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Metabolism and Synthesis of Indole-3-Acetic Acid (IAA) in *Zea mays*[1]

Levels of IAA during Kernel Development and the Use of in Vitro Endosperm Systems for Studying IAA Biosynthesis

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Kernels of *Zea mays* on an intact plant accumulate indole-3-acetic acid (IAA) at the rate of 190 ng g^{-1} fresh weight h^{-1}. Of the IAA synthesized, 97% is in the esterified form and less than 3% remains as the free acid. The site of biosynthesis of the IAA, whether synthesized in the leaf and transported to the kernel, or in the kernel and remaining in the kernel, has not been established. In an attempt to determine the locus of synthesis, we grew isolated kernels on agar media not containing tryptophan or other possible aromatic precursors of IAA and observed IAA synthesis of 99 ng g^{-1} fresh weight h^{-1}, approximately 52% of the in situ rate. Thus, the kernel contains all of the enzymes required for de novo aromatic biosynthesis of IAA and its ester conjugates. Furthermore, endosperm cells in suspension culture, grown on hormone-free media and in the absence of aromatic precursors, are able to synthesize IAA at a rate of 9.2 ng g^{-1} fresh weight h^{-1}, or 4.8% of the in situ rate. This finding establishes that all of the enzymes of IAA biosynthesis occur in the endosperm and that the endosperm is a site of IAA biosynthesis. Isolated endosperm, prepared from developing kernels, synthesized IAA from labeled anthranilate at a rate of 8.6 ng g^{-1} fresh weight h^{-1}, or 4.5% of the in situ rate. Frozen endosperm preparations maintained the ability to synthesize labeled IAA from labeled anthranilate. The identity of the synthesized IAA was established by mass spectral analysis. We suggest that endosperm preparations of *Z. mays* are suitable for study of the mechanism(s) of IAA biosynthesis because they (a) have high rates of synthesis; (b) show stability to freezing, enabling enzyme storage; (c) provide a system with a known rate of in situ synthesis; and (d) are available in large amounts for use as an enzyme source.

Conjugation of plant hormones in developing seeds is a general phenomenon (Avery et al., 1942a, 1942b; Hatcher, 1943; Cohen and Bandurski, 1978; Miller et al., 1987; Bialek and Cohen, 1989) The stored hormones are available as sources of hormone for the developing seedling plant (Epstein et al., 1980), are protected against oxidation (Cohen and Bandurski, 1978), serve as transport forms (Nowacki and Bandurski, 1980), and ultimately serve in hormonal homeostasis (Bandurski et al., 1988). Definitive chemical studies began with Cholodny (1935), who recognized that the endosperm of cereals was a rich source of growth hormone following moistening to permit enzymatic hydrolysis of bound forms. Pohl (1935), Laibach and Meyer (1935), Hatcher and Gregory (1941), and Avery et al. (1942a, 1942b) (for reviews, see Thimann and Went, 1937; Cohen and Bandurski, 1982) showed that the seed hormone precursor was readily converted to the same hormone found in the tip of grass coleoptiles. Finally, Heyn (1935) and Skoog (1937) demonstrated that removal of the endosperm makes the seedling more responsive to applied IAA, thus developing the concept of a seed auxin precursor.

Despite the long history of research on IAA, there is as yet no certainty as to a well-defined pathway for IAA biosynthesis demonstrably able to produce IAA in amounts sufficient for a plant's needs. A biosynthetic pathway of IAA involving Trp has been well established in several different bacterial species (Sequeira and Williams, 1964; Kosuge et al., 1966; Thomashaw et al., 1984). In plants Trp has also been shown to be a major precursor for IAA in bean seeds (Bialek et al., 1992). However, several lines of evidence, including lack of growth induction by Trp (Winter, 1966; Thimann and Grochowska, 1968) and questions about microbial contamination (Libbert et al., 1966), suggest that Trp conversion to IAA may not be the only route. Recently, this laboratory (Bandurski et al., 1992) showed by means of labeling with deuterium oxide that Trp synthesis can proceed without incorporating label into IAA. This established that the two processes are separable in time. Conclusive evidence for the existence of two pathways to IAA, one of which does not pass through Trp, has been presented recently (Wright et al., 1991; Michalczuk et al., 1992; Normanly et al., 1993). It seems certain that there are at least two pathways for the biosynthesis of IAA, one involving a pathway not through Trp (Wright et al., 1991; Normanly et al., 1993; Rekoslavskaya and Bandurski, 1994) and a second pathway through Trp (Bialek et al., 1992; Michalczuk et al., 1992).

Working with rye in the 1940s, Hatcher (1943) proposed that the increase in IAA during seed development was not the result of transport from the mother plant but that IAA

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Abbreviation: DAP, days after pollination.

was synthesized within the seed. *Zea mays* endosperm suspension cultures have been initiated and grown for many years without the need for hormone supplementation (Shannon, 1982). The in vitro culture of *Z. mays* kernels also proceeds on hormone-free media (Gegenbach, 1977). Since these two cell culture systems grow without the need for hormone supplementation, it is reasonable to conclude that these systems can make all of the hormones required for sustained growth.

