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Metabolism of indole-3-acetic acid in rice: Identification and characterization of *N*-β-D-glucopyranosyl indole-3-acetic acid and its conjugates

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Abstract

A search was made for conjugates of indole-3-acetic acid (IAA) in rice (*Oryza sativa*) using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) in order to elucidate unknown metabolic pathways for IAA. *N*-β-D-Glucopyranosyl indole-3-acetic acid (IAA-*N*-Glc) was found in an alkaline hydrolysate of rice extract. A quantitative analysis of 3-week-old rice demonstrated that the total amount of IAA-*N*-Glc was equal to that of IAA. A LC–ESI–MS/MS-based analysis established that the major part of IAA-*N*-Glc was present as bound forms with aspartate and glutamate. Their levels were in good agreement with the total amount of IAA-*N*-Glc during the vegetative growth of rice. Further detailed analysis showed that both conjugates highly accumulated in the root. The free form of IAA-*N*-Glc accounted for 60% of the total in seeds but could not be detected in the vegetative tissue. An incorporation study using deuterium-labeled compounds showed that the amino acid conjugates of IAA-*N*-Glc were biosynthesized from IAA-amino acids. IAA-*N*-Glc and/or its conjugates were also found in extracts of *Arabidopsis*, *Lotus japonicus*, and maize, suggesting that *N*-glucosylation of indole can be the common metabolic pathway of IAA in plants.

Keywords: Rice; *Oryza sativa*; Gramineae; Indole-3-acetic acid; *N*-β-D-Glucopyranosyl indole-3-acetic acid; *N*-Glucosylation; Metabolism; LC–ESI–MS/MS

1. Introduction

The plant hormone indole-3-acetic acid (IAA, **1**) (Fig. 1) is an important signal molecule that regulates many aspects of growth and development in plants. The concentration of free IAA (**1**) at the tissue or cellular level is thought to be controlled exquisitely through a fine balance of the activities of biosynthesis, metabolism, and inter-cellular or -tissue transport, referred to as IAA homeostasis. To date, the mechanisms involved have been studied extensively, although many details remain unelucidated (Normanly, 1997; Normanly and Bartel, 1999; Ljung et al., 2002; Woodward and Bartel, 2005).

It has been suggested that most of the IAA (**1**) in plants is present in conjugated forms, since a large amount of IAA (**1**) is released by hydrolysis of plant extracts. The conjugates are thought to be storage or transport forms of the hormone, and in some cases, to act as intermediates in the catabolic processing of IAA (**1**). IAA (**1**) conjugates are usually classified based on the distinct linked form via the carboxy group into ester-type conjugates, including IAA-Glc (**2**), -inositol, and -glucans, and amide-type conjugates such as IAA-amino acids (Asp, **3**; Glu, **4**) and IAA-peptides. In *Arabidopsis*, it has been estimated that ester- and amide-conjugates of IAA (**1**) account for as

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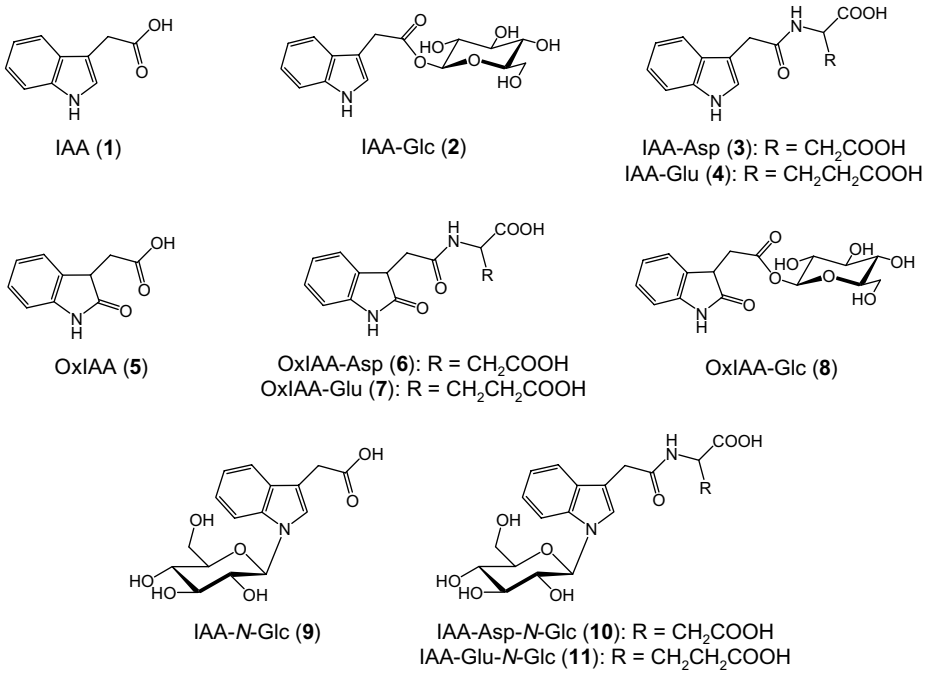

Fig. 1. Structures of IAA (**1**) and its metabolites.

much as about 99% of the total IAA (**1**) pool (Tam et al., 2000).

Oxidation is also considered to play an important role in the metabolism of IAA (**1**). The presence of 2-oxoindole-3-acetic acid (OxIAA, **5**) has been demonstrated in a wide variety of plants, although only limited data is available about the enzymological background of its formation *in planta*. It has been shown that the oxidation of IAA (**1**) to OxIAA (**5**) by corn endosperm enzyme preparation requires molecular oxygen, but none of the cofactors/cosubstrates of peroxidases, mixed function oxygenases or intermolecular dioxygenases stimulates the oxidation (Reinecke and Bandurski, 1988). OxIAA (**5**) is also present in conjugated forms, among which are the amino acid conjugates, such as OxIAA-Asp (**6**) and OxIAA-Glu (**7**) (Normanly, 1997; Normanly and Bartel, 1999; Ljung et al., 2002; Woodward and Bartel, 2005). Recently, we have shown that a glucose conjugate of OxIAA (OxIAA-Glc, **8**) is highly accumulated in the seedlings of *Arabidopsis* (Kai et al., 2007). We also have demonstrated that the amino acid conjugates of IAA (**1**) undergo oxidative conversion via hydroxylation at C-6 of the indole ring. Further conversions of OxIAA (**5**) have been reported in several plant species, although, in some cases, the products were detected only when IAA (**1**) was supplied to the plants Nonhebel and Bandurski, 1984; Nonhebel et al., 1985; Tateishi and Yamashita, 1998; Lewer, 1987; Lewer and Bandurski, 1987; for review, see Normanly, 1997; Ljung et al., 2002).

