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Arabidopsis thaliana auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid

(auxin/gas chromatography–selected ion monitoring–mass spectrometry)

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ABSTRACT **We used tryptophan auxotrophs of the dicot *Arabidopsis thaliana* (wall cress) to determine whether tryptophan has the capacity to serve as a precursor to the auxin, indole-3-acetic acid (IAA). Quantitative gas chromatography–selected ion monitoring–mass spectrometry (GC-SIM-MS) revealed that the *trp2-1* mutant, which is defective in the conversion of indole to tryptophan, accumulated amide- and ester-linked IAA at levels 38-fold and 19-fold, respectively, above those of the wild type. Tryptophan and free IAA were isolated from the *trp2-1* mutant grown in the presence of [^{15}N]anthranilate and [$^{2}H_5$]tryptophan, and the relative ^{15}N and $^{2}H_5$ enrichments of tryptophan and IAA were determined via GC-SIM-MS. The ^{15}N enrichment of tryptophan, 13% ± 4%, was less than the ^{15}N enrichment of the IAA pool, 39% ± 4%; therefore, IAA biosynthesis occurs via a tryptophan-independent pathway. The amount of $^{2}H_5$ incorporated by the plant into IAA from tryptophan (9% ± 4%) was low and only slightly above the level of spontaneous, nonenzymatic conversion of [$^{2}H_5$]tryptophan to [$^{2}H_5$]IAA. These results show that the dicot *Arabidopsis* is similar to the monocot *Zea mays* in that the major route of IAA biosynthesis does not occur through tryptophan.**

The phytohormone auxin plays a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation, and differentiation to tropic responses, fruit development, and senescence (1). The biosynthetic route to the synthesis of the most abundant plant auxin, indole-3-acetic acid (IAA), has been the subject of controversy for decades. The observation that radiolabeled tryptophan, when applied to watermelon slices, yielded radiolabeled IAA (2) was the first of numerous biochemical studies from which several pathways involving the conversion of tryptophan to IAA have been proposed (Fig. 1). In most of the *in vitro* studies where radiolabeled tryptophan was converted to IAA, at best only a few percent of the total radioactivity was recovered as IAA. This inefficient conversion of tryptophan to IAA could be accounted for by contaminating epiphytic bacteria (4, 5). Indeed, maize coleoptiles do not convert tryptophan to IAA when incubated in sterile conditions (6). More recently, Baldi *et al.* (7) showed that feeding tryptophan in high concentration to *Lemna gibba* (duckweed) increased the pool size of tryptophan but had negligible effects on IAA levels. Additionally, feeding *Lemna* [^{15}N]tryptophan to the extent that 99% of the tryptophan pool was ^{15}N-labeled did not result in a significant ^{15}N enrichment of IAA. Bandurski *et al.* (8) showed that maize seedlings grown in the presence of deuterium incorporate label into tryptophan but not IAA.

The isolation of tryptophan auxotrophs in both maize and *Arabidopsis* makes it possible to determine whether tryptophan is indeed the precursor to IAA. The orange pericarp (*orp*) mutant of maize does not make tryptophan due to a defect in tryptophan synthase β activity (9), yet it has 50-fold higher levels of IAA (10). We have used tryptophan auxotrophs of the dicot *Arabidopsis thaliana* (11–13) to determine whether tryptophan is the sole precursor to IAA. In this study we show that there is a tryptophan-independent IAA biosynthetic pathway. Our data indicate that the precursor is likely to be a compound earlier in the tryptophan biosynthetic pathway, between anthranilate and indole.