In this study we describe a time course for the biosynthesis of free and ester IAA in the developing endosperm of *Z. mays*. These data demonstrate that free IAA is esterified as rapidly as it is synthesized in the kernel. Free IAA levels do increase, but the amounts of free IAA are small and can be accounted for by the relationship between the free energy changes of the first two conjugation reactions (Kesy and Bandurski, 1990). In much earlier studies using less exacting analytical methods, Avery et al., (1942a), Teas and Newton (1951), and Corcuera (1967) for maize and Hatcher for rye (1943) described the time course for IAA accumulation. Brief reports of conclusions of this work have appeared previously (Jensen and Bandurski, 1991; Jensen et al., 1992).

A second objective of this study was to determine whether endosperm suspension cultures and cultured kernels produce IAA de novo. We examined the feasibility of using homogenized *Z. mays* endosperm to study IAA biosynthesis. To test this system, [^{14}C]anthranilic acid was used as a putative early precursor, and we demonstrated the attractiveness of the maize endosperm as an experimental system for studies of IAA biosynthesis.

MATERIALS AND METHODS

Plant Material

For the developmental study, experiments were conducted on sweet corn (*Zea mays* L., cv Silver Queen) grown under field conditions. Ear shoots were bagged prior to silk emergence and then sib pollinated when the silks emerged. In an attempt to compensate for variability due to environmental conditions, two groups of ears were pollinated on different dates. For IAA determinations, two ears from each pollination group were harvested every 5 d from 0 until 45 DAP. Additional ears were harvested 58 DAP and allowed to dry at room temperature. Free and total IAA determinations were made on fresh tissue. Each sample consisted of kernels from one ear for a total of four samples per age group. To examine the conversion of [^{14}C]anthranilate to IAA, additional ears were harvested between 22 and 30 DAP and used immediately.

Suspension cultures of endosperm cells derived from the *Z. mays* inbred line A636 were kindly provided by Dr. Jack Shannon of Pennsylvania State University. This cell line was established in 1986 from kernels 10 DAP. A complete description of the initiation and characterization of maize endosperm suspension cultures has been previously reported (Shannon, 1982; Felker et al., 1989). The cells were grown on liquid media, pH 5.6, containing Murashige-Skoog salts (Murashige and Skoog, 1962), 3% Suc, 2 g/L Asn, and 0.4 mg/L thiamine HCl (Shannon, 1982). No auxin addition is required for sustained growth. Cultures were grown in the dark at 29°C on a rotary shaker at 120 rpm. Two 1-L stock cultures grown for 7 d after subculturing served as a source of inoculum for the experimental flasks. Each stock flask was subcultured into a set of six 250-mL flasks, each containing 80 mL of medium. Every 2 d after subculture one flask from each set was harvested, fresh weight was determined, and the tissue was lyophilized for later analysis. *Z. mays* kernels (hybrid B73 × LH51) grown in vitro were provided by Dr. Fred Below of University of Illinois. The kernels were grown on solid medium containing Murashige-Skoog salts, 15% Suc, 2 g/L Asn, and 0.4 mg/L thiamine HCl. No hormones or aromatic amino acid were added to the media. Kernels were harvested 20 and 32 DAP and lyophilized.

Feeding of Labeled Precursors

Ears of corn were surface sterilized for 10 min in a 1:10 dilution of commercial bleach containing 5% NaOCl and then rinsed four times with sterile water. The endosperm was excised from the kernels, and two endosperm were placed in sterile microfuge tubes and gently homogenized with a glass rod. Additional endosperm were frozen at −80°C for later analysis to determine the stability of the system to freezing. Alternatively, many endosperm were placed in a sterilized mortar, homogenized, and then transferred to sterile microfuge tubes in amounts approximately equal to two endosperm. To each tube approximately 250,000 dpm (8.6 nmol) of [^{14}C]anthranilic acid was added. Control samples were boiled for 5 min and cooled prior to the addition of the radiolabel. The samples were allowed to incubate for 0, 4, 12, and 24 h. To stop the reactions an equal volume of 2 N NaOH was added to inactivate the enzymes and hydrolyze the IAA conjugates. The samples were then analyzed immediately or frozen with liquid nitrogen and stored at −80°C for later analysis.

Sample Extraction

For the determination of free and total IAA in the developmental study, 15 to 90 fresh kernels, depending on age, were extracted with aqueous acetone (final concentration 70%, v/v) after being frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. An internal standard of [$^{13}C_6$]IAA (100 μg/sample) and approximately 80,000 cpm of [^{3}H]IAA, to facilitate peak detection, were added to the samples. The samples were allowed to extract for 18 h at 4°C and then centrifuged for 5 min in a benchtop centrifuge at 1300*g*; the supernatant liquid was divided for free and total IAA analysis.

