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Promotion of Indoleacetic Acid Oxidase Isoenzymes in Tobacco Callus Cultures by Indoleacetic Acid[1]

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ABSTRACT

Indoleacetic acid oxidase in tobacco callus cultures (*Nicotiana tabacum* L., cv. White Gold) was composed of at least two groups of isoenzymes, which were distinctly different in electrophoretic mobilities and in responses to growth substances. Indoleacetic acid had dual effects; at low concentrations it promoted the development of two fast-migrating indoleacetic acid oxidase isoenzymes, but at high concentrations it increased the level of other indoleacetic acid oxidase isoenzymes with low and moderate electrophoretic mobilities. However, indoleacetic acid was not unique in such effects; 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were effective at concentrations lower than that of indoleacetic acid.

A correlation was apparent between the relative levels of the two groups of indoleacetic acid oxidase isoenzymes and the rate and type of growth, as affected by auxins. The development of the fast-migrating anionic indoleacetic acid oxidase isoenzymes was accompanied by a type of growth characterized by rapid growth rate, high water content, and friable tissues. On the other hand, a further increase in the isoenzymes of slower migrating rate was associated with growth retardation.

The indoleacetic acid-mediated increase of the fast-migrating indoleacetic acid oxidase isoenzymes was dependent on the level of kinetin, suggesting a multiple control by different types of growth substances. The inhibition of the formation of the fast-migrating isoenzymes by actinomycin D and cycloheximide suggests a requirement for both RNA and protein synthesis.

Previous work (9) has shown the influence of cytokinins on IAA oxidase in tobacco callus cultures, but the development of two fast-migrating IAA oxidase isoenzymes in response to low concentrations of cytokinins required the presence of IAA, suggesting that IAA was also a regulatory factor. In this paper I present evidence for the regulation of IAA oxidase isoenzymes by IAA and by other auxins.

Peroxidase has long been known to catalyze the oxidation of IAA (3, 5, 7). The IAA oxidase isoenzyme fractions obtained in this study all possessed peroxidase activity; however, the relative distribution of isoperoxidases, as affected by growth substances, did not parallel that of IAA oxidase, perhaps due to difference in affinities for substrates. Repression of certain isoperoxidases by IAA was reported in dwarf pea stem sections (12), tobacco and *Pelargonium* pith tissues (4, 8), and oat coleoptile sections (15). On the other hand, promotion of other isoperoxidases by IAA was observed in tobacco and *Pelargonium* pith tissues (4, 8, 14). Recently, IAA at a high concentration was shown to induce the synthesis of two anionic isoperoxidases in lentil roots (13). Nevertheless, a quantitative evaluation of the effect of IAA on the level of IAA oxidase has not previously been reported.

MATERIALS AND METHODS

Materials. Tobacco callus tissue was used as plant material. The tissue was originally derived from the pith of 3-month-old tobacco plant (*Nicotiana tabacum* L., cv. White Gold) and cultured *in vitro* for 5 years with frequent transfer on Linsmaier and Skoog's medium (11). This tissue required both exogenous IAA and kinetin to maintain growth. The concentrations of IAA and kinetin were 6 and 2 μM respectively for the stock culture but varied in the experiments as indicated in the tables and figures. The stock solutions of IAA, actinomycin D, and cycloheximide were sterilized by filtration. The cultures were grown at 28 C for 20 to 30 days under weak light (20 ft-c) on 16-hr day and 8-hr night cycles.

Enzyme Preparation. Two to five grams of the fresh tissue were homogenized in a Potter-Elvehjem tissue homogenizer with 4 to 10 ml of 50 mM phosphate buffer, pH 7.5, containing 50 mM sodium ascorbate and 0.8 M KCl (1). After centrifuging at 20,000g for 10 min, the residue was re-extracted twice with fresh buffer, and the mixture was centrifuged. The volume of the combined extract was 5 ml/g fresh weight of tissue. A portion was then dialyzed against 4 liters of deionized water for 22 hr. All operations were done at 2 to 4 C.

Electrophoresis. Immediately before electrophoresis, the extract was diluted with cold deionized water and then mixed with a 60% (w/v) sucrose solution (2:1) so that the aliquot used per gel was equivalent to 1 mg dry weight of the tissue. The gel column was made of 4.5% (w/v) polyacrylamide in the upper 10-mm section and 5.5% polyacrylamide in the lower 55-mm section with a diameter of 5 mm. A Buchler analytical polyacrylamide gel electrophoresis apparatus was used and electrophoresis was carried out by the method of Davis (2). During electrophoresis the temperature of the buffer surrounding the gels was kept at 4 C and the current applied was 2 ma per gel. Immediately after electrophoresis the gel was sliced into 1-mm sections, and each section was extracted separately with 2 ml of 30 mM phosphate buffer at pH 6 on a shaker at 2 C for 20 hr.