MATERIALS AND METHODS

A. thaliana ecotype Columbia carrying mutations *trp1-1*, *trp2-1*, and *trp3-1* have been described (11–13). Seeds were surface sterilized (11) and sown onto plant nutrient medium with 0.5% sucrose (PNS; ref. 14) solidified with 0.75% bacteriological grade agar (ICN) with or without added L-tryptophan to a final concentration of 50 μM. Plates were wrapped with Parafilm and incubated at 22°C under continuous illumination for 13 days. High light is defined here as 100–160 $\mu E \cdot m^{-2} \cdot s^{-1}$ [1 einstein (E) = 1 mol of photons]. Low light is defined here as 20–30 $\mu E \cdot m^{-2} \cdot s^{-1}$. For stable isotope labeling of *trp2-1*, [^{15}N]anthranilate (ICON Isotopes, Summit, NJ; 99 atom %) was added to PNS/0.75% agar, to a final concentration of 80 μM, and [$^{2}H_5$]tryptophan (L-tryptophan, indole $^{2}H_5$; Cambridge Isotope Laboratories, Cambridge, MA; 98 atom %) was added to a final concentration of 50 μM. After 13 days the seedlings were weighed, frozen in liquid nitrogen, and stored at −80°C.

Isolation of IAA, Indole-3-acetonitrile (IAN), Indole, and Tryptophan. Free IAA, ester-linked IAA, and amide-linked IAA were isolated as described (15) with the following modifications: 500 ng of [$^{13}C_6$]IAA (Cambridge Isotope Laboratories; 99 atom %) and 500 ng of [^{13}C]IAN (labeled in the side chain α carbon) per 200 mg of tissue were added as internal standards, and ≈100,000 cpm of [^{3}H]IAA (25.4 Ci/mmol; 1 Ci = 37 GBq; Amersham) was added as a radiotracer. [^{13}C]IAN was synthesized by Nebojsa Ilic (University of Maryland) using the method of Stowe (16). After the isopropanol was removed, one-third of the sample was set aside for isolation of IAN (see below). The amino column step was omitted from the protocol (15) for the isolation of ester-linked IAA. The HPLC column was a Phenomenex (Belmont, CA) Ultracarb 5 ODS 30 (50 × 4.6 mm). The mobile phase was 25% methanol/1% acetic acid.

The fraction that had been set aside from the IAA isolation was adjusted to 2 ml with water and passed over an amino column [solid-phase extraction (SPE), 500 mg; J & W Sci-

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Abbreviations: IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile; GC-SIM-MS, gas chromatography–selected ion monitoring–mass spectrometry; IGP, indole-3-glycerol phosphate.

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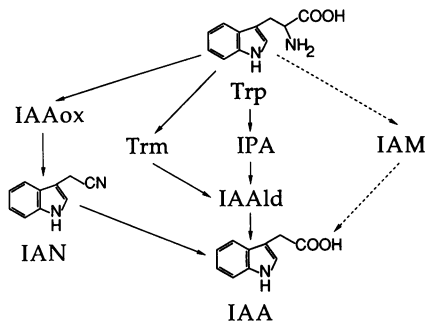

FIG. 1. Proposed routes of IAA biosynthesis from tryptophan. The pathway utilized by microbes is indicated by dashed arrows (reviewed in ref. 3). Trp, tryptophan; IPA, indole-3-pyruvic acid; IAAld, indole-3-acetaldehyde; Trm, tryptamine; IAAox, indole-3-acetaldoxime; IAN, indole-3-acetonitrile; IAM, indole-3-acetamide.

entific, Rancho Cordova, CA] as described (15). The flowthrough was collected and applied to a C18 column (Prep-Sep SPE, 300 mg; Fisher) conditioned with methanol and water. IAN was eluted with 2.5 ml of acetonitrile, evaporated to dryness, resuspended in 50% methanol, and injected onto the HPLC column described above. IAN was eluted with 25% methanol/1% acetic acid. The fractions collected for gas chromatography–selected ion monitoring–mass spectrometry (GC-SIM-MS) analysis were determined by first establishing the elution time of an IAN standard and collecting fractions with the same retention time. Immediately prior to GC-SIM-MS analysis, samples were evaporated to dryness with a stream of nitrogen gas, resuspended in 30 μl of bis(trimethylsilyl)trifluoroacetamide plus 1% chlorotrimethylsilane (Supelco), and incubated at 45°C for 30 min.

In the isolation of indole, 500 ng of [$^2H_6$]indole (Cambridge Isotope Laboratories; 78 atom %) per 200 mg of tissue was added as an internal standard. The procedure was the same as for the isolation of free IAA described previously (15) until the amino column step. For indole isolation, the diluted sample was passed over a conditioned amino column, and the flowthrough was applied under vacuum to a Prep-Sep C_{18} column (SPE, 300 mg; Fisher) conditioned with methanol and water. Indole was eluted with 6 ml of pentane. Pentane was removed by rotary evaporation, and the indole was resuspended in ethyl acetate.

