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Regulation of Indole-3-Acetic Acid Biosynthetic Pathways in Carrot Cell Cultures[1]

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ABSTRACT

2,4-Dichlorophenoxyacetic acid (2,4-D) promotes the accumulation of tryptophan-derived indole-3-acetic acid (IAA) in carrot cell cultures during callus proliferation by a biosynthetic pathway that is apparently not active during somatic embryo formation. The effects of 2,4-D were examined by measuring the isotopic enrichment of IAA due to the incorporation of stable isotope-labeled precursors (deuterium oxide, [^{15}N]indole, and $^{2}H_5$-L-tryptophan). Enrichment of IAA from deuterium oxide is similar in both cultured hypocotyls and cell suspension cultures in the presence and absence of 2,4-D, despite the large differences in absolute IAA concentrations. The enrichment of IAA due to the incorporation of [^{15}N]indole is also similar in callus proliferating in the presence of 2,4-D and in embryos developing in the absence of 2,4-D. The incorporation of $^{2}H_5$-L-tryptophan into IAA, however, is at least 7-fold higher in carrot callus cultures proliferating in the presence of 2,4-D than in embryos developing in the absence of 2,4-D. Other experiments demonstrated that this differential incorporation of $^{2}H_5$-L-tryptophan into IAA does not result from differential tryptophan uptake or its subsequent compartmentation. Thus, it appears that differential pathways for IAA synthesis operate in callus cultures and in developing embryos, which may suggest that a relationship exists between the route of IAA biosynthesis and development.

The proliferation of carrot (*Daucus carota* L.) suspension cells and the formation of somatic embryos in these suspensions depends upon both endogenous and exogenous auxin. Despite an absolute requirement for auxin supplementation for sustained growth, cultured plant cells produce substantial amounts of the native auxin, IAA (12, 21, 22). This endogenous auxin seems to be essential for normal embryo formation during somatic embryogenesis as demonstrated by antiauxin and inhibitor studies (1, 12, 29). In carrot cells, exogenous 2,4-D, a synthetic auxin, actually stimulates the accumulation of large amounts of endogenous IAA, which seems to maintain callus proliferation and prevent the development of somatic embryos (19). The removal of 2,4-D from the medium and the subsequent decline in the native auxin levels permit the maturation of somatic embryos. However, only those cells that have had a specific minimum time of exposure to 2,4-D can become competent to form somatic embryos (1).

The ability of 2,4-D to induce the competence of suspension cells to form somatic embryos is, therefore, critical to the overall mechanism of somatic embryo development. This condition of competency appears to be closely associated with the severalfold increase in IAA levels associated with the presence of 2,4-D (19). To understand how 2,4-D promotes elevated endogenous IAA levels and is involved in the mechanism of somatic embryo formation, we wished to determine the nature of the changes in IAA metabolism in response to 2,4-D. The possible influences of 2,4-D were investigated here by feeding carrot cells with various labeled precursors that would mark different points of the proposed pathways for IAA biosynthesis (Fig. 1). The incorporation of these precursors into IAA was then measured, and comparisons were made between the incorporation observed in callus cultures proliferating in the presence of 2,4-D and embryo cultures developing in the absence of 2,4-D.

^{2}H oxide has been used as a nonspecific precursor for demonstrating de novo synthesis of the indole ring (4, 11, 20, 25). The incorporation of ^{2}H atoms into the indole ring of IAA was used to monitor the early stages of IAA biosynthesis to determine the influence of 2,4-D on indolic metabolism in hypocotyls and in suspension cultures. [^{15}N]Indole was used in a similar manner as an intermediate in both the IAA and tryptophan biosynthetic pathways to determine the effects of 2,4-D on the two proposed pathways for IAA biosynthesis. Finally, the influence of 2,4-D on the incorporation of $^{2}H_5$-L-tryptophan into IAA was found to change in conjunction with the formation of somatic embryos. Uptake and compartmentation of this possible precursor do not appear to contribute to the differences in metabolism associated with the presence of 2,4-D.

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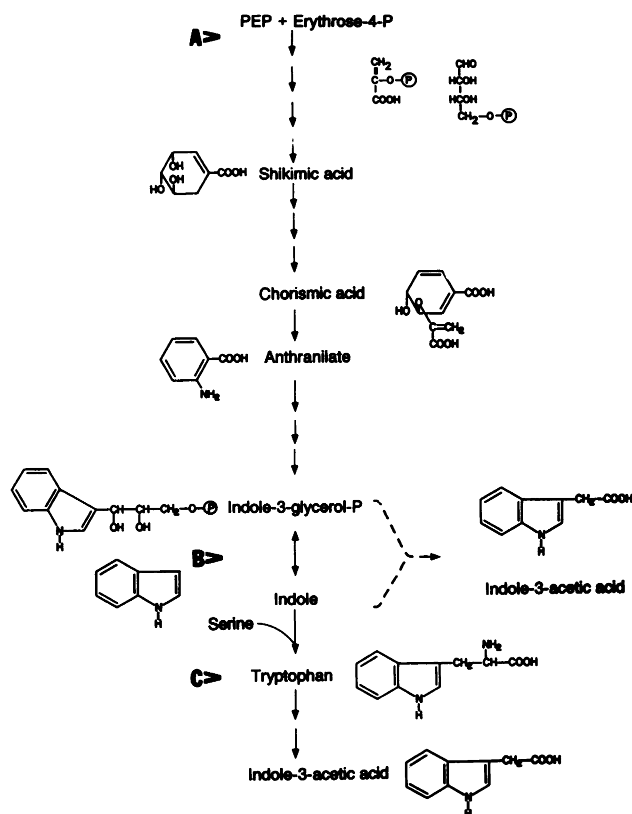

