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Indole-3-acetic acid biosynthesis in isolated axes from germinating bean seeds: The effect of wounding on the biosynthetic pathway

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Abstract

A facile radiotracer method for discriminating between the activities of tryptophan-dependent and tryptophan-independent pathways for IAA biosynthesis is described. This method utilized the simultaneous exposure of plants to [^{14}C]anthranilate in the presence or absence of excess unlabeled tryptophan in order to determine if tryptophan feeding can affect the relative enrichment of the IAA pool. Using this radiotracer method, the activities of the two biosynthetic pathways were analyzed in isolated axes of germinating bean seedlings at various times after cotyledon excision. Unlabeled tryptophan suppressed [^{14}C]anthranilate conversion into IAA in isolated axes of different ages immediately following cotyledon excision. On the other hand, tryptophan feeding did not inhibit [^{14}C]IAA accumulation in isolated axes 36 or 120 h after cotyledon excision. Thus, this method was able to resolve time-dependent differences following cotyledon excision in the biosynthetic activities of the two pathways. Moreover, the present results lend further support to the emerging consensus that the tryptophan-dependent pathway acts to maintain very high IAA levels required for mediating rapid cell proliferation in wounded tissues and, as previously shown, young embryos.

Introduction

Conventional wisdom views the indolic amino acid tryptophan as the most likely precursor of indole-3-acetic acid (IAA). Indeed, considerable research on IAA biosynthesis has repeatedly demonstrated that plant parts, including excised organs, tissue sections, cultured cells, and cell-free preparations, can readily convert tryptophan into IAA (Müller and Weiler 2000; Nonhebel et al. 1993; Sembdner et al. 1981; Wildman et al. 1947). These results were taken to imply that the tryptophan-dependent (TD) pathways represent the primary pathways for IAA biosynthesis in plants (Eckardt 2001). Recent analyses have also shown that the TD pathways are primarily responsible for the IAA biosynthesis occurring in isolated axes of germinating bean seedlings (Bialek et al. 1992), embryogenic cells in carrot cultures (Michalczuk et al. 1992), and excised maize coleoptiles (Koshiba et al. 1995).

A different interpretation about the primary pathway for IAA biosynthesis has, however, emerged from analytical studies on intact seedlings or entire plants. For example, in *Lemna gibba* plants, exogenous tryptophan does not label the IAA pool fast enough to account for IAA levels, thereby suggesting that the IAA must be synthesized via another pathway that does not use tryptophan as an intermediate (Baldi et al. 1991). Moreover, auxin analyses using stable isotopic tracers of *trp-2* and *trp-3* mutants of *Arabidopsis thaliana* (Normanly et al. 1993) and of the *orp* mutant of *Zea mays* (Wright et al. 1991) have directly demonstrated that tryptophan-independent (TI) pathways are primarily responsible for IAA biosynthesis in intact plants. These mutants lack one or two functional genes coding for tryptophan synthase β but can nevertheless synthesize IAA *de novo*, ac-

cumulate much higher levels of IAA conjugates than wild-type seedlings, and, in labeling studies with seedlings of these mutant plants, the free IAA pool becomes enriched from [^{15}N]anthranilate to a greater extent than the enrichment of the tryptophan pool. In addition, *in vitro* enzyme preparations from normal and *orp* seedlings of maize are able to convert indole to IAA directly without involvement of tryptophan (Östin et al. 1999). Finally, it appears that somatic carrot embryos employ a stage-dependent switch from the TD to the TI pathway in order to carry out the organized growth necessary for embryo development (Michalczuk et al. 1992).

The methodology we have previously used for measuring the relative activities of the two pathways was to label the plant material with potential precursors of tryptophan and/or IAA (usually [^{15}N]anthranilate and [$^{2}H_5$]tryptophan), with the key comparison being the relative enrichment of the tryptophan and IAA pools following the exposure to the labeled precursors. Similar enrichment of both pools is taken to indicate that tryptophan is potentially being used as the intermediate for IAA biosynthesis. Because a precursor should have a higher enrichment than its metabolic product product, when a higher enrichment of the IAA pool versus the tryptophan pool is found this is an indication that a measurable amount of IAA is being synthesized via a TI pathway.