For the determination of free IAA from the lyophilized endosperm cells, 5% of each sample, based on dry weight, was extracted with 70% acetone in vacuo for 2 h at room temperature. Internal standards were added as above. After 2 h the samples were centrifuged as above, and the supernatant was reduced in volume to an aqueous phase and diluted with 2 mL of water in preparation for column chromatography.

For the determination of total IAA from the lyophilized tissue, an additional 5% of the sample, plus internal standard,

was extracted and simultaneously hydrolyzed with 1 N NaOH in vacuo for 2 h at room temperature. The sample was then acidified to pH 2.5 with 1 N HCl in preparation for column chromatography.

IAA Purification

Free and total IAA were purified using a modification of a method developed by Chen et al. (1988). Quantification was accomplished through the use of stable isotope-labeled IAA as an internal standard (Magnus et al., 1980; Cohen et al., 1986). The extract was applied to a DEAE-Sephadex A-25 column (acetate form, 1.5 mL bed volume; Pharmacia) packed in a 3-mL syringe fitted with a polypropylene frit. Column washing and elution were as described by Chen et al. (1988). After elution, the samples were dried in vacuo, taken up in solvent, and chromatographed using an HPLC (LDC/Milton Roy model CM 4000 pump with a model 3000 Spectro monitor variable UV detector set at 280 nm) equipped with a C_{18} column (5-μm ODS, 25 × 4.6 cm, Val-U-Pak; Regis, Morton Grove, IL) using 20% acetonitrile containing 1% acetic acid as the mobile phase. The 3-mL fraction at the retention time of IAA was checked for radioactivity and dried in vacuo. The sample was then methylated with diazomethane and subjected to a second C_{18} HPLC step using 45% acetonitrile as the mobile phase. The free IAA fraction was then dried in vacuo and dissolved in acetonitrile for GC-MS analysis.

Alternatively, extracts from the lyophilized tissues were loaded by means of a vacuum manifold onto a preconditioned 10- × 10-mm semiprep guard column (Upchurch Scientific, Oak Harbor, WA) containing C_{18} pellicular packing (Whatman, Clifton, NJ). After the precolumn was washed with 10 mL of water, it was inserted in place of the injector loop on the HPLC and further purified as above.

For the determination of total IAA in the developing endosperm, 2 mL of the extract was base hydrolyzed and placed on a C_{18} column as described by Chen et al. (1988). The sample was then dried in vacuo, taken up in solvent, and purified by HPLC as described above for free IAA.

To determine the incorporation of ^{14}C from anthranilate into IAA, 80,000 cpm of [^{3}H]IAA were added to the sample, which was then acidified to pH 2.5 with 1 N HCl. The labeled IAA was added for determining recoveries, aiding in peak detection and as a purity check following isolation. The samples were then purified as above through the first HPLC step. Some samples were additionally methylated and further purified as above to confirm the identity of the labeled compound.

GC-MS-Selected Ion-Monitoring Analysis

GC-MS-selected ion-monitoring analysis of the purified methylated IAA was conducted on a Hewlett-Packard 5890 GC with a capillary direct interface to a model 5970 mass selective detector. The GC column was a 15.0 m DB-17 fused silica capillary column (0.25 mm i.d., 0.25-μm film thickness; J & W Scientific). The GC temperature program consisted of an initial 1-min hold at 100°C, followed by a ramp to 230°C at 30°C/min. The ions at m/z 130, 136, 189, and 195 were monitored and peak areas measured to determine the $^{13}C/^{12}C$ ratio. Additional samples were scanned from m/z 50 to 350 to check for cleanliness.

RESULTS

IAA Levels during Kernel Development

Developing kernels of *Z. mays* contain levels of IAA several orders of magnitude greater than reported for vegetative tissues (Bandurski and Schulze, 1977). For the first 10 DAP IAA levels remain constant at approximately 0.018 μg kernel^{-1} (445 ng g^{-1} fresh weight). Beginning 10 to 15 DAP, there is nearly a 5000-fold increase in the amount of total IAA, reaching a maximum of 92 μg kernel^{-1} (160,000 ng g^{-1} fresh weight) about 45 DAP and then decreasing by approximately one-half in the dry seed (Fig. 1A). The change in IAA levels parallels the tissue fresh weight (Fig. 1B), indicating that the tissue monitors concentration and not amount per kernel. The increase of 92 μg kernel^{-1} between 10 and 45 DAP translates to an average IAA accumulation rate of 190 ng g^{-1} fresh weight h^{-1}. This value may or may not equal the kernel's IAA biosynthetic rate because it is not known whether all of the IAA made is conjugated and stored, whether there are losses during development, or whether some IAA is imported into the kernel from the mother plant.

Plotting free and total IAA on a per gram dry weight basis indicates that IAA levels increase at a rate independent of the change in dry weight until d 20 and then decline (Fig.