IAA Oxidase Assay. The extract of each 1-mm gel slice was assayed for IAA oxidase by incubation with 0.15 mM IAA, 0.1 mM 2,4-dichlorophenol, and 0.1 mM $MnCl_2$ in a shaking water bath at 37 C for 30 min. Then the amount of IAA remaining was estimated with the modified Salkowski reagent (6). The IAA oxi-

[1] Contribution No. 471.

dase activity was expressed as μg of IAA destroyed per mg dry weight of tissue at 37 C in 30 min.

The detailed methods have been previously described (9, 10).

RESULTS

Increase of IAA Oxidase by IAA. The tobacco stock callus tissue at the outset of the experiments had four anionic IAA oxidase isoenzymes but had little activity of the fast-migrating isoenzymes A_5 and A_6. In the presence of 0.2 μM kinetin, however, IAA at 10 μM promoted the development of two fast-migrating anionic IAA oxidase isoenzymes, A_5 and A_6, whereas in the control culture without IAA there was little activity of A_5 and A_6 (Fig. 1). To test whether these increases required RNA and protein synthesis, actinomycin D and cycloheximide were added to the culture media. As a result, the development of A_5 and A_6 was repressed (Table I). This suggests an involvement of both DNA-dependent RNA and protein synthesis in the IAA-promoted increase of the two fast-migrating IAA oxidase isoenzymes, although it provided no proof for *de novo* synthesis of these isoenzymes.

Effects of IAA Concentration. Experiments with a series of concentrations of IAA showed a minimum of 0.1 μM being required for a significant increase in IAA oxidase A_5 (Fig. 2). The increase of IAA oxidase isoenzyme A_6 in response to IAA concentration paralleled that of isoenzyme A_5, although the level of A_6 was lower than that of A_5. The optimal concentration of IAA for the increase of these two IAA oxidase isoenzymes was 10 μM. Increasing the IAA concentration from 10 to 100 μM, or beyond,

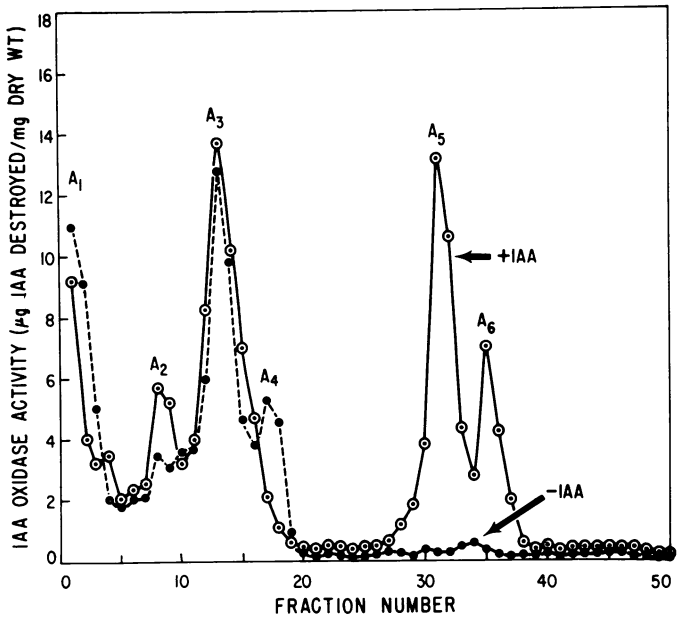

FIG. 1. Promotion of IAA oxidase isoenzymes A_5 and A_6 by IAA in tobacco callus tissues. The IAA level was 10 μM, and the media with and without IAA all contained 0.2 μM kinetin. The growth period was 20 days. The IAA oxidase activity was expressed as μg of IAA destroyed per mg dry weight of tissue at 37 C in 30 min.

Table I. *Inhibition of IAA-promoted Fast-migrating IAA Oxidase Isoenzymes A_5 and A_6 by Actinomycin D and Cycloheximide*

The growth media contained 10 μM IAA and 0.2 μM kinetin. The concentrations of actinomycin D and cycloheximide were 5 and 1 mg/l, respectively. The growth period was 25 days.

