

# UGT74D1 Catalyzes the Glucosylation of 2-Oxindole-3-Acetic Acid in the Auxin Metabolic Pathway in *Arabidopsis*

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IAA is a naturally occurring auxin that plays a crucial role in the regulation of plant growth and development. The endogenous concentration of IAA is spatiotemporally regulated by biosynthesis, transport and its inactivation in plants. Previous studies have shown that the metabolism of IAA to 2-oxindole-3-acetic acid (OxIAA) and OxIAA-glucoside (OxIAA-Glc) may play an important role in IAA homeostasis, but the genes involved in this metabolic pathway are still unknown. In this study, we show that UGT74D1 catalyzes the glucosylation of OxIAA in *Arabidopsis*. By screening yeasts transformed with *Arabidopsis* UDP-glycosyltransferase (UGT) genes, we found that OxIAA-Glc accumulates in the culture media of yeasts expressing UGT74D1 in the presence of OxIAA. Further, we showed that UGT74D1 expressed in *Escherichia coli* converts OxIAA to OxIAA-Glc. The endogenous concentration of OxIAA-Glc decreased by 85% while that of OxIAA increased 2.5-fold in *ugt74d1*-deficient mutants, indicating the major role of UGT74D1 in OxIAA metabolism. Moreover, the induction of UGT74D1 markedly increased the level of OxIAA-Glc and loss of root gravitropism. These results indicate that UGT74D1 catalyzes a committed step in the OxIAA-dependent IAA metabolic pathway in *Arabidopsis*.

**Keywords:** *Arabidopsis* • Auxin • Glucosyltransferase • Metabolism • Plant hormone.

**Abbreviations:** EST,  $\beta$ -estradiol; GH3, GRETCHEN HAGEN 3; GST, glutathione S-transferase; GUS,  $\beta$ -glucuronidase; IAA-Asp, IAA-aspartate; IAA-Glc, 1-O-(indol-3-ylacetyl)- $\beta$ -D-glucose; IAA-Glu, IAA-glutamate; IBA, indole-3-butylic acid; IBA-Glc, 1-O-(indol-3-ylbutanoyl)- $\beta$ -D-glucose; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; MS, Murashige and Skoog; MS/MS, tandem mass spectrometry; OxIAA, 2-oxindole-3-acetic acid;

OxIAA-Glc, OxIAA-glucoside; RT-PCR, reverse transcription-PCR; UGT, UDP-glycosyltransferase; UPLC, ultra performance liquid chromatography.

## Introduction

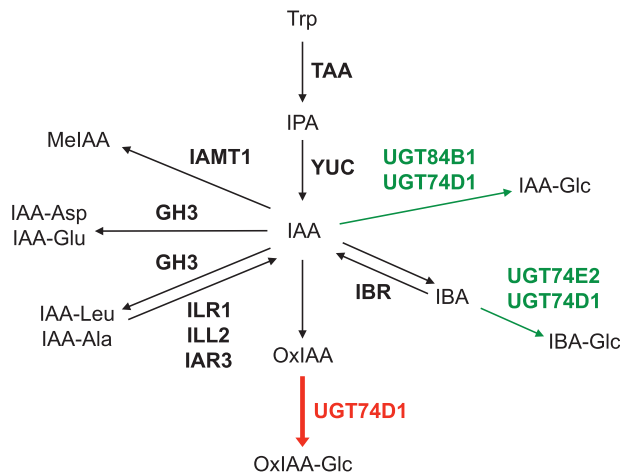
The phytohormone auxin plays a key role in many aspects of a plant's life cycle, including embryogenesis, organ formation and environmental responses (Woodward and Bartel 2005, Zhao 2010). Auxin regulates cell elongation and differentiation in a concentration-dependent manner. A naturally occurring auxin, IAA, is mainly synthesized from tryptophan via indole-3-pyruvate (IPA) by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) flavin-containing monooxygenases in *Arabidopsis* (Fig. 1) (Mashiguchi et al. 2011, Stepanova et al. 2011, Won et al. 2011). The TAA and YUC family genes are regulated spatiotemporally and contribute to the uneven distribution of IAA in plants (Cheng et al. 2006, Cheng et al. 2007, Stepanova et al. 2008, Tao et al. 2008, Yamada et al. 2009). Plants modulate cellular IAA concentrations using membrane-localized proteins, including PIN efflux carriers, AUX1/LAX influx transporters and ABCB transporters (Hayashi 2012). PIN efflux carriers play a crucial role in the regulation of polar auxin transport (Vanneste and Friml 2009). A rapid change in PIN localization by vesicle trafficking determines the direction of auxin flow in plant organs (Grunewald and Friml 2010).

