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Bound Form Indole-3-acetic Acid Synthesis in Tumorous and Nontumorous Species of *Nicotiana*[1]

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ABSTRACT

The synthesis of H_2O-soluble and NaOH-hydrolyzable bound forms of indole-3-acetic acid (IAA) in petiole slices of *Nicotiana glauca*, *Nicotiana langsdorffii*, and their tumorous and nontumorous hybrids in the presence of exogenous ^{14}C-IAA was investigated. The synthesis of conjugates progressively increased during 6 hours of incubation in ^{14}C-IAA. The results showed that the rate of synthesis of IAA conjugates was higher in tumorous hybrids supplied exogenous IAA than in the parental species similarly supplied, and the rate of synthesis was higher in amphidiploid tumor plants than in a nontumorous mutant. It was also found that after 10 to 12 hours of incubation, 45% of the IAA taken up by F1 hybrids was in conjugated form whereas only 10 to 25% of the IAA taken up by a nontumorous mutant, *N. langsdorffii*, or *N. glauca* was conjugated. An F1 hybrid and an amphidiploid hybrid were found equally efficient in conjugating exogenously supplied IAA. It is postulated on the basis of these and other findings that IAA conjugates play an important role in tumorigenesis in *Nicotiana*.

The tumorigenesis of *Nicotiana glauca* × *Nicotiana langsdorffii* hybrids has been investigated for many years. The concept that abnormal phytohormone relationships might be a key factor in tumor formation was first brought up by Kehr (15). The results of subsequent studies appear to support this hypothesis (2 and the references cited therein). Tissue culture studies also showed that exogenous IAA and kinetin were not required for growth of cells from tumor-prone hybrids. Tumor formation occurred when endogenous levels of IAA were low (1, 2, 15); hence tumorigenesis cannot be attributed solely to the presence of excess auxin in tumor-prone hybrids. Bayer and Hagen (6, 7) suggested that the availability of free IAA rather than total IAA content was important for tumorigenesis.

The IAA content of plants is affected by IAA oxidases and peroxidases. In addition, conjugated forms of IAA have been thought to regulate the amount of free IAA in plants by storing some of it (5, 8). Bound forms of IAA include IAA-aspartic acid identified in pea (3, 12, 25), barley (20), and other plants (12, 21). IAA has also been reported bound with different carbohydrates such as with glucose found in pea (25), and with inositol, arabinose, and glucan polysaccharide detected in *Zea mays* (18, 22). The isolation and identification of IAA conjugates as well as enzyme studies on them have been reported extensively by Bandurski and co-workers (4, 16, 18). Recently the kinetics of conjugated IAA synthesis in pea was determined by Davies (11), and some new types of conjugated IAA were found in crown gall callus tissue (13).

The purpose of our study was to determine if there is synthesis of conjugated IAA in the presence of exogenously applied ^{14}C-IAA in tumorous and nontumorous species of tobacco and to determine if there is a correlation between the capacity to synthesize IAA conjugates and tumorigenesis.

MATERIALS AND METHODS

Plant materials used were petiole tissue slices from GG,[2] LL, GL, GGLL, and GGLL-NTM derived from X-ray-irradiated GGLL (14, 24). Four to eight leaves from the tops of the plants were harvested and soaked in 95% ethyl alcohol for 30 sec. All subsequent steps through incubation were done under sterile conditions. The petiole tissue was excised with a razor blade and sliced with a polyacrylamide gel slicer to 1-mm-thick pieces. The slices were incubated in basal medium containing 0.6 μM IAA and 1.8 μM ^{14}C-IAA (58 mCi/mmol, New England Nuclear) in 30 mM sucrose-0.02 M phosphate buffer (pH 6.2) at 25 C. After incubation, the slices were rinsed with distilled H_2O on a Büchner funnel under vacuum. One piece was put in 0.5 ml of 80% NCS tissue solubilizer (Amersham/Searle) for determination of total radioactivity in the tissue. Other pieces were subjected to Davies' method (11) for differential extraction of free and bound form IAA using some small modifications as follows. Each of the pieces was extracted successively for 24 hr at 20 to 25 C in the dark in 1 ml of 100% ethyl alcohol, 1 ml of H_2O, and 0.5 ml of 1 N NaOH; finally the pieces of tissue were placed in 0.5 ml of 80% NCS in glass scintillation vials for 3 hr at 60 C. Between each transfer, the slices were rinsed briefly with distilled H_2O.