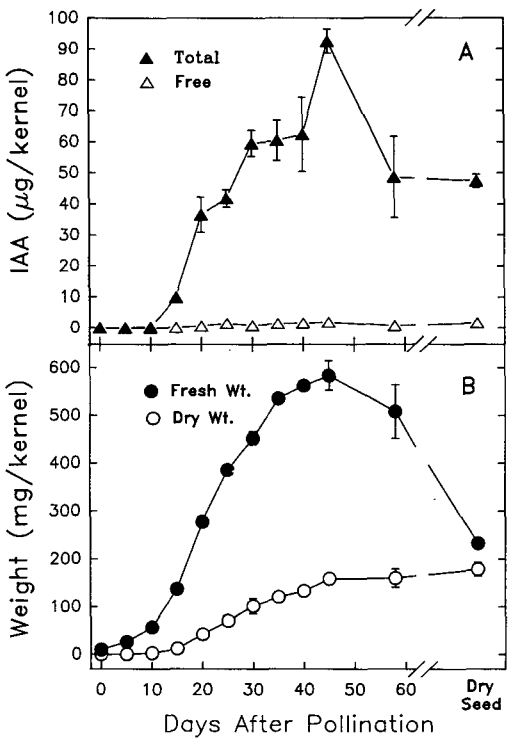

Figure 1. Comparison of the levels of free and total IAA (A) and the accumulation of fresh and dry weight (B) during growth and maturation of *Z. mays* kernels in situ. Vertical bars indicate the SE of four replicate samples.

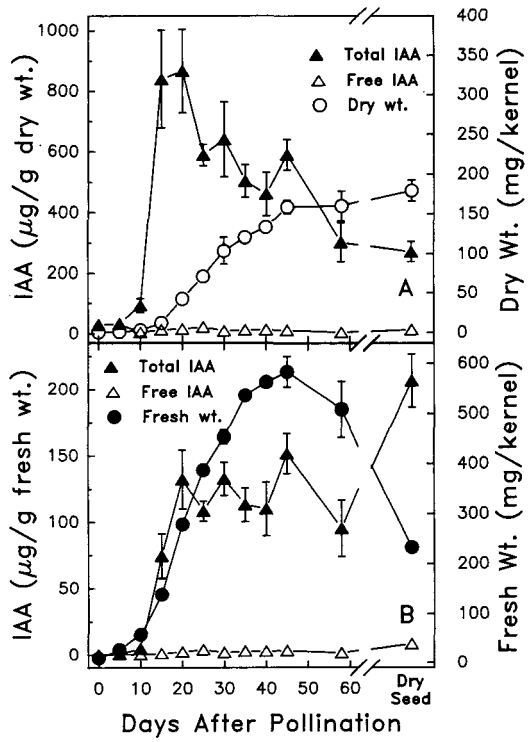

Figure 2. Comparison of the levels of free and total IAA when calculated on a per gram dry weight basis (A) and a per gram fresh weight basis (B) during the growth and maturation of *Z. mays* kernels in situ. Vertical bars indicate the SE of four replicate samples.

2A). When the levels of free and total IAA are plotted on a per gram fresh weight basis, it can be seen that the concentration of IAA remains relatively constant after d 20 (Fig. 2B).

Compared to the amount of total IAA, the free IAA levels remain relatively constant at about 1 to 2% of the total IAA. Considered alone, the free IAA undergoes a large increase between 5 and 20 DAP from 0.018 μg kernel^{-1} (2.5 μM) to approximately 1.00 μg kernel^{-1} (22.5 μM), a 50-fold increase on a μg kernel^{-1} basis but only a 10-fold increase on a molar basis (Fig. 3). The amount of free IAA then remains at a relatively constant level for the rest of the growth period. A plot of molar IAA concentration versus time is similar in shape to Figure 3. During the period of rapid increase in total IAA the ratio of ester to free IAA remains constant (Fig. 4) when computed on a fresh weight, dry weight, or per kernel basis. Free IAA constitutes approximately 1 to 2% of the total, the remainder being ester conjugates. There was no detectable amide-linked IAA (data not shown).

IAA Levels in Corn Endosperm Cell-Suspension Cultures and in Vitro Cultured Corn Kernels

The growth curves (fresh and dry weights) of the *Z. mays* endosperm cells in suspension culture during the course of the culture cycle are shown in Figure 5. The curves are typical for cells in culture inoculated at a high density. The levels of free IAA during the growth cycle are shown in Table I. Because of the variability of the data during the first 6 d of

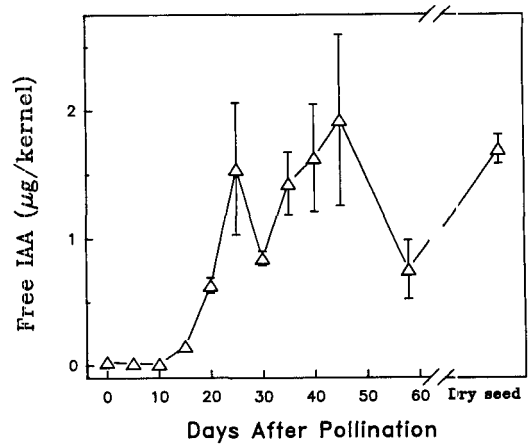

Figure 3. The levels of free IAA per kernel during development of *Z. mays* kernels in situ. Vertical bars indicate the SE of four replicate samples.

growth, the time of maximum free IAA cannot be conclusively determined, although the data suggest a maximum of approximately 4 d after subculture. There is clearly a decrease in free IAA when the culture stops growth.