We wished to develop an alternative to the stable isotope method that could be employed for high throughput screens to discriminate between the activities of TD and TI pathways for IAA biosynthesis. The method involves exposing plant material to radiolabeled anthranilate, which serves as a precursor of both tryptophan and IAA, in the presence or absence of excess unlabeled tryptophan, which should increase the tryptophan pool size. This experiment has two potential outcomes: 1) the addition of unlabeled tryptophan does not significantly decrease the amount of labeled IAA synthesized from labeled anthranilate, thereby indicating that the TI pathway is acting as the primary pathway for IAA biosynthesis; or 2) unlabeled tryptophan does significantly decrease the amount of labeled IAA, thereby suggesting that the TD pathway is primarily responsible for IAA biosynthesis (for further discussion, see Sztein et al. (2000)). Using this alternative method, the activities of the two biosynthetic pathways were analyzed in isolated axes of bean seedlings at various times after cotyledon excision. Our results demonstrate that this tryptophan dilution-test method can resolve time-dependent differences in the activities of the two IAA biosynthetic pathways. In addition, the results lend further support to the emerging consensus that the TD pathway acts to maintain very high IAA levels for initiating rapid rates of cell proliferation during development, such as in embryos (Michalczuk et al. 1992), and in wounded tissues.

Materials and methods

Plant material

Green bush beans (*Phaseolus vulgaris* L. cv. Burpee's Stringless) were prepared as follows: about 200 seeds were thoroughly washed under running tap water, sterilized with two 15-min immersions in 0.5 l of a freshly prepared solution of 10% commercial bleach (5% sodium hypochlorite) with 0.01 % Triton X-100 (Sigma, St. Louis, MO, USA), and rinsed with five sequential 5-min washes with sterile distilled water. Five to six seeds per plate were then placed into sterile 100 × 15 mm tissue culture plates lined with sterile Whatman #1 filter paper wetted with sufficient sterile distilled water to cover the bottom. The plates were sealed with Parafilm strips and placed in darkness for 12 h at 24 °C to allow for seed imbibition. In all experiments except pretreatment D (see below and Table 1), embryonic axes were immediately dissected from the imbibed seeds. A scalpel furnished with a sterile #10 blade was used to cut and peel off the seed coat and then to excise the cotyledons from the axis. Immediately after excision, embryonic axes were placed at a density of 5 axes per plate in 60 × 15 mm Petri dishes prepared with Murashige and Skoog salt medium (LifeTechnologies, Inc., Grand Island, NY, USA) supplemented with 3% sucrose (Boehringer, Mannheim, Germany), 1 ml/l Murashige and Skoog vitamins (Sigma), and 1 ml/l of formulation VII micronutrient solution (Basile 1978), adjusted to pH 5.7, and solidified with 1.5% Bactoagar (Difco Labs, Detroit, MI, USA). This medium is hereafter referred to as the complete MS medium. Dishes were sealed with Parafilm strips, placed in a light-tight cardboard box in the dark and pretreated as follows:

Pretreatment A: Axes were grown for 120 h, then transferred to MS medium with [^{14}C]anthranilate with or without unlabeled tryptophan for 12 h.

Pretreatment B: Axes were immediately transferred to MS medium with [^{14}C]anthranilate plus or minus unlabeled tryptophan for 12 h.

Table 1. The effect of simultaneous tryptophan feeding on the ability of radioactive anthranilate to label the IAA pool. After the seeds were imbibed in distilled water for 12 h, the seeds and/or the isolated axes were subjected to various pretreatments prior to the isolated axes being exposed to [^{14}C]anthranilate for 12 h in the presence or absence of 3.5 μmol/ml of unlabeled tryptophan. The results are presented as the mean of 3 or 4 replicates ± the standard error. Using pair-wise t-tests, the mean value marked with an asterisk is significantly different from the other mean value in the same row at the 0.05 level. Pretreatments indicated by a letter (A-D) were as outlined in Materials and Methods

Pretreatment	Intact seed on MS medium (h)	Isolated axes on MS medium (h)	[^{14}C]IAA labeling from [^{14}C]anthranilate in absence of tryptophan (ng/g FW/h)	[^{14}C]IAA labeling from [^{14}C]anthranilate in presence of tryptophan (ng/g FW/h)
A	0	120	935 ± 60	916 ± 91
B	0	0	710 ± 12*	144 ± 19*
C	0	36	716 + 70	839 ± 162
D	36	0	378 ± 59*	225 ± 24*

Pretreatment C: Axes were cultured for 36 h, then transferred to MS medium with [^{14}C]anthranilate plus or minus unlabeled tryptophan for 12 h.