A mixture of toluene (Eastman Kodak, scintillation grade)-Omnifluor (New England Nuclear)-Triton X-100 (Rohm and Haas) 3.75l:15g:1.88l) was used as scintillation fluid and the samples were counted in an Intertechnique SL-30 liquid scintillation counter. The external standard channel ratio method of quench correction was used and background counts were subtracted. The counting efficiencies were about 85 to 90%.

For cell counting, the HCl-chromic acid method (9) was used. The cells were counted with a counting slide (0.06 mm^2 × 0.2 mm deep). Xylem tissue was disregarded and only the intact cells were counted. All of the experiments were repeated at least 10 times. The morphology of different cells was recorded.

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[2] Abbreviations: GG: *Nicotiana glauca*; LL: *N. langsdorffii*; GL: F1 of GG × LL; GGLL: amphidiploid hybrid; GGLL-NTM: nontumorous mutant of GGLL.

RESULTS

Morphology of Cells. The number of cells in all five species of tobacco used in our experiments increased linearly with the fresh weight. The GGLL and GGLL-NTM cells were larger than GL, GG, LL cells and GG had the smallest cells of all of the species examined. The average weights of cells and numbers of cells/unit weight for the different species of tobacco are shown in Table I.

Ion Exchange Chromatography of IAA and IAA Conjugates. About 20 to 30 slices from GGLL were incubated in basal medium under constant light at 25 C for 24 hr. The IAA and bound form IAA were differentially extracted from slices by successive treatments with ethanol, water, and 1 N NaOH to give extracts A, B, and C, respectively. The ethyl alcohol extract (A) was evaporated to dryness in a stream of N_2 and the residue was dissolved in 3 ml of 0.1 M sodium phosphate-citric acid buffer (pH 3). The H_2O extract (B) was divided into two equal portions. One portion was adjusted to pH 3 to give extract B-1; the other portion was treated with an equal volume of 10 N NaOH and incubated for 4 hr in a 37 C water bath. The pH was then adjusted to 3 to give extract B-2. The 1 N NaOH extract (C) was adjusted to pH 3 without further treatment.

In order to distinguish between free and bound forms of IAA, each of the above extracts was applied to a SP-Sephadex C-25 column (1 × 10 cm) in 0.1 M sodium phosphate-citric acid buffer (pH 3). After samples were applied to the columns, 5 ml of the phosphate-citric acid buffer were added and the material was eluted with 60 ml of 0 to 0.5 M NaCl linear gradient. Radioactivities of the eluate fractions were determined and when such results for the ethyl alcohol and NaOH extracts were plotted (not shown here), only one major peak was observed and that was in the position coinciding with standard IAA. Identification of IAA in this peak was also made by gas chromatography by a detailed procedure described elsewhere (19). However, the H_2O extract yielded material characterized by one major peak and several minor ones including a negligible peak in the IAA region (Fig. 1). When an aliquot of the H_2O extract was treated with NaOH before fractionation on the SP-Sephadex column, only one major peak was observed but this time it coincided with IAA (Fig. 1). Therefore, the H_2O extract appeared to contain bound form IAA from which IAA was released by alkali. The chemical structure of these conjugates was not further identified.

On a cell basis a rapid uptake of IAA occurred in the first 30 min of incubation and the amount of free IAA found in the hybrid GL was about 2- to 3-fold higher than in the parental species (GG and LL) after 6 hr of incubation (Fig. 2). The concentration of water-soluble conjugates in GL was about four times higher than LL, and nine times higher than GG after 6 hr (Fig. 2B). The concentrations of NaOH-hydrolyzable conjugates were more than 10 times higher than those in GG and LL (Fig. 2C). Thus, the rate of synthesis of IAA conjugates in hybrid cells in the presence of exogenous IAA was substantially higher than in the cells of the parental species. This conclusion also holds if a fresh weight basis is used (Fig. 2, D, E, F).