For the stable isotope labeling experiment, 500 ng of [^{13}C]IAN and 500 ng [$^{13}C_6$]IAA were added per 200 mg of harvested tissue as internal standards. The sample was divided equally for the isolation of free IAA and IAN (as described above) and tryptophan.

For tryptophan isolation, ≈100,000 cpm of [^{3}H]tryptophan (27 Ci/mmol; Amersham) was added as a radiotracer. The sample was brought to 2 ml with water and applied to a Dowex 50X2-100 (Sigma) column, and the tryptophan was eluted as described (17). The sample was evaporated to dryness by rotary evaporation and resuspended in 0.2 ml of 1 M sodium phosphate (pH 8.3 at 37°C), 0.1 ml of pyridoxal phosphate (Sigma; 1 mg/5 ml), and 1.2 ml of water. Two-tenths milliliter of tryptophanase (E.C. 4.1.99.1) (4 mg/ml; Sigma) in 1 M sodium phosphate (pH 8.3 at 37°C) was added, and the sample was incubated at 37°C for 1.5–2 hr. Free indole, enzymatically released from the isolated tryptophan, was extracted with three volumes of pentane; the pentane fraction was evaporated to dryness and resuspended in ethyl acetate.

GC-SIM-MS. GC-SIM-MS analysis was carried out as described (18). In experiments where IAA was quantified, molecular ions with m/z 189, 190, and 195 were monitored. To quantitate IAN, the molecular ions with m/z 228 and 229 were monitored, and for the quantitation of indole, the molecular ions with m/z 117 and 122 were monitored. Corrections for the natural abundance of ^{13}C and ^{15}N were calculated with theoretical values (19). In the double-labeling experiment with stable isotopes, the relative abundance of unlabeled, ^{15}N-labeled, and $^2H_5$-labeled free IAA, IAN, and indole derived from tryptophan via tryptophanase was determined as follows. For IAA the molecular and quinolinium ions with m/z 130, 131, 135, 136, 189, 190, 194, and 195 were monitored. For IAN the ions monitored had a m/z of 129, 130, 134, 228, 229, and 233. For indole derived from tryptophan via tryptophanase the molecular ions at m/z 117, 118, and 122 were monitored. Corrections were made for the natural abundance of the heavy isotopes ^{13}C and ^{15}N as well as for the actual isotopic enrichments of [$^2H_5$]tryptophan, [$^{13}C_6$]IAA, and [^{13}C]IAN using experimentally determined values. For a discussion of these calculations see ref. 18.

Mass Fragmentation Analysis. The conditions used to isolate amide-linked IAA and ester-linked IAA result in the conversion (100% and 6%, respectively) of IAN to IAA (B. Bartel and G.R.F., unpublished results). [^{13}C]IAN added to the sample upon extraction was used to distinguish between endogenous IAA and that derived from IAN. IAN was isolated from one-half of the sample under conditions of neutral pH, which did not result in the interconversion of IAN to IAA. The ratio of [^{13}C]IAN to unlabeled IAN was determined via GC-SIM-MS as described above, and the abundance at m/z 228 (unlabeled IAN) was divided by the abundance at m/z 229 (labeled IAN). This value was termed the IAN correction factor. The remainder of the sample was used to isolate amide-linked IAA or ester-linked IAA and was made up of [^{13}C]IAA (from [^{13}C]IAN) and unlabeled IAA (which was a mixture of IAA derived from unlabeled IAN as well as endogenous IAA). The ^{13}C-labeled IAA was represented by the ions with m/z of 190 and 130. The unlabeled IAA from both sources was represented by ions with m/z of 189 and 130. The values at m/z 189, 190, and 195 were corrected for natural abundance of heavy isotopes; then the value at m/z 189 representing endogenous IAA was determined by subtracting the amount of m/z 189 that was due to IAN breakdown into IAA (the IAN correction factor multiplied by the abundance at m/z 190).

