Characterization of Auxin Conjugates in Arabidopsis. Low Steady-State Levels of Indole-3-Acetyl-Aspartate, Indole-3-Acetyl-Glutamate, and Indole-3-Acetyl-Glucose[1]

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Amide-linked indole-3-acetic acid (IAA) conjugates constitute approximately 90% of the IAA pool in the dicot Arabidopsis, whereas ester-linked conjugates and free IAA account for approximately 10% and 1%, respectively when whole seedlings are measured. We show here that IAA-aspartate Asp, IAA-glutamate (Glu), and IAA-glucose (Glc) are present at low levels in Arabidopsis. Nine-day-old wild-type Arabidopsis seedlings yielded 17.4 ± 4.6 ng g^{-1} fresh weight IAA-Asp and 3.5 ± 1.6 ng g^{-1} fresh weight IAA-Glu, and IAA-Glc was present at 7 to 17 ng g^{-1} fresh weight in 12-d-old wild-type seedlings. Total IAA content in 9-d-old Arabidopsis seedlings was 1,200 ± 178 ng g^{-1} fresh weight, so these three IAA conjugates together made up only 3% of the conjugate pool throughout the whole plant. We detected less than wild-type levels of IAA-Asp and IAA-Glu (7.8 ± 0.4 ng g^{-1} fresh weight and 1.8 ± 0.3 ng g^{-1} fresh weight, respectively) in an Arabidopsis mutant that accumulates conjugated IAA. Our results are consistent with IAA-Asp, IAA-Glu, and IAA-Glc being either minor, transient, or specifically localized IAA metabolites under normal growth conditions and bring into question the physiological relevance of IAA-Asp accumulation in response to high concentrations of exogenous IAA.

Plants maintain free indole-3-acetic acid (IAA) levels through a complex network of environmentally and developmentally responsive pathways that achieve IAA transport, degradation, conjugation, conjugate hydrolysis, and, sometimes, conversion to indole butyric acid (for review, see Normanly, 1997; Cohen and Slovin, 1999; Normanly and Bartel, 1999). IAA conjugation is a ubiquitous process in both higher and lower land plants (Cohen and Bandurski, 1982; Sztein et al., 1995, 1999), and IAA-conjugates form rapidly when IAA homeostasis is perturbed, either from applied IAA or in mutant or transgenic lines in which IAA synthesis is de-regulated. For example, the maize Trp synthase β double mutant *orange pericarp* has 51-fold higher levels of IAA-conjugates than wild type (Wright et al., 1991), and the Arabidopsis conditional Trp auxotroph *trp2-1* (also a Trp synthase β mutant) accumulates 38-fold higher levels of conjugated IAA when grown in non-permissive conditions (Normanly et al., 1993). Other examples include the Arabidopsis *sur1* mutant, which is defective in IAA homeostasis and accumulates free and conjugated IAA (Boerjan et al., 1995; King et al., 1995), and transgenic tobacco overexpressing microbial IAA biosynthetic genes that accumulate IAA conjugates (Sitbon et al., 1992).

The role of IAA-conjugates is still incompletely defined, but the conjugated moiety may well dictate the metabolic fate of the attached IAA; either release from the conjugate or oxidation and likely other forms of metabolism that have yet to be determined (for review, see Normanly, 1997). The enzymes responsible for amide-linked IAA conjugate formation have yet to be isolated, but considerable progress has been made in the isolation and characterization of enzymes and corresponding genes for ester-linked IAA conjugate formation and IAA conjugate hydrolysis (Szerszen et al., 1994; Bartel and Fink, 1995; Bandurski et al., 1998; Davies et al., 1999). IAA-amino acid hydrolases in Arabidopsis are encoded by a gene family with at least six members. These conjugate hydrolases have different amino acid specificities both in vivo and in vitro when tested with a battery of IAA-amino acid conjugates (Bartel and Fink, 1995; Davies et al., 1999). They are also differentially expressed, implying that a variety of IAA conjugates exist and serve different roles at varied locations throughout the plant. Determination of the native substrates for these enzymes will be an important component to defining their role in IAA homeostasis. The IAA conjugates have not been quantified in Arabidopsis, although labeling studies have revealed that IAA-Asp and IAA-Glu are early metabolites that form in response to micromolar concentrations of exogenous IAA (Östin et al., 1998; Barratt et al., 1999). Labeling studies with less exogenous IAA (0.5 μM) do not yield these conjugates, instead IAA is converted to OxIAA and OxIAA-

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Table I. *IAA analysis in 9- and 12-d-old wild-type and trp2-1 Arabidopsis*

Values represent means ± SE of three replicates except where indicated.