Previous studies indicate that various conjugation and degradation enzymes also play important roles in the regulation of IAA concentration (Fig. 1). Several GRETCHEN HAGEN 3 (GH3) genes encode enzymes that catalyze the formation of IAA-amino acid conjugates (Staswick et al. 2005). Each GH3 enzyme has a slightly different substrate specificity and probably contributes to the formation of several types of

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**Fig. 1** Main IAA biosynthetic pathway and proposed IAA metabolic pathways in *Arabidopsis*. Genes encoding IAA biosynthetic and metabolic enzymes identified in *Arabidopsis* are shown in bold. The red arrow indicates a novel reaction step catalyzed by UGT74D1. The green arrows indicate the proposed reaction steps catalyzed by previously characterized UGTs.

IAA–amino acid conjugates (Staswick et al. 2005). The overexpression of *GH3* genes results in reduced auxin phenotypes, such as dwarfism, short hypocotyls and fewer lateral roots in *Arabidopsis* (Nakazawa et al. 2001, Takase et al. 2003, Takase et al. 2004). In contrast, *gh3*-deficient mutants of *Arabidopsis* show slightly increased sensitivity to IAA (Staswick et al. 2005). Previous studies using  $^{14}\text{C}$ -labeled IAA demonstrated that IAA is metabolized to IAA-Asp and IAA-Glu in *Arabidopsis* (Fig. 1) (Östin et al. 1998, LeClere et al. 2002). In addition, IAA-Asp and IAA-Glu levels are substantially increased in the IAA-overproducing *supreroor1* and *supreroor2* mutants (Barlier et al. 2000, Mashiguchi et al. 2011, Novák et al. 2012). These results indicate that the GH3-mediated formation of IAA-Asp and IAA-Glu plays an important role in the inactivation of IAA.

Similar to IAA, treatment with some IAA–amino acid conjugates, such as IAA-Leu, IAA-Ala and IAA-Phe, inhibits root growth in *Arabidopsis*. Loss-of-function mutants of IAA-LEUCINE RESISTANT 1 (ILR1) and IAA-ALANINE RESISTANT 3 (IAR3) exhibit reduced sensitivity to IAA-Leu and IAA-Ala, respectively (Bartel and Fink 1995, Davies et al. 1999). ILR1, ILR1-LIKE 2 (ILL2) and IAR3 are endoplasmic reticulum (ER)-localized amidohydrolases that generate IAA from IAA–amino acid conjugates (Fig. 1) (LeClere et al. 2002). Previous studies demonstrated that *ilr1 iar3 ill2* triple mutants exhibit low-auxin phenotypes, and the endogenous levels of IAA are reduced in these mutants (LeClere et al. 2002, Rampey et al. 2004). These reports suggest that IAA–amino acid conjugates function as auxin storage forms, and the GH3 and amidohydrolase families play a role in the regulation of IAA concentration.

IAA CARBOXYL METHYLTRANSFERASE 1 (IAMT1) catalyzes the methylation of the carboxyl group, which inactivates IAA to its methyl ester (MeIAA) (Fig. 1) (Zubieta et al. 2003).

Ectopic expression of *IAMT1* leads to the low-auxin phenotype, including upward curling leaves and disrupted gravitropism, and reduced IAA sensitivity in *Arabidopsis*. Suppression of *IAMT1* by RNA interference (RNAi) results in epinastic small leaves and dwarfism. Analysis of a GUS ( $\beta$ -glucuronidase) reporter gene under the *IAMT1* promoter suggests the spatiotemporal regulation of *IAMT1* in *Arabidopsis* (Qin et al. 2005). *IAMT1* may also play an important role in leaf morphogenesis (Qin et al. 2005).