Alternatively, whole seeds were allowed to develop until just before incubation with label:
Pretreatment D: After the initial 12 h of germination on filter paper, the imbibed seeds were transferred to complete MS medium for 36 h, cotyledons excised, and then transferred to MS medium with [^{14}C]anthranilate plus or minus unlabeled tryptophan for 12 h.

Uptake experiments

These experiments closely followed the procedures described in Sztein et al. (2000). In order to quantify tryptophan uptake, ca. 120 mg of embryonic axes following a 36 h incubation on the complete MS medium were placed at a density of 3 to 5 axes per plate in 60 × 15 mm Petri dishes and exposed to 3.5 μmol/ml of filter sterilized (0.2 μm syringe filter, Nalge Co.) unlabeled L-tryptophan (Sigma), 17 pmol/ml of L-[5-^{3}H]tryptophan (specific activity: 33 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ, USA) tracer, and 40 μl of a 0.01% solution of the surfactant Tween 80 (Sigma) in 7 ml of complete MS liquid medium. The concentration of unlabeled tryptophan corresponded to 500 times the amount of endogenous tryptophan present in wild-type *Lemna gibba* L. fronds (7 nmol/g FW; Tam et al. (1995)). The plates were incubated at 24 °C on a rotary shaker set at 70 rpm under 20 μmol m^{-2} s^{-1} of cool-white fluorescent lights for 1 h. The axes were then thoroughly rinsed three times with distilled water, and the amount of radioactivity incorporated was measured in Econo-Safe counting cocktail (Research Products International Corp., Mt. Prospect, IL, USA), using a Beckman LS 6500 liquid scintillation counter.

The same procedure was used to measure anthranilate uptake except that the tryptophan was replaced with [ring ^{14}C-(U)] anthranilic acid (specific activity: 56 mCi/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO, USA) at a final concentration of 11.2 nmol/ml. The potential competition between tryptophan and anthranilate uptake was studied by using the same protocol with 3.5 μmol/ml of unlabeled tryptophan, 17 pmol/ml of L-[5-^{3}H] tryptophan tracer, and 11.2 nmol/ml [ring ^{14}C-(U)] anthranilic acid. All treatments were replicated three times.

Analysis of [^{14}C]IAA synthesized from [^{14}C]anthranilate

The culture procedures described above for the anthranilate uptake experiments was also used to study the conversion of [^{14}C]anthranilate to [^{14}C]IAA in ca. 250 mg of embryonic axes (10 axes per plate) except for the following changes. The incubation medium consisted of 40 μl of 0.01% Tween 80 solution (Sigma) and 64 nmol/ml of [ring ^{14}C-(U)]anthranilic acid in 7 ml of complete MS liquid medium. A supplement of 3.5 μmol/ml of a filter-sterilized solution of L-tryptophan was added in parallel experiments to determine potential isotopic dilution through tryptophan. The axes were incubated in the appropriate medium for 12 h. All treatments were replicated three times.

Due to low levels of [^{14}C]IAA synthesis, these experiments required several modifications from the standard protocol used to analyze endogenous IAA levels in plants (Sztein et al. 2000). The axes from each replicate plate were blotted on absorbent paper, weighed, rinsed three times with distilled water, re-