Arabidopsis Line (Age in Days Post Germination)	Free	Ester-Linked[a]	Total[b]	IAA-Asp	IAA-Glu	IAA-Glc
			ng g^{-1} fresh wt			
Wild type (9)	12 ± 3	91 ± 38	1,200 ± 178	17.4 ± 4.6	3.5 ± 1.6[c]	n.d.[d]
trp2-1 (9)	30 ± 9	180 ± 81	1,753 ± 346	7.8 ± 0.4	1.8 ± 0.3[e]	n.d.
Wild type (12)	2.1 ± 0.2[e]	35 ± 6	373 ± 81	n.d.	n.d.	12[f]

[a] Ester-linked includes free IAA. [b] Total includes free, ester-linked, and amide-linked IAA. [c] Five replicates. [d] n.d., Not determined. [e] Four replicates. [f] Two replicates.

hexose (Östin et al., 1998). We quantified IAA-Asp, IAA-Glu, and IAA-Glc in wild-type Arabidopsis and a conjugate-accumulating mutant, *trp2-1*. These conjugates were present at very low levels in wild type, and IAA-Asp and IAA-Glu levels decreased in the *trp2-1* mutant.

RESULTS

Free, ester-linked, and total IAA were measured in 9-d-old Arabidopsis, both in wild type and in the Trp synthase β conditional mutant *trp2-1* (Table I). The same measurements were also made in 12-d-old wild-type Arabidopsis (Table I). The values for ester-linked and total IAA have been corrected for the conversion of indole-3-acetonitrile to IAA during alkaline hydrolysis (Ilic et al., 1996). The values obtained for free IAA levels were in agreement with those from previous work (Normanly et al., 1993). The total IAA values were generally consistent with those of previous work (Normanly et al., 1993, 1997; Ilic et al., 1996), but did vary more than the free IAA values. We attribute this to the increased complexity of the method for total IAA analysis and the fact that the propagation methods used in this study were significantly different from the previous method. Here seedlings were propagated hydroponically, whereas in previous experiments seedlings were grown on solidified agar medium. The composition of the growth medium affects the physiological state of the seedlings as well. We noticed significant differences in growth rate with the two types of liquid medium that were used to culture the 9- and 12-d-old seedlings used in this study (see "Materials and Methods").

The *trp2-1* mutant was previously shown to accumulate conjugated IAA, in amounts dependent on the growth conditions (Normanly et al., 1993). In high light (approximately 100 μE m^{-2} s^{-1}) IAA conjugate levels in this mutant can be as much as 38-fold above wild type, whereas the low-light conditions that we used here resulted in approximately 1.5-fold accumulation of IAA conjugates (Table I). IAA-Asp and IAA-Glu were purified separately from 9-d-old wild-type and *trp2-1* tissue and IAA-Glc was purified from 12-d-old wild-type tissue. Figures 1 and 2 show the full scan spectra of Arabidopsis extracts to which [$^{13}C_6$]IAA-Asp and [$^{13}C_6$]IAA-Glu, respectively, had been added. The molecular ions and major fragment ions for methylated [$^{13}C_6$]IAA-Asp were *m/z* = 324 and *m/z* = 163 and 136, respectively, whereas the molecular ion and major fragment ions for methylated endogenous IAA-Asp were *m/z* = 318 and *m/z* = 157 and 130, respectively. The molecular ions and major fragment ions for methylated [$^{13}C_6$]IAA-Glu were *m/z* = 338 and *m/z* = 163 and 136, respectively,