The level of total IAA increases up to a maximum approximately 6 to 8 d after subculture and then decreases by approximately one-half by d 10 (Table I). Table I also shows the levels of IAA on a per gram fresh weight basis and on a per gram dry weight basis. Presenting the data on a ng per flask basis clearly shows that *Z. mays* endosperm cells in suspension cultures, without aromatic nutrients, are producing IAA. During the 6 d from 2 to 8 d after subculturing, approximately 9.5 μg of IAA were produced per flask, yielding a biosynthetic rate of approximately 2.5 ng g^{-1} fresh weight h^{-1}. This is 1.3% of the observed accumulation rate for intact kernels on the plant. Preliminary experiments indicate that a large amount (up to 20 μg/flask) of IAA is excreted into the media. Therefore, it is likely that the bio-

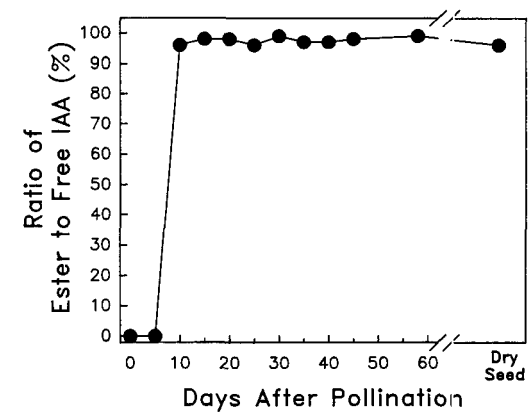

Figure 4. Average percentage of total IAA in the ester form during development of *Z. mays* kernels in situ. The remaining IAA is in the free acid form.

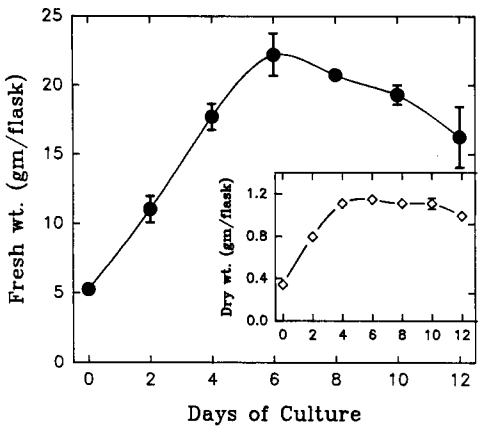

Figure 5. Fresh and dry weight of *Z. mays* endosperm suspension cultures during the culture cycle. Cultures were inoculated at a high density to obtain a rapid growth rate. Vertical bars indicate the SE of four replicate samples.

synthetic rate may be as much as 9.2 ng g^{-1} fresh weight h^{-1} or 4.8% of that of the intact kernel.

The amount of free and total IAA found in in vitro cultured *Z. mays* kernels is shown in Table II. Total IAA in the cultured kernels doubled in 12 d from 16 to 33 μg/kernel. Thus, the biosynthetic rate in this system is 99 ng g^{-1} fresh weight h^{-1} or 52% of the rate for kernels on the plant. Free IAA content was 3.5 μg/kernel in the cultured kernels, in contrast to the 1 to 2 μg/kernel in the intact kernel.

IAA Biosynthesis in Corn Endosperm Homogenates

Providing [^{14}C]anthranilate to homogenates of corn endosperm harvested 20 to 30 DAP resulted in the biosynthesis of [^{14}C]IAA. The amount of radioactivity found in the IAA HPLC peak is shown in Table III. After 24 h of incubation, as much as 42,500 dpm was found to be associated with the IAA HPLC peak. This corresponds to a 4.5 to 8% conversion of applied radioactivity into the putative IAA. Approximately 1.2 μg of anthranilic acid were added per experiment. Thus, using an average percentage conversion of 6% (for the 24-h incubation values), approximately 72 ng of labeled IAA were produced in 24 h for a rate of 8.6 ng g^{-1} fresh weight h^{-1}. This rate is 4.5% of the observed accumulation rate for intact kernels on the plant. This rate does not take into consideration the amount of anthranilic acid present in the endogenous pool, which would dilute the applied radioactivity and, therefore, result in underestimation of the rate. The calculated

Table II. *Synthesis of IAA and IAA esters by in vitro cultured kernels of Z. mays*

	IAA	
	d 20	d 32
	μg/kernel	
Free	3.5	3.5
Total	16	33

IAA biosynthetic rates of the various in vitro endosperm systems examined as compared to the rate observed for the intact kernel on the plant are summarized Table IV. As shown by the data of Figure 6, radioactivity from anthranilic acid was incorporated into the IAA fraction at an initially rapid rate and then as a linear function of time between 5 and 25 h. In all cases [^{3}H]IAA was added at the beginning of purification to aid in peak location and for determination of recoveries. In addition, it was possible to use the ratio of ^{14}C/^{3}H in the samples as an index of purification. The ratio decreased as radioactive impurities were removed from the sample. After two HPLC steps the ratio remained unchanged, indicating an essentially pure IAA fraction, since it is unlikely that a contaminant would co-elute with both the free acid and methylated form of IAA from an HPLC column (data not shown). GC-MS analysis of the IAA fraction resulted in a single peak (Fig. 7A) with a spectrum (Fig. 7B) identical with that of an IAA standard (Fig. 7C).