Glucosylation is probably implicated in the inactivation of IAA, although its physiological roles are still unknown. UDP-glucosyltransferases (UGTs) are one of the largest families of glycosyltransferases in plants. There are 107 UGTs in *Arabidopsis* (Yonekura-Sakakibara and Saito 2009). Some UGTs catalyze the glucosylation of plant hormones, including auxin, ABA, cytokinins and salicylic acid, using UDP-glucose as a co-substrate (Lim and Bowles 2004, Gachon et al. 2005, Yonekura-Sakakibara and Saito 2009). UGT84B1 catalyzes the conversion of IAA to 1-O-(indol-3-ylacetyl)- $\beta$ -D-glucose (IAA-Glc) (Fig. 1) (Jackson et al. 2001), which widely exists in higher plants (Cohen and Bandurski 1982, Sitbon et al. 1993, Jakubowska and Kowalczyk 2004, Iyer et al. 2005). Overexpression of *UGT84B1* leads to enhanced shoot branch formation, gravitropism defects and decreased sensitivity to auxin in *Arabidopsis* (Jackson et al. 2002, Lim and Bowles 2004). It has been shown that UGT74E2 catalyzes the formation of 1-O-(indol-3-ylbutanoyl)- $\beta$ -D-glucose (IBA-Glc) from indole-3-butylic acid (IBA), which is probably generated from IAA in plants (Tognetti et al. 2010). Overexpression of *UGT74E2* results in short stature, compressed rosette, enhanced shoot branching and high osmotic stress tolerance in *Arabidopsis*. A recent study demonstrated that UGT74D1 converts both IAA and IBA to their corresponding glucosides *in vitro*, implicating it in the glucosylation of both IAA and IBA *in planta* (Jin et al. 2013). However, the phenotypes of *UGT74D1*-overexpressing plants were different from those observed in *UGT84B1*- or *UGT74E2*-overexpressing plants (Jin et al. 2013), suggesting the distinct functions of UGT74D1 in *Arabidopsis*.

The oxidation of IAA to 2-oxindole-3-acetic acid (OxIAA) is one type of IAA inactivation reaction in plants (Fig. 1). This IAA inactivation reaction is irreversible, and OxIAA does not show growth-promoting activity (Reinecke and Bandurski 1983, Normanly 1997, Szein et al. 1999, Chamarro et al. 2001, Kowalczyk and Sandberg 2001, Ljung et al. 2002, Woodward and Bartel 2005). In *Zea mays* tissue extracts, IAA was enzymatically converted to OxIAA under aerobic conditions (Reinecke and Bandurski 1988), and the levels of OxIAA increased significantly in plants that overproduce IAA (Novak et al. 2012, Sairanen et al. 2012). It has been demonstrated that IAA is metabolized to OxIAA and its glucoside in *Arabidopsis* seedlings (Östin et al. 1998). The endogenous levels of OxIAA glucoside, 1-O-(2-oxindol-3-ylacetyl)- $\beta$ -D-glucose (OxIAA-Glc), are much higher than that of IAA-Asp, IAA-Glu, IAA-Glc and OxIAA in *Arabidopsis* (Kai et al. 2007), suggesting that the OxIAA pathway is an important metabolic pathway for IAA

homeostasis in plants (Ljung et al. 2002, Rosquete et al. 2012, Ljung 2013). However, the genes involved in the OxIAA metabolic pathway are still unknown.

Although various metabolic pathways have been proposed for the regulation of the IAA level, the main IAA metabolic pathway in plants remains unclear. Östin et al. (1998) demonstrated that IAA was primarily metabolized to IAA-Asp and IAA-Glu by the application of a relatively high concentration of IAA in *Arabidopsis*. Interestingly, most of the IAA applied was metabolized to OxIAA and OxIAA-Glc and only a small portion was conjugated with Asp or Glu when a relatively low concentration of IAA was applied. These results suggest that the GH3 and OxIAA pathways may have critical but distinct roles in IAA homeostasis. Thus, identification of the genes involved in the OxIAA pathway may provide the key to elucidate the main IAA metabolic pathway in plants.

In this study, we show that UGT74D1 plays an important role in the OxIAA pathway in *Arabidopsis* (Fig. 1). UGT74D1 heterologously expressed in *E. coli* cells converted OxIAA to OxIAA-Glc in the presence of UDP-glucose. The endogenous level of OxIAA-Glc is dramatically reduced in *ugt74d1*-deficient mutants. Moreover, the induction of UGT74D1 resulted in increased level of OxIAA-Glc and loss of root gravitropism in *Arabidopsis*. In conclusion, UGT74D1 catalyzes a committed step in the OxIAA pathway, and contributes to IAA homeostasis in *Arabidopsis*.