Treatment	IAA Oxidase Isoenzymes	
	A_5	A_6
	μg IAA destroyed/mg dry wt·30 min	
Control	39.5	15.5
Actinomycin D	9.3	3.2
Cycloheximide	3.2	1.9

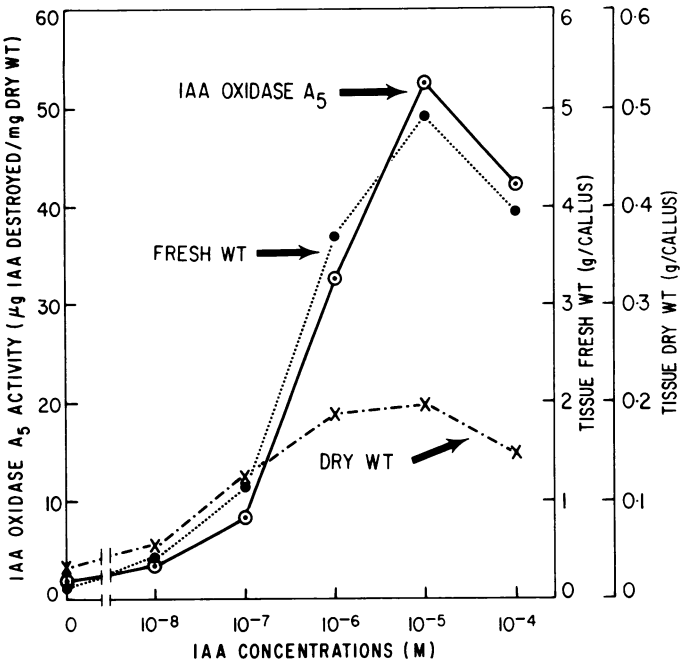

FIG. 2. Correlation of IAA oxidase isoenzyme A_5 with growth of tobacco callus tissue in response to different concentrations of IAA. The media contained 0.2 μM kinetin. The growth period was 28 days.

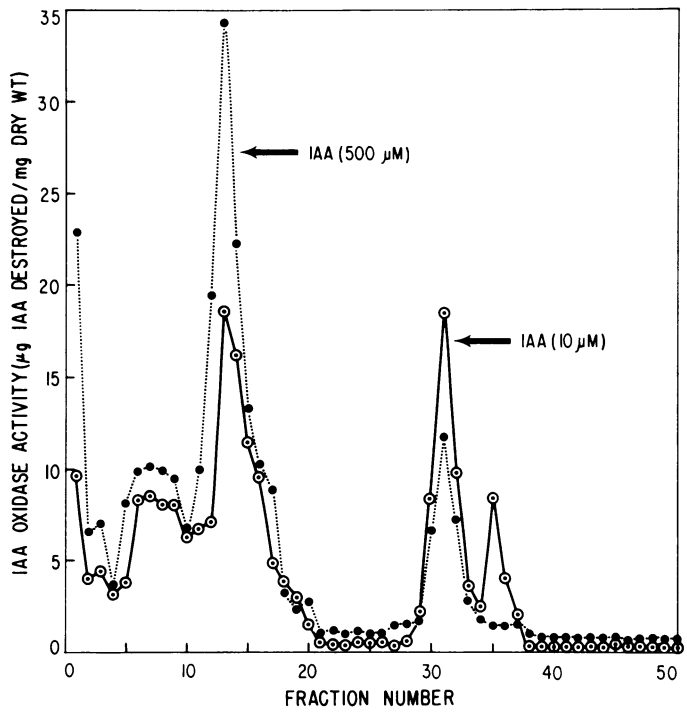

FIG. 3. Effect of high concentration of IAA on IAA oxidase isoenzymes in tobacco callus tissues. The media contained 0.2 μM kinetin. The growth period was 27 days. The IAA oxidase activity was expressed as μg of IAA destroyed per mg dry weight of tissue at 37 C in 30 min.