DISCUSSION

Accumulation of IAA in the developing kernel can be divided into three phases: (a) the period of vascular development of the cob (0–15 DAP), (b) kernel filling (15–45 DAP), and (c) desiccation and dormancy induction (45+ DAP). During the first 10 DAP, IAA is present in the kernel in quantities greater than that typical of vegetative tissues (445 ng g^{-1} fresh weight versus 1–50 ng g^{-1} fresh weight; Bandurski and Schulze, 1977). IAA conjugate accumulation does not begin until about 10 DAP, but the reason for this delay in IAA ester accumulation is not known. We hypothesize that IAA from the kernel has a physiological role in the development of the cob similar to that observed for the strawberry receptacle (Nitsch, 1950) in that the cob is a rachis inflorescence and is morphologically analogous to the receptacle of a strawberry. This view is supported by the fact that cultured endosperm cells secrete IAA into the culture medium and, by analogy, we presume, the intact endosperm would secrete IAA into the cob tissue supporting the kernels.

Table I. *Levels of free and total IAA in Z. mays endosperm cells in suspension culture at various ages after subculturing*

Days after Subculture	ng flask^{-1}		ng g^{-1} fresh wt		ng g^{-1} dry wt	
	Free	Total	Free	Total	Free	Total
2	816 ± 290	2349 ± 169	73 ± 17	204 ± 42	1026 ± 38	2943 ± 157
4	1143 ± 42	7278 ± 487	64 ± 8	434 ± 36	1032 ± 74	6726 ± 421
6	865 ± 249	10542 ± 3318	40 ± 15	469 ± 103	754 ± 219	9190 ± 2881
8	890 ± 163	11704 ± 714	43 ± 9	568 ± 33	813 ± 195	11028 ± 294
10	656 ± 93	6018 ± 421	35 ± 6	312 ± 38	597 ± 123	5458 ± 738

Table III. *Conversion of [^{14}C]anthranilate to [^{14}C]IAA by homogenates of Z. mays endosperm from kernels 20 to 30 DAP*

dpm in the IAA-containing fraction following incubation for 12 and 24 h.

Sample No.	Incubation Time	
	12 h	24 h
	dpm[a]	*dpm*[a]
1	17,850 (3.5)[b]	29,891 (4.9)
2	11,200 (2.0)	42,292 (7.6)
3	20,050 (3.5)	28,053 (6.4)
4		42,539 (7.9)
5		40,997 (7.6)

[a] Value for boiled controls has been subtracted from these results. [b] Value in parentheses is percentage of applied ^{14}C converted to IAA. The actual amount of ^{14}C applied was measured at the time of sample extraction.

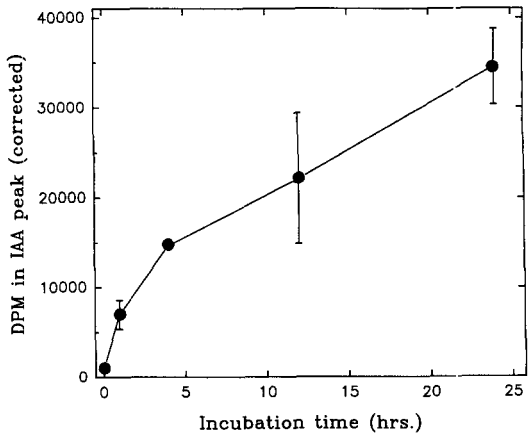

Figure 6. Time course of labeled IAA synthesis when [^{14}C]anthranilate is incubated with homogenized endosperm from *Z. mays* kernels 24 DAP. Vertical bars indicate the SE (n = 3).

In contrast to the time course observed for IAA, cytokinins reach maximum levels during early stages of development approximately 10 DAP (Morris et al., 1993). At this early stage, rapid cell division is occurring in the endosperm. By the time IAA begins to accumulate, cell division has ceased and growth occurs through cell expansion, an effect that is usually attributed to IAA. Beginning with the period of rapid cell expansion, large amounts of ester IAA appear in the kernels, and the level of total IAA in the developing kernel increases as the kernel matures. In vegetative tissues of *Z. mays* seedlings and other plants, approximately 88% of the total IAA exists as esters and the remainder as free IAA (Bandurski and Schulze, 1977). In contrast, while the kernel is rapidly growing, esters of IAA account for 97 to 99% of the total IAA.