In addition to the hitherto described metabolic conversion of IAA (**1**), it is also likely that the indole ring of IAA (**1**) undergoes modifications, such as halogenation, acylation, prenylation, and glycosylation (Somei and Yamada, 2003). In this regard, the formation of *N*-glucosides of IAA (**1**) was suggested to occur in the seedlings of a woody plant, Scots pine, after IAA (**1**) was supplied, although the compound's structural identity was not fully established (Ljung et al., 2001a). In the present study, we searched for indole ring-modified metabolites of IAA (**1**) in a monocot, rice (*Oryza sativa*). We report here the chemical identification of *N*-β-D-glucopyranosyl IAA (IAA-*N*-Glc, **9**) found in a hydrolysate of the plant extract, and demonstrate that this compound is derived from *N*-glucosides of IAA (**1**) amides with aspartate, IAA-Asp-*N*-Glc (**10**), and glutamate, IAA-Glu-*N*-Glc (**11**), that are actually present in the vegetative tissues of rice. Quantification using internal standards is described, revealing a characteristic distribution in plants. Also included are the results of a comparative study which suggest that these metabolites can be found in different plant species.

2. Results

2.1. Search for IAA (1) conjugates using LC–ESI–MS/MS

In the present study, we targeted metabolites of IAA (**1**) in which the indole ring is modified with biological components such as isoprenoids and saccharides. So we first treated the plant extract with a strong alkaline to liberate the conjugated carboxy group of IAA (**1**) in all the metabolites into the free form. Assuming that any metabolites having the indole-3-acetyl moiety will provide a quinolinium ion at *m/z* 130 in ESI^+-MS/MS, we searched for precursor ions

of *m/z* 130 with a mass range of 200 to 800 in a strong-alkaline hydrolysate of rice plants. As a consequence, more than 20 compounds were detected (data not shown), and their structure was analyzed by MS/MS. Compound **9** (t_R, 7.69 min; molecular weight, 337) yielded product ions, *m/z* 218, 176, and 130, in the positive mode (Fig. 2), which was similar to the fragmentation pattern of IAA-Glc (**2**) in the negative mode. However, considering that compound **9** was found in the strong alkaline hydrolysate, it was unlikely to be IAA-Glc (**2**), which should be decomposed under such conditions. Hence, we assumed that compound **9** was a glucoside of IAA (**1**) with the glucose moiety linked to the indole ring or C-2′ position of the acetic acid part in IAA (**1**). The MS/MS analysis of compound **9** showed that the product ion at *m/z* 176 was abundant, as shown in Fig. 2. This suggested that the glucosyl bond is relatively cleavable, and therefore, we predicted that the glucosyl moiety of compound **9** is linked to the N-1 position of IAA (**1**).

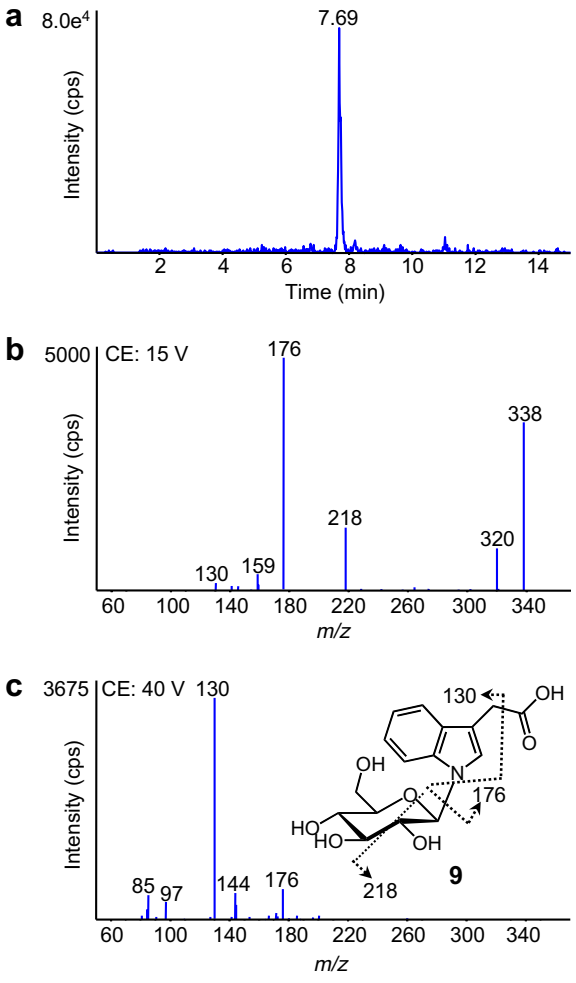

Fig. 2. LC–ESI–MS/MS analysis of the alkaline hydrolysate of rice extract. Compound **9** was detected as a precursor of *m/z* 130 (a). The product ion spectrum was obtained with two sets of collision energy (CE) (15 V, b; 40 V, c). The proposed fragmentation of compound **9** is also described.

The proposed fragmentation in MS/MS is described in Fig. 2.

Preliminary quantitative analysis, however, demonstrated that **9** was not present as its free form in the vegetative tissues of rice (see below). We thus assumed that most of **9** in plants is bound to an additional component. Because **9** was not released after mild-alkaline hydrolysis of the plant extracts in a significant amount (data not shown), we presumed that compound **9** was present in amide forms with amino acids, probably via the carboxy group of **9**. We then established a multiple reaction monitoring (MRM)-based method for detecting the putative amino acid conjugates, and tried to detect them in the extract of 2–3-week-old plants. As shown in Fig. 3, compounds **10** (t_R, 5.86 min) and **11** (t_R, 6.47 min) were detected, and were thought to be Asp and Glu conjugates, respectively, from their product-ion spectra. Proposed assignments of their product ions are shown in Fig. 3.

2.2. Chemical synthesis of IAA (*1*) conjugates

We assumed that **9** was *N*-β-D-glucopyranosyl IAA as described above. In order to confirm this, an authentic compound was chemically synthesized (Fig. 4). First, the indole ring of IAA methyl ester (**12**) was partially reduced to indoline **13** to enhance the basicity of the nitrogen atom. Condensation of **13** with D-glucose was accomplished using the previously reported method (Iwaki et al., 2003), with which *N*-β-D-glucoside had been formed. The product was then acylated and oxidized to regenerate the indole ring of IAA (**1**). After deprotection, IAA-*N*-Glc (**9**) was obtained in 25% yield from IAA (**1**). The coupling constant in the signal of the anomeric proton at δ_H 5.62 ppm was 9.1 Hz in the ^{1}H NMR spectrum of synthetic IAA-*N*-Glc (**9**), confirming that glucose was linked to the indole ring via the β-form. Other signals in ^{1}H and ^{13}C NMR spectra also supported such a structure. The chromatographic behavior and mass spectrum of this synthetic sample agreed with those of the naturally occurring compound (Table 1), and thus, **9** was identified as IAA-*N*-Glc.

Subsequently, IAA-*N*-Glc (**9**) was derived into the amides with Asp and Glu as shown in Fig. 4. LC–ESI–MS/MS analysis showed that these synthetic standards also had identical LC characteristics and mass spectra with naturally occurring **10** and **11** (Table 1), and therefore, they were identified as the double conjugates of IAA (**1**) with glucose and amino acids, Asp and Glu, respectively.

Using the same procedure, we synthesized IAA-*N*-[6,6-^{2}H$_2$]Glc (**9**-d_2), IAA-Asp-*N*-[6,6-^{2}H$_2$]Glc (**10**-d_2), and IAA-Glu-*N*-[6,6-^{2}H$_2$]Glc (**11**-d_2) as the internal standards for the quantification using LC–ESI–MS/MS.