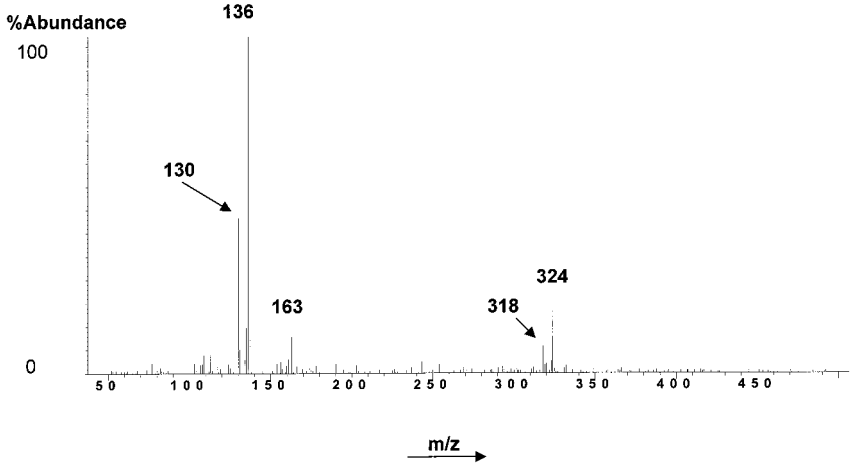

Figure 1. The 70-eV electron impact full scan mass spectrum from *m/z* 50 to 510 of methyl [$^{13}C_6$]IAA-Asp (internal standard) and methyl IAA-Asp isolated from Arabidopsis.

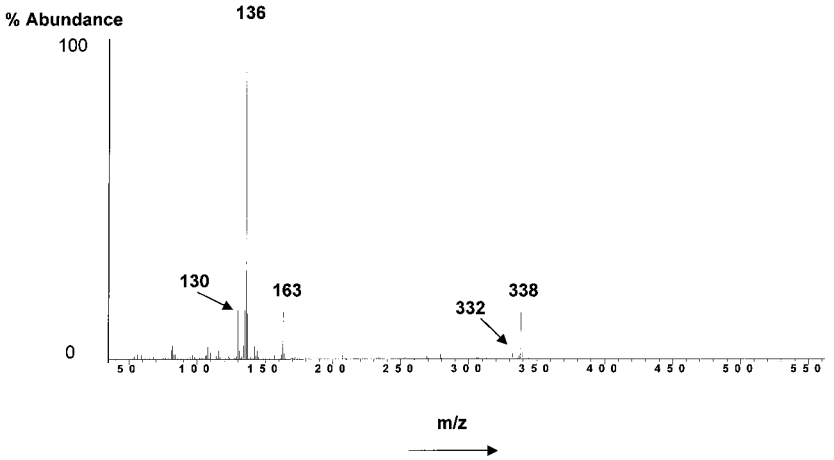

Figure 2. The 70-eV electron impact full scan mass spectrum from *m/z* 50 to 550 of methyl [$^{13}C_6$]IAA-Glu (internal standard) and methyl IAA-Glu isolated from Arabidopsis.

whereas the molecular ion and major fragment ions for methylated endogenous IAA-Glu were *m/z* = 332 and *m/z* = 157 and 130, respectively. Figure 3 is the full scan spectra of acetyl IAA-Glc isolated from Arabidopsis (no standard added). The molecular ion and major fragment ion for acetyl [$^{13}C_6$]IAA-Glc were *m/z* = 511 and 136, respectively, and the molecular ion and major fragment ion for acetylated endogenous IAA-Glc were *m/z* = 505 and *m/z* = 130, respectively. The molecular ions and major fragment ions corresponding to IAA-Asp and IAA-Glu were present in good abundance (Figs. 1 and 2). For IAA-Glc analysis we did not see significant isomerization from 1-*O*-IAA-Glc to 4-*O*- or 6-*O*-IAA-Glc during preparation or storage, and thus monitored 1-*O*-IAA-Glc by gas chromatography (GC)-selected ion monitoring-mass spectroscopy (MS). Calibration plots (Tam and Normanly, 1998) of [$^{13}C_6$]IAA-Asp and [$^{13}C_6$]IAA-Glu revealed a linear relationship between peak area ratio and molar ratio when the molar ratio between $^{13}C_6$-labeled standard and the corresponding endogenous compound ranged from 5:1 to 50:1 for IAA-Asp and 10:1 to 100:1 for IAA-Glu (data not shown). Therefore, the molecular ions and major fragment ions of the $^{13}C_6$-labeled compounds are reliable internal standards and can be used in the isotope dilution equation (Cohen et al., 1986) to calculate endogenous levels of IAA-Asp and IAA-Glu. Table I summarizes the levels of IAA-Asp and IAA-Glu in wild-type and *trp2-1* seedlings. IAA-Glc was analyzed in wild-type seedlings (12-d-old; Table I) that had been grown in a different liquid medium than the 9-d-old seedlings.