Table I. *Number of Cells per Unit Fresh Weight and Average Fresh Weights of Cells in Petioles of Different Species of Tobacco*

Nicotiana Species*	cells/mg	µg/cell
GG	1383	0.72
LL	698	1.43
GL	639	1.56
GGLL	294	3.40
GGLL-NTM	289	3.46

*See Materials and Methods for key to abbreviations

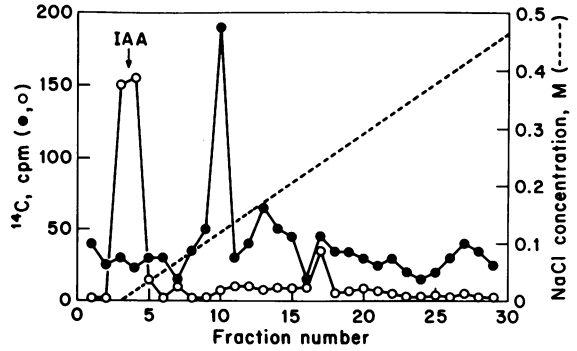

FIG. 1. SP-Sephadex ion exchange chromatography of water-soluble IAA conjugates from GGLL before and after alkaline hydrolysis. ●——●: Unhydrolyzed extract B-1 (see under "Materials and Methods"); ○——○: extract B-2 (extract B following alkaline hydrolysis). Samples were eluted with a 0 to 0.5 M linear NaCl gradient.

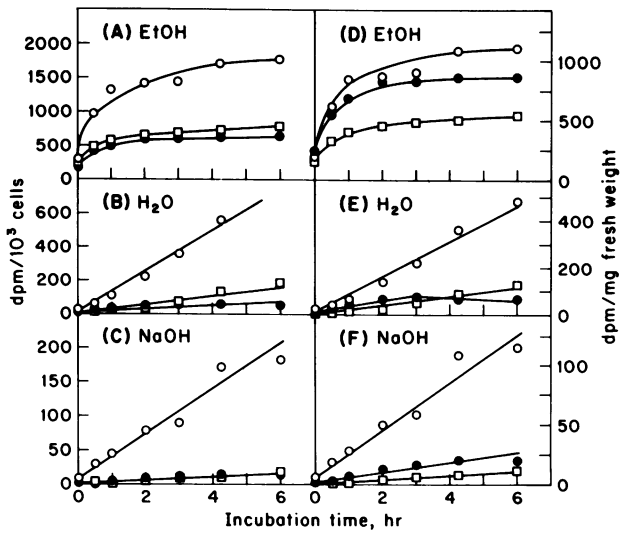

FIG. 2. A, D: Uptake of IAA with time by petiole slices from three species of *Nicotiana* as measured following extraction with ethyl alcohol. B, E: Concentration of water-soluble IAA conjugates in petiole slices from three species of *Nicotiana* after various times of incubation. C, F: Concentration of NaOH-hydrolyzable IAA conjugates in petiole slices of *Nicotiana* after various times of incubation. □——□, LL; ●——●, GG; ○——○, GL.

The efficiency of synthesis of IAA conjugates based on the amount of ^{14}C-IAA taken up by the slices of the various species of tobacco was also evaluated. The results (Fig. 3A) indicate that after 12 hr of incubation 45% of the IAA of the hybrid cells was in water-soluble and NaOH-hydrolyzable conjugates whereas the figures were only 25% and 10% for the parental LL and GG cells, respectively. The amounts of bound form IAA found in GL and GGLL were several times higher after a 6-hr incubation than nontumorous species. The differences cannot be attributed to some anomaly in the distribution of radioactivity derived from the applied ^{14}C-IAA since the residual counts solubilized by NCS after the various other extractions were less for parental species than for the hybrid (4% of the total IAA for GG and LL and 10% for GL).

When the efficiencies of synthesis of bound form IAA by tumorous tetraploid (GGLL) and the nontumorous mutant (GGLL-NTM) cells were compared, it was found that GGLL was more efficient than GGLL-NTM (Fig. 3B), but was about the same as for the diploid GL (Fig. 3A). This experiment was repeated several times with essentially the same results.

The rates of synthesis of bound form IAA in different species were also compared with the results summarized in Table II. It appears that H_2O-soluble conjugated IAA was synthesized at a greater rate in tumorous species than in nontumorous species (GG, LL, GGLL-NTM). NaOH-hydrolyzable IAA was also synthesized at a greater rate in tumorous species than in parental species (GG and LL), but apparently not at a greater rate than in the nontumorous mutant (GGLL-NTM).