blotted, placed into 1.5-ml microcentrifuge tubes (Kontes, Vineland, NJ, USA), and frozen in liquid nitrogen. A small sample of the axes was monitored by liquid scintillation counting to check for sufficient anthranilate uptake. The axes in each microcentrifuge tube were then ground in 200 μl of freshly made 80% acetone with the addition of 5 μl of a 1 μg/ml unlabeled indole-3-acetic acid (Sigma) solution in ethyl acetate and ca. 10 mg of glass beads (Superbrite, 66–160 mm, 3M) using plastic pestles (Kontes) after which they were centrifuged for 10 min at 13,000 g in a microcentrifuge. The supernatant was placed in a 1.5 ml-screw-cap clear glass vial, and the pellet was reextracted with 100 μl of 80% acetone and recentrifuged as above. The supernatants from all extractions of a given replicate were pooled in a glass vial with 500 μl of a 100 mm phosphate buffer adjusted to pH 2.5. This solution was partitioned against 700 μl of ethyl acetate, equilibrated for 30 min at room temperature, and placed in a freezer at −18 °C for a minimum of 2 h (Östin et al. 1999). The ethyl acetate phase containing the IAA was recovered, placed in a 5 ml teardrop flask, dried *in vacuo* prior to the final purification step and resuspended in 100 μl of 50% methanol with the addition of ~3,000 dpm of [^{3}H]IAA (specific activity: 20.8 Ci/mmol; American Radiolabeled Chemicals) as a secondary radioactive tracer. The sample was partially purified using C_{18} high performance liquid chromatography as described in Sztein et al. (2000). Fractions containing IAA were collected, 200 ng of [$^{13}C_6$]IAA (Cohen et al. 1986) added, the sample was mixed well, and then divided in two subsamples. The larger subsample (90% of the total) was placed in a scintillation vial and measured directly in a scintillation counter using a dual isotope program to determine the amount of [^{14}C]IAA present in the sample. The smaller subsample was used to evaluate percent recovery of the [^{14}C]IAA by using the reverse isotope dilution method (Bialek et al. 1983), which compares the amount of unlabeled IAA added at the beginning of the extraction versus the amount remaining at the end of the purification procedure to determine the percentage of radioactive IAA lost during purification. In brief, the subsample was evaporated in vacuo, resuspended in 100 μl methanol, methylated with an ethereal solution of diazomethane as previously described, and resuspended in 50 μl of ethyl acetate prior to being analyzed by gas chromatography-mass spectrometry (GC-MS). Selected ion spectra for the samples were determined in a Hewlett-Packard GC 6890/5973 MS (Hewlett-Packard Co., Palo Alto, CA, USA) equipped with a 30 m × 0.25 mm i.d. 0.25 μm fused silica capillary column of cross-linked 5% phenyl methyl siloxane (HP-5MS, Hewlett-Packard). Chromatographic parameters were as follows: injector temperature 250 °C, initial oven temperature 70 °C, and then a ramp at 20 °C /min to 280 °C. The monitored ions were m/z 130, 136 (quinolinium ion, m + 6), 189, 195 (molecular ion, m + 6).

Percent recovery of the cold carrier IAA was then used to calculate the amount of radioactive IAA present in the original sample. The total dpm obtained by direct counting were proportionally adjusted for the percentage used in the recovery determination (~10%), and that number adjusted for recovery calculated by the use of [$^{13}C_6$]IAA in a stable isotope dilution protocol (Chen et al. 1988). This value was then converted into ng [^{14}C]IAA per sample, and finally expressed as pg [^{14}C]IAA/mg FW.

Results

Two preliminary uptake experiments were required in order to use the tryptophan-feeding method as the basis for determining the relative activities of different IAA biosynthetic pathways. First of all, the exogenous tryptophan concentration should be high enough to effectively label the tryptophan pool. Isolated embryonic axes were incubated for 36 h on MS medium before being exposed to 3.5 μmol/ml of unlabeled tryptophan plus radioactive tryptophan tracer for 12 h. Treated axes had an uptake rate of 173.1 ± 4.7 pmol tryptophan/mg FW/h, which is a much higher uptake rate than has been observed in other plants (Rapparini et al. 1999; Sztein et al. 2000). Thus, all subsequent experiments used this concentration of unlabeled tryptophan. Secondly, the exogenous tryptophan in the medium should not significantly inhibit anthranilate incorporation. Isolated axes were incubated for 36 h on MS medium before being exposed to radioactive anthranilate in the presence or absence of 3.5 μmol/ml of unlabeled tryptophan for 12 h (Table 1). In the absence of exogenous tryptophan, the axes incorporated anthranilate at a rate of 2.4 ± 0.4 pmol/mg FW/h. Other axes that were simultaneously exposed to 3.5 μmol/ml exogenous tryptophan exhibited an anthranilate incorporation rate of 2.1 ± 0.4 pmol/mg FW/h, which shows that anthranilate incorporation was not significantly affected by the presence of tryptophan.

