that of transferred cells in that both types of explant require a low level of IAA for growth initiation. That further growth of these explants is promoted by newly synthesized IAA is suggested by the detection of enzymatic activity converting tryptophan to IAA *in vitro.* It is also implied that cellular growth itself is insufficient to cause the appearance of enzymatic activity since in culture the explants from *N. glauca* have an absolute IAA requirement for growth and have no detectable IAA synthetic activity in actively growing cells. It seems, therefore, that IAA participates in two aspects of cellular regulation: it acts as a phytohormone and also as an inducer of IAA synthetase, thus causing continued production of IAA.

Since the hormonal effect of IAA on plants is immediate growth stimulation, it is difficult to distinguish it from a reaction coupled to cell division due to some other biochemical event such as the formation of IAA synthetases. Therefore, the results obtained with transferred cells do not permit differentiation between these two processes. However, the growth-promoting effect of the nonhormonal compounds indole and

Table III. *Enzymatic Conversion of ^{14}C-Tryptophan to ^{14}C-IAA by Extracts Prepared from Parent and Hybrid Pith Cells*

Pith tissues of the parent and hybrid were cultured on a defined medium at 28 C in the dark as indicated. Assay for the biosynthesis of ^{14}C-IAA by extracts is described under "Materials and Methods."

Source of Extract	Culture Conditions	Protein in Extract	Radioactivity in IAA	Specific Activity
		mg	*cpm*	*cpm/mg protein*
Parent	a. 14 days with IAA	0.90	112	124
	b. 10 days with IAA; 4 days without	0.85	289	340
	c. Fresh pith tissue	...	196	...
Hybrid	d. 12 days with IAA	1.35	1544	1144
	e. 4 days with IAA; 8 days without	1.30	2178	1663
	f. Fresh pith tissue	...	230	...
Parent (2.0 ml)	g. 8 days with IAA; 4 days without	0.85	267	315
Hybrid (2.0 ml)	h. 7 days with IAA; 4 days without	1.35	2092	1550
Parent (1.0 ml) + hybrid (1.0 ml)	i. Equal portions of g and h	1.10	1160	1160

tryptophan on hybrid cells makes it possible to dissociate enzyme induction from that of cell division. The action of indole and tryptophan on hybrid cells may involve a two-step reaction, *i.e.*, induction of IAA synthetases, which is reflected in the rather long initial lag period (6 days) compared with that of cells cultured with IAA (2 days), and subsequently the enzymatic production of IAA needed for promotion of cell growth. These and other observations seem to indicate that the induction of IAA synthetases occurs prior to cell division.

It is noteworthy that indole was more effective than tryptophan in the promotion of cell growth. A possible explanation is that the tryptophan present in the culture medium was preferentially incorporated into newly synthesized proteins by the pith explants and consequently limited the amount of tryptophan available for promoting cell growth.

The induction of IAA synthetases may involve activation at the level of gene transcription. A possible interpretation of the results obtained here is based on the unique genetic composition of the parental and hybrid cells. In the parental system, the regulation of gene function regarding the formation of IAA synthetases seems to be stringently controlled, and therefore cells have a tendency to remain in the repressed state. However, because of the tumorous nature of hybrid cells, it seems reasonable to assume that in them the transcriptional processes for production of IAA synthetases are easily activated. Once the genes of the hybrid are derepressed by pretreatment with IAA, indole, or tryptophan, these genes are maintained in the functional state.

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