2.3. Endogenous levels of IAA (*1*) conjugates

Endogenous levels of IAA (**1**) and its conjugates in 1-, 2- and 3-week-old rice plants and seeds were determined by LC–ESI–MS/MS using the deuterium-labeled internal

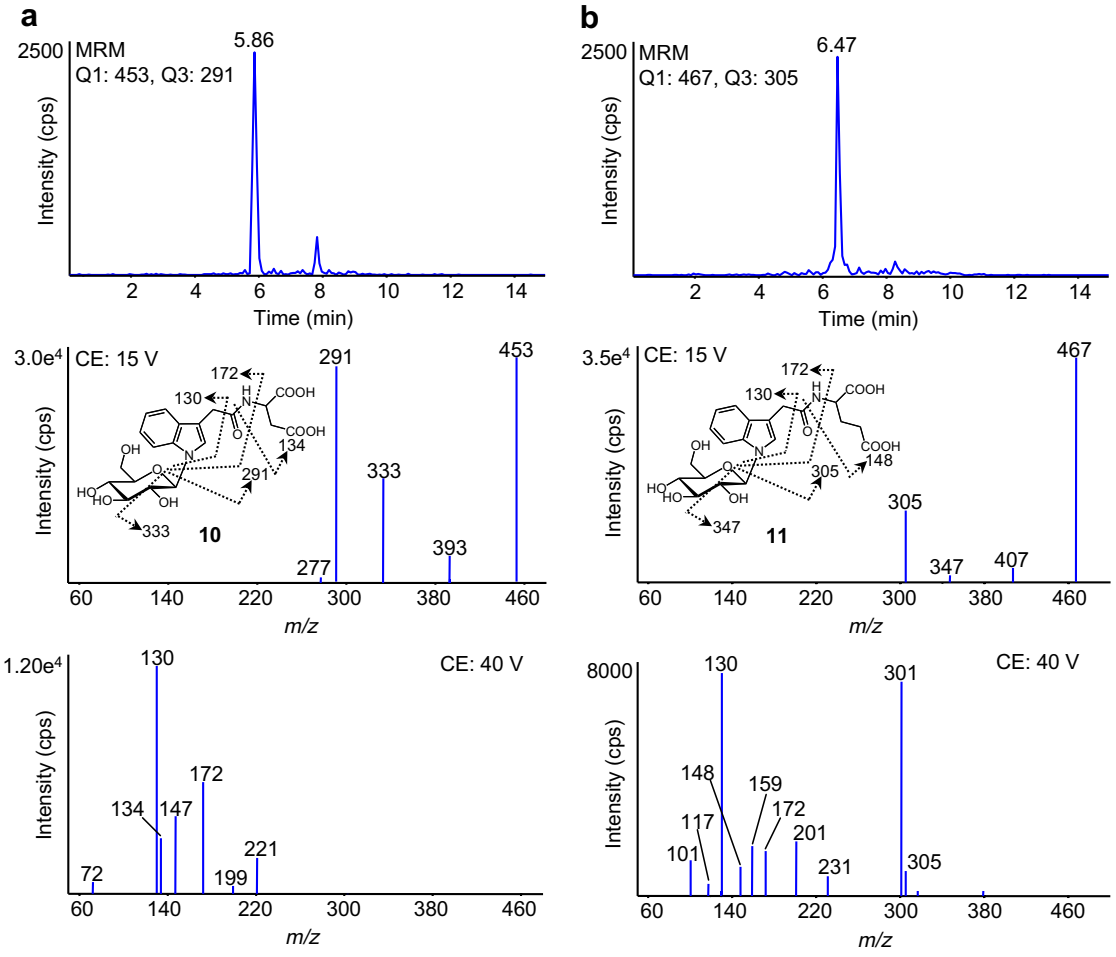

Fig. 3. LC–ESI–MS/MS analyses of compounds **10** (a) and **11** (b) in the extract of rice plants. These compounds were detected in the MRM mode (top). Subsequently, their product ion spectra were measured with two sets of collision energy (middle and bottom). These results indicate that compounds **10** and **11** are IAA-Asp-*N*-Glc and IAA-Glu-*N*-Glc, respectively.

standards. As shown in Fig. 5, the total amount of IAA-*N*-Glc (**9**) increased slightly during vegetative growth, which contrasts with the decrease in the total amount of IAA (**1**). In 3-week-old plants, the total amount of IAA-*N*-Glc (**9**) was 0.50 nmol g^{-1} fresh wt, which was comparable to that of IAA (**1**). The free form of IAA-*N*-Glc (**9**) was not detected in the vegetative tissues of rice, whereas it accounted for 60% of the total IAA-*N*-Glc (**9**) pool in seeds.

Amounts of amide forms of IAA-*N*-Glc (**9**) were much greater than those of IAA-amino acids (**3**, **4**), which have been considered to be the major low molecular weight metabolites in rice (Matsuda et al., 2005). Of the two types of IAA-*N*-Glc (**9**) amides, the Asp-bound compound **10** was present in larger amounts than the Glu-bound form **11**, which was the same as the relationship between the amounts of IAA-Asp (**3**) and IAA-Glu (**4**). The sum of IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) well accounted for the total amount of IAA-*N*-Glc (**9**) in 2- and 3-week-old plants. IAA-Asp-*N*-Glc (**10**) was also present in seeds, whereas no IAA-Glu-*N*-Glc (**11**) was detected. The total amount of IAA-*N*-Glc (**9**) in seeds could not be accounted for in this experiment, and thus the presence of an unknown conjugate in seeds was suggested.

2.4. Biosynthesis of amide forms of IAA-N-Glc (9)

To elucidate how IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) are biosynthesized in plants, precursor administration experiments were performed using deuterium-labeled IAA-Asp (**3**-d_2), IAA-Glu (**4**-d_2), and IAA-*N*-Glc (**9**-d_2). The labeled compounds were administered in vivo by growing rice plants hydroponically in water containing each sample. After 2 days, the plants were extracted to be analyzed by LC–ESI–MS/MS in the MRM mode, where a definite level of uptake was confirmed for each compound. As shown in Fig. 6, significant incorporation of IAA-Asp (**3**) into IAA-Asp-*N*-Glc (**10**) and IAA-Glu (**4**) into IAA-Glu-*N*-Glc (**11**) was observed. The structural identity of the incorporated compounds was confirmed from their product-ion spectra (Table 2). In contrast, no incorporation of IAA-*N*-Glc (**9**) was observed (data not shown). These results indicate that IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) are likely to be biosynthesized