Figure 1. Schematic representation of the IAA biosynthetic pathway showing where the incorporation of ^2H_2O (A), [^{15}N]indole (B), and $^2H_5$-L-tryptophan (C) can occur resulting in labeled IAA. The proposed pathway branch point, which results in IAA synthesis without tryptophan as an intermediate, is also shown. PEP, Phosphoenolpyruvate.

MATERIALS AND METHODS

^2H Incorporation into IAA in Excised Carrot Hypocotyls Cultured on Medium Containing ^2H_2O

Carrot seeds (*Daucus carota* L., cv Danvers 126; Meyer Seed Company, Baltimore, MD) were germinated under sterile conditions as previously described (28). The excised hypocotyls were placed on Murashige and Skoog medium (24) with or without 2,4-D (1 mg/L) that had been supplemented with 3% sucrose and solidified with 1% agar. The media were prepared with either distilled water or 30% ^2H_2O (Cambridge Isotope Laboratories). After 2 weeks, hypocotyls cultured on medium with ^2H_2O were harvested, and the IAA was extracted with 70% isopropanol/0.2 M imidazole buffer, pH 7. After the organic phase was removed in vacuo, the extract was submitted to alkaline hydrolysis (7 N NaOH, 100°C, 3 h under nitrogen) to release the free IAA from conjugates and to remove ^2H from the acetic acid side chain and other exchangeable positions (9, 16). After hydrolysis, IAA was purified on a Fisher Prep-Sep C_{18} column, on a Fisher Prep-Sep amino column, and by HPLC on a Waters Nova-pak C_{18} reverse phase radial compression cartridge (5 × 100 mm) from which IAA was eluted with 15% acetonitrile/water containing 1% acetic acid (8). Purified IAA was methylated with ethereal diazomethane, and selected ion spectra were determined on a Hewlett-Packard 5890 GC/5971 MS equipped with a 15 m x 0.237 mm DB-1701 fused silica capillary column (J & W Scientific). Chromatographic parameters were as follows: injector temperature 250°C, initial oven temperature 140°C for 1 min followed by a ramp at 20°C/min to 280°C. The monitored ions were: m/z[3] 130, 131, 132, 133, 134, 135, 136 (quinolinium ion and m + 1, m + 2, m + 3, m + 4, m + 5, and m + 6) and 189, 190, 191, 192, 193, 194, and 195 (molecular ion and m + 1, m + 2, m + 3, m + 4, m + 5, and m + 6). In parallel cultures grown on media prepared with distilled water, the levels of IAA (free and conjugated) were determined by GC-MS-SIM using [$^{13}C_6$]IAA as the internal standard, as previously described (8, 9).

Incorporation of Label into IAA from Deuterated Water and Labeled Putative Precursors in Carrot Cell-Suspension Cultures

Suspension cultures obtained from a cell line with high embryogenic potential were fractionated through stainless steel sieves of different mesh sizes. The fraction collected between 43- and 109-μm filters was washed with 2,4-D-free medium and resuspended in Murashige and Skoog medium with or without 2,4-D (1 mg/L) at a density of 10^3 cell clusters/mL. The media were prepared with either 30% ^2H_2O, distilled water, distilled water supplemented with 2 mg/L of [ring 2,4,5,6,7-$^2H_5$]L-tryptophan (MSD Isotopes) and 10 μCi/L of L-[5-^3H]L-tryptophan as a tracer (27 Ci/mmol, Amersham), or distilled water supplemented with 2 mg/L of [^{15}N]indole (Cambridge Isotope Laboratories). After 2 weeks, the cultures were harvested by centrifugation (1000*g*) and ground in 4 mL/g of cell of 70% isopropanol/0.2 M imidazole buffer, pH 7. The IAA in the extracts from cultures grown on the medium containing deuterated water was purified, and ^2H incorporation was determined as described above.

