Biosci. Biotechnol. Biochem., **71** (8), 1946–1954, 2007

Facile Preparation of Deuterium-Labeled Standards of Indole-3-Acetic Acid (IAA) and Its Metabolites to Quantitatively Analyze the Disposition of Exogenous IAA in *Arabidopsis thaliana*

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Received March 15, 2007; Accepted May 13, 2007; Online Publication, August 7, 2007
[doi:10.1271/bbb.70151]

[2′,2′-$^2H_2$]-indole-3-acetic acid ([2′,2′-$^2H_2$]IAA) was prepared in an easy and efficient manner involving base-catalyzed hydrogen/deuterium exchange. 1-*O*-([2′,2′-$^2H_2$]-indole-3-acetyl)-β-D-glucopyranose, [2′,2′-$^2H_2$]-2-oxoindole-3-acetic acid, and 1-*O*-([2′,2′-$^2H_2$]-2-oxoindole-3-acetyl)-β-D-glucopyranose were also successfully synthesized from deuterated IAA, and effectively utilized as internal standards in the quantitative analysis of IAA and its metabolites in *Arabidopsis thaliana* by using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The use of this technique shows that these metabolites were accumulated in the roots of *Arabidopsis* seedlings. Dynamic changes in the metabolites of IAA were observed in response to exogenous IAA, revealing that each metabolic action was regulated differently to contribute to the IAA homeostasis in *Arabidopsis*.

Key words: *Arabidopsis thaliana*; indole-3-acetic acid; metabolism; internal standard; quantitative analysis

Indole-3-acetic acid (IAA, **1**) is an auxin, a plant hormone, which regulates various processes in plant growth and development. Its endogenous level is controlled by the relative rates of biosynthesis and metabolism, as well as by inter-cellular and/or -tissue transport. It is generally accepted that metabolism plays a key role in this regulation, because the total amounts of IAA metabolites found in plants are usually greater than those of free IAA.[1–4)]

To date, two major types of catabolic reaction have been demonstrated for IAA in plants (Fig. 1). One is conjugation with a variety of plant components. Forms of IAA bound with glucose (IAA-Glc, **2**), amino acids (IAA-Asp, **3**; IAA-Glu, **4**), cyclitol, glucan, and peptides have been described for various plant species.[4)] Some of these conjugates are thought to be storage forms that reversibly release free IAA depending on the need. The other is oxidation, typically represented by the conversion of IAA into 2-oxoindole-3-acetic acid (OxIAA, **5**).[4)] It has been proposed that **5** further undergoes conjugation[1)] and in this regard we recently identified 1-*O*-(2-oxoindole-3-acetyl)-β-D-glucopyranose (OxIAA-Glc, **6**) as the result of screening oxidized IAA metabolites in *Arabidopsis thaliana*.[5)] The homeostatic regulation of IAA level in a plant is most likely established by the coordinated control of these multiple conversions, although the level of each quantitative contribution remains unclear.

To fully understand the role of metabolism in the homeostasis of IAA, an accurate quantification of the levels of IAA and its metabolites in plant tissues is essential. Several methods have been developed for this purpose, based on mass spectrometry coupled with GC or LC,[6–11)] in which isotope-labeled preparations of IAA and IAA metabolites are used as the internal standards. While a standard for IAA is commercially available as [$^{13}C_6$]IAA ([$^{13}C_6$]-**1**) or [2,4,5,6,7-$^2H_5$]IAA (**1**-d_5), major IAA metabolites **3**–**5** have had to be derived from either of these two labeled preparations by chemical or enzymatic means.[9,10)] Thus, a sufficient and inexpensive supply of isotope-labeled IAA is needed for synthesizing the isotope-labeled metabolites of IAA. [2′,2′-$^2H_2$]IAA (**1**-d_2) is a suitable material for this purpose, since it can be easily prepared by base-catalyzed hydrogen/deuterium exchange at C-2′ of **1** by using an NaOD/D_2O solution.[12)] However the deuterium content of 96% reported in the literature is rather unsatisfactory, so we examined the reaction conditions to synthesize **1**-d_2 with a higher deuterium content.

We present in this paper a facile method for synthesizing IAA labeled with deuterium at the C-2′

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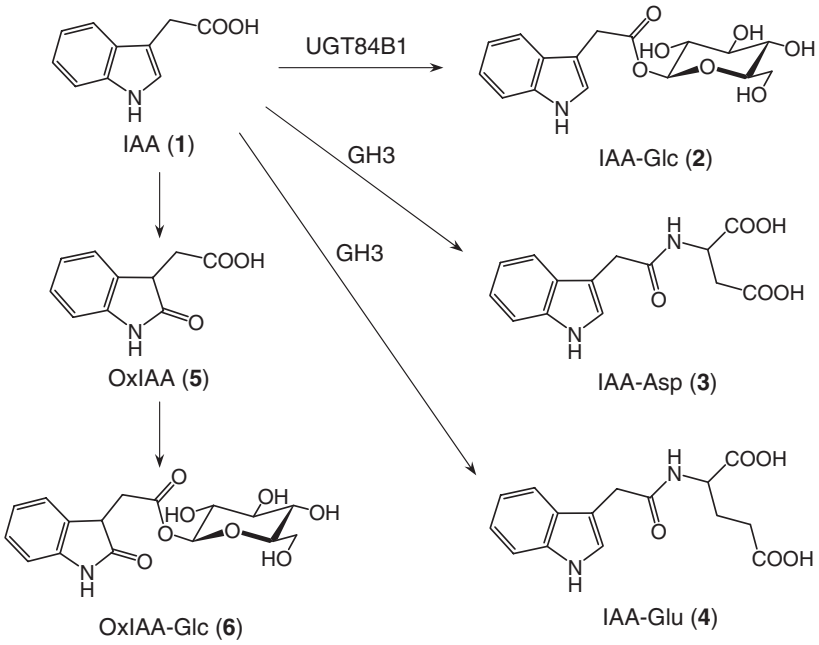