Effects of Glucose, Inositol, and Aspartic Acid on Bound Form IAA Synthesis. Since IAA has been found to conjugate with myoinositol (5, 16, 18), glucose (16, 22, 32), and aspartic acid (3) in certain plants, we postulated that the addition of those compounds might increase bound form IAA synthesis. This was tested by incubation of tissue in basal medium containing 2.3×10^{-2} mM of either glucose, *myo*-inositol, or aspartic acid under light at 25 C. No enhancement of bound form IAA synthesis was detected in 2 hr. Since this negative result might have been caused by high endogenous levels of carbohydrates and amino acids present during the photosynthesis, another experiment was performed in which the tissue was preincubated in the dark for 4 to 6 hr in 30 mM sucrose and 0.02 M phosphate buffer (pH 6.2). After this preincubation, the tissue was transferred into basal medium without any additions (control) and into basal medium containing either glucose, *myo*-inositol, or aspartic acid and incubated under light for 2 hr. Under these conditions, both H_2O-soluble and NaOH-hydrolyzable conjugates were increased above the control about 20% in GG and LL, 100% in GL and GGLL, 60% in GGLL-NTM for all three substrates.

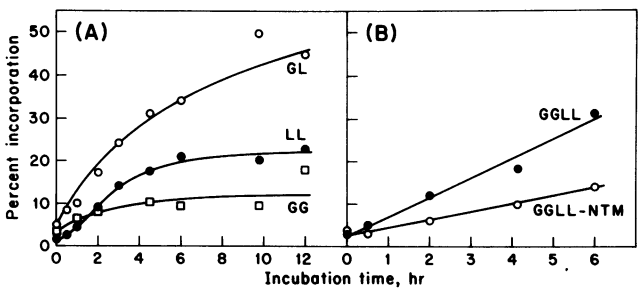

FIG. 3. Per cent of added ^{14}C-IAA appearing in conjugated form (summation of water-soluble and NaOH-hydrolyzable conjugates) in petiole slices from five species of *Nicotiana* after various times of incubation. A: □——□, GG; ●——●, LL; ○——○, GL. B: ●——●, GGLL; ○——○, GGLL-NTM..

Effect of Some Phenolic Compounds on Bound Form IAA Synthesis. Scopoletin, a phenolic compound, has been noted in tumorous tobacco plants (2). This substance has also been demonstrated to be a competitive inhibitor of IAA peroxidase (23). Consequently, it appeared of interest to investigate the effects of polyphenols on bound form IAA synthesis. The compounds tested at the 1 mM level were ferulic acid, caffeic acid, and scopoletin. They were tested on the GGLL and GGLL-NTM species of tobacco. All of the phenolic compounds tested enhanced bound form IAA synthesis (Table III).

DISCUSSION

The data of Table I enable us to relate IAA concentrations to numbers of cells and not just to fresh weight as is commonly done. Relating the amount of IAA to fresh weight provides a measure of the concentration of IAA in a tissue whereas expressing IAA content per cell unit provides an estimate of the absolute amount in a cell which in turn reflects the genetic capability of the cell. We have found both ways of expressing the results useful depending on the concept we wish to examine.

Our data indicate that there may be different IAA uptake systems in the cells of the hybrid species tested (GL, GGLL, and GGLL-NTM). When the average cell surface areas are calculated from the morphology experiments, the relative average cell surface areas are as follows: GG, 1.00; LL, 1.58; GL, 1.86; GGLL, 2.82; and GGLL-NTM, 2.84. As shown in Table IV, the uptake of IAA in hybrids on a per unit area basis were about equal and about the sum of GG and LL. Thus, it seems that the IAA uptake system of the hybrid species may represent the summation of different uptake mechanisms of the parental species rather than gene amplification. The significance of this finding still needs to be determined.