Cultures containing labeled tryptophan or indole were analyzed for tryptophan and IAA as follows. Extracts were concentrated on a rotary evaporator under vacuum to approximately one-third of the initial volume and applied to an 8-mL bed volume Dowex 50W-X2, 200- to 400-mesh, H^+ form column. The column was washed with 3 bed volumes of distilled water and eluted with 20 mL of 2 N NH_4OH. The eluate was concentrated in vacuo and applied to a Baker amino SPE disposable column, equilibrated with 2 mL of each of methanol, distilled water, and 0.2 M imidazole buffer, pH 7. The column was washed with 10 mL of distilled water, and the IAA was eluted with 4 mL of 0.1 N HCl in methanol. Both the acidic methanol eluate and column washings were saved. The methanol eluate, which contained free and amide-bound IAA, was neutralized with 0.1 N NaOH, evaporated to near dryness, and hydrolyzed/proton exchanged in 7 N NaOH at 100°C under nitrogen for 3 h, and incorporation of

[3] Abbreviations: m/z, mass to charge ratio; SIM, selected ion monitoring.

label into IAA was determined as described above. The efflux not retained on the column, which contained tryptophan, was evaporated to dryness, derivatized with acetic anhydride to form the methyl ester of *N*-acetyl tryptophan, and purified by HPLC (18). GC-MS analysis was performed as described above except that the ramp rate was 30°C/min. Under these conditions, the retention time for the methyl ester of *N*-acetyl tryptophan was 6.08 min. The monitored ions were m/z 130, 131, 132, 133, 134, 135, and 136 (quinolinium ion and m + 1, m + 2, m + 3, m + 4, m + 5, m + 6) and m/z 261, 262, 263, 264, 265, 266 (molecular ion and m + 1, m + 2, m + 3, m + 4, m + 5, m + 6). The uptake of tryptophan was calculated on the basis of the loss of radioactivity of the [^{3}H]tryptophan tracer from the culture medium.

Measuring the Labeling Efficiency of the Plastid-Localized Tryptophan Pool

Carrot suspension cultures were first maintained in 2,4-D-free medium for at least 6 weeks, with weekly transfers, before the experiment to remove as much residual 2,4-D as possible. At this stage, cells growing in the absence of 2,4-D proliferated in a manner similar to the original cultures grown with 2,4-D, probably due to the high cell density used. Approximately 20 g of packed cells were washed in Murashige and Skoog medium and resuspended in 100 mL of Murashige and Skoog media supplemented with $^{2}H_5$-L-tryptophan (50 mg/L) either with or without 2,4-D. After 2 weeks of growth, the cells were removed by centrifugation (1000*g*) and resuspended in 50 mL of buffer (1 mM NaP_2O_7, 50 mM Hepes, 330 mM sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$, pH 6.8) at 4°C as has been used for amyloplast isolation (26). The cells were fractionated in a cold Waring blender equipped with razor blades using three 5-s pulses of low speed. Plastids were then purified by filtration through Miracloth and a 43-μm screen and differential centrifugation to separately pellet the cellular debris and the plastids (25*g* and 650*g*, respectively). The integrity of the plastids was monitored by visual observation through a phase contrast microscope.

RESULTS

IAA Biosynthetic Pathway

A generalized scheme of IAA biosynthesis given in Figure 1 illustrates the rationale for the labeling experiments. The points of incorporation of the labeled precursors are designated as A, B, and C, and the proposed branch point of the IAA biosynthetic pathway is shown (32).

Hypocotyl Culture on $^{2}H_2O$ Media

Carrot hypocotyls were cultured on solid media containing either 0 or 30% $^{2}H_2O$ in either the presence or the absence of 2,4-D. Undifferentiated callus proliferated over the entire hypocotyl cultured in the presence of 2,4-D during a 4-week period (Fig. 2B). Hypocotyls cultured in the absence of 2,4-D, in contrast, formed only small quantities of callus at the basal end in 2 weeks. This callus was then able to regenerate only roots in the subsequent 2 weeks (Fig. 2A). There was no evident inhibition of callus growth or root formation by the $^{2}H_2O$ in the media as noted by visual comparison with cultures not containing $^{2}H_2O$.

The concentrations of endogenous IAA in the cultured hypocotyls were separately determined using cultures grown without $^{2}H_2O$ because the incorporation of the ^{2}H atoms into the indole ring of the IAA could interfere with quantitative measurements using the [$^{13}C_6$]IAA internal standard. The total IAA concentration (free plus conjugated forms) in the hypocotyls cultured on 2,4-D-free medium (90 ng of total IAA/g fresh weight, Fig. 3C) was somewhat lower than that of the freshly excised hypocotyls (150 ng of total IAA/g fresh weight, Fig. 3A). Hypocotyls cultured in medium containing 2,4-D, however, were characterized by a dramatic increase in IAA concentration (650 ng of total IAA/g fresh weight, Fig. 3B). The predominant form of IAA for each of the treatments was the amide conjugate (Fig. 3, A–C). The incorporation of ^{2}H atoms into nonexchangeable positions of IAA in hypocotyls exposed to 30% $^{2}H_2O$ was measured as an indication of de novo synthesis by a shift in the natural isotopic abundance of the quinolinium ion (from m/z 130 to m/z $\leq$ 131). The same relative shift in isotopic abundance was seen for the m/z 189 of the molecular ion. The mass distribution of the quinolinium ion of naturally occurring IAA is m/z 130 (100%), 131 (10.24%), and 132 (0.4%) (31). Calculations involving enrichment of endogenous molecules by stable isotope labels account for this natural abundance by subtraction. The figures (4–7) show actual selected ion spectra and are not corrected for ion current due to natural abundance of heavy isotopes. The ^{2}H enrichment of IAA from hypocotyls cultured in the presence of 2,4-D (57%) was similar to the enrichment found in hypocotyls cultured in the absence of 2,4-D (59%; Fig. 4, A and B).