The different ratios of ester to free IAA in seedlings and developing kernels is noteworthy. The large difference suggests that alternative control mechanisms operate in the kernel and seedling. Figure 8 represents our working hypothesis regarding the pathways by which the concentration of free IAA might be regulated. We propose that in the developing kernel the concentration of free IAA is regulated only by the rate constants k_0, k_1, and k_2. This hypothesis fits the theoretical and experimental results observed in the developing kernel. The levels of indol-3-acetyl-1-*O*-β-D-Glu synthase, the first enzyme involved in conjugate formation, mirrors that of total IAA on a per kernel basis in developing kernels (Pawlak and Bandurski, 1991). It seems likely that production of IAA is regulated by as yet unknown means and it is the availability of free IAA that regulates the levels of indol-3-acetyl-1-*O*-β-D-Glu synthase and other enzymes involved in conjugation. In seedlings all of the rate constants, k_0 through k_6, would be involved in regulating free IAA levels, thus leading to a difference in the ester to free IAA ratios in developing kernels versus seedlings. To summarize, the ratio of ester to free IAA in the developing kernel is regulated by conjugation (k_1 versus k_2). and the amount of total IAA is determined by the rate constant k_0. An important question remaining to be answered is, what regulates k_0?

Table IV. *Comparison of the IAA biosynthetic rates calculated for several in vitro corn endosperm systems to that of the intact kernel*

Tissue	Rate	Activity[a]
	ng g^{-1} fresh wt h^{-1}	%
Intact kernel	190	100
In vitro kernel	99	52
Endosperm suspension culture	9.2	4.8
Homogenized endosperm	8.6	4.5

[a] Activities are normalized to that of the intact kernel.

IAA Levels in Culture Systems

Stable suspension cultures derived from maize endosperm have proven useful for the study of endosperm physiology

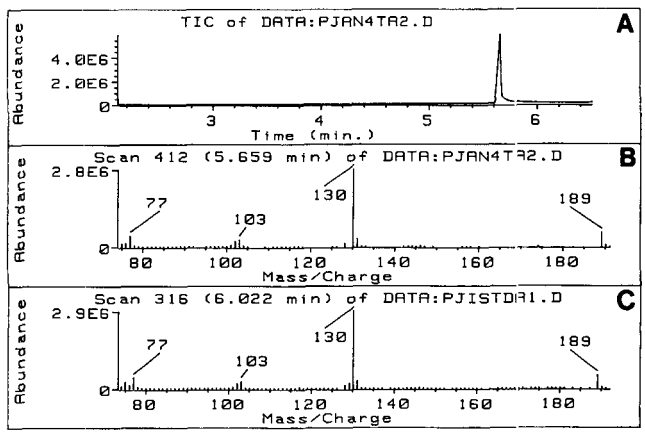

Figure 7. A, GC total ion chromatogram (TIC) of HPLC fraction containing the methylated IAA purified from homogenized endosperm incubated with [^{14}C]anthranilic acid. B, Mass spectrum of the single peak observed in A. C, Mass spectrum of methylated IAA.

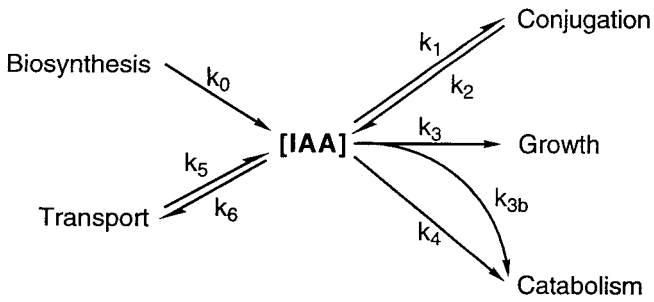

Figure 8. Inputs and outputs to the pool of free IAA in plant tissues. The symbols k_0 through k_6 represent the rate constants for the indicated reactions. The concentration of free IAA is determined by the rate constants for inputs and outputs operating in the system being examined.

and biochemistry (Shannon, 1982). A graph of IAA levels in endosperm cultures is very similar to that for IAA levels in developing kernels (Jensen and Bandurski, 1991) (Fig. 1A; Table I); therefore, the cultured cells appear to retain the characteristics of the intact kernel with respect to IAA dynamics. In the developmental study the whole seed was assayed; however, it has been shown that only trace amounts of IAA occur in the embryo and scutellum. Although IAA biosynthetic capacity is only 4.8% of that of the intact kernel on a per gram fresh weight basis, this is sufficient to make cultured endosperm cells a suitable system for the study of IAA metabolism. A major difference between intact kernels and the cultured cells is the percentage of IAA in the ester form. In the developing kernels, ester IAA is maintained at approximately 98% of the total for most of the developmental period (Fig. 4). In the suspension cultures, the ester IAA accounts for approximately 65% of the total initially and then varies between 88 and 92% of the total IAA for the remainder of the culture cycle. The percentage of ester after 4 d is similar to that in vegetative tissue. This suggests that in the cell-suspension cultures all of the rate constants k_0 through k_6 from Figure 8 are involved in regulating the free IAA concentration, in contrast to that proposed for the kernel. This may be the result of IAA being utilized during cell division. In the kernel cell division stops approximately 10 DAP. This difference in ratios, however, has no impact on the usefulness of this system in examining the biosynthetic pathway because of the large amount of IAA produced by the in vitro grown cells. When studying regulation, however, there is clearly a difference in how the two systems regulate IAA levels. With the in vitro grown kernels the higher proportion of free IAA may be a consequence of growing in culture or the different variety of maize used.