DISCUSSION

Free IAA and IAA conjugates with a single amino acid or sugar moiety are readily extracted from plant tissue with alcohol, acetone, or ethyl ether, and most IAA analysis procedures incorporate one of these organic solvents in the extraction protocol (Sandberg et al., 1987). Mild alkaline conditions hydrolyze IAA from ester-linked conjugate moieties, whereas stronger alkaline conditions at high temperature release IAA from amide-linked conjugate moieties (Slovin et al., 1999). In Arabidopsis, the measurement of ester-linked and amide-linked IAA is complicated by the presence of large amounts of IAN, which is nonenzymatically converted to IAA under even mild alka-

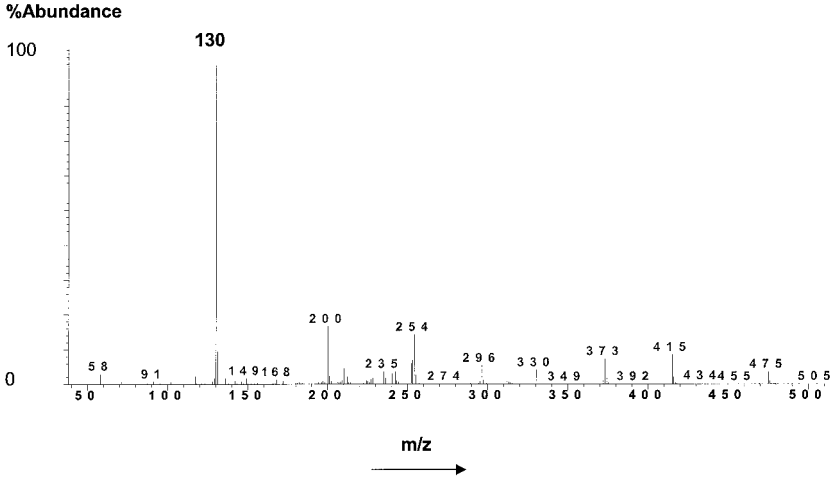

Figure 3. The 70-eV electron impact full scan mass spectrum from *m/z* 50 to 510 of acetyl IAA-Glc isolated from Arabidopsis (no internal standard added).

line conditions. A method to correct for IAN has been devised (Ilic et al., 1996) but it increases the complexity of the entire IAA analysis procedure for Arabidopsis. Knowing the identity of the IAA-conjugates in Arabidopsis would circumvent this added step, because these conjugates could be purified and quantified without employing alkaline hydrolysis.

IAA conjugates have been identified in a number of species and soybean and maize seeds have been the most extensively studied (Slovin et al., 1999). The entire complement of IAA conjugates in vegetative tissue has not been determined for any plant species. Labeling studies from a variety of dicots including Arabidopsis indicate that IAA-Asp and IAA-Glu are likely to be ubiquitous amide conjugates (Slovin et al., 1999). Arabidopsis has some ester-linked conjugates (approximately 10% of the total IAA pool) and IAA-Glc is a likely constituent of this pool.

From labeling studies in a variety of plant species, IAA-Asp appears to be a precursor to catabolic pathways (for review, see Normanly, 1997). Additionally, a cell line of *Hyoscyamus muticus* (henbane) with an apparent defect in IAA-Asp formation is temperature sensitive, but not IAA deficient, implying that IAA-Asp is required for a function other than as a source of free IAA (Oetiker and Aeschbacher, 1997). IAA-Asp formation has been shown to be auxin-inducible in various plant species (Venis, 1972; Slovin et al., 1999), and Arabidopsis appears to have this pathway as well. Arabidopsis rapidly converts exogenous IAA (1–500 μM) to IAA-Glu and IAA-Asp (Östin et al., 1998; Barratt et al., 1999). Interestingly, lower concentrations of exogenous IAA are preferentially converted to OxIAA and OxIAA-hexose (Östin et al., 1998). Using $^{13}C_6$-labled IAA-Asp, IAA-Glu, and IAA-Glc as internal standards, we found that IAA-Asp, IAA-Glu, and IAA-Glc are indeed native compounds in Arabidopsis (Table I). IAA-Glc represents approximately 34% of the ester-linked pool (measured in whole seedlings), whereas IAA-Asp and IAA-Glu combined represent a little over 2% of the amide conjugate pool in whole seedlings of wild-type Arabidopsis. We expected that IAA-Asp and IAA-Glu should make up a significant portion of the IAA conjugates that accumulate in the *trp2-1* mutant. We were surprised to find that under growth conditions that resulted in approximately 1.5-fold accumulation of amide-linked conjugates, IAA-Asp and IAA-Glu combined represented only 1% of the amide conjugate pool, one-half the amount in wild type. We cannot rule out the possibility that these conjugates accumulate locally and in a manner that would go undetected in whole seedling analysis. It would appear, however, that the conjugation pathway that is activated by high concentrations of exogenous IAA (Barratt et al., 1999) is not being utilized in the *trp2-1* mutant, thereby bringing into question the physiological relevance of this pathway. Under normal growth conditions, IAA-Asp and IAA-Glu may be very short-lived metabolites, or they could be localized to a discrete region of the plant. Additionally, the levels of these conjugates may fluctuate over the life span of the plant.