$^{2}H_2O$ Labeling in Embryogenic Suspension Cultures

The incorporation of $^{2}H_2O$ into IAA was also measured in suspension cells after 2 weeks of incubation in liquid media containing 30% $^{2}H_2O$. After the initial 2 weeks of culturing in medium without 2,4-D, most of the initial cell clusters had developed into somatic embryos (primarily "heart" and "torpedo" stages; 19, 29). Embryogenesis was associated with at least a 10-fold decrease in the level of IAA (30 ng/g fresh weight total, Fig. 3F) from that of the initial cell clusters (497 ng/g fresh weight total, Fig. 3D). The cell clusters cultured for 2 weeks on medium with 2,4-D proliferated with no signs of embryo formation and maintained a high IAA concentration (548 ng/g fresh weight total, Fig. 3E) comparable to levels in the initial cell clusters. As with hypocotyls, most of the IAA of the suspension-cultured cells was isolated as the amide-conjugated form (Fig. 3, D–F). The enrichment of IAA due to the incorporation of ^{2}H atoms from the medium into nonexchangeable positions was similar in both the embryogenic cultures devoid of 2,4-D (55%, Fig. 4D) and in the proliferating cultures with 2,4-D (53%, Fig. 4C).

[^{15}N]Indole Labeling of Tryptophan and IAA

[^{15}N]Indole was incorporated into tryptophan and IAA in both embryogenic cultures with no 2,4-D and in proliferating

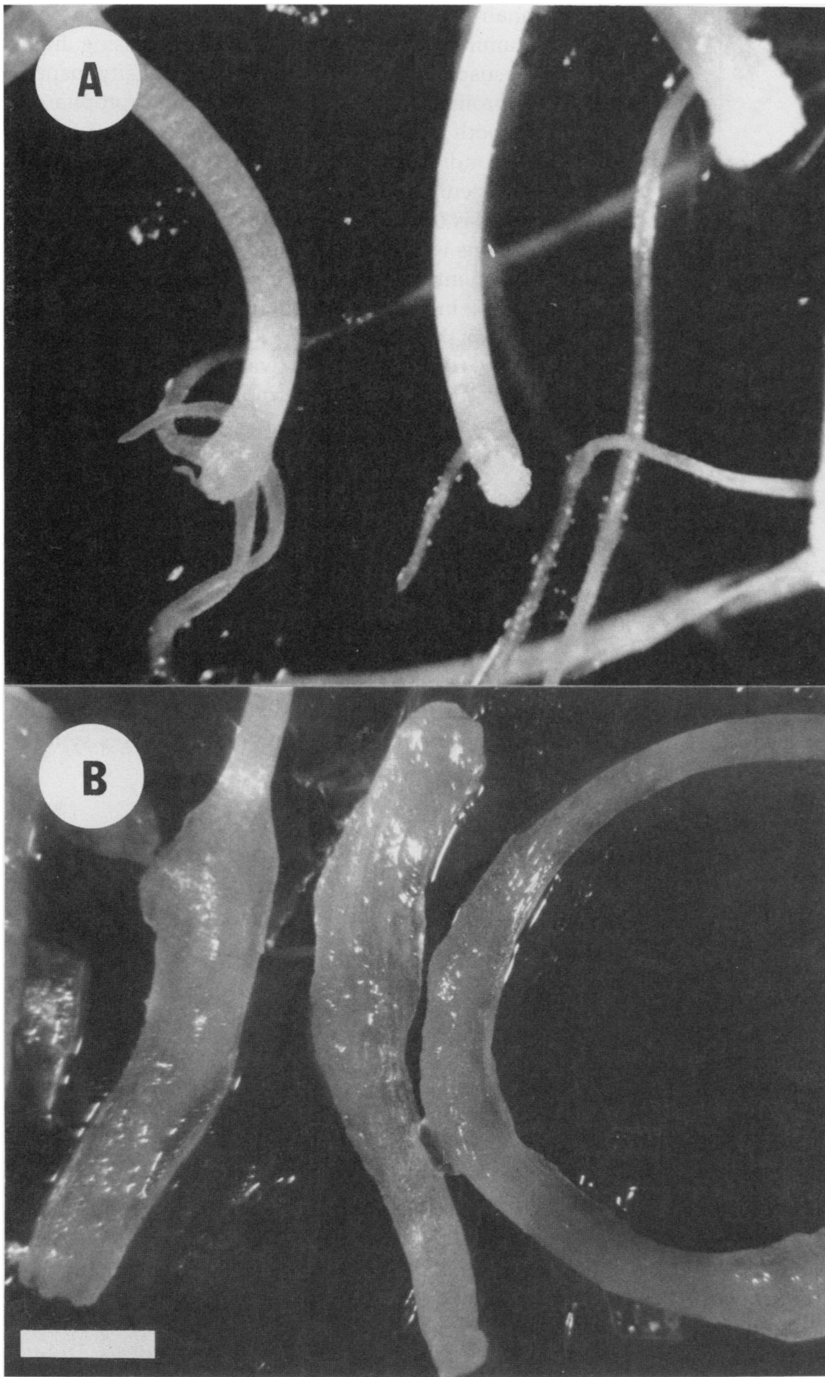

Figure 2. Photomicrograph of excised hypocotyls after 4 weeks of culturing on Murashige and Skoog medium (A) and on the same medium supplemented with 2,4-D (B). Bar, 2 mm.