RESULTS

***Arabidopsis* Tryptophan Auxotrophs Accumulate IAA.** The *Arabidopsis* tryptophan biosynthetic pathway (Fig. 2) is likely to be the same as that described for microbial systems

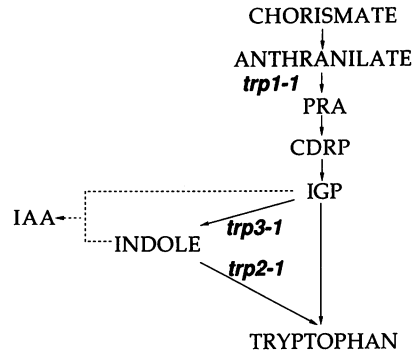

FIG. 2. Tryptophan biosynthetic pathway. *A. thaliana* mutants defective in enzyme activity for anthranilate phosphoribosyltransferase (*trp1-1*), tryptophan synthase α (*trp3-1*), and tryptophan synthase β (*trp2-1*) are indicated (11, 12). A tryptophan-independent biosynthetic route to IAA from either indole or IGP is indicated by dashed lines. PRA, *N*-phosphoribosylanthranilate; CDRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose phosphate; IGP, indole-3-glycerol phosphate.

based upon the Trp$^-$ mutants, cloned *TRP* genes, and enzyme activities identified in this plant [reviewed by Last (13)]. The *trp1-1* mutant is defective in anthranilate phosphoribosyltransferase activity (11). The *trp2-1* mutant is deficient in tryptophan synthase β activity (15% of wild-type levels) (12), and the *trp3-1* mutant has been deduced to be deficient in tryptophan synthase α activity (11). These mutants are conditional auxotrophs—i.e., in the nonpermissive condition of high light (see *Materials and Methods*), they require tryptophan for growth, whereas in the permissive condition of low light, they are viable without added tryptophan.

IAA exists in three general forms in plants, either unconjugated (free IAA), conjugated via ester linkages to sugars or *myo*-inositol (ester-linked IAA), or conjugated via amide linkages to amino acids or peptides (amide-linked IAA) (20). To determine the effect of these mutations on IAA levels, wild-type, *trp2-1*, and *trp3-1* seedlings were grown in sterile nutrient agar in permissive and nonpermissive conditions, with and without added tryptophan, and the amounts of free IAA, ester-linked IAA, and amide-linked IAA were quantified using isotope dilution GC-SIM-MS analysis. The levels of free IAA did not differ significantly among wild type, *trp2-1*, and *trp3-1* (Table 1). However, there was a dramatic elevation of IAA level in the *trp2-1* mutants grown in high light without added tryptophan—levels of amide-linked and ester-linked IAA were 38-fold and 19-fold higher, respectively, as compared with wild type. Under the same conditions, the levels of amide-linked IAA and ester-linked IAA in the *trp3-1* mutant were 11-fold and 6-fold higher, respectively, than wild type. In a separate experiment, the levels of free IAA and amide-linked IAA were determined for the *trp1-1* mutant and were not distinguishable from wild-type levels (data not shown). Quantitation of indole in the *trp2-1* and *trp3-1* mutants showed a dramatic increase in indole levels (31-fold) over wild type in *trp2-1* plants grown in high light without added tryptophan (Table 1). No change in indole level was observed in the *trp3-1* mutant. These results are intriguing in light of the controversy over whether tryptophan is the primary precursor to IAA in plants. *trp2-1* is deficient in the enzyme activity that converts indole to tryptophan and not surprisingly leads to an accumulation of indole. The accumulation of IAA in the *trp2-1* and *trp3-1* mutants suggests that there may be another route to IAA biosynthesis from indole or indole-3-glycerol phosphate (IGP) that bypasses tryptophan (Fig. 2).