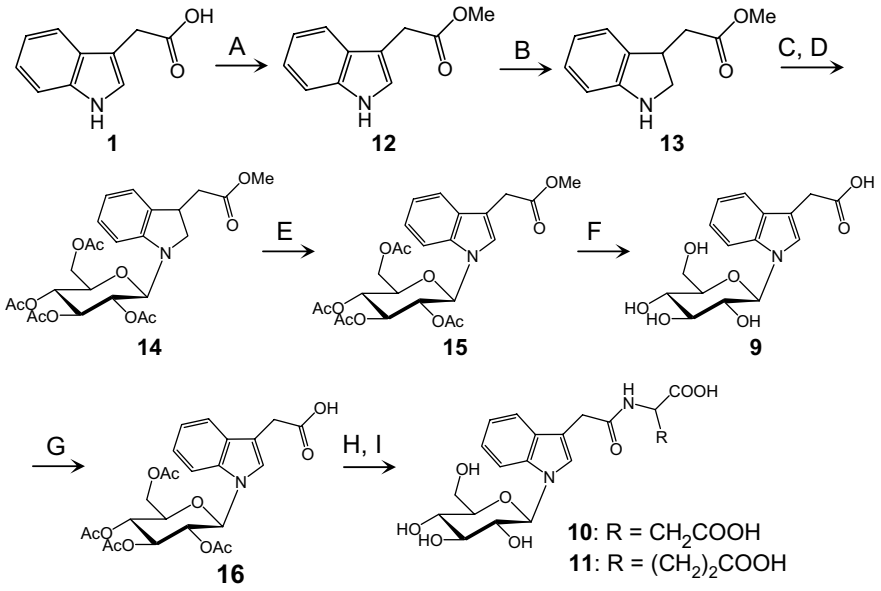

Fig. 4. Scheme for the synthesis of IAA-*N*-Glc (**9**), IAA-Asp-*N*-Glc (**10**), and IAA-Glu-*N*-Glc (**11**). For details see "Experimental". A, methyl iodide, K_2CO_3/*N*,*N*-dimethylformamide; B, triethylsilane/trifluoroacetic acid, 60 °C; C, D-glucose/MeOH, reflux; D, acetic anhydoride/pyridine; E, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone/1,4-dioxane; F, 1M KOH; G, acetic anhydride/pyridine; H, amino acid methyl ester, *N*,*N*′-dicyclohexylcarbodiimide, 4-dimethylaminopyridine/pyridine; I, 1M KOH.

Table 1
Tabulated LC–ESI–MS/MS analysis of natural and synthetic compounds product ion spectra of each compound were obtained with two sets of collision energy (CE) values in the positive mode

Compound		Retention time (min)	Precusor ion (*m*/*z*)	Ion (*m*/*z*) (abundance)
IAA-*N*-Glc (**9**)	Natural	7.69	338	CE 15, 338 (72), 320 (24), 218 (22), 176 (100), 130 (6); CE 40, 176 (15), 144 (13), 130 (100), 85 (18)
	Synthetic	7.66	338	CE 15, 338 (12), 320 (9), 302 (4), 218 (26), 176 (100), 130 (2); CE 40, 176 (8), 172 (13), 144 (12), 130 (100), 85 (14)
IAA-Asp-*N*-Glc (**10**)	Natural	5.86	453	CE 15, 453 (81), 333 (46), 291 (100); CE 40, 172 (64), 134 (36), 130 (100)
	Synthetic	5.81	453	CE 15, 453 (21), 333 (31), 291 (100); CE 40, 172 (41), 144 (11), 134 (25), 130 (100), 116 (7)
IAA-Glu-*N*-Glc (**11**)	Natural	6.47	467	CE 15, 467 (100), 347 (15), 305 (43); CE 40, 172 (42), 148 (45), 130 (100)
	Synthetic	6.50	467	CE 15, 467 (17), 347 (12), 305 (100); CE 40, 172 (25), 148 (22), 130 (100), 102 (5)

from IAA-Asp (**2**) and IAA-Glu (**3**), respectively, by the glucosylation of indole at the N-1 position.

2.5. Accumulation of amide forms of IAA-N-Glc (*9*) in root

It has been demonstrated that IAA (**1**) and its metabolites accumulated in different parts during the vegetative growth of plants (Kowalczyk and Sandberg, 2001; Ljung et al., 2001b, 2005; Bhalerao et al., 2002; Matsuda et al., 2005). To examine whether IAA-*N*-Glc (**9**) amides have such a tissue-dependent distribution, we quantified their amounts in the aerial parts and roots of 2-week-old rice. While the level of IAA (**1**) was relatively high in the aerial parts, all the metabolites of IAA (**1**) examined were accumulated in the roots (Fig. 7). In particular, levels of IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) were 22- and 24-fold higher in the roots than aerial parts, respectively. These observations suggest that amide forms of IAA-*N*-Glc (**9**) could be mainly biosynthesized in the roots, although distribution of a compound within a plant is of course associated with its inter-tissue movement.

2.6. Hormonal activity of IAA-N-Glc (*9*) and its amide forms (*10, 11*)

The presence of the free form of IAA-*N*-Glc (**9**) only in seeds, suggested that it is one of the storage forms of auxin. In order to verify whether the *N*-glucosyl bond of IAA-*N*-Glc (**9**) and its amides (**10**, **11**) is hydrolysable in vivo, we investigated the inhibitory effect on elongation of the shoot and seminal root in rice seedlings. As shown in Fig. 8, IAA-*N*-Glc (**9**) and its amino acid-bound derivatives (**10**, **11**) showed no significant inhibition. Taking into consideration that the methyl ester of IAA (**1**) has shown more biological activity than IAA (**1**) in some bioassay experiments (Zimmerman and Hitchcock, 1937; Qin et al., 2005), we also

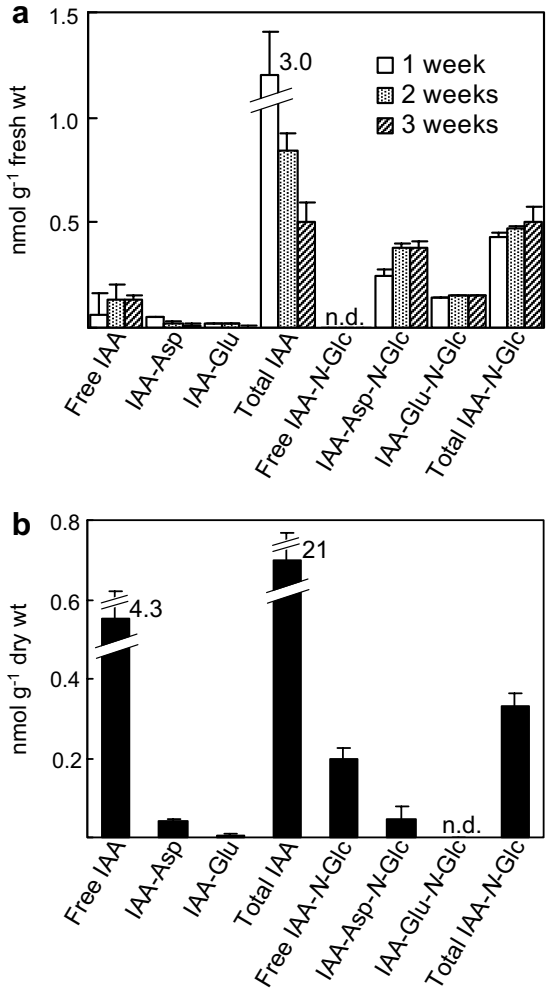

Fig. 5. Quantification of IAA (**1**) metabolites in 1–3-week-old plants (a) and seeds (b). The error bars indicate the standard deviations of three replicates.