callus cultures with 2,4-D. The presence of the indole in the media had no visible effects on the growth of the somatic embryos or the proliferation of the suspension cultures. The incorporation of [^{15}N]indole was measured as a shift in the quinolinium ion from the ion of m/z 130 to m/z 131 for both IAA and tryptophan. If the naturally occurring heavy isotopes are taken into account, 25% of the tryptophan molecules from proliferating cultures and 29% of the tryptophan from embryogenic cultures were labeled with ^{15}N derived from the exogenous indole (data not shown). The IAA was similarly labeled by [^{15}N]indole as shown in Figure 5, with an enrichment of 21% in cultures proliferating in the presence of 2,4-D and an enrichment of 25% in embryogenic cultures growing without 2,4-D.

$^{2}H_5$-L-Tryptophan Labeling of the Tryptophan Pool and IAA

$^{2}H_5$-L-tryptophan was used to label IAA produced in both embryogenic and proliferating cultures. The incorporation of

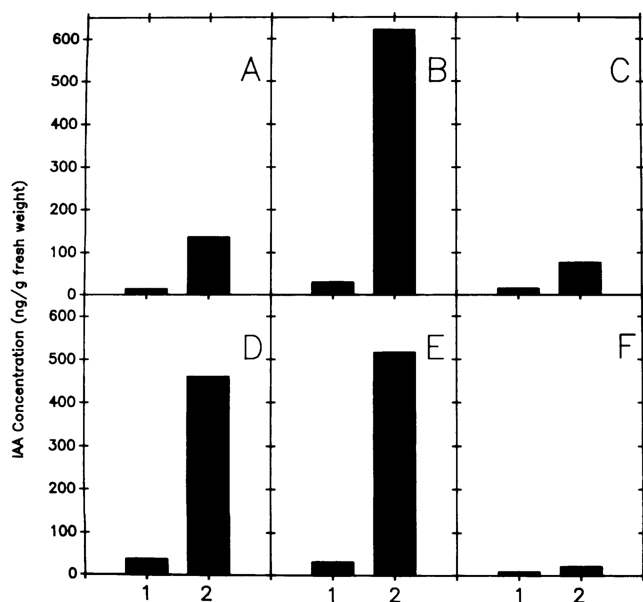

Figure 3. The concentrations of free (1) and amide-conjugated IAA (2) in isolated hypocotyls (A), isolated hypocotyls cultured on medium with 2,4-D (B), isolated hypocotyls cultured on medium without 2,4-D (C), isolated 39- to 109-μm cell clusters grown in medium with 2,4-D (D), callus cell cultures proliferating in medium with 2,4-D (E), and embryos growing in medium in the absence of 2,4-D (F). No ester-conjugated IAA was detectable in any of the samples. Each value is representative of three to five individual replications with variation that did not exceed 15%.

$^2H_5$-L-tryptophan into IAA was measured as a shift in the quinolinium ion of m/z 130 to m/z 135 and in the molecular ion of m/z 189 to m/z 194. In proliferating cultures grown in the presence of 2,4-D, approximately 35% of the IAA molecules were labeled by the $^2H_5$-L-tryptophan, but in the embryo cultures growing in the absence of 2,4-D only 4.7% of the IAA molecules were labeled (Fig. 6). The incorporation of ^2H_2O in this system shows that the difference in labeling is not due to the absence of IAA production.

To determine whether this differential incorporation was attributable to the uptake of labeled tryptophan from the media, both the uptake of tryptophan and its ability to label endogenous tryptophan pools were examined. During the 2 weeks of culturing, both cultures took up almost identical quantities of tryptophan from the media based on the uptake of [^{3}H]tryptophan tracer. The cells proliferating in the presence of 2,4-D removed 76% of the tryptophan from the medium (21.8 μg/g fresh weight) during the 2-week experimental period, and the embryos differentiating in the absence of 2,4-D removed 83% (21.3 μg/g fresh weight) of the tryptophan from the medium during the same time. The labeling of the bulk cellular tryptophan pool was measured as a shift from m/z 130 to m/z 135 of the quinolinium ion of the tryptophan extracted from the cells. The exogenous $^2H_5$-L-tryptophan represented nearly 51% of the tryptophan extracted from proliferating cells and 55% of the tryptophan extracted from embryos.