Since IAA-Glu, IAA-Asp, and IAA-Glc are present in such low amounts, approximately 98% of the amide-linked IAA conjugates in Arabidopsis remain unidentified. Arabidopsis has large amounts of indole-3-methyl glucosinolate (Haughn et al., 1991), but this compound is not converted to IAA upon base treatment (Ilic et al., 1996) and so it does not contribute to the IAA conjugate pool that is quantified by way of base hydrolysis. It is formally possible that there are as-yet-unidentified indolic compounds that are not IAA conjugates, but that like IAN are converted nonenzymatically to IAA upon base treatment. However, the multiplicity of IAA conjugate hydrolases in Arabidopsis, with varying amino acid specificities (Davies et al., 1999) is suggestive that more amide-linked IAA conjugates exist in this plant.

IAA-peptides have been identified as the major amide conjugates in bean (Bialek and Cohen, 1986), and polyclonal antibodies to the peptide moiety of one of these conjugates cross-react with proteins from Arabidopsis (J.D. Cohen, personal communication). Measurement of bulk amide-linked IAA in protein fractions of Arabidopsis is complicated by the need to accurately quantify and subtract IAN from these fractions. Initial attempts to measure amide-linked IAA in ammonium sulfate fractions and SDS-treated fractions in Arabidopsis produced highly variable results (data not shown), so it will be necessary to isolate protein fractions that are free of IAN and then test for the presence of amide-linked IAA. Similar work is ongoing with bean (J.D. Cohen, personal communication), and antibody to the bean peptides may prove useful in the purification of IAA-peptides in Arabidopsis.

MATERIALS AND METHODS

Chemicals

The internal standard for IAA-Asp analysis was a mixture of [$^{13}C_6$]IAA-Asp and [^{3}H]IAA-Asp (50 μCi/μmol). Similarly, the internal standard for IAA-Glu analysis was a mixture of [$^{13}C_6$]IAA-Glu and [^{3}H]IAA-Glu (50 μCi/μmol). Both were gifts from Jerry Cohen (for synthesis description, see Cohen, 1981). HPLC grade solvents were from VWR (Bridgeport, NJ). C_{18} PrepSep columns were purchased from Fisher Scientific (Pittsburgh). Amino columns and resin were from J&W Scientific (Folsom, CA). Sephadex LH-20 and [^{3}H]IAA (26 Ci/mmol) were obtained from Amersham-Pharmacia Biotech (Piscataway, NJ). [$^{13}C_1$]IAN was synthesized by Nebosja Ilic as described previously (Ilic et al., 1996). [$^{13}C_6$]IAA was from Cambridge Isotope Labs (Cambridge, MA).

Plant Material

Wild-type Arabidopsis ecotype Columbia and the *trp2-1* mutant (Last and Fink, 1988) were used as analytical plant tissue. For analysis of 9-d-old seedlings, seeds were surface sterilized for 15 min in 20% (v/v) commercial bleach, 0.001% (w/v) SDS, then washed five times with sterile water, and placed in a 250-mL flask containing 80 mL of liquid plant nutrient medium with Suc (Haughn and Somerville, 1986). Seedlings were grown under continuous illumination (30 μE m^{-2} s^{-1}) from cool-white fluorescent lamps at room temperature and with gentle shaking (75–100 rpm). Tissue was harvested, gently blotted dry, weighed, frozen in liquid nitrogen, and stored at −80°C. For analysis of 12-d-old seedlings, wild-type Arabidopsis seeds were surface sterilized and rinsed as above and placed in 125-mL flasks containing 50 mL of sterile Murashigi and Skoog salts medium (Life Technologies/Gibco-BRL, Grand Island, NY) with 1% (w/v) Suc. The seedlings were grown for 12 d under cool-white fluorescent lights (25 μE m^{-2} s^{-1}) on a shaker (100 rpm).