Stable Isotope Labeling of the *trp2-1* Mutant. To determine whether tryptophan or an earlier intermediate in the pathway is the precursor to IAA, we grew *trp2-1* seedlings in the presence of [$^2H_5$]tryptophan and [^{15}N]anthranilate. Once incorporated into a ring structure, the stable isotopes were nonexchangeable (18, 21), so any deuterium incorporated into the indole ring of IAA was the result of synthesis from [$^2H_5$]tryptophan and any ^{15}N incorporation into either tryptophan or IAA was due to *de novo* synthesis from [^{15}N]anthranilate. Furthermore, the isotopic enrichment of any given precursor should be reflected in the product. If tryptophan were the major precursor to IAA, then the percent incorporation of both heavy isotopes into tryptophan must be greater than or equal to their percent incorporation into IAA. However, if the percent isotopic incorporation into IAA is greater than that of tryptophan, the IAA must be derived from another precursor, in order to account for the higher level of isotope enrichment in IAA.

Table 2. Isotopic enrichment of free IAA, tryptophan, and IAN in plants labeled with [^{15}N]anthranilate and [$^2H_5$]tryptophan

Parameter	Free IAA	Tryptophan	IAN
	Wild type		
% unlabeled	23 ± 1	9*	22†
% ^{15}N labeled	68 ± 1	54*	61†
% $^2H_5$ labeled	9 ± 2	37*	18†
	trp2-1		
% unlabeled	53 ± 6	12 ± 3	25 ± 3
% ^{15}N labeled	39 ± 4	13 ± 4	31 ± 3
% $^2H_5$ labeled	9 ± 5	76 ± 7	45 ± 3

Samples were prepared in triplicate unless indicated otherwise.
*Duplicate samples.
†Single sample.

The pattern of ^{15}N incorporation into tryptophan and IAA revealed that there is a pathway to IAA that does not utilize tryptophan (Table 2). Incorporation of ^{15}N into tryptophan (13% ± 4%) was not sufficient to account for the percent incorporation of ^{15}N into IAA (39% ± 4%). The extent of $^2H_5$ incorporation into IAA (9% ± 5%) indicated that a small amount of tryptophan (76% ± 7% $^2H_5$ labeled) was converted into IAA *in vivo*. These data do not preclude a tryptophan to IAA pathway; however, the amount of $^2H_5$ incorporation into IAA from tryptophan was very low for both mutant and wild type. Moreover, we have observed significant nonenzymatic conversion of [$^2H_5$]tryptophan to [$^2H_5$]IAA. In a control experiment we found that [$^2H_5$]tryptophan, methylated and analyzed via GC-SIM-MS, was made up of 3–8% [$^2H_5$]IAA. Since we added 50 μM [$^2H_5$]tryptophan in our labeling experiment, there was a considerable amount (700 nM) of [$^2H_5$]IAA present in the medium, which, if taken up by the plant would appear in the final pool measurement. Although we cannot rule out tryptophan as a precursor, the difference between the "enzymatic" and nonenzymatic conversion of tryptophan was so small that its significance could be questioned.

Is IAN an Intermediate in the IAA Biosynthetic Pathway? Several members of the family Brassicacae (to which *Arabidopsis* belongs) encode enzymes that convert tryptophan to

Table 1. Isotope dilution GC-SIM-MS quantitation of IAA, indole, and IAN in tryptophan biosynthetic mutants

Genotype	Free IAA	Ester-linked IAA	Total IAA	Amide-linked IAA	Indole	IAN
			Low light minus tryptophan			
Wild type	0.025 ± 0.012	ND	0.06*		0.04 ± 0.02	0.75 ± 0.03
trp2-1	0.020 ± 0.002	ND	2.11 ± 0.08*		0.07 ± 0.01	0.98 ± 0.25
trp3-1	0.020 ± 0.01	ND	0.43 ± 0.07*		0.04 ± 0.01	0.47 ± 0.12
			High light minus tryptophan			
Wild type	0.021 ± 0.007	0.06 ± 0.08†		0.58 ± 0.12	0.007 ± 0.003	0.34 ± 0.10
trp2-1	0.016 ± 0.009	0.82 ± 0.14†		22.29 ± 5.33	0.217 ± 0.027	3.77 ± 0.52
trp3-1	0.017 ± 0.002	0.84 ± 0.05†		11.08 ± 1.03	0.009 ± 0.003	2.00 ± 0.21
			High light plus tryptophan			
Wild type	ND	0.05 ± 0.04†		0.86 ± 0.09	0.007 ± 0.003	0.37 ± 0.04
trp2-1	ND	0.23 ± 0.05†		3.48 ± 0.95	0.241 ± 0.020	0.75 ± 0.25

Values given are μg/g of fresh weight. ND, Not determined.
*Includes free IAA, ester-linked IAA, and amide-linked IAA.
†Also contains free IAA.