The ability of $^2H_5$-L-tryptophan to label the plastid-localized tryptophan pool as well as the bulk cellular tryptophan pool was examined in a separate experiment using high-density carrot suspension cultures. Such high-density suspension cultures proliferated as callus with $^2H_5$-L-tryptophan in the media in both the presence and the absence of 2,4-D. High-density conditions were used for this experiment to produce a sufficient amount of cellular material for the isolation and analysis of intact plastids. Even though the experimental conditions were different from those described in the previous experiment, the bulk cellular tryptophan pools were similarly labeled in cultures proliferating in the presence (72%; Fig. 7A) or the absence (67%; Fig. 7C) of 2,4-D. The plastid-localized tryptophan pool was labeled slightly less efficiently than the bulk cellular tryptophan pool but was also labeled similarly in the presence (53%, Fig. 7B) or the absence (55%, Fig. 7D) of 2,4-D.

DISCUSSION

Carrot hypocotyl tissue and suspension cells synthesize substantial amounts of IAA de novo in both the presence and absence of 2,4-D as shown by the incorporation of label from ^2H_2O in both excised hypotocyls and in suspension-cultured cells. Excised hypocotyls were used in addition to the suspension cultures because they represent cells that have had no prior exposure to 2,4-D. Even though IAA is synthe-

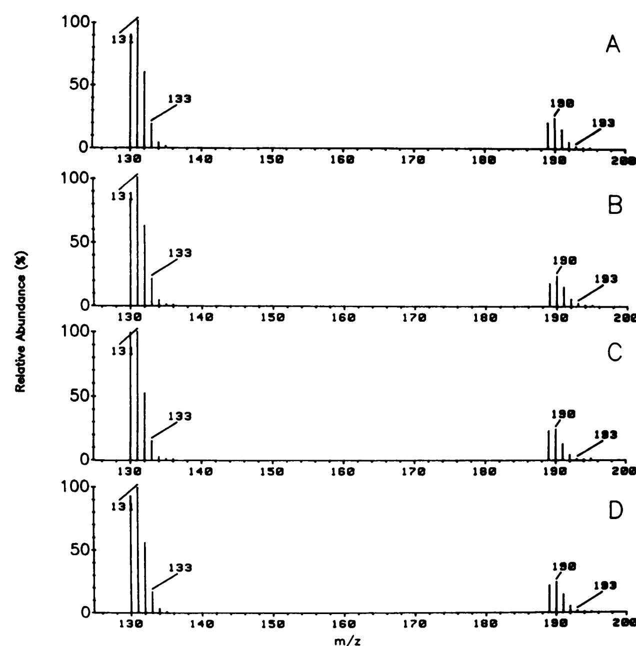

Figure 4. Selected ion spectra of IAA showing the relative isotopic enrichment from the incorporation of ^2H_2O from the media (30%) into either one, two, three, or four positions of the indole ring. Excised hypocotyls cultured in medium supplemented with 2,4-D (A) show a relative enrichment of 57%, and hypocotyls cultured in the absence of 2,4-D (B) show an enrichment of 59%. Callus cultures proliferating in the presence of 2,4-D (C) show a relative enrichment of 53%, and embryos growing in the absence of 2,4-D (D) show an enrichment of 55%. Each value is representative of three to five individual replications.

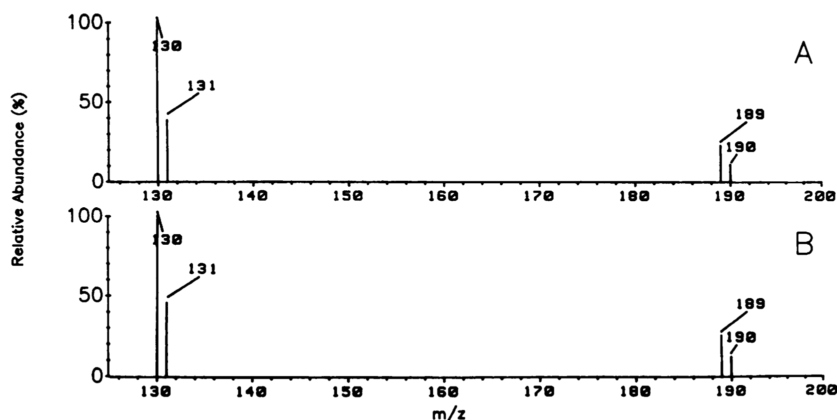

Figure 5. Selected ion spectra of IAA showing the relative isotopic enrichment from the incorporation of [^{15}N]indole from the media in callus cultures proliferating in the presence of 2,4-D (A) and in embryos growing in the absence of 2,4-D (B). After the naturally occurring isotopes were subtracted, the enrichment in A is 21% and in B is 25%. Each value is representative of three to five individual replications.

sized in the absence of 2,4-D, the presence of 2,4-D is necessary for the maintenance of callus proliferation. One hypothesis about the exogenous auxin dependency of tissue cultures is that the endogenous concentrations of auxin are too low to support growth (13). It has been demonstrated, however, that habituated cell lines, which proliferate callus in the absence of exogenous auxin, did not differ in auxin content from comparable lines that cease to grow in the absence of exogenous auxin (13). Higher auxin levels were consistently observed only in cell lines transformed by the Ti plasmid of *Agrobacterium*, which codes for enzymes of an IAA biosynthetic pathway distinct from routes postulated for higher plants (23).