## Results

### Isolation of an OxIAA glucosyltransferase gene in *Arabidopsis*

*Arabidopsis* UGTs are divided into 14 groups (groups A–N) (Li et al. 2001, Ross et al. 2001). Group L contained the UGTs (UGT84B1, UGT74E2 and UGT74D1) that catalyze the glucosylation of IAA and IBA (Fig. 1; Supplementary Fig. S1) (Jackson et al. 2001, Tognetti et al. 2010, Jin et al. 2013). In addition, UGT74F1, UGT74F2, UGT84A2 and UGT74B1 in group L mediate the glucosylation of various aromatic compounds: salicylic acid, anthranilate, sinapic acid and indole-glucosinolate (Lim et al. 2002, Messner et al. 2003, Quiel and Bender 2003, Grubb et al. 2004, Sinlapadech et al. 2007). From these results, we anticipated that OxIAA glucosyltransferases would also be present in group L.

To identify the genes that encode OxIAA glucosyltransferases in *Arabidopsis*, we used a screening system that involves budding yeast *Saccharomyces cerevisiae* transformed with each UGT gene in group L. Budding yeasts produce high amounts of UDP-glucose for the synthesis of cell wall components,  $\beta$ -1,3-glucan and  $\beta$ -1,6-glucan (Douglas et al. 1994, Shahinian and Bussey 2000, Oka and Jigami 2006). We expressed the 13 UGT genes individually in budding yeasts and carried out screening of the yeasts that produced OxIAA-Glc by supplementing the culture media with OxIAA (Table 1). Some UGTs (UGT74E1, UGT74E2, UGT75B2 and UGT84B2) are not tested in this study

**Table 1** Screening of *Arabidopsis* OxIAA glucosyltransferases in *S. cerevisiae* in the presence of OxIAA

Gene	AGI code	OxIAA-Glc
UGT74B1	AT1G24100	ND
UGT74F1	AT2G43840	ND
UGT74F2	AT2G43820	ND
UGT74C1	AT2G31790	ND
UGT74D1	AT2G31750	Detected
UGT74E1	AT1G05675	NT
UGT74E2	AT1G05680	NT
UGT75B1 <sup>a</sup>	AT1G05560	ND
UGT75B2 <sup>a</sup>	AT1G05530	NT
UGT75D1	AT4G15550	ND
UGT75C1	AT4G14090	ND
UGT84A1	AT4G15480	ND
UGT84A2	AT3G21560	ND
UGT84A3	AT4G15490	ND
UGT84A4	AT4G15500	ND
UGT84B1	AT2G23260	ND
UGT84B2 <sup>a</sup>	AT2G23250	NT

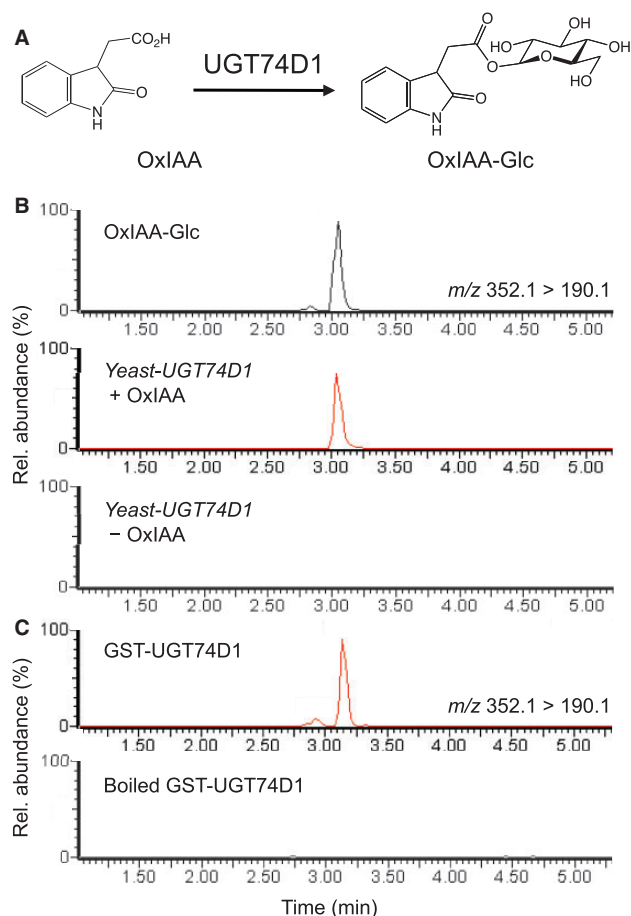
<sup>a</sup> No conversion of OxIAA to OxIAA-Glc was detected in the previous study (Jackson et al. 2001).