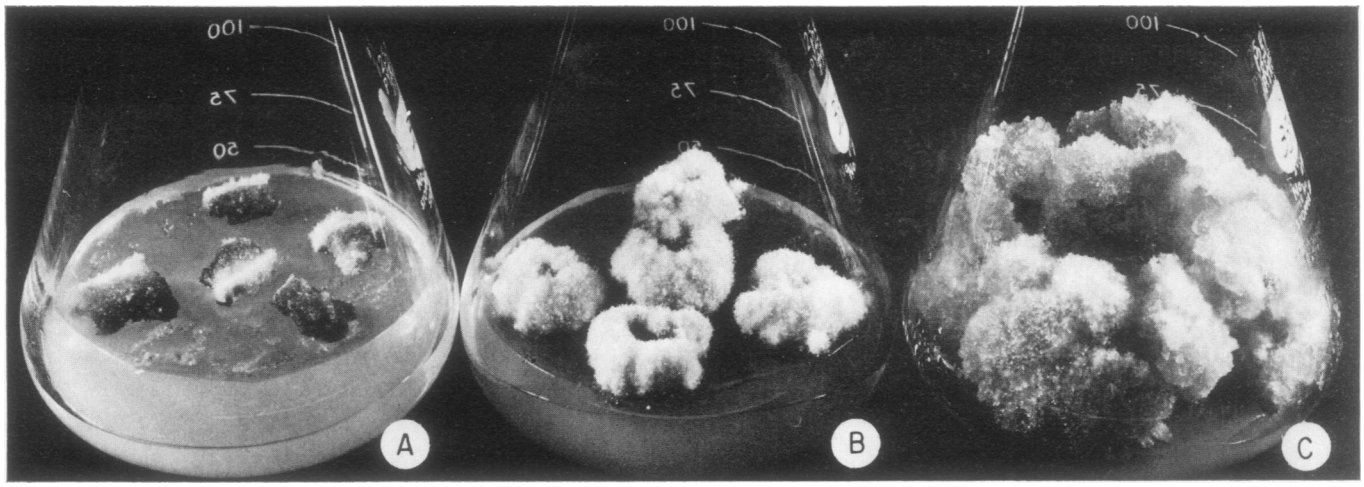

FIG. 4. Difference in rate and type of growth promoted by different concentrations of IAA in tobacco callus cultures. A: No IAA; B: 0.1 μM IAA; C: 10 μM IAA. The media contained 0.2 μM kinetin. The growth period was 28 days.

Table II. *Dependence of IAA-promoted Fast-migrating IAA Oxidase Isoenzyme A_5 on Kinetin Levels*

The growth period was 24 days.

IAA Concn	Kinetin Concn		
	0.2 μM	1.0 μM	5.0 μM
μM	*μg IAA destroyed/mg dry wt·30 min*		
1	28.2	6.7	0
10	46.2	21.2	0
100	36.5	22.5	0

Table III. *Effects of 2,4-D, 2,4,5-T, and 2,4,6-T at Two Concentrations on IAA Oxidase Isoenzymes and Growth of Tobacco Callus Tissues*

The media contained 0.2 μM kinetin. The growth period was 25 days.

Treatment	IAA Oxidase Isoenzymes			Tissue Dry Wt
	A_5	A_1	A_3[1]	
	μg IAA destroyed/mg dry wt·30 min			*mg/callus*
2,4-D, 1 μM	20.4	28.6	21.6	108
10 μM	3.5	31.9	27.7	52
2,4,5-T, 1 μM	19.9	18.0	27.5	134
10 μM	3.1	33.5	37.4	62
2,4,6-T, 1 μM	2.2	14.7	11.7	22
10 μM	1.2	13.3	13.5	18
IAA 10 μM	40.0	15.7	13.2	203
Control	2.3	12.6	14.3	23

[1] For isoenzyme A_3, only the peak reading is shown because this fraction was not completely separated from the adjacent fractions.

repressed the development of the fast-migrating isoenzymes, but increased the level of others with low and moderate electrophoretic mobilities. With IAA at 500 μM, the level of IAA oxidase A_5 was decreased by 30% relative to that with an optimal level of IAA and the development of A_6 was inhibited almost completely. In contrast, the level of A_1 and A_3 was significantly increased (Fig. 3). Apparently the IAA oxidase isoenzymes were differentially affected by IAA depending upon its concentration.

Of particular interest was the development of isoenzyme A_5, which became one of the major IAA oxidases under optimal growth conditions. The development of this isoenzyme paralleled the fresh weight increase of the tobacco callus tissue under the influence of increasing concentrations of IAA (Fig. 2). With IAA at the optimal concentration of 10 μM both the level of A_5 and the fresh weight of tissue were the highest, but before reaching this point there was a distinct transition in the 0.1 to 1 μM concentration range. In this range, equally significant was the transformation from one to another type of growth. This change is partly reflected in the fresh and dry weight curves shown in Figure 2. With IAA at concentrations above 1 μM up to 100 μM, the cells of the tobacco callus tissue were larger, more friable and loosely packed, and contained more water than those with IAA at lower concentrations (Fig. 4). The two types of growth were apparently different.