Preparation of Isotope-Labeled IAA-Glc Internal Standard

The liquid endosperm of sweet corn (*Zea mays* [L.] cv Silver Queen, obtained from a local market at the table ready stage, approximately 25–30 d after pollination) was collected by first cutting the rows of kernels with a razor blade, then expelling the kernel contents by pressing the kernels against the rim of a prechilled beaker. The liquid endosperm tissue was squeezed through two layers of cheesecloth and the resulting liquid was frozen in liquid nitrogen and stored at −80°C for later use. To isolate IAA-Glc synthetase activity, 50 mL of 50 mM Tris (Tris[hydroxymethyl]-aminomethane)-HCl, pH 7.6, was added to 60 g of frozen corn endosperm that had been thawed on ice, $(NH_4)_2SO_4$ to 85% saturation (610 g L^{-1}) was added while stirring with pH adjustment to 7.6. The preparation was centrifuged at 10,000*g* for 10 min and the resulting pellet suspended in 0.01 M Tris-HCl buffer, pH 7.1. The sample was dialyzed against water at 3°C overnight with constant stirring. Following dialysis the dialysis bag was placed in a dry beaker and covered with anhydrous flake polyethylene glycol (M_r 20,000, Aquacide III, Calbiochem, La Jolla, CA) for 5 h. The concentrated solution was centrifuged at 10,000*g* for 10 min and the supernatant, which contained the IAA-Glc synthetase activity, was used to produce the [^{3}H]/[$^{13}C_6$]IAA-Glc standard (Michalczuk and Chisnell, 1982). The reaction was carried out by mixing 0.5 mL of the enzyme preparation, 0.3 mL of buffer containing 50 mM Tris-HCl, 8.3 mM $MgCl_2$, 8.3 mM glutathione, pH 7.6, 10 mg of uridine-5-diphospho-Glc (Sigma-Aldrich, St. Louis), 100 μg of [$^{13}C_6$]IAA, and 25 μCi of [^{3}H]IAA. The reaction was incubated for 24 h at 35°C and then 2 mL of isopropanol was added to stop the reaction. The reaction product was purified on a 1- × 30-cm column of Sephadex LH-20, equilibrated, and run in 50% (v/v) isopropanol/water. The quantity of IAA-Glc in the pooled fractions was determined by hydrolyzing an estimated 250 ng of [$^{13}C_6$]/[^{3}H]IAA-Glc in the presence of 250 ng of IAA in 4 mL of 1 N NaOH for 1 h at room temperature. Following hydrolysis, the sample was brought to pH 2.5 and purified by C_{18}-HPLC, methylated, and analyzed by GC-MS in the selected ion mode. The ion intensities at *m/z* 136 and 130 were used to calculate the amount of [$^{13}C_6$]/[^{3}H]IAA-Glc relative to the peak of unlabeled IAA standard.

IAA-Glc Analysis

Arabidopsis seedlings (10–20 g) were extracted with 60% (v/v) isopropanol, 40% (v/v) 200 mM imidazole buffer, pH 7.0, containing 200 ng of [$^{13}C_6$]/[^{3}H]IAA-Glc. The sample was purified using ethyl acetate partitioning at pH 2.5 to remove lipids and organic acids, followed by chromatography on a 1.5- × 45-cm column of Sephadex LH-20 run in 50% (v/v) isopropanol/water. IAA-Glc eluted between 46 and 52 mL. The sample was then adjusted to pH 7, brought to dryness, and acylated at 60°C for 1 h using a 1:1 mixture (v/v) of acetic anhydride and 1% (w/v) dimethylaminopyridine in pyridine (Chisnell, 1984). The solvents were removed by rotary evaporation and the acylated IAA-Glc was dissolved in 200 μL of 10% (v/v) acetonitrile/water. The sample was purified by C_{18} HPLC on a 25-cm × 4.6-mm UltraSphere column (Phenomenex, Torrance, CA) using a programmed linear gradient of 10% (v/v) acetonitrile/water to 100% (v/v) acetonitrile over a 1-h period. Under these conditions the acylated IAA-Glc had a retention volume of 44 to 52 mL. The acylated IAA-Glc fractions were pooled, reduced to dryness, and re-suspended in 25 μL of ethyl acetate for GC-MS.