ND, not detected. NT, not tested.

due to the limited availability of *Arabidopsis* full-length clones (Table 1; Supplementary Table S1). However, it has been shown previously that UGT75B2 and UGT84B2 do not convert OxIAA to OxIAA-Glc *in vitro* (Jackson et al. 2001). After incubation of the budding yeasts with OxIAA, we analyzed OxIAA metabolites in the yeast cells and the culture media using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). OxIAA-Glc was identified by using a product ion at  $m/z$  190.1 (3.14 min) generated from the parent ion at  $m/z$  352.1 on an MS/MS chromatogram (Fig. 2B). Our analysis showed accumulation of OxIAA-Glc in the culture media of yeasts transformed with UGT74D1 (*Yeast-UGT74D1* + OxIAA), suggesting that UGT74D1 may convert OxIAA to OxIAA-Glc (Fig. 2A, B). It is important to note that a product ion peak at  $m/z$  190.1 for OxIAA-Glc was not detected in the culture medium of yeast cells expressing UGT74D1 without supplementation of the OxIAA (*Yeast-UGT74D1*—OxIAA in Fig. 2B). Similarly, no OxIAA-Glc was detected in the culture of yeast cells transformed with the other UGT genes tested in this study (Table 1).

### UGT74D1 catalyzes the conversion of OxIAA to OxIAA-Glc

Recently, Jin et al. (2013) demonstrated that UGT74D1 converts IBA, indole-3-propionic acid, IAA, 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-carboxylic acid to their corresponding glucosides *in vitro*. However, OxIAA was not investigated as a naturally occurring



**Fig. 2** Conversion of OxIAA to OxIAA-Glc by UGT74D1. (A) Enzymatic reaction catalyzed by UGT74D1. (B) The MS/MS chromatogram for authentic OxIAA-Glc and metabolites of yeast expressing *UGT74D1* in the culture medium with OxIAA (*Yeast-UGT74D1* + OxIAA) or without OxIAA (*Yeast-UGT74D1* – OxIAA). (C) The MS/MS chromatogram for the reaction products of GST-UGT74D1 and boiled GST-UGT74D1.

substrate (Jin et al. 2013). To investigate directly if UGT74D1 exhibits glucosyltransferase activity against OxIAA, we expressed glutathione *S*-transferase (GST) fused with UGT74D1 (GST-UGT74D1) in *E. coli* for use in an enzyme assay. From the enzymatic reactions containing the GST-UGT74D1 fusion protein, we detected a product ion at *m/z* 190.1, representing OxIAA-Glc, on the MS/MS chromatogram (Fig. 2C), indicating that the GST-UGT74D1 protein efficiently converted OxIAA to OxIAA-Glc using UDP-glucose as a co-substrate. The formation of OxIAA-Glc was abolished in the absence of GST-UGT74D1 proteins (boiled GST-UGT74D1 in Fig. 2C). Consistent with the results of Jin et al. (2013), GST-UGT74D1 also catalyzed the conversion of IAA to IAA-Glc in our assay conditions (Supplementary Fig. S2). To determine the substrate preference of UGT74D1, we analyzed the  $K_m$  values of GST-UGT74D1 for OxIAA and IAA (Table 2). The  $K_m$  value of GST-UGT74D1 for OxIAA (15.98  $\mu\text{M}$ ) was 5.5 times lower than that for IAA (88.27  $\mu\text{M}$ ), indicating that UGT74D1 has

**Table 2** Kinetic parameters for the glucosyltransferase activity of GST-UGT74D1 towards OxIAA and IAA

Substrate	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1}\text{min}^{-1}$ )
OxIAA	15.98	58.31	3.65
IAA	88.27	48.56	0.55