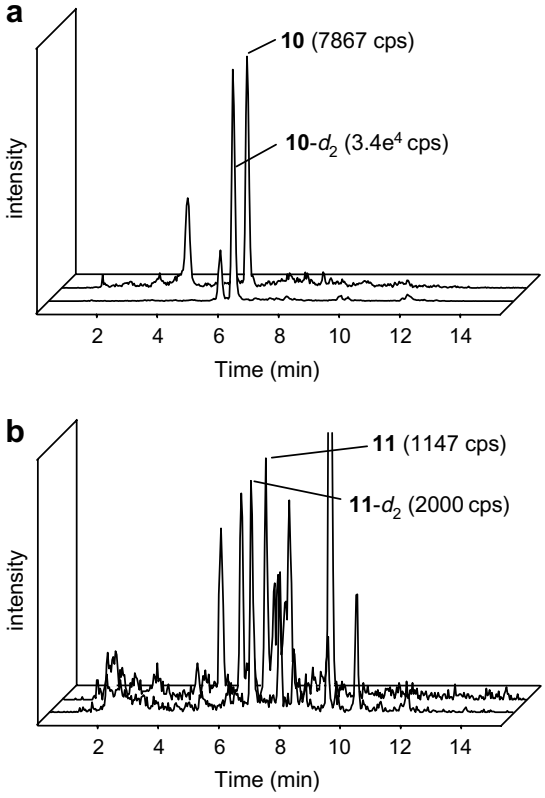

Fig. 6. Administration of [2′,2′-$^2H_2$]IAA-Asp (**3**-d_2) (a) and [2′,2′-$^2H_2$]IAA-Glu (**4**-d_2) (b) to vegetative growing rice. After 48 h of incubation, the extracts of plants were analyzed by LC–ESI–MS/MS. The incorporation of deuterium into Asp and Glu-bound forms of IAA-*N*-Glc (**9**) was observed.

prepared a methyl ester of IAA-*N*-Glc (**9**) and examined its activity. However, no significant activity was observed either. These results suggest that *N*-glucosylation is likely to be an inactivation process for IAA (**1**) in rice plants.

2.7. Occurrence of IAA-N-Glc (9) and its bound forms in other plants

To examine whether the *N*-glucosylation of IAA (**1**) occurs in other plants, the presence of IAA-*N*-Glc (**9**) and its bound forms was investigated in *Arabidopsis*, *Lotus japonicus*, and maize (Table 3). In *Arabidopsis* and *L. japonicus* seedlings, the total amount of IAA-*N*-Glc (**9**) was estimated to be 0.13 and 0.38 nmol g^{-1} fresh wt, respectively, although the free form of IAA-*N*-Glc (**9**) was not found in either plant. On the other hand, free IAA-*N*-Glc (**9**) was detected at 0.17 nmol g^{-1} fresh wt in seedlings of maize, where the total amounted to 0.44 nmol g^{-1} fresh wt. No detailed characterization of the bound form of IAA-*N*-Glc (**9**) in these plants was performed in the present study.

3. Discussion

As a result of searching for new metabolites in rice plants, we have identified IAA-*N*-Glc (**9**) and two amino acid-bound derivatives, IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**). The formation of *N*-glucosides of IAA (**1**) as well as of IAA-Asp (**3**) has been suggested in a woody plant, Scots pine, after an exogenous application of [^{14}C]IAA (^{14}C-**1**), although their structures were not fully established (Ljung et al., 2001a). In our study, the identity was attained by comparing the naturally occurring compounds with the standard samples, which were chemically synthesized from IAA (**1**) and D-glucose. The synthesis of IAA-*N*-Glc (**9**) proceeded effectively according to the scheme shown in Fig. 4: the formation of a *N*-β-D-glucoside bond was confirmed by the NMR analysis. The IAA-*N*-Glc (**9**) could be converted to its amides with Asp and Glu by a conventional method without problems. The synthetic compounds were identical to the naturally occurring compounds with respect to both LC behavior and MS/MS characteristics. Thus, this is the first report to demonstrate that endogenous IAA (**1**) undergoes *N*-glucosylation in herbaceous plants. The reaction may be closely related to the formation of L-tryptophan-*N*-glucoside, which has been

Table 2
Summary of LC–ESI–MS/MS analysis of metabolites from labeled IAA-Asp and IAA-Glu in rice plants product ion spectra of each compound were obtained with two sets of collision energy (CE) values in the positive mode

Supplied compound	Retention time (min)	Deuterium labeled metabolite	Precursor ion (*m*/*z*)	Ion (*m*/*z*) (abundance)
[2′,2′-$^2H_2$]IAA-Asp (**3**-d_2)	5.98	[2′,2′-$^2H_2$]IAA-Asp-*N*-Glc (**10**-d_2)	455	CE 15, 455 (32), 437 (21), 335 (65), 293 (100); CE 40, 174 (50), 132 (100)
[2′,2′-$^2H_2$]IAA-Glu (**4**-d_2)	6.56	[2′,2′-$^2H_2$]IAA-Glu-*N*-Glc (**11**-d_2)	469	CE 15, 469 (80), 349 (32), 307 (100); CE 40, 174 (72), 132 (100)

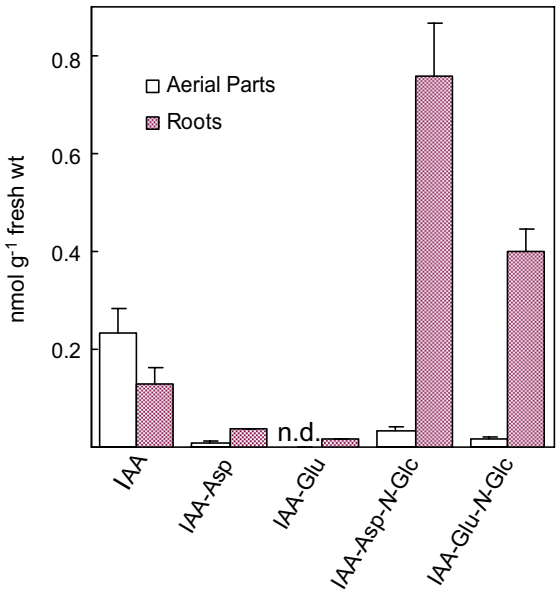

Fig. 7. Tissue-dependent distribution of IAA (**1**) and its metabolites in rice. One- to three-week-old plants were divided into aerial parts and roots. The error bars indicate the standard deviations of three replicates.