IAA-Asp and IAA-Glu Analysis

IAA-Asp and IAA-Glu were isolated according to a protocol similar to that described by Cohen (1982). Frozen plant tissue was ground with glass beads (150–212 μm; Sigma-Aldrich) in a mortar that had been chilled with liquid nitrogen. Four milliliters of extraction buffer (35% [v/v] 0.2 M imidazole, pH 7.0, and 65% [v/v] isopropanol) per gram fresh weight and internal standard (either a mixture of 0.27 μg of [$^{13}C_6$]IAA-Asp and 6 × 10^{-4} μCi of [^{3}H]IAA-Asp or 0.5 μg of [$^{13}C_6$]IAA-Glu and 0.012 μCi of [^{3}H]IAA-Glu) were added and allowed to equilibrate on ice for 1 h. Cellular debris was removed by centrifugation in a clinical centrifuge for 15 min at 4°C and the supernatant was collected. The pellet was washed with extraction buffer and centrifuged two times. The supernatants were pooled and the isopropanol was removed by rotary evaporation. The aqueous phase was adjusted to pH 3 with 2 N HCl. The sample was applied to an amino column (0.4 g resin g^{-1} fresh weight tissue) that had been conditioned with hexane, acetonitrile, and water. The column was washed with hexane, ethyl acetate, and acetonitrile and IAA-Asp or IAA-Glu was eluted with 3% (v/v) formic acid/isopropanol. The sample was reduced in volume by rotary evaporation to approximately 1 mL, applied to a Sephadex LH-20 column (2 × 40 cm), equilibrated, and run in 50% (v/v) isopropanol/water. IAA-Asp and IAA-Glu each eluted as single peaks at approximately 42.5 and 69

mL, respectively. The samples were dried to a residue by rotary evaporation and dissolved in methanol. Two volumes of ethereal diazomethane was added and immediately evaporated under a stream of oxygen-free nitrogen gas. The sample was dissolved in 50% (v/v) methanol/water and then centrifuged for 5 min at room temperature prior to HPLC purification. HPLC was performed on an LC system (model 5000, Varian, Palo Alto, CA) equipped with an UV detector set at 278 nm. The reversed-phase C_{18} column (50 × 4.6 mm, Ultracarb 5 ODS) was obtained from Phenomenex. The mobile phase was a linear gradient from 15% (v/v) methanol/water to 100% (v/v) methanol in 15 min, with a flow rate of 1 mL min^{-1}. The retention time for IAA-Asp and IAA-Glu was approximately 9 min. Fractions corresponding to [^{3}H]IAA-Glu were collected, dried to a residue by rotary evaporation, and dissolved in ethyl acetate. In a separate experiment, fractions corresponding to [^{3}H]IAA-Asp were collected, dried to a residue by rotary evaporation, dissolved in 50% (v/v) methanol/water, and subjected to HPLC a second time using the same conditions, except the flow rate was 0.7 mL min^{-1} with a linear gradient for the mobile phase from 30% (v/v) methanol/water to 75% (v/v) methanol/water in 15 min.

IAA Analysis

Steady-state levels of free, ester-linked (includes ester-linked and free IAA), and total IAA (includes free, ester-linked, and amide-linked) were determined as described by Chen et al. (1988). Fifty to 500 mg of plant material was used per sample and the internal standard was [$^{13}C_6$]IAA. Twenty-five nanograms of internal standard was added to extracts for free IAA analysis and 200 ng was used for ester and total IAA analysis. Approximately 75,000 dpm of [^{3}H]IAA was used as a tracer during purification. For ester and total IAA analysis, IAN levels were determined simultaneously as described by Ilic et al. (1996) using 250 ng of [$^{13}C_1$]IAN as an internal standard. HPLC purification was as described by Chen et al. (1988) using a C_{18} column (50 × 4.6 mm, Ultracarb 5 ODS, Phenomenex). The mobile phase was a 72% (v/v) solvent A (1% [v/v] acetic acid/water) and 28% (v/v) solvent B (100% [v/v] methanol). The retention time of IAA was approximately 6.5 min.