Fig. 1. Schematic Representation of the Metabolism of IAA (**1**) in *Arabidopsis* Seedlings. GH3, IAA-amino acid synthase; UGT84B1, IAA-Glc synthase.

position (**1**-d_2) based on a simple base-catalyzed proton exchange reaction. **1**-d_2 was converted into **2**-d_2, **5**-d_2, and **6**-d_2, and using these labeled preparations as internal standards, an LC-ESI-MS/MS-based method to quantify trace amounts of the major IAA metabolites in *Arabidopsis* was established. The method was used to monitor the influence of exogenous IAA on the endogenous levels of IAA metabolites in order to better understand the IAA homeostasis in plants.

Results and Discussion

*Synthesis of [2′,2′-$^2H_2$]IAA (**1**-d_2), [2′,2′-$^2H_2$]IAA-Glc (**2**-d_2), [2′,2′-$^2H_2$]OxIAA (**5**-d_2), and [2′,2′-$^2H_2$]OxIAA-Glc (**6**-d_2)*

IAA was stirred in a $NaOD/D_2O$ solution, and the replacement of the α-hydrogenes with deuterium was traced by monitoring the change in the signal intensity assigned to α-methylene protons in the ^{1}H-NMR spectrum. High isotopic purity (>99%) was obtained by 1.0 M $NaOD/D_2O$ for 4 days at 80 °C and in 2.0 M $NaOD/D_2O$ for 2 days at 80 °C. Other NMR signals of **1**-d_2 were identical with those of unlabeled IAA. The occurrence of degradation products of IAA was observed after 6 days, indicating that the longer reaction time was unfavorable. The reaction at higher temperature (>80 °C) with 2 M $NaOD/D_2O$, which has been employed in the literature,[12] was also inappropriate, causing a significant degradation of IAA, along with a decrease in isotopic purity. We conclude, therefore, that satisfactory deuteration of IAA was achieved under the milder conditions of 1.0 M $NaOD/D_2O$ for 4 days at 80 °C and 2.0 M $NaOD/D_2O$ for 2 days at 80 °C. Finally, the treatment of IAA with 1.0 M $NaOD/D_2O$ for 4 days at 80 °C afforded [2′,2′-$^2H_2$]IAA (**1**-d_2) in a 66% yield with >99% isotopic purity, this being used as the substrate in subsequent reactions. The use of a lower concentration of $NaOD/D_2O$ for a longer time (up to 4 days) was apparently effective for improving the isotopic purity, compared to the method reported in the literature.[12] To date, deuterated IAA has also been obtained by the hydrolysis of indole-3-acetonitrile by $NaOD/D_2O$ with 95% isotopic purity.[6] Compared to this, the method employed in the present study has an advantage in terms of the isotopic purity achieved.

[2′,2′-$^2H_2$]IAA-Glc (**2**-d_2) was synthesized from **1**-d_2 in accordance with the method of Schmidt *et al.* with some modifications (Fig. 2).[13] By using 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate as the glucosyl donor, the β-anomer of the protected glucose ester of **1**-d_2 was obtained in an 87% yield. Subsequent deprotection of the benzyl groups was conducted by catalytic hydrogenation with Pd-C. Glucoside **2**-d_2 was obtained without any loss of deuterium at C-2′, although the yield was low (22%). To improve this, we attempted to synthesize **2**-d_2 through the condensation of **1**-d_2 with 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose using *N*,*N*′-dicyclohexylcarbodiimide (DCC) under basic conditions followed by deprotection. The reaction proceeded well to afford the glucosyl ester in a good yield, but was inadequate because a significant level of deuterium exchange occurred under the basic conditions employed, causing a decrease in the isotopical purity (data not shown).

[2′,2′-$^2H_2$]OxIAA (**5**-d_2) was synthesized by oxidation under strong acidic conditions (Fig. 3).[14] Treatment

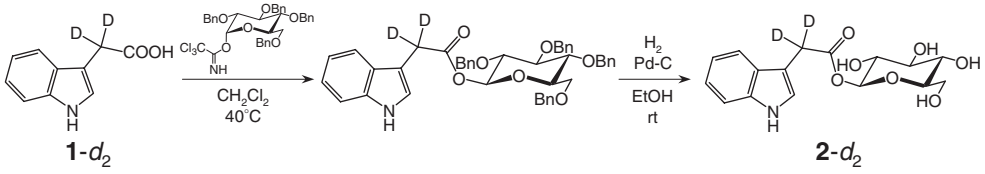

Fig. 2. Scheme for Synthesizing [2′,2′-$^2H_2$]IAA-Glc (**2**-d_2).

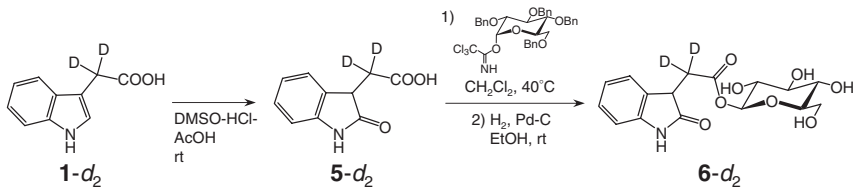

Fig. 3. Scheme for Synthesizing [2′,2′-$^2H_2$]OxIAA (**5**-d_2) and [2′,2′-$^2H_2$]OxIAA-Glc (**6**-d_2).

of **1**-d_2 with DMSO/HCl/AcOH afforded **5**-d_2 in a 79% yield. No substitution of the deuterium in the product was apparent by ^{1}H-NMR or by LC-ESI-MS/MS. [2′,2′-$^2H_2$]OxIAA-Glc (**6**-d_2) was synthesized from **5**-d_2 according to the same method as that used for **2**-d_2 (34% yield in 2 steps) (Fig. 3). No loss of deuterium in **6**-d_2 was apparent.