In our experiments, both excised carrot hypocotyls and cell suspensions are able to synthesize IAA in the absence of 2,4-D (Fig. 4), but this endogenous IAA alone is unable to support unorganized callus proliferation under these conditions (19). On the other hand, the low levels of IAA produced by embryogenic cultures appear to be necessary to support organized growth resulting in carrot somatic embryogenesis (19). Therefore, it seems as though the high levels of exogenous 2,4-D change the growth pattern from the organized growth of hypocotyls before excision to callus proliferation, and low levels of endogenous IAA seem to be necessary for the differentiation of normal embryos. Thus, 2,4-D may be acting either directly as a strong auxin or indirectly by perturbing endogenous IAA metabolism, which then causes callus proliferation. This is supported by the fact that 2,4-D increases endogenous IAA accumulation (19), and 2,4-D has also been shown to increase the level of auxin-binding proteins in the membranes of carrot cells (15).

The apparent influence of 2,4-D on the growth pattern and IAA metabolism in carrot cells suggests that 2,4-D may be influencing the endogenous IAA biosynthetic pathway. The labeling experiments with ^2H_2O and [^{15}N]indole suggest that the steps of IAA synthesis before indole glycerol phosphate (see Fig. 1) are occurring in a similar manner in both 2,4-D-treated callus and in 2,4-D-free embryos. However, the extent to which tryptophan acted as a precursor to IAA depended on the presence or the absence of 2,4-D. When we consider the labeling efficiency of the tryptophan pool and the enrichment of IAA from labeled tryptophan, it is possible to calculate from our data that 9% of the IAA in embryogenic cultures growing in the absence of 2,4-D and 68% of the IAA in callus cultures proliferating in the presence of 2,4-D were derived from tryptophan. Because of uncertainties in any labeling experiment (i.e. multiple pools, compartmentation, change in rates of production), these numbers must be viewed as only relative indicators of actual biosynthesis.

Several different reasons could explain why this differential incorporation of tryptophan into IAA was correlated with the presence or absence of 2,4-D. The possibility that tryp-

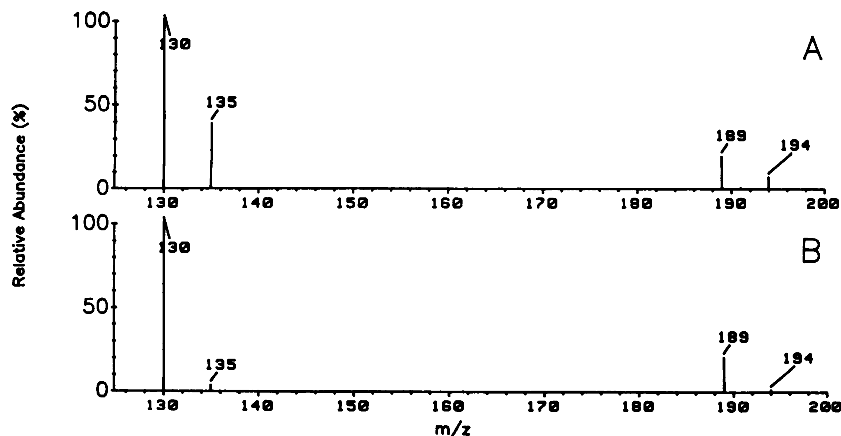

Figure 6. Selected ion spectra of IAA showing the relative isotopic enrichment from the incorporation of $^2H_5$-L-tryptophan from the media in callus cultures proliferating in the presence of 2,4-D (A) and in embryos growing in the absence of 2,4-D (B). The enrichment in A is 35% and in B is 4.7%. Each value is representative of three to five individual replications.