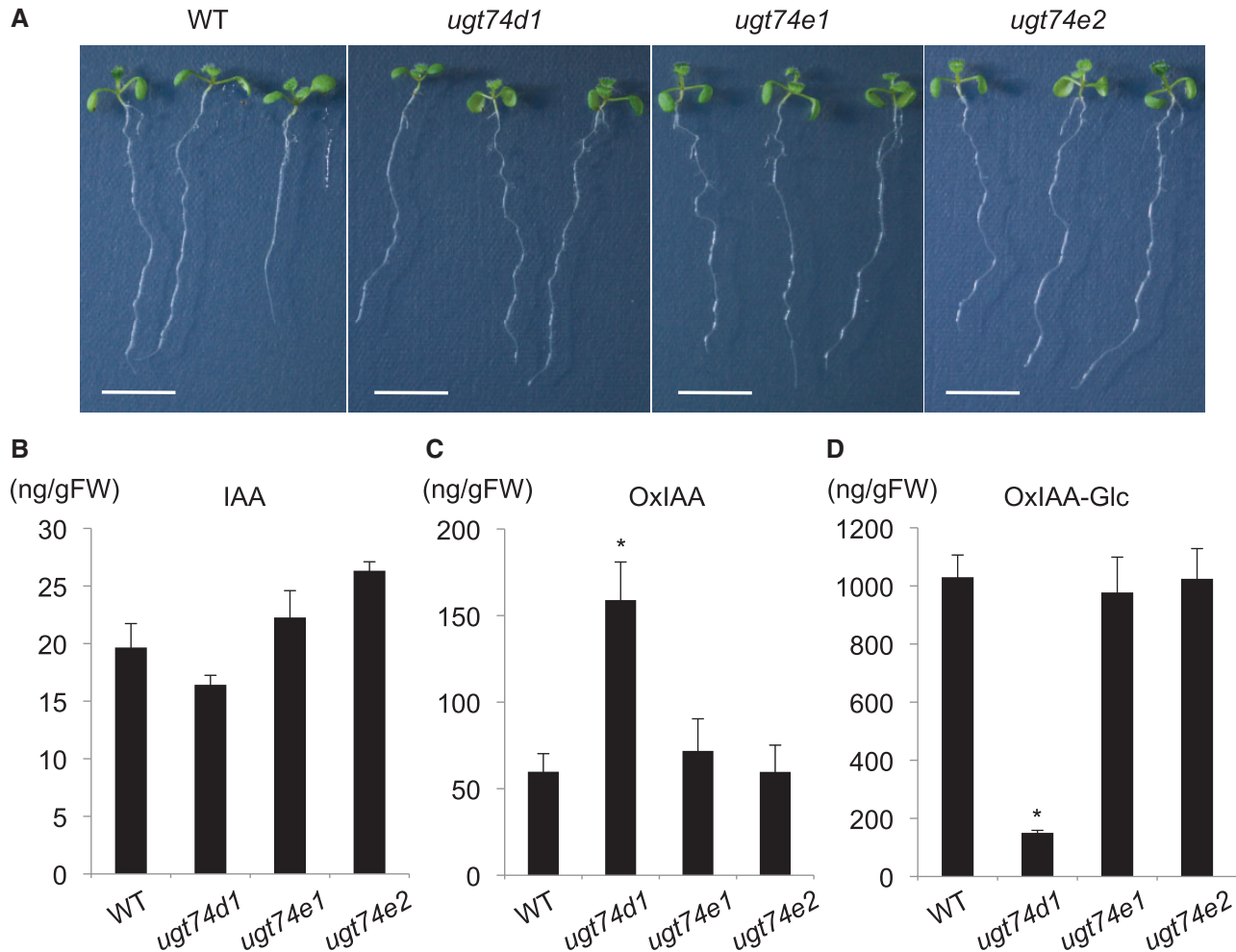
higher affinity for OxIAA than IAA. The catalytic efficiency ( $k_{\text{cat}}/K_m$ ) of GST-UGT74D1 for OxIAA (3.65  $\mu\text{M}^{-1}\text{min}^{-1}$ ) was 6.6 times higher than that for IAA (0.55  $\mu\text{M}^{-1}\text{min}^{-1}$ ) (Table 2). These results indicate that UGT74D1 has a higher specificity towards OxIAA than IAA.

### OxIAA-Glc is mainly produced by UGT74D1 in *Arabidopsis*

To investigate whether *UGT74D1* mainly contributes to OxIAA metabolism in *Arabidopsis*, we analyzed the endogenous levels of IAA, OxIAA and OxIAA-Glc in the *ugt74d1*-deficient mutant. The transcripts of *UGT74D1* were not detected in the T-DNA insertion mutant, *ugt74d1* (SALK\_004870) (Supplementary Fig. S3). The amounts of IAA metabolites in the wild type were found to be in the following order, OxIAA-Glc > OxIAA > IAA (Fig