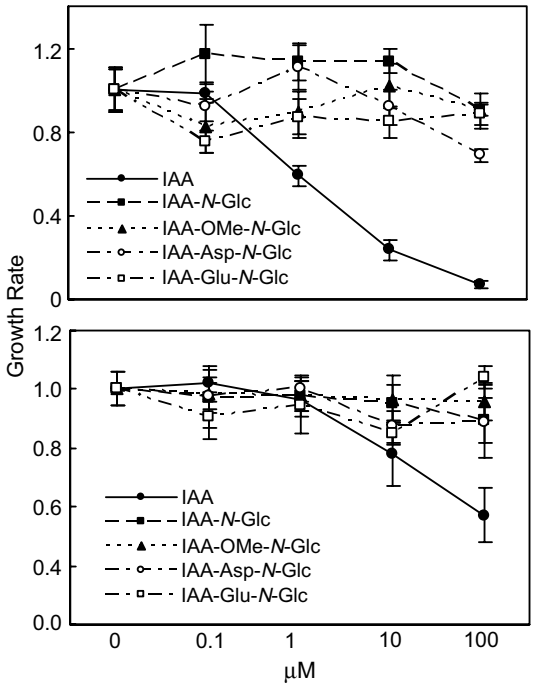

Fig. 8. Hormonal activity of IAA (**1**), IAA-*N*-Glc (**9**), and its derivatives was investigated in terms of inhibition of the growth of the aerial parts (upper) and seminal root (lower). Germinated rice seeds were incubated on a floating plastic net in the solution containing test compounds. The error bars indicate the standard deviations of five replicates.

reported to be present in various fruits such as pears and apples (Diem et al., 2000).

The catabolism as well as biosynthesis of IAA (**1**) has been less studied in rice than in other plants, in spite of the importance of this crop. To date, the presence of oxidized IAA and IAA-inositol in kernels has been reported (Hall, 1980; Tateishi and Yamashita, 1998). We also demonstrated that IAA-Asp (**3**) and IAA-Glu (**4**) are the major amino acid conjugates in rice plant seedlings by a quantitative analysis using LC–ESI–MS/MS (Matsuda et al., 2005). Their levels, however, were relatively low suggesting the possible occurrence of some yet unknown major metabolic pathways. We also found recently that the glucose ester of OxIAA (**5**) is present in much larger amounts than the amino acid conjugates of IAA (**1**) in *Arabidopsis* (Kai et al., 2007), while this compound was scarcely detected in rice seedlings (data not shown). In contrast, the present study has demonstrated that IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) occur in considerable amounts in the vegetative tissues of rice plants. The total amount of IAA-*N*-Glc (**9**) in 3-week-old plants, which almost equals the sum of the amounts of IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**), was approximately the same as that of free IAA (**1**), suggesting that the two conjugates play an important role in the metabolism of IAA (**1**). However, the increase in the total amount of IAA-*N*-Glc (**9**) from 1 to 3 weeks after the germination appeared to be too moderate to account for the drastic decrease in the total amount of IAA (**1**) in this period (Fig. 5). Although the conjugates of IAA-*N*-Glc (**9**) could be further metabolized

Table 3
Survey of plants for the presence of IAA-*N*-Glcs Data shown are the mean ±SD of three replicates

Plant	Concentration (nmol g^{-1} fresh wt)	
	Free IAA-*N*-Glc (**9**)	Total IAA-*N*-Glc (**9**)
Arabidopsis (1 week)	n.d.	0.13 ± 0.059
L. japonicus (4 weeks)	n.d.	0.38 ± 0.023
Maize (1 week)	0.17 ± 0.050	0.44 ± 0.052

n.d., not detected.

in rice plants, it is also possible that there may be other major pathways for the metabolism of IAA (**1**) in young rice plants, which represents an interesting subject of future research.

In vivo precursor administration experiments established that IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) in rice seedlings were not synthesized from IAA-*N*-Glc (**9**), but from IAA-Asp (**3**) and IAA-Glu (**4**), respectively (Fig. 6). Considering the low endogenous levels of these amino acid conjugates of IAA (**1**), they are likely to rapidly undergo *N*-glucosylation after their formation from IAA (**1**) during vegetative growth. This may suggest that the free form of IAA-*N*-Glc (**9**) present in seeds in a significant amount could be synthesized from its amino acid-bound forms via the hydrolysis of their amide bonds. The enzyme(s) that catalyzes such a hydrolytic cleavage might be induced specifically during seed development and/or maturation to give rise to the accumulation of free IAA-*N*-Glc (**9**). However, the occurrence of direct *N*-glucosidation of IAA (**1**) cannot be ruled out, as was suggested by an incorporation experiment in young seedlings of Scots pine (Ljung et al., 2001a). In the present study, although no incorporation of ^{13}C into IAA-*N*-Glc (**9**) was observed in the rice seedlings fed with [$^{13}C_6$]IAA ($^{13}C_6$-**1**) (data not shown), it is still possible that the direct *N*-glucosylation of IAA (**1**) occurs specifically in the developing seeds. It has been well documented that the metabolism of IAA (**1**) in plants is dependent on the stage of growth and/or development (Normanly, 1997; Ljung et al., 2002; Woodward and Bartel, 2005). Little is known, however, about *N*-glucosylation of IAA (**1**), and further studies are required.

IAA-*N*-Glc-related metabolites were found not only in rice plants, but in *Arabidopsis*, *L. japonicus*, and maize (Table 3). Further analyses will likely reveal their distribution in a wider variety of plant species. IAA-*N*-Glc (**9**) and its derivatives showed no significant auxin activity against rice seedlings (Fig. 8) or against *Arabidopsis* (data not shown). Although these results strongly suggest that *N*-glucosylation is an irreversible inactivation process for IAA (**1**) in plants, the function and significance of free IAA-*N*-Glc (**9**) in rice seeds remain unexplained. Some IAA-*N*-Glc-specific glucosidase(s) might emerge during a very limited period in the germination process to regenerate IAA (**1**) that plays a special role in the differentiation. After the IAA-*N*-Glc (**9**) in seeds is used up, no significant accumulation seems to occur in the vegetative tissues until the development of seeds in the next generation.

The glucose ester of IAA (**1**), IAA-Glc (**2**), has been well documented as a metabolite of IAA (**1**) conjugated with a sugar molecule in plants. It has been demonstrated that IAA-Glc (**2**) is mainly accumulated in seeds and is readily hydrolyzed in vivo, and therefore this metabolite has been considered one of the storage forms of auxin that provides active auxin during the early development of seedlings (Cohen and Bandurski, 1982; Normanly, 1997; Ljung et al., 2002). In addition to the storage function, it has been demonstrated that IAA-Glc (**2**) also plays an important role in IAA (**1**) homeostasis in the vegetative tissues. In *Arabidopsis* overexpressing the gene *UGT84B1* encoding IAA (**1**) *O*-glucosyl transferase, significant fluctuations in the levels of IAA (**1**) and its metabolites caused a morphologically abnormal phenotype (Jackson et al., 2002). The localized occurrence of IAA-*N*-Glc (**9**) in seeds indicates that it may supplement the function of IAA-Glc (**2**) during the germination process, as was suggested in the case of Scots pine seeds (Ljung et al., 2001a). However, IAA-*N*-Glc (**9**) is unlikely to play a role in the control of homeostasis in rice seedlings, because it was absent in the vegetative tissues.

4. Conclusion

In conclusion, we have demonstrated the presence of significant amounts of *N*-glucosylated metabolites of IAA (**1**) in rice plants as well as seeds. The *N*-glucosylation of plant components is a relatively uncommon process, except for the biosynthesis of nucleosides, and thus, its biochemical background has not been well elucidated. The identification of gene(s) encoding enzyme(s) that catalyze *N*-glucosylation of IAA (**1**) is under investigation.