The low yield of glucosides in the cases of **2**-d_2 and **6**-d_2 was due to the concomitant reduction of the indole- and 2-oxoindole-ring, respectively, during deprotection of the benzyl groups by catalytic hydrogenation. The yield could be improved by using unprotected glucosyl donors such as *O*-glucosyl trichloroacetimidate.[15)]

Optimization of the ESI-MS/MS parameters for quantification

In order to find an optimum multiple reaction monitoring (MRM) transition for quantification, comparative ESI-MS/MS-based analyses of deuterium-labeled and unlabeled compounds were performed for **2**, **5** and **6** (Fig. 4). In the negative mode, the deprotonated molecule $[M-H]^-$ of **2**-d_2 gave major product ions at m/z 176 and 132 by neutral loss of the Glc moiety and decomposition into quinolinium ions, respectively. Non-labeled **2** underwent the same ion transformation, providing the corresponding ions with m/z values smaller by 2 mass units at the same intensity. In the case of the pair **5**-d_2 and **5**, both compounds strongly yielded 2-oxoquinolinium ions at m/z 148 and 146, respectively, from the protonated molecule $[M+H]^+$ in the positive mode. Deuterium-labeled **6**-d_2 gave product ions at m/z 192 and 148 from $[M-H]^-$ with high abundance in the negative mode, which was also the case for **6**. In all experiments, the deuterium label in these compounds was stable enough not to cause any loss of deuterium during the fragmentation of $[M+H]^+$ or $[M-H]^-$ into major product ions during ESI-MS/MS. Based on these results, we selected the following pairs of ions for MRM-based quantification: **2** (336→174), **2**-d_2 (338→176), **5** (192→146), **5**-d_2 (194→148), **6** (352→190), and **6**-d_2 (354→192). By optimizing the MRM parameters, good sensitivity was achieved, as well as good linearity between the relative amount and the response ratio for each pair of unlabeled and labeled compounds (data not shown). The detection limits for compounds **2**, **5** and **6** were 0.85, 1.6 and 0.4 fmol, respectively.

*Stability of the deuterium labels of [2′,2′-$^2H_2$]IAA-Glc (**2**-d_2), [2′,2′-$^2H_2$]OxIAA (**5**-d_2) and [2′,2′-$^2H_2$]OxIAA-Glc (**6**-d_2)*

In earlier studies, [2′,2′-$^2H_2$]IAA (**1**-d_2) was used as an internal standard for GC–MS.[16–18)] However, the deuterium atoms were replaced with hydrogen atoms during the purification and conversion processes, leading to inaccurate results.[8,16,19)] In the case of LC-ESI-MS/MS, highly selective monitoring of a compound is possible under relatively mild conditions without any complicated treatment. Therefore, the deuterium label in the standard compounds used in this study was expected to be stable during the analyses.

To investigate the stability of the deuterium label in **2**-d_2, **5**-d_2 and **6**-d_2 during the analytical procedures, each labeled compound was subjected to a purification process (see "Materials and Methods") and subsequent an analysis by LC-ESI-MS/MS. The MRM chromatograms obtained are shown in Fig. 5. In all cases, only the deuterium-labeled compound could be detected by MRM, showing that no replacement of deuterium by hydrogen occurred. It was thus concluded that deuterium-labeled **2**-d_2, **5**-d_2 and **6**-d_2 were stable enough to be used as internal standards in the quantification of these IAA metabolites in plants. The peak detected by MRM (192→146) at 7.5 min (Fig. 5B) did not agree with that for **6**, being due to the presence of a contaminant during the purification procedure.

It has been documented that 1-*O*-IAA-Glc (**2**) is easily isomerized into 2-*O*-, 4-*O*- and 6-*O*-IAA-Glc *via* acyl-migration.[20)] As shown in Fig. 5, however, no conversion was observed during the experimental procedures