Our data demonstrate that petiole slices of the two tumorous tobacco species examined (GL and GGLL) synthesize bound form IAA at a higher rate (Fig. 2 and Table II) and to a greater extent (Fig. 3, A and B) than nontumorous species (GG, LL, and GGLL-NTM). Since the GGLL-NTM was obtained from X-ray-irradiated GGLL seeds, the genetic relationships between the two species should be very close. In addition, from the morphological point of view, GGLL and GGLL-NTM are very similar except for the presence in one case and absence in the

Table II. *Rate of Conjugation of Labeled IAA into H_2O-soluble and NaOH-hydrolyzable Bound Forms in Petiole Slices from 5 Species of Tobacco*

Nicotiana Species	H_2O-soluble		NaOH-hydrolyzable	
	dpm/1000 cells/min*	dpm/mg/min*	dpm/1000 cells/min	dpm/mg/min
GG	0.18	0.25	0.05	0.07
LL	0.43	0.30	0.05	0.03
GL	1.93	1.23	0.55	0.35
GGLL	2.10	0.62	0.88	0.26
GGLL-NTM	1.03	0.30	0.87	0.25

*The rate of conjugation was calculated from the data in Figure 3.

Table III. *The Effects of Phenolic Compounds on Bound-form IAA Synthesis--Percent of Control Amounts of IAA Found in H_2O and 1N NaOH Extracts of Tumorous (GGLL) and Nontumorous Mutant (GGLL-NTM) Amphidiploid Species*

	GGLL		GGLL-NTM	
	H_2O	NaOH	H_2O	NaOH
control	100%	100%	100%	100%
ferulic acid	186%	174%	148%	361%
caffeic acid	276%	325%	215%	273%
scopoletin	189%	924%	147%	231%

Table IV. *Uptake of Labeled IAA with Time by Cells of Different Species of Nicotiana on a Unit Area Basis*

Nicotiana Species	0 hr[†] cpm/unit area	1 hr cpm/unit area	3 hrs cpm/unit area
GG	165±49*	480± 55	600± 96
LL	117±13	354± 57	422± 84
GL	180±72	691± 76	913±102
GGLL	39± 4	940±158	872±194
GGLL-NTM	88±22	1092±104	951±114
GG+LL[+]	282	837	1022

[†]The experiment was done as follows: The petiole discs were dipped in ^{14}C-IAA containing incubation medium for the specified time interval, and then were rinsed with distilled water. The extraction procedures are described in Materials and Methods.

*Standard deviation

[+]GG+LL = summation of GG and LL

other of the ability to develop spontaneous tumors. So the absence of tumors in GGLL-NTM plants cannot be attributed in some way to a lack of "hybrid vigor" in these plants, as might be argued for GL *versus* GG and LL. It is tempting to speculate that a lower capacity to synthesize IAA conjugates is directly or indirectly related to the loss of tumorigenesis in GGLL-NTM plants.

The existence of a high amount of bound form IAA in the cells of tumor-prone hybrids (Fig. 2) after exposure to exogenous IAA may help to account for the ability of such cells to survive and grow on tissue culture media without added IAA whereas parental cells cannot. Cheng (10) has provided evidence that IAA acts to induce its own synthesis in IAA-deprived cells of hybrid (GL) plants but not in nontumorous parent cells. In the case of pith explants from the basal regions of hybrid plants, some exogenous IAA is required to initiate synthesis of IAA but this exogenous IAA is not required by pith transplants from upper regions of the hybrid plant. The inducing IAA in this case appears to be preexisting, free IAA or, we postulate, IAA arising by shift of an equilibrium between bound and free IAA. If correct, this hypothesis would predict that, in line with the generally observed auxin gradient in plants, there is a high concentration of bound form IAA in apical than in basal cells.

Bound form IAA may also be involved in tumorigenesis according to the following scheme. It has been noted that genetic tumors appear most often on mature *Nicotiana* hybrids apparently in response to a signal to cells in potential centers of growth (17). Such cells respond by becoming meristematic. The signal appears to be a reduction in the endogenous level of IAA (1, 2) and the response on the molecular level is the activation of an IAA synthetase system, such activation occurring readily in tumorous hybrid plants and essentially not at all in parental species (10). We hypothesize that the release of IAA from bound form IAA plays an important role in this activation mechanism. This hypothesis is described in detail elsewhere (19). However, further experiments are required to demonstrate to what extent IAA conjugates may serve as IAA reservoirs and to what extent they may act in a detoxifying mechanism or to demonstrate that they play some other role. In the meantime, the concept that conjugates are involved in tumorigenesis suggests a new direction for investigation of tumorigenesis in genetic hybrid plants.

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