5. Experimental

5.1. Plant material

Rice (*Oryza sativa* cv. Nipponbare), *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia), *Lotus japonicus* (ecotype Gifu B-129, a gift from Legume Base, NBRP, Department of Agriculture, Miyazaki University, Miyazaki, Japan), and maize (*Zea mays* cv. Snowdent 108, Yukijirushi Seeds and Plants Co., Sapporo, Japan) were grown with established procedures.

5.2. Search for IAA-conjugates using LC–ESI–MS/MS

Two- to three-week-old rice plants were homogenized in liquid N_2, and subsequently extracted with acetone–H_2O (4:1, v/v) containing 2.5 mM diethyldithiocarbamic acid. After the extraction procedure was repeated, the combined extract was concentrated under reduced pressure. To the concentrate, 7 M NaOH was added in order to release carboxy groups of the metabolites at 100 °C for 3 h. The reaction mixture was then cooled in an ice-bath, acidified with 3 M HCl, and partially purified with Sep-Pak Plus C18 (Waters, Massachusetts, USA). The prepared sample was analyzed using a LC–ESI–MS/MS system consisting of an Agilent 1100 HPLC system coupled to an API3000 triple-quadrupole-stage mass spectrometer (Applied Biosystems/MDS Sciex, Ontario, Canada), operated in either the precursor-ion scan or product-ion scan mode. The conditions for HPLC were as follows: column, Cadenza CD-

C18 (75 × 2 mm, Imtakt, Kyoto, Japan); flow rate, 200 μl/min; solvent, 0.05% (v/v) acetic acid (A) and MeOH (B), 10–90% (v/v) B/(A + B) for 15 min; temperature, 30 °C. The data obtained were processed with Analyst 1.3 software (Applied Biosyntems).

5.3. Chemical synthesis

^{1}H and ^{13}C NMR spectra were obtained with a Bruker Avance 400 or Bruker ARX 500 spectrometer with tetramethylsilane (CD_3CN, dimethylsulfoxide) or sodium 3-(trimethylsilyl)-1-propansulfonate (D_2O) as an internal standard. Assignments of ^{1}H and ^{13}C signals were carried out using ^{1}H–^{1}H COSY, HMQC, and HMBC spectroscopic analysis. High-resolution mass spectra were recorded with a LCMS-IT-TOF (Shimadzu, Kyoto, Japan). Optical rotations were measured with a JASCO P-1010 polarimeter.

*IAA-N-Glc (**9**).* IAA (**1**) was methylated with 1.0 equivalent of methyl iodide and K_2CO_3 in *N*,*N*-dimethylformamide. Treatment of IAA methyl ester (IAA-OMe, **12**) with 2.1 equivalents of triethylsilane under strong-acidic conditions gave 2,3-dihydroIAA-OMe (**13**) in 89% yield. Condensation of **13** with excess D-glucose in MeOH, followed by acetylation with Ac_2O-pyridine (1:2, v/v) gave 2,3-dihydroIAA-OMe-*N*-AcGlc (**14**) in 58% yield. Oxidation of **14** with 1.0 equivalent of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone produced IAA-OMe-*N*-AcGlc (**15**) in 49% yield, which was deprotected in mild alkaline to give IAA-*N*-Glc (**9**) as a colorless solid quantitatively. $[\alpha]_D^{22}$: +3.9 (EtOH; c0.16). ^{1}H NMR (400 MHz, D_2O): δ7.61 (2H, *d*, *J* = 8.1 Hz, IAA, H-4,7), 7.43 (1H, *s*, IAA, H-2), 7.34 (1H, *dd*, *J* = 7.9, 7.4 Hz, IAA, H-6), 7.24 (1H, *d*, *J*= 7.7, 7.5 Hz, IAA, H-5), 5.60 (1H, *d*, *J* = 9.1 Hz, Glc, H-1), 4.05 (1H, *t*, *J* = 9.1 Hz, Glc, H-2), 3.87 (1H, *m*, Glc, H-6), 3.86 (2H, *s*, $CH_2C{=}O$), 3.69–3.77 (3H, *m*, Glc, H-3,4,6), 3.60 (1H, *m*, Glc, H-5). ^{13}C NMR (100 MHz, D_2O): δ179.8 (C=O), 139.2 (IAA, C-7a), 130.7 (IAA, C-3a), 127.2 (IAA, C-2), 125.6 (IAA, C-6), 123.4 (IAA, C-5), 121.8 (IAA, C-4), 113.2 (IAA, C-7), 112.3 (IAA, C-3), 87.2 (Glc, C-1), 81.0 (Glc, C-3 or 4), 79.1 (Glc, C-3 or 4), 74.5 (Glc, C-2), 72.0 (Glc, C-5), 60.1 (Glc, C-6), 33.2 ($CH_2C{=}O$). HRESI-MS: *m*/*z* 338.1239 $[M+H]^+$ (calc. for $C_{16}H_{20}NO_7$, 338.1239).