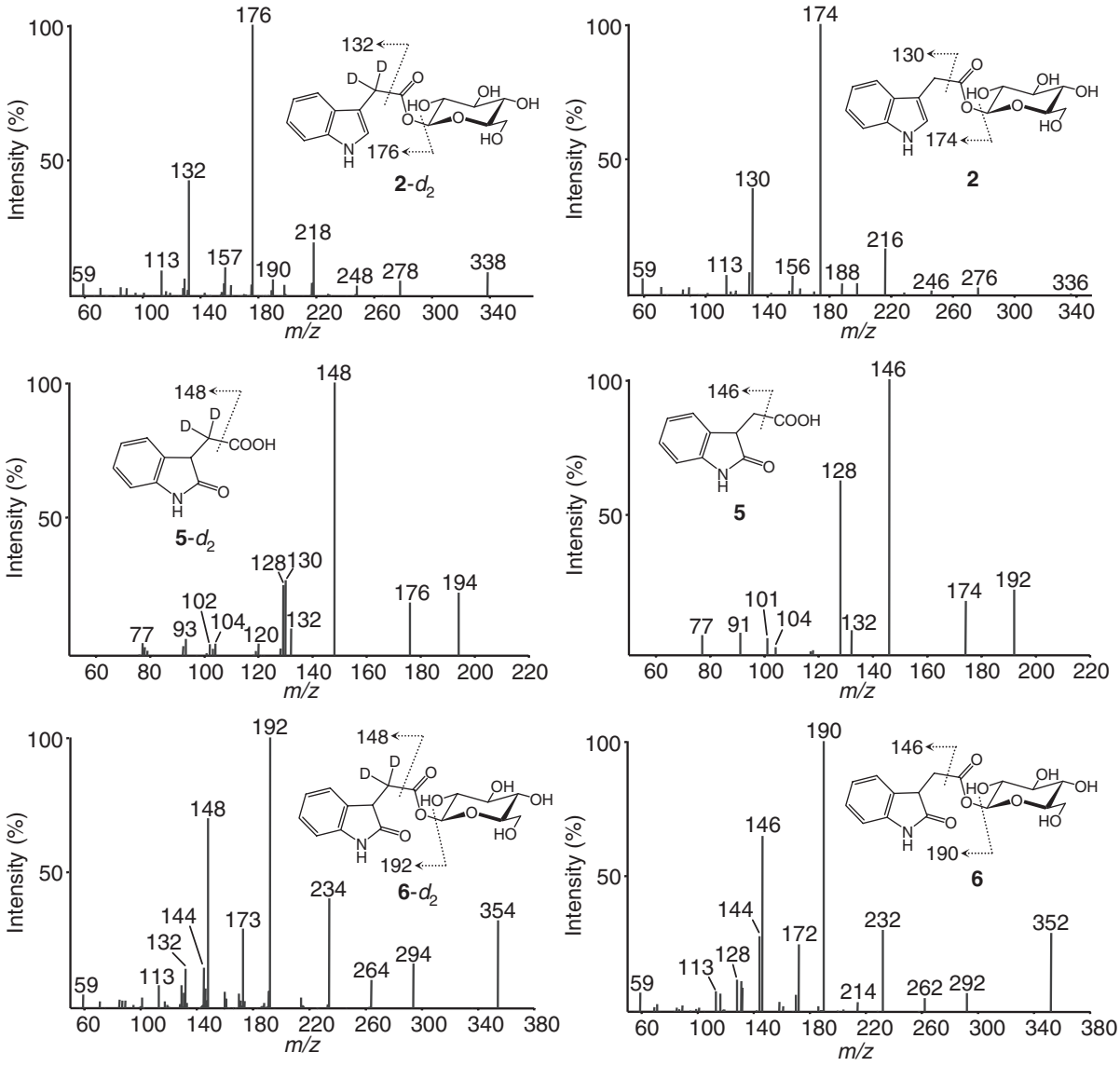

Fig. 4. Product Ion Analyses of Deuterium-Labeled and Non-Labeled IAA-Glc (top), OxIAA (middle), and OxIAA-Glc (bottom). The characteristic fragmentation of each metabolite is described in the spectra.

used in the present study, while we did apparent isomerization in an acidic aqueous solution after a long period of preservation at room temperature. In this regard, we have previously found that isomers of **6** were formed during purification of the extract of *Arabidopsis* seedlings by preparative HPLC.[5] Since such isomerization causes the production of artifact peaks during LC and consequently disturbs the quantification, a certain degree of care during the analytical procedures must be taken.

Quantification of IAA and its metabolites in Arabidopsis seedlings

In order to determine the levels of **1**, **2**, **5** and **6** in *Arabidopsis*, 2-week-old seedlings grown in a Murashige-Skoog (MS) medium were separated into their aerial parts and roots, and extracted with 80% (v/v) acetone-2.5 mM diethyldithiocarbamic acid in the presence of internal standards. Quantification with LC-ESI-MS/MS under the optimized conditions already described showed that **1**, **2** and **5** were accumulated in the roots as shown in Fig. 6, which is consistent with previous reports.[10,11] Among the compounds quantified, **6** had the highest levels and was 4-fold more abundant in the roots than in the aerial parts. Metabolite **6** has been shown to be derived from **5** in the seedlings of *Arabidopsis*.[5] The present data suggest that **6** was produced mainly in the roots.

Effect of exogenous IAA on its endogenous metabolites in Arabidopsis

In spite of the many studies on IAA metabolites, little is known about the relative contribution of each metabolic pathway to the homeostatic level of free IAA in plants. Therefore, IAA was fed to seedlings of *Arabidopsis* through the roots and harvested at various

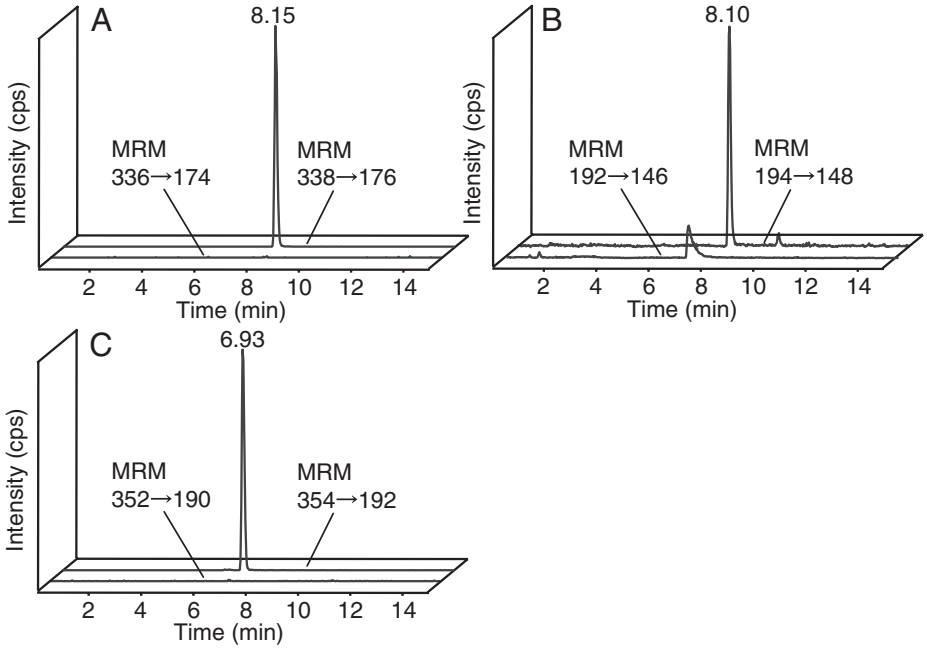

Fig. 5. MRM Chromatograms of [2′,2′-$^2H_2$]IAA-Glc (**2**-d_2) (A), [2′,2′-$^2H_2$]OxIAA (**5**-d_2) (B) and [2′,2′-$^2H_2$]OxIAA-Glc (**6**-d_2) (C) in the Eluates of Solid-Phase Cartridges.

No loss of deuterium in any compound was apparent during the purification or ionization process.

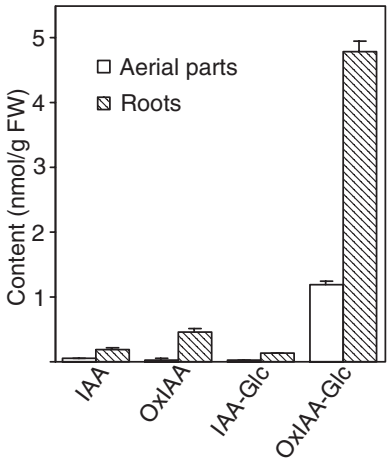

Fig. 6. Quantification of IAA (**1**), IAA-Glc (**2**), OxIAA (**5**) and OxIAA-Glc (**6**) in the Aerial Parts and Roots of *Arabidopsis*.

Two-week-old seedlings were divided into their aerial parts and roots. The error bars indicate the standard deviation of three replicates.

times from 2 to 24 h post-treatment to quantify IAA and its metabolites. The resulting metabolic dynamics are shown in Fig. 7. After feeding with 100 nM IAA, the levels of **3**–**6** increased in a time-dependent manner, whereas those of **1** and **2** remained almost constant. Under these conditions, the plants appeared to be able to maintain homeostasis in terms of the IAA level. Throughout this experiment, there was much more **6** than any other metabolite, indicating that oxidation and subsequent glucosylation were the main metabolic pathway for exogenous IAA in the seedlings of *Arabidopsis*. On the other hand, when the concentration of IAA was increased to 10 μM, a significant rise in the levels of **1** and **2** was observed. However, both compounds decreased gradually thereafter to normal levels. This suggests that the level of **2** was determined by that of endogenous IAA. The increase in the level of **6** was again remarkable, with a moderate but significant increase of **5** in this case. However, the accumulation of **3** and **4** under these conditions was much more prominent, which is in marked contrast with the result obtained after feeding with 100 nM IAA. Thus, the major metabolic pathway changed to conjugation with amino acids when an excess of IAA was given.

It has been demonstrated that *GH3* genes encoding IAA-amino acid synthetases were rapidly expressed in response to exogenous auxin.[21–24] UGT84B1 has also been identified as a conjugate synthetase for **2**,[20] although the gene encoding this enzyme was not auxin-inducible ["AtGenExpress Visualization Tool" (http://jsp.weigelworld.org/expviz/expviz.jsp)]. The regulation of other types of metabolic conversion as well as of the reverse regeneration of IAA from the metabolites remains ambiguous. The present study has shown that the metabolic profile concerning IAA depended on its concentration, most likely due to the activation of multiple metabolic reactions in individually different ways in response to the IAA level. This reflects a complicated aspect of the regulation of IAA level in plants, or homeostasis. A variety of T-DNA *Arabidopsis* mutants are now available ("SIGnAL" supplied by SALK Institute Genomic Analysis Laboratory) which have defects in the genes putatively involved in IAA metabolism.[25–27] The profiling of IAA metabolites in

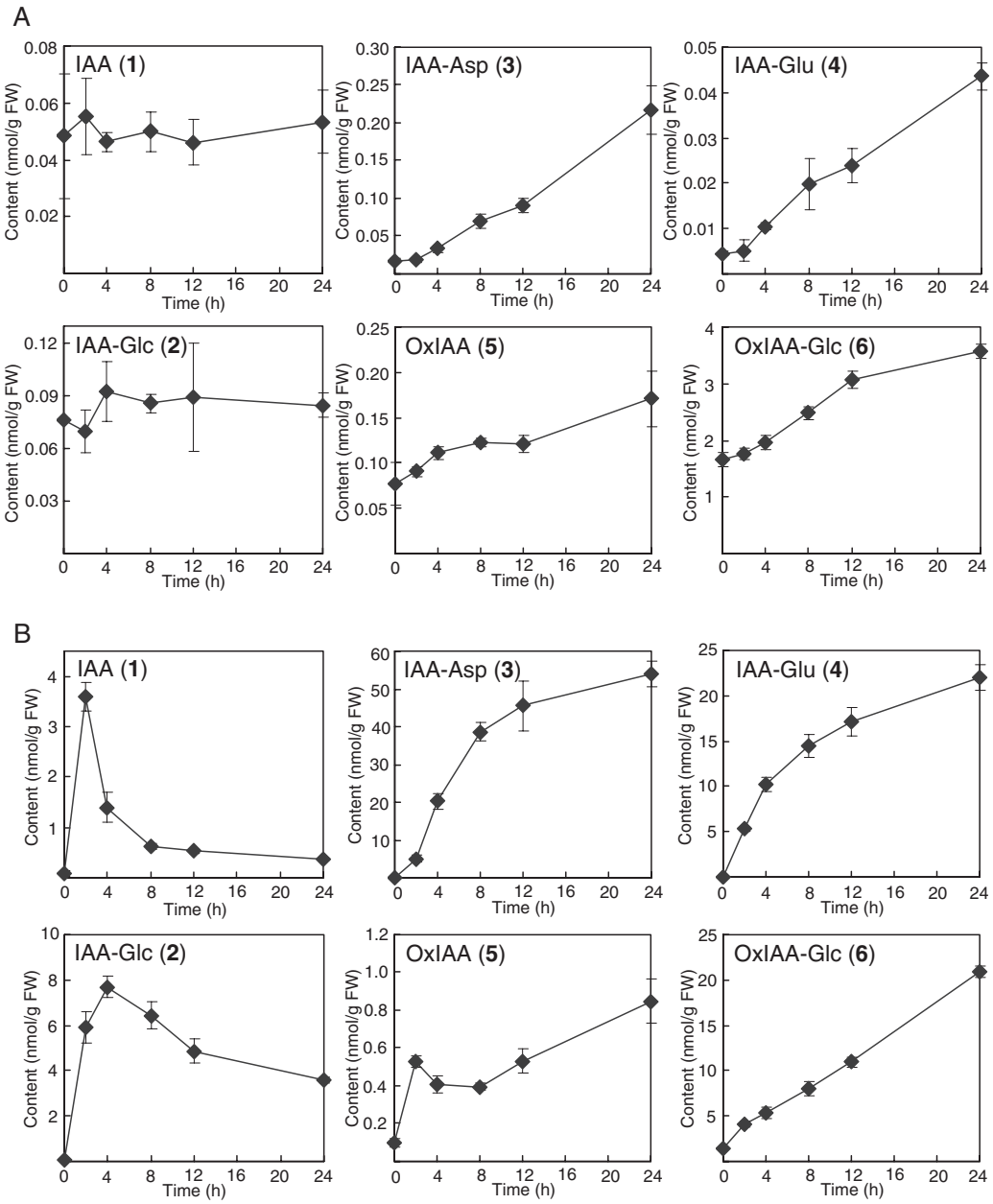

Fig. 7. Time-Course Study of the Disposition of Exogenous IAA in *Arabidopsis*.
The seedlings were incubated in a liquid MS medium containing either 100 nM (A) or 10 μM (B) IAA. The error bars indicate the standard deviation of three replicates.

those mutants is in progress in order to identify the enzyme(s) that plays a critical role in regulating the level of each metabolite, and to better understand the complex mechanism of IAA homeostasis in plants.

Materials and Methods

General. ^{1}H- and ^{13}C-NMR spectra were recorded by a Bruker Avance 400 spectrometer with TMS as the internal standard. Assignment of the ^{1}H and ^{13}C signals was carried out by using ^{1}H–^{1}H COSY, HMQC and HMBC spectra. The LC-ESI-MS/MS data were collected with an Agilent 1100 HPLC system coupled to an API3000 triple-quadrupole-stage mass spectrometer (Applied Biosystems/MDS Sciex, Ontario, Canada). The ESI-MS data were measured with the same mass spectrometer. The HRFAB-MS analysis was conducted with a JMS 700 spectrometer (JEOL, Tokyo, Japan) using glycerol as the matrix. Column chromatography was conducted with Kieselgel 60 (Merck, Darmstadt, Germany) as the adsorbent, and preparative HPLC was performed with a LC-10A HPLC system (Shimadzu, Kyoto, Japan).

Chemicals. NaOD [40% (w/w), in D_2O, 99.5 atom % D] was purchased from Aldrich (Milwaukee, WI, USA)

and D_2O (100.0 atom % D) from Acros (New Jersy, USA). [$^{13}C_6$]IAA ($^{13}C_6$-**1**), an internal standard for IAA (**1**), was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). IAA-Glc (**2**), OxIAA (**5**), and OxIAA-Glc (**6**) were synthesized in a previous study.[5] The samples of [2′,2′-$^2H_2$]IAA-Asp (**3**-d_2) and [2′,2′-$^2H_2$]IAA-Glu (**4**-d_2) were kindly presented by Dr. Jun Hiratake of the Institute for Chemical Research at Kyoto University. All other chemicals were obtained commercially.

*Study for the deuteration of IAA (**1**).* IAA (100 mg, 0.570 mmol) was dissolved in D_2O (5 ml) containing the specified concentration of NaOD and the mixture was stirred under specified conditions. Every 2 days, *ca.* 0.5 ml of the reaction mixture was analyzed by ^{1}H-NMR spectroscopy.

Chemical synthesis.

*[2′,2′-$^2H_2$]-indole-3-acetic acid (**1**-d_2).* IAA (1 g, 5.70 mmol) was dissolved in 50 ml of 1 M NaOD/D_2O, and the mixture was stirred for 4 days at 80 °C. After cooling to room temperature, the mixture was acidified with 2 M HCl to form crystals. The crystals were washed with water and recrystallized from EtOAc to yield a white yellow substance (669 mg, 66%). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 7.02 (1H, *m*, H-5), 7.10 (1H, *m*, H-6), 7.30 (1H, $J = 2.4$ Hz, H-2), 7.39 (1H, *d*, $J = 8.1$ Hz, H-7), 7.61 (1H, *d*, $J = 7.9$ Hz, H-4), 10.21 (1H, *br*, H-1), 10.62 (1H, *br*, COO*H*). ESI-MS: m/z 178 ($M + H^+$).

*1-O-([2′,2′-$^2H_2$]-indole-3-acetyl)-β-D-glucopyranose (**2**-d_2).* **1**-d_2 (100 mg, 0.564 mmol) and 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate (386 mg, 0.564 mmol) were dissolved in 3 ml of dry CH_2Cl_2, and the solution was stirred overnight at 40 °C. The reaction mixture was diluted with 10 ml of $CHCl_3$, successively washed with a saturated aqueous $NaHCO_3$ solution and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue was purified by column chromatography (hexane-EtOAc stepwise elution) to give 1-*O*-([2′,2′-$^2H_2$]-indole-3-acetyl)-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose as an orange paste (342 mg, 87%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.52–3.58 (2H, *m*), 3.65–3.76 (4H, *m*), 4.34 (1H, *d*, $J = 9.2$ Hz), 4.44–4.52 (3H, *m*), 4.60 (1H, *d*, $J = 12.1$ Hz), 4.75–4.84 (3H, *m*), 5.65 (1H, *d*, $J = 8.1$ Hz, Glc, H-1), 6.98 (2H, *dd*, $J = 1.9$, 5.9 Hz), 7.02 (1H, *d*, $J = 2.4$ Hz), 7.07–7.32 (21H, *m*), 7.59 (2H, *d*, $J = 7.8$ Hz, IAA, H-4), 7.99 (1H, *br*, IAA, H-1). ESI-MS: m/z 701 ($M + H^+$).

1-*O*-([2′,2′-$^2H_2$]-indole-3-acetyl)-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose (342 mg, 0.488 mmol) and 10% (w/w) Pd-C (136 mg) were suspended in 30 ml of EtOH, and the suspension was stirred for 6 h at room temperature in an H_2 atmosphere. The precipitate was removed by filtration, and the filtrate was purified by column chromatography ($CHCl_3$:MeOH = 90:10). Subsequent preparative HPLC [column, Inertsil ODS-3 10 × 250 mm (GL Science, Tokyo, Japan); solvent, water: MeCN =75:25; flow rate, 3.20 ml/min; detection, 280 nm)] gave [2′,2′-$^2H_2$]IAA-Glc (**2**-d_2) as a colorless oil (37 mg, 22%). ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.30–3.65 (4H, *m*), 3.66 (1H, *m*), 3.81 (1H, *d*, $J = 12.0$ Hz), 5.52 (1H, *d*, $J = 7.8$ Hz, Glc, H-1), 7.01 (1H, *m*, IAA, H-5), 7.09 (1H, *m*, IAA, H-6), 7.19 (1H, *s*, IAA, H-2), 7.34 (1H, *d*, $J = 8.1$ Hz, IAA, H-7), 7.55 (1H, *d*, $J = 7.8$ Hz, IAA, H-4). ^{13}C-NMR (100 MHz, CD_3OD) δ: 62.2 (Glc, C-6), 71.0 (Glc, C-2 or 4 or 5), 74.0 (Glc, C-2 or 4 or 5), 77.9 (Glc, C-3), 78.8 (Glc, C-2 or 4 or 5), 95.5 (Glc, C-1), 107.8 (IAA, C-3), 122.7 (IAA, C-7), 119.5 (IAA, C-4 or 5), 119.9 (IAA, C-4 or C-5), 122.5 (IAA, C-6), 124.9 (IAA, C-2), 128.6 (IAA, C-3a), 137.9 (IAA, C-7a), 172.9 (IAA, C-1′). ESI-MS: m/z 338 ($M - H^-$). FAB-MS: m/z 362 ($M + Na^+$), 339 (M^+). HRFAB-MS: m/z 339.1285 (M^+, calcd. for $C_{16}H_{17}D_2NO_7$, 339.1287).

*[2′,2′-$^2H_2$]-2-oxoindole-3-acetic acid (**5**-d_2).* **1**-d_2 (400 mg, 2.27 mmol) was dissolved in 6 ml of DMSO/conc. HCl/AcOH (1:5:10, v/v), and the solution was stirred for 3 h at room temperature. The mixture was quenched in 10 ml of ice-water and then extracted with EtOAc (3 × 15 ml). The combined EtOAc solution was successively washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was crystallized from $CHCl_3$ to give a white pink crystal (346 mg, 79%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.61 (1H, *s*, H-3), 6.81 (1H, *d*, $J = 7.7$ Hz, H-8), 6.92 (1H, *m*, H-5), 7.16 (1H, *m*, H-6), 7.22 (1H, *d*, $J = 7.3$ Hz, H-7), 10.3 (1H, *s*, H-1), 12.3 (1H, *s*, COOH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 41.8 (C-2′), 109.3 (C-4), 121.3 (C-5), 123.8 (C-7), 127.9 (C-6), 129.5 (C-3a), 143.1 (C-7a), 172.4 (C-1′), 178.3 (C-2). ESI-MS: m/z 194 ($M + H^+$). HRFAB-MS: m/z 194.0784 ($M + H^+$, calcd. for $C_{10}H_8D_2NO_3$, 194.0786).

*1-O-([2′,2′-$^2H_2$]-2-oxoindole-3-acetyl)-β-D-glucopyranose (**6**-d_2).* **5**-d_2 (100 mg, 0.518 mmol) and 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate (425 mg, 0.621 mmol) were dissolved in 3 ml of dry CH_2Cl_2, and the solution was stirred overnight at 40 °C. The reaction mixture was diluted with 10 ml of $CHCl_3$, successively washed with a saturated aqueous $NaHCO_3$ solution and brine, and then dried over anhydrous Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue was purified by column chromatography ($CHCl_3$–MeOH stepwise elution) to give 1-*O*-([2′,2′-$^2H_2$]2-oxoindole-3-acetyl)-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose as a yellow powder (149 mg, 40%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.54–3.81 (7H, *m*), 4.47–4.89 (9H, *m*), 5.62 (0.5H, *d*, $J = 8.1$ Hz, Glc, H-1), 5.67 (0.5H, *d*, $J = 8.1$ Hz, Glc, H-1), 6.87–7.33 (24H, *m*). ESI-MS: m/z 717 ($M + H^+$).

1-*O*-([2′,2′-$^2H_2$]2-oxoindole-3-acetyl)-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose (149 mg, 0.208 mmol) and 10% (w/w) Pd-C (59.6 mg) were suspended in 10 ml of EtOH, and the suspension was stirred for 4 h at room temperature in an H_2 atmosphere. The precipitate was

Table 1. Multiple Reaction Monitoring (MRM) Parameters to Quantify Metabolites **2**, **5** and **6**

Compound	Polarity	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
2	Negative	−51	−260	−10	−14	−11
5	Positive	36	230	10	17	8
6	Negative	−46	−300	−10	−24	−11

DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential

removed by filtration, and the filtrate was purified by column chromatography ($CHCl_3$–MeOH stepwise elution) and subsequent preparative HPLC (column, Inertsil ODS-3 10×250 mm; solvent, water:MeCN = 82.5:17.5; flow rate, 3.20 ml/min; detection, 280 nm) to give [2′,2′-$^2H_2$]OxIAA-Glc (**6**-d_2) as a colorless paste (63.4 mg, 86%). This preparation was a mixture of keto-enol tautomers. ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.31–3.32 (3H, *m*, Glc. H-2,4,5), 3.40 (1H, *m*, Glc, H-3), 3.66 (1H, *dd*, $J = 4.4$, 12.0 Hz, Glc, C-6), 3.78–3.83 (2H, *m*, Glc-H-6, OxIAA-H-3), 5.47 (0.5H, *d*, $J = 8.0$ Hz, Glc, H-1), 5.51 (0.5H, *d*, $J = 8.0$ Hz, Glc, H-1), 6.89 (1H, *d*, $J = 7.7$ Hz, OxIAA, H-4), 6.98 (1H, *m*, OxIAA, H-5), 7.18 (1H, *m*, OxIAA, H-6), 7.30 (1H, *d*, $J = 7.3$ Hz, OxIAA, H-7). ^{13}C-NMR (100 MHz, CD_3OD) δ: 43.27 (OxIAA, C-3), 43.37 (OxIAA, C-3), 62.27 (Glc, C-6), 70.96 (Glc, C-2 or 4 or 5), 73.88 (Glc, C-2 or 4 or 5), 73.93 (Glc, C-2 or 4 or 5), 77.80 (Glc, C-3), 78.80 (Glc, C-2 or 4 or 5), 78.85 (Glc, C-2 or 4 or 5), 96.01 (Glc, C-1), 96.06 (Glc, C-1), 110.85 (OxIAA, C-4), 123.33 (OxIAA, C-5), 123.38 (OxIAA, C-5), 125.27 (OxIAA, C-7), 129.30 (OxIAA, C-6), 130.23 (OxIAA, C-3a), 130.28 (OxIAA, C-3a), 143.63 (OxIAA, C-7a), 171.57 (OxIAA, C-1′), 181.05 (OxIAA, C-2), 181.11 (OxIAA, C-2). ESI-MS: m/z 354 ($M - H^-$). HRFAB-MS: m/z 356.1321 ($M + H^+$, calcd. for $C_{16}H_{18}D_2NO_8$, 356.1314).

ESI-MS/MS analysis. Specified amounts of **2**, **2**-d_2, **5**, **5**-d_2, **6** and **6**-d_2 were dissolved in 60% (v/v) MeOH. The solutions were directly introduced into an API3000 mass spectrometer to obtain product-ion spectra. The parameters for mass spectrometry related to the ionization and fragmentation were optimized by using Analyst 1.3 software (Applied Biosystems) as listed in Table 1.

Plant material. Seeds of *Arabidopsis thaliana* ecotype Columbia were surface sterilized in 5% (v/v) sodium hypochlorite containing 0.05% (v/v) Tween 20 for 5 min, and then washed three times with sterile water. The seeds were germinated in plastic Petri dishes containing an agar-solidified MS medium. The plates were kept in darkness at 4 °C for 2 days to synchronize germination, and then grown at 22 °C with a 16-h photoperiod (light intensity, 3000 lux).

Quantitative analysis. The analytical samples were prepared as described previously with some modifications.[5] Plant tissues were homogenized in liquid N_2 and subsequently extracted with 80% (v/v) acetone containing 2.5 mM diethyldithiocarbamic acid and the respective internal standards (0.1–0.5 nmol) for 2 h. After the extraction procedure had been repeated, the combined extract was concentrated under reduced pressure. The aqueous concentrate was subjected to solid-phase extraction after adjusting the pH value to approximately 2.6. After removing the solvent, the sample was analyzed by LC-ESI-MS/MS operated in the MRM mode to quantify IAA and its metabolites. The following transitions from precursor to product ions were monitored: **1**, 176→130; [$^{13}C_6$]-**1**, 182→136; **3**, 291→130; **3**-d_2, 293→132; **4**, 305→130; **4**-d_2, 307→132; **2**, 336→174; **2**-d_2, 338→176; **5**, 192→146; **5**-d_2, 194→148; **6**, 352→190; **6**-d_2, 354→192. Quantification was based on the ratio of the peak area for each metabolite to that for the internal standard.

IAA treatment. Two-week-old seedlings were transferred into a liquid MS medium containing the appropriate concentration of IAA and incubated for 24 h at 22 °C under continuous light. After the seedlings had been washed with water, a quantitative analysis was performed as described above.

We thank Dr. Jun Hiratake (Institute for Chemistry, Kyoto University, Kyoto, Japan) for advice regarding the synthesis of deuterium-labeled compounds and for the HRFAB-MS measurements.

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