The effects of simultaneous tryptophan feeding on the ability of radioactive anthranilate to label the IAA pool of isolated bean axes are shown in Table 1. After the seeds were imbibed for 12 h, the seeds and/or the isolated axes were subjected to various pretreatments prior to the isolated axes being exposed to [^{14}C]anthranilate for 12 h in the presence or absence of 3.5 μmol/ml of unlabeled tryptophan. Pretreatment A was designed to prepare 6-day-old isolated axes similar to the 6-day-old axes analyzed in Bialek et al. (1992). To our surprise, no significant difference in [^{14}C]IAA accumulation was observed in the axes in pretreatment A that were treated with [^{14}C]anthranilate alone vs. those exposed to both [^{14}C]anthranilate and unlabeled tryptophan. Thus, it appeared that exogenous tryptophan did not affect IAA biosynthesis under these conditions, which argues that axes of this age are utilizing a TI pathway as the primary pathway for IAA biosynthesis.

Since this result appeared to contradict the interpretation of Bialek et al. (1992), isolated axes were subjected to other pretreatments in an effort to identify the cause of the seemingly different result. Pretreatment B involved excising the cotyledons from imbibed seeds and then immediately transferring the isolated axes into either anthranilate or the anthranilate-plus-tryptophan treatment. In these axes, the addition of unlabeled tryptophan into the [^{14}C]anthranilate solution caused an 80% reduction in the radioactive enrichment of the IAA, as compared to the anthranilate control. Thus, the surgical isolation of embryonic axes from imbibed seeds had apparently induced high levels of IAA biosynthesis via the TD pathway.

Pretreatment C inserted a 36 h interval between cotyledon excision and the start of anthranilate labeling. The amount of [^{14}C]IAA accumulation was not significantly different in these axes following the anthranilate versus the anthranilate-plus-tryptophan exposures. Thus, it appeared that the IAA biosynthetic pathway had switched from the TD pathway in isolated axes immediately following cotyledon excision (pretreatment B) to the TI pathway in isolated axes in 36 h following cotyledon excision (pretreatment C).

Pretreatment D was designed to evaluate whether or not the effect observed in pretreatment C might be attributable to the age of the isolated axes. Intact seed following imbibition were transferred to culture medium for 36 h, after which the cotyledons were excised and the axes transferred to either anthranilate or anthranilate-plus-tryptophan treatment. Consequently, although the axes in pretreatments C and D were the same age from the start of seed imbibition, the surgical isolations occurred at different times. In pretreatment D, the inclusion of unlabeled tryptophan in the anthranilate medium did result in a significant reduction in [^{14}C]IAA accumulation, which implies that the TD pathway was activated in these axes in response to surgical isolation, as was observed in the axes in pretreatment B. However, the rates of [^{14}C] accumulation on a mg FW basis are much lower in pretreatment D than the other pretreatments. The observed differences in accumulation rates can partially be explained by the observation that the axes remaining attached to the cotyledons (pretreatment D) grew almost 40% faster than the axes growing in culture medium (other pretreatments) (data not shown). In addition, the interpretation of the results from pretreatment D is further complicated by the observation of Bialek et al. (1992) that the free IAA levels are significantly higher in attached axes in intact seeds than in isolated axes during the first days following seed imbibition.