GC-MS Analysis

GC-MS analysis was carried out with a GC (model 6890, Hewlett-Packard, Palo Alto, CA) fitted with a DB-1701 capillary column (15-m × 0.25-mm i.d.; J&W Scientific) coupled to a mass selective detector (model 5973, Hewlett-Packard). The GC-MS was controlled by Chemstation software (Hewlett-Packard). For both IAA and IAN analysis the injector temperature was 280°C and the helium carrier gas was set at a flow rate of 2 mL min^{-1}. For IAA analysis the inlet pressure was initially 9.87 pounds per square inch (psi) and increased to 16.8 psi by the end of the program. The temperature program for the GC oven started at 140°C with a 2-min hold and increased 30°C min^{-1} to 260°C with a 2-min hold and then 30°C min^{-1} to 280°C with hold of 5 min at the end of the program. The molecular ions for methylated IAA, methyl [$^{13}C_1$]IAA, and methyl [$^{13}C_6$]IAA (*m/z* 189, 190, and 195, respectively) were monitored after electron impact ionization (70 eV). For IAN analysis the inlet pressure was initially 12.78 psi with a 2-min hold, then decreasing at a rate of 98 psi min^{-1} to 4.73 psi, and held constant for the duration of the program. The temperature program for the GC oven started at 140°C with a 2-min hold, then increased 30°C min^{-1} to 280°C with a final hold of 5 min. The molecular ions of silylated IAN and silylated [$^{13}C_1$]IAN (*m/z* 228 and 229, respectively) were monitored. For IAA-Asp and IAA-Glu analysis, the injector temperature was 280°C and the helium carrier gas was set at a flow rate of 0.8 mL min^{-1}. The inlet pressure held constant at 9.87. The temperature program for the GC oven started at 140°C with a 1-min hold, then increased 30°C min^{-1} to 260°C with a 3-min hold, and 30°C min^{-1} to 280°C with a 5-min hold at the end of the program. For methyl-IAA-Asp and methyl [$^{13}C_6$]IAA-Asp the molecular ions (*m/z* 318 and 324, respectively) were monitored along with the corresponding major fragment ions of *m/z* 130 and 157 for endogenous methyl IAA-Asp and *m/z* 136 and 163 for the methylated internal standard. The molecular ions for methyl IAA-Glu and methyl [$^{13}C_6$]IAA-Glu (*m/z* 332 and 338, respectively) were monitored along with the corresponding major fragment ions (same as for methyl IAA-Asp). For IAA-Glc analysis, the GC was fitted with a HP-5 column (0.21 mm × 30 m, Hewlett-Packard), the injector temperature was 280°C, and the column was initially at 70°C for 2 min, followed by a temperature program at 20°C min^{-1} to 280°C and a 5-min hold at 280°C. The molecular ions for acetyl IAA-Glc and acetyl [$^{13}C_6$]IAA-Glc were *m/z* 505 and 511, respectively, and the major fragment ions were *m/z* 130 and 136.

To correct for the natural abundance of ^{13}C in both the internal standards and the endogenous compounds that we were measuring, we derived the correction factor *R* as described (Cohen et al., 1986). For example, the *R* value for IAA-Asp was the ratio of corrected natural abundance for methylated IAA-Asp to the corrected natural abundance for methylated [$^{13}C_6$]IAA-Asp. The corrected natural abundance for methylated IAA-Asp was the ratio of ion abundance for a major fragment ion (*m/z* 130) to the ion abundance of adjacent ions of methylated IAA-Asp (*m/z* 130 + *m/z* 131 + *m/z* 132 + *m/z* 133 + *m/z* 134). Similarly, the corrected natural abundance for methylated [$^{13}C_6$]IAA-Asp was determined by the ratio of ion abundance for a major fragment ion of methylated [$^{13}C_6$]IAA-Asp (*m/z* 136) to the ion abundance of adjacent ions of the same compound (*m/z* 134 + *m/z* 135 + *m/z* 136+ *m/z* 137 + *m/z* 138). Once the *R* values were determined for each compound the isotope dilution equation described by Cohen et al. (1986) was used to calculate the levels of these compounds in Arabidopsis.

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