IAA via IAN (refs. 3 and 22 and references therein). Since IAN is thought to be derived from tryptophan (Fig. 1), we expected that IAN levels in the Trp^- mutants would be lower than those in wild type. We found instead, an 11-fold accumulation of IAN in the *trp2-1* mutant and a 6-fold accumulation in the *trp3-1* mutant (Table 2). This result suggests that IAN might give rise to IAA in a tryptophan-independent pathway. If so, then in the double-labeling experiment described above, the ^{15}N incorporation into IAN should be greater than or equal to the ^{15}N incorporation into IAA. Table 2 shows that IAN is 31% ± 3% ^{15}N-labeled, which is slightly less than the 39% ± 4% ^{15}N incorporation into IAA. Although the percent ^{15}N enrichment of IAN is less than expected for a precursor to IAA, the percent isotopic enrichments are so similar that it is difficult to rule out IAN as an intermediate in IAA biosynthesis (see *Discussion*). The $^2H_5$ incorporation into IAN (45% ± 3%) is less than the $^2H_5$ incorporation into tryptophan (76% ± 7%) but more than the 9% ± 5% $^2H_5$ incorporation observed for IAA. If any of the $^2H_5$-labeled IAA that we observe arose from synthesis as opposed to mere degradation of [$^2H_5$]tryptophan, our data are consistent with IAN being an intermediate in a tryptophan → IAA pathway.

DISCUSSION

We have used tryptophan auxotrophs of *A. thaliana* to determine whether tryptophan is the primary precursor to IAA in plants. Both the *trp2-1* and *trp3-1* mutants, defective in tryptophan biosynthesis, have elevated levels of amide-linked IAA and ester-linked IAA under conditions where the plants require tryptophan for growth. This observation has formed the basis for our hypothesis that there is an IAA biosynthetic pathway that does not utilize tryptophan. Our results are similar to those of Wright *et al.* (10), who found that the maize *orp* mutant, defective in tryptophan synthase β activity, accumulates IAA to a level 50-fold above wild type. The double-labeling experiment with stable isotopes further validates this hypothesis. In both wild-type and mutant, the incorporation of ^{15}N from anthranilate into tryptophan is not sufficient to account for the incorporation of ^{15}N into IAA, which argues against tryptophan being the precursor to IAA. The *trp3-1* mutant, defective in the conversion of IGP to indole, also accumulates IAA, which is consistent with the notion that IGP is the precursor to IAA. If IGP were the precursor, then a *trp2-1/trp2-1 trp3-1/trp3-1* double mutant should incorporate less $^2H_7$ from exogenous [$^2H_7$]indole than would a *trp1-1/trp1-1 trp2-1/trp2-1* double mutant. This experiment could not be carried out since the *trp2-1/trp2-1 trp3-1/trp3-1* double mutant could not be constructed. Analysis of F_2 and F_3 progeny from a cross of the *trp2-1* and *trp3-1* mutants indicates that the double mutant is lethal (data not shown).

When plants are labeled with [$^2H_5$]tryptophan, we do detect some conversion of [$^2H_5$]tryptophan to [$^2H_5$]IAA; however, the extent of conversion is only slightly higher than the nonenzymatic conversion that we observed. If there is a route to IAA from tryptophan, it is likely to be the indoleacetaldoxime → IAN pathway shown in Fig. 1 (3). The amount of $^2H_5$ incorporation into IAN from tryptophan that we observe is sufficient to account for the $^2H_5$ enrichment of IAA and so is consistent with IAN being an intermediate in a tryptophan → IAA pathway. However, our observation that IAN accumulates in the *trp2-1* and *trp3-1* mutants is unexpected in light of the hypothesis that tryptophan is the precursor to IAN and suggests that IAN could (*i*) be derived from indole or IGP, and (*ii*) be an intermediate in the tryptophan-independent IAA biosynthetic pathway. The ^{15}N enrichment of IAN (31% ± 3%) is less than would be expected (39% ± 4%) for a direct precursor to IAA, yet the values for percent enrichment are so close that it is difficult to definitively rule out IAN as a precursor to IAA. One possible explanation for the lower enrichment of IAN could be that after 13 days (the duration of the labeling period) ^{15}N from anthranilate is limiting and becomes diluted by unlabeled anthranilate synthesized by the plant. The *trp2-1* and *trp3-1* mutants are capable of synthesizing IAN (as evidenced by its accumulation in both mutants), which over time would become progressively less ^{15}N-enriched. Preliminary experiments examining the relative incorporation of ^{15}N into IAA and IAN over time have indicated that this may be the case.