Discussion

The radiotracer method employed in these experiments is an attractive alternative for determining the relative activities of the two biosynthetic pathways responsible for synthesizing IAA in plants that is adaptable to rapidly screening different species or for characterization of selected mutants. The use of reverse isotope dilution methods makes it adaptable for use in laboratories with various analytical capabilities. For example, in the final quantification of the carrier unlabeled IAA, we used GC-MS analysis because it was the most accurate method available to us. Because the unlabeled tracer is added in large quantities, however, other methods of analysis such as HPLC or ELISA could be substituted. This feature of the procedure makes analysis of such pathways a routine procedure as compared to the stable isotope protocols we previously described. It also brings a method for IAA pathway analysis to laboratories without ready access to mass spectrometry instrumentation and is adaptable for the analysis of diverse species (Sztein et al. 2000) or as a high through-put screen for selection of mutant lines of a single species. Nevertheless, it does not provide the same detailed analysis of the isotopomers that is possible with

stable isotope mass fragmentation methods and thus the two procedures are complementary.

The results from pretreatment A in Table 1 demonstrated that the TI pathway is primarily responsible for IAA biosynthesis in 6-day-old axes from germinating bean seedlings. This conclusion appeared to contradict the interpretations of Bialek et al. (1992). In their experiments, however, isolated axes were exposed to continuous tryptophan labeling for 132 h following imbibition, and then exhibited equivalent enrichment in the tryptophan and IAA pools. Thus they concluded from this result that the predominant pathway in isolated axes is a TD pathway. These contradictory interpretations can be reconciled by hypothesizing that both pathways were active at different times during the longer labeling period used in Bialek et al. (1992). Apparently, the isolated axes used in Bialek et al. (1992) carried out high rates of TD IAA biosynthesis for a short period after cotyledon excision, as was also observed in the pretreatment B in Table 1. Subsequently, the axes switched to lower rates of TI biosynthesis like the axes in pretreatment C. Given the predicted difference in the biosynthetic rates of the two pathways, then long-term labeling experiments would necessarily result in most of the IAA being derived from tryptophan. This explanation is entirely consistent with other work on IAA biosynthesis, which suggests that the TD pathway is capable of much higher rates of IAA biosynthesis than the TI pathway (Michalczuk et al. 1992).

Furthermore, the most straightforward interpretation from pretreatments B and D is that wounding induces the transient activation of the TD pathway. This interpretation would also explain why cultured plant cells and tissue explants are frequently observed to exhibit high levels of tryptophan conversion to IAA (Michalczuk et al. 1992; Ribnicky et al. 1996). It appears that unorganized cells and wounded explants activate the TD pathway in order to generate the high auxin levels needed for rapid cell proliferation. By contrast, intact plants appear to use the low-capacity TI pathway in order to maintain the low auxin levels required for organized development (Michalczuk et al. 1992).

The effects of biological and environmental stress on indolic intermediates of the tryptophan pathway in general has received considerable attention over the last several years thanks to an abundance of useful *Arabidopsis* mutants and concerted efforts to look at pathogen stress and pathogen responses in this model system. This has resulted in sufficient information to propose that an important aspect of plant responses to stress involves a remodeling of these indolic pathways. For example, Zhao and Last (1996) showed that the accumulation of the indolic phytoalexin camalexin induced by abiotic elicitors, plant pathogens, and in spontaneous lesions in the cell death mutant *acd2* were all accompanied by the coordinate induction of mRNAs for the tryptophan biosynthetic pathway enzymes. This was confirmed by a recent report (Hagemeir et al. 2001) that showed a range of tryptophan-derived secondary products accumulate following infection of *Arabidopsis* by *Pseudomonas*. This is an important finding since camalexin, like TI-derived IAA, appears to be produced from indole directly and not through tryptophan (Zook 1998). In maize, which does not produce camalexin, the remodeling of indolic metabolism also occurs and two genes, *bx1* and *igl*, produce free indole independent of tryptophan synthase (EC 4.2.1.20). The first yields the defense compound DIMBOA (Frey et al. 1997), and the second responds to a herbivore elicitor (Frey et al. 2000). Collectively, these discoveries on stress effects on indolic metabolic events in plants are consistent with our data on wounding effects on IAA biosynthesis. Of course, developmental, in addition to stress signals, appear to control the activity of the TD-IAA biosynthesis pathway resulting in increased levels of IAA in the target tissues (Michalczuk et al. 1992).

In summary, this perspective concerning the distinct developmental and stress roles of the two IAA pathways should help to resolve the conflicting reports about the nature of the primary pathway for IAA biosynthesis in plants.

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