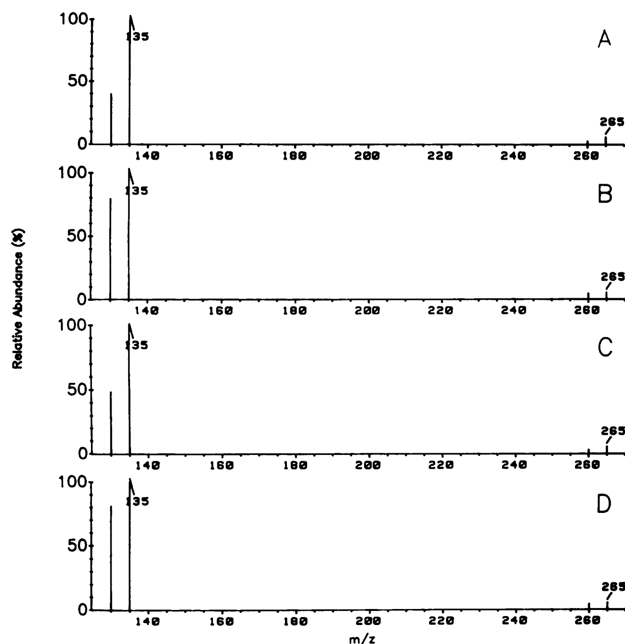

Figure 7. Selected ion spectra of tryptophan showing the efficiency of exogenous $^2H_5$-L-tryptophan to label the bulk cellular tryptophan pool (A) and the plastid-localized tryptophan pool (B) in the presence of 2,4-D and the bulk cellular tryptophan pool (C) and plastid-localized tryptophan pool (D) in the absence of 2,4-D. The enrichment of the cellular pools was 72% (A) and 67% (C), and the enrichment of the plastid-localized pools was 53% (B) and 55% (D). These are representative values of three replicate experiments showing similar results.

tophan uptake was affected by the presence of 2,4-D was eliminated because similar rates of radioactive tryptophan uptake were observed in both cultures. A second possibility was that despite the long-term feeding, which should allow equal saturation of different pools, 2,4-D changes the compartmentation of tryptophan in the cells, which could then lead to the differential incorporation into IAA.

It has been suggested that tryptophan (5, 14) and perhaps IAA (7, 17, 27) are both synthesized in the plastids. If IAA biosynthesis used only tryptophan that was newly synthesized in the plastid as its immediate precursor in carrot cells, then the precursor for IAA would be primarily unlabeled tryptophan. If so, the differential incorporation of tryptophan into IAA (i.e. high in callus, low in embryos) could have resulted from a decreased efficiency of $^2H_5$-L-tryptophan to label the plastid-localized pool in embryos. This possibility is unlikely, however, because the ability of $^2H_5$-L-tryptophan to label the plastid-localized tryptophan pool and the bulk cellular tryptophan pool was virtually unaffected by 2,4-D.

Although the experiments in which the labeling efficiency of the plastid by exogenous tryptophan was evaluated were performed using different tissue culture conditions from those used in typical and embryogenic experiments, it does illustrate that the plastid-localized tryptophan pool can be effectively labeled by exogenous $^2H_5$-L-tryptophan. Finally, the results of experiments in this paper are consistent with the hypothesis that 2,4-D promotes the incorporation of tryptophan into IAA in callus cells and that it is a nontryptophan pathway (32) that is active in normally developing somatic embryos.

Although the conversion of tryptophan into IAA has been demonstrated many times in bacteria (23) and plants (30), the role of tryptophan as the obligatory precursor of IAA has now been seriously questioned (3, 32). The enzymes thought to be involved in the conversion of tryptophan into IAA have not been shown to be specific, leaving open the possibility that such a conversion would not be a controlling factor in normal IAA biosynthesis (10). It might be expected that IAA biosynthesis would be controlled in part by the concentration of its precursor, but there seems to be no correlation between tryptophan concentration and IAA concentration (3, 13, 32). Early precursors in the indolic pathway, such as anthranilate and indole, show biological auxin activity, but tryptophan is inactive in such assays (2) except in some tissue-cultured cells that have had previous exposure to exogenous auxin (6). Moreover, the conversion of tryptophan into IAA was much lower than would be expected for a direct precursor as shown in studies with *Lemna* (3). Compelling experimental evidence against tryptophan as the direct precursor of IAA was recently obtained using the *orp* mutant of maize (32). This mutant, which results from recessive mutations in two unlinked trpB loci, was shown to contain low levels of tryptophan but 50-fold more de novo synthesized IAA than wild-type seedlings (32), thus demonstrating that tryptophan is not the obligatory precursor of IAA.

The present results concerning IAA metabolism in developing carrot embryos suggest that the nontryptophan pathway is acting as the primary mode of IAA biosynthesis during somatic embryogenesis, whereas tryptophan conversion predominates in undifferentiated suspension cultures. The functioning of more than one active IAA biosynthetic pathway under possible developmental control could have profound significance for the regulation of IAA activity and plant growth in general. It is worth noting that excised carrot hypocotyls are fully capable of synthesizing IAA but that this tissue can produce only roots without 2,4-D. Induction of the embryogenic potential requires treatment with exogenous auxin (1), and such a treatment seems to alter cellular IAA metabolism. One can speculate, as a working hypothesis, that 2,4-D does not induce embryogenesis directly by acting as a strong auxin but, rather, by altering the metabolism of endogenous IAA.

It should be noted, however, that the absence of 2,4-D cannot be uncoupled from the formation of somatic embryos under the conditions of the experiment. It is possible that the removal of 2,4-D may not be influencing the incorporation of tryptophan into IAA as much as it causes the differentiation of the carrot cells into embryos, which could, in turn, lead to a switch in the IAA biosynthetic pathway. Therefore, IAA metabolism may affect the developmental transition from callus to embryo, or the development of embryos may regulate the preferential pathway of IAA biosynthesis. Whatever the mechanism of action of 2,4-D, it is clear that the inducation and progression of embryogenesis in carrot cell suspension cultures is correlated with significant changes in

indolic metabolism involved in the formation of endogenous IAA.

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