Corn Endosperm as a Model System for Studying IAA Biosynthesis

When one evaluates the physiological importance of any proposed IAA biosynthetic pathway, the ability of the pathway to satisfy the needs of the plant must be determined. The pool sizes of the potential precursors and products should be determined so that the relative importance of a compound as a precursor can be determined. There is a vast amount of data demonstrating the conversion of Trp to IAA (for reviews, see Schneider and Wightman, 1974; Marumo, 1986), but the relative importance of this conversion has not been demonstrated. In a recent report, Baldi et al. (1991) grew *Lemna* on media containing L-[^{15}N]Trp and demonstrated the appearance of ^{15}N in IAA only when the level of Trp was 400 to 1600 times that of the normal pool size. When D-[^{15}N]Trp was fed, no conversion was observed at any feeding level. On the basis of pool size and the amount of labeled IAA produced they concluded that Trp was not a significant precursor of IAA in *Lemna*. In most in vitro experimental systems studied thus far, less than 0.05% of the applied radioactive precursors were converted to IAA. Using the in vitro homogenized corn endosperm, we observed conversion rates of up to 8%.

Many of the early studies of IAA biosynthesis did not use rigorous methods to positively identify the metabolite(s) of interest. As a result, conclusive proof that the radioactivity associated with that metabolite is actually due to labeled metabolite has often not been presented. When one uses radioactive precursors, the product(s) first needs to be purified to a constant specific activity to ensure that the radioactivity is associated with a specific peak. The compound must then be positively identified by mass spectral or some other qualitative analysis. By adding [^{3}H]IAA to our sample during extraction and then purifying to a constant ^{3}H/^{14}C ratio, we conclude that we have indeed observed the metabolic conversion of [^{14}C]anthranilic acid to [^{14}C]IAA. Mass spectral analysis provided the final proof as to the identity of the ^{14}C-labeled IAA.

The Kernel as the Site of IAA Production

The seed as a site of auxin production has been proposed for many years (Nutman, 1939; Muir, 1942) but has largely been ignored as a tissue for use in IAA biosynthesis experiments. Hatcher (1943), working with rye, concluded that the grain was the site of auxin production based on the fact that prematurely harvested grains continued to accumulate auxin. We believe the evidence in this paper strongly supports the contention that the large amount of IAA appearing in the kernel is produced within the endosperm. This conclusion is based on the following data. First, in vitro cultured kernels were able to accumulate large amounts of IAA. Since only inorganic salts, Suc, thiamine, and Asn are present in the media, the increase in the levels of IAA is direct proof that de novo synthesis has occurred in situ.

Second, the biosynthetic rate observed for the homogenized endosperm was 4.5% of the intact kernel and is thus a substantial fraction of the in situ rate (Table IV). This value was determined under assay conditions of limited substrate availability and with no correction for isotope dilution and is, thus, an underestimate of the actual biosynthetic rate.

Contrast between a Monocotyledonous and a Dicotyledonous Plant

It has been reported by Bialek and Cohen (1989) that during bean seed development the level of total IAA on a

per seed basis remains fairly constant, while the percentages of free, ester, and amide IAA change. This is different from conclusions presented here for maize. A possible explanation for the lack of change in the amount of total IAA in bean seeds could be that the IAA is produced elsewhere in the plant and then transported to the seed. For example, in another dicotyledonous plant, a study of the location of indole alkaloid biosynthesis in *Ipomoea* concluded that alkaloids were produced in the leaves and then transported to the seed (Mockaitis et al., 1973). We believe that in maize the indole ring is produced in the kernel, and this may be an intrinsic difference between monocots and dicots. More recently, Bialek et al. (1992) showed that de novo synthesis provides the IAA for the developing bean seedling. In this system, IAA conjugates in the seed have little or no role in supplying IAA for the seedling (Bialek et al., 1992). Again, this is different from our findings for maize seedlings in which there is no de novo biosynthesis and the seedling is totally reliant on IAA from conjugates stored in the seed (Jensen and Bandurski, 1990). This may explain the large difference in total IAA found in bean versus corn seeds (170 ng/seed versus >160,000 ng/seed, respectively).

Maize Endosperm as a System for Study of IAA Biosynthesis

Because of the high rates of synthesis, the stability of the biosynthetic system to freezing, the known rates of in situ synthesis, and the availability of unlimited amounts of enzymatic material, we suggest that the endosperm of *Z. mays* is a suitable material for study of the mechanism(s) of IAA biosynthesis.

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