The conditions used to isolate amide-linked IAA and ester-linked IAA result in the nonenzymatic conversion of IAN to IAA, and we have used mass fragmentation analysis to distinguish between the endogenous IAA and IAA derived from IAN. The existence of IAN as a native component of plants has been questioned, due to the fact that myrosinases are known to catalyze the breakdown of glucosinolates to IAN upon tissue damage (23). We do not believe that the IAN we quantified in *Arabidopsis* is an artifact due to the myrosinase-catalyzed breakdown of glucosinolates, because myrosinases are not active above pH 5, and our extraction was carried out at neutral pH. It is formally possible that other compounds present in plant extracts could be nonenzymatically degraded to IAA in the alkaline conditions used for isolating conjugated IAA. For example, indolylmethylglucosinolate, a glucosinolate in *Arabidopsis*, has been shown to break down into IAN (24, 25). However, <0.1% of indolylmethylglucosinolate was converted to IAA when incubated in the conditions used to isolate either ester-linked IAA or amide-linked IAA (N. Ilic and J.D.C., unpublished results). In the double-labeling experiment, we isolated free IAA, which is not subject to contamination from the breakdown of either IAN or indolylmethylglucosinolate.

There is evidence to suggest that tryptophan and IAA are synthesized in plastids (26–29), and it has been argued (30) that a discrepancy between the heavy isotope enrichments of tryptophan and IAA could be explained if (*i*) IAA is made only from newly synthesized tryptophan and (*ii*) there is an unlabeled pool of tryptophan, inaccessible to IAA biosynthesis. We discount this interpretation of our double-labeling experiment for two reasons. If the unlabeled tryptophan is subtracted from the total tryptophan pool, the amount of newly synthesized tryptophan as evidenced by ^{15}N incorporation is 15%, which is still insufficient to account for the 39% ^{15}N incorporation observed for IAA. Secondly, Michalczuk *et al.* (31) demonstrated that [$^2H_5$]tryptophan was able to label the plastid tryptophan pool only slightly less efficiently than the bulk cellular pool (53% vs. 67%, respectively); therefore, the low incorporation of $^2H_5$ into IAA (9% ± 5%) is not likely to be due to inefficient uptake of the added [$^2H_5$]tryptophan by the plastids, the presumed site of IAA synthesis.

Our results, along with those from *in vivo* studies in *Lemna*, maize, and cultured carrot cells (7, 8, 10, 31) provide strong evidence that there is a tryptophan-independent pathway to IAA biosynthesis in plants. There appear to be differences in the role of tryptophan as a precursor among the species examined so far. In the case of maize, there is no evidence for a tryptophan-dependent IAA biosynthetic pathway (10), yet Michalczuk *et al.* (31) demonstrated that in carrot cell cultures there are two IAA biosynthetic pathways, one of which is tryptophan independent, and another in which tryptophan is the precursor. We have clear evidence for a tryptophan-independent pathway in *Arabidopsis*. The evidence for the existence of a tryptophan-dependent pathway is less convincing because there is significant noncatalytic conversion of tryptophan to IAA. In other plants, genes encoding the enzyme activities for several of the enzymatic steps in the postulated tryptophan to IAA biosynthetic pathways have been cloned (22, 32). The *Arabidopsis* homologs can be engineered into antisense constructs to generate

defects in the putative pathway from tryptophan to IAA, revealing the existence and function of such a pathway in *Arabidopsis*.

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