Dependence of IAA-Effect on Kinetin. The promotion of IAA oxidase isoenzymes A_5 and A_6 by low concentrations of IAA was dependent on the level of kinetin (Table II). The level of IAA oxidase isoenzymes A_5 and A_6 was higher with kinetin at 0.2 μM than at 1 μM. With kinetin at 5 μM, the development of these two IAA oxidase isoenzymes was completely repressed. This inhibition was not reversible by increasing concentration of IAA.

2,4-D and Related Compounds. To answer the question of whether IAA was the specific auxin required for the development of IAA oxidases A_5 and A_6, 2,4-D, 2,4,5-T, 2,4,6-T, and phenoxyacetic acid at various concentrations were tested. The results showed that at 10 μM, the optimal level for IAA to promote the development of the fast-migrating IAA oxidase isoenzymes, 2,4-D or 2,4,5-T used alone or in combination with IAA repressed the development of IAA oxidase isoenzymes A_5 and A_6 almost completely (Table III). At 1 μM, however, 2,4-D or 2,4,5-T increased the level of A_5 as compared with that in the absence of auxin, although the increase was less than that promoted by IAA at the optimal concentration of 10 μM. 2,4-D or 2,4,5-T at 1 μM also promoted growth of tobacco callus tissues (Table III). On the other hand, these compounds at higher concentrations increased the level of other IAA oxidases such as A_1 and A_3 but decreased the rate of growth. Structurally related compounds without growth-promoting activity, such as 2,4,6-T and phenoxyacetic acid, were without effect.

Thus the development of IAA oxidase isoenzyme A_5 was not specifically associated with IAA; other auxins at concentrations which stimulated growth also increased the level of this isoenzyme.

DISCUSSION

Clearly, IAA had a dual effect, depending upon its concentration, on the development of IAA oxidase isoenzymes in tobacco callus tissues. This tissue also responded to the increasing concentrations of IAA not only in the rate of growth but also in the type of growth. Whether these corresponding changes bear any causal relationship is an intriguing question for which no answer is available at present. Galston *et al.* (4) observed both promotion and repression of peroxidase isoenzymes by IAA in tobacco pith tissues cultured *in vitro* and suggested a possible causal relation of the induced changes in peroxidase and subsequent growth pattern. From the data presented in this paper, apparently the development of the two fast-migrating IAA oxidase isoenzymes was associated with a type of growth characterized by rapid growth rate, high water content, and friable tissues. Interestingly, this type of growth was similar to that promoted by GA_3, with which the levels of three fast-migrating IAA oxidase isoenzymes were significantly increased (10).

Promotion of the fast-migrating IAA oxidase isoenzymes was not unique for IAA. It was affected not only by cytokinin (9) and gibberellin (10) but also by other auxins (Table III). 2,4-D and 2,4,5-T at suitable concentrations increased the level of IAA oxidase isoenzyme A_5, although they were not as effective as IAA. Ritzert and Turin (14) reported promotion of two isoperoxidases in tobacco cells by IAA but not by 2,4-D, probably due to the high concentration (2 mg/l) of 2,4-D used.

In contrast to the positive association of IAA oxidase isoenzyme A_5 with rapid growth, a further increase in the level of other IAA oxidase isoenzymes, such as A_1 and A_3, with slow- and moderate-migrating rate appeared to be accompanied by growth retardation. This was observed in various experiments under different conditions, such as that in the presence of 10 μM or higher concentrations of 2,4-D and 2,4,5-T or a high concentration of IAA. However, these isoenzymes were usually present in the tissue under normal growth conditions, and hence it is difficult to interpret these observations in simple terms.

The IAA oxidase system in tobacco callus tissues apparently was composed of at least two groups of isoenzymes which were distinctly different not only in electrophoretic mobilities, but also in responses to different types and different concentrations of growth regulators and in association with growth. Although any causal relationship between IAA oxidase and plant growth is not known at present, the corresponding changes in the concentrations of IAA oxidase isoenzymes and in the rate and type of growth are consistent and suggestive of possible significance. Perhaps the two groups of IAA oxidase isoenzymes function in different capacities under different conditions and their relative levels, not the over-all concentration, regulate the pattern of growth.

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