*IAA-Asp-N-Glc (**10**) and IAA-Glu-N-Glc (**11**).* IAA-*N*-Glc (**9**) was acetylated with excess acetic anhydride, and subsequently condensed with L-amino acid methyl esters to give *N*-glucosides of IAA-amide (yield: Asp, 38%; Glu, 54%). Treatment of respective *N*-glucosides of IAA-amide with 1M KOH afforded IAA-Asp-*N*-Glc (**10**) and IAA-Glu-*N*-Glc (**11**) as colorless solids quantitatively. Physicochemical data for IAA-Asp-*N*-Glc (**10**). $[\alpha]_D^{22}$: +12.5° (EtOH; c0.22). ^{1}H NMR (400 MHz, D_2O): δ7.61 (1H, *d*, *J* = 8.4 Hz, IAA, H-7), 7.59 (1H, *d*, *J* = 8.1 Hz, IAA, H-4), 7.46 (1H, *s*, IAA, H-2), 7.34 (1H, *m*, IAA, H-6), 7.23 (1H, *m*, IAA, H-5), 5.62 (1H, *d*, *J* = 9.2 Hz, Glc, H-1), 4.78 (1H, *m*, Asp, H-α), 4.06 (1H, *t*, *J* = 9.2 Hz, Glc, H-2), 3.88 (1H, *m*, Glc, H-6), 3.72–3.81 (5H, *m*, Glc H-3,4,6, $CH_2C{=}ONH$), 3.65 (1H, *dd*, *J* = 9.4, 9.2 Hz, Glc, H-5), 2.89 (2H, *m*, Asp, H-β). ^{13}C NMR (100 MHz, D_2O): δ177.3 (C=O), 177.0 (C=O), 176.6 (C=O), 139.4 (IAA, C-7a), 130.6 (IAA, C-3a), 127.5 (IAA, C-2), 125.7 (IAA, C-6), 123.5 (IAA, C-5), 121.8 (IAA, C-4), 113.2 (IAA, C-7), 112.4 (IAA, C-3), 87.1 (Glc, C-1), 81.0 (Glc, C-4), 79.2 (Glc, C-3), 74.4 (IAA, C-2), 72.0 (IAA, C-5), 63.3 (IAA, C-6), 51.9 (Asp, C-α), 38.1 (Asp, C-β), 34.7 ($CH_2C{=}ONH$). HRESI-MS: *m*/*z* 453.1510 $[M+H]^+$ (calc. for $C_{20}H_{25}N_2O_{10}$, 453.1509). Physicochemical data for IAA-Glu-*N*-Glc (**11**). $[\alpha]_D^{22}$: +4.2° (EtOH; c0.23). ^{1}H NMR (400 MHz, D_2O): δ7.62 (2H, *d*, *J* = 8.3 Hz, IAA, H-4,7), 7.47 (1H, *s*, IAA, H-2), 7.34 (1H, *dd*, *J* = 8.2, 7.2 Hz, IAA, H-6), 7.24 (1H, *dd*, *J* = 7.7, 7.3 Hz, IAA, H-5), 5.62 (1H, *d*, *J* = 9.1 Hz, Glc, H-1), 4.31 (1H, *dd*, *J*= 10.4, 4.9 Hz, Glu, H-α), 4.07 (1H, *dd*, *J* = 9.2, 9.1 Hz, Glc, H-2), 3.88 (1H, *m*, Glc, H-6), 3.73–3.81 (5H, *m*, Glc, H-3,4,6, $CH_2C{=}ONH$), 3.66 (1H, *m*, Glc, H-5), 2.36 (2H, *t*, *J* = 7.3 Hz, Glu, H-γ), 2.17 (1H, *m*, Glu, H-β), 1.92 (1H, *m*, Glu, H-β). ^{13}C NMR (100 MHz, D_2O): δ179.7 (Glu, COOH), 177.8 (C=O), 177.6 (C=O), 139.4 (IAA, C-7a), 130.6 (IAA, C-3a), 127.5 (IAA, C-2), 125.7 (IAA, C-6), 123.5 (IAA, C-5), 121.8 (IAA, C-4), 113.2 (IAA, C-7), 112.5 (IAA, C-3), 87.1 (Glc, C-1), 81.0 (Glc, C-4), 79.2 (Glc, C-3), 74.4 (Glc, C-2), 72.0 (Glc, C-5), 63.3 (Glc, C-6), 54.8 (Glu, C-α), 34.7 ($CH_2C{=}ONH$), 32.6 (Glu, C-γ), 28.3 (Glu, C-β). HRESI-MS: *m*/*z* 467.1684 $[M+H]^+$ (Calc. for $C_{21}H_{27}N_2O_{10}$, 467.1665).

*IAA-OMe-N-Glc (**17**).* The carboxy group of IAA-*N*-Glc (**9**) was methylated with (trimethylsily)diazomethane to afford **17** in 73% yield. ^{1}H NMR (500 MHz, CD_3CN): δ7.53 (1H, *d*, *J* = 7.9 Hz), 7.51 (1H, *d*, *J* = 8.3 Hz), 7.33 (1H, *s*), 7.21 (1H, *ddd*, *J* = 8.2, 7.1, 0.8 Hz), 7.12 (1H, *ddd*, *J* = 7.9, 7.3, 0.7 Hz), 5.43 (1H, *d*, *J* = 9.1 Hz), 3.83 (1H, *t*, *J* = 8.9 Hz), 3.77 (2H, *s*), 3.76 (1H, *dd*, *J* = 12.1, 2.5 Hz), 3.66 (3H, *s*), 3.61 (1H, *dd*, *J* = 12.0, 5.4 Hz Hz), 3.50–3.56 (2H, *m*), 3.45 (1H, *dd*, *J* = 9.3, 9.2 Hz). ^{13}C NMR (125 MHz, CD_3CN): δ173.6, 137.9, 129.4, 125.4, 123.2, 121.0, 120.0, 111.5, 110.0, 85.9, 79.9, 78.6, 73.2, 71.1, 62.6, 52.6, 31.5. HRESI-MS: *m*/*z* 352.3586 $[M+H]^+$ (Calc. for $C_{17}H_{22}NO_7$, 352.3591).

*IAA-N-[6,6-$^2H_2$]Glc (**9**-d_2), IAA-Asp-N-[6,6-$^2H_2$]Glc (**10**-d_2), and IAA-Glu-N-[6,6-$^2H_2$]Glc (**11**-d_2).* **9**-d_2 was synthesized using the same procedure as IAA-*N*-Glc (**9**), except for the use of [6,6-$^2H_2$]D-glucose. **10**-d_2 and **11**-d_2 were synthesized from **9**-d_2 as described above. Their structures were confirmed by NMR spectroscopy and mass spectrometry.

5.4. Quantitative analysis

Plant tissues were homogenized in liquid N_2, to which acetone–H_2O (4:1 v/v) containing 2.5 mM diethyldithiocarbamic acid and the internal standards were added. Extraction was conducted at 4 °C for 2 h twice, and the combined extract was evaporated to remove the solvent.

Aqueous concentrate was acidified to pH 3.0, and applied to a Sep-Pak Plus C18 cartridge. The eluate with CH_3CN–H_2O (7:3, v/v) was concentrated under reduced pressure. The following transitions from the precursor ($[M+H]^+$) to product ions were used for MRM: IAA (**1**), 176 → 130; [$^{13}C_6$]IAA ($^{13}C_6$-**1**), 182 → 136; IAA-Asp (**3**), 291 → 130; [2′,2′-$^2H_2$]IAA-Asp (**3**-d_2), 293 → 132; IAA-Glu (**4**), 305 → 130; [2′,2′-$^2H_2$]IAA-Glu (**4**-d_2), 307 → 132; IAA-*N*-Glc (**9**), 338 → 176; IAA-*N*-[6,6-$^2H_2$]Glc (**9**-d_2), 340 → 176; IAA-Asp-*N*-Glc (**10**), 453 → 291; IAA-Asp-*N*-[6,6-$^2H_2$]Glc (**10**-d_2), 455 → 291; IAA-Glu-*N*-Glc (**11**), 467 → 305; IAA- Glu-*N*-[6,6-$^2H_2$]Glc (**11**-d_2), 469 → 305. The HPLC conditions were as described above. These compounds were quantified using the ratio of the peak area of each analyte to that of its internal standard. Total amounts of IAA (**1**) and IAA-*N*-Glc (**9**) were analyzed after alkaline-hydrolysis of plant extracts by the same procedure described above.

5.5. Feeding experiments

Three-week-old rice plants grown in Kimura's B solution were transferred to a solution containing 10 μM of each deuterium-labeled compound, [2′,2′-$^2H_2$]IAA-Asp (**3**-d_2), [2′,2′-$^2H_2$]IAA-Glu (**4**-d_2), and IAA-*N*-[6,6-$^2H_2$]Glc (**9**-d_2), respectively. After 48 h with a 16-h photoperiod at 28 °C, the samples for analysis were prepared with the same procedure described above. The incorporation of deuterium into metabolites was analyzed by LC–ESI–MS/MS.

5.6. Shoot- and root-elongation assay

Surface-sterilized rice seeds were transferred to a floating plastic net and incubated hydroponically in an appropriate concentrate of test compounds under continuous light at 28 °C for 6 days. The water culture was refreshed every day to avoid depletion of the test compounds. The lengths of the shoot and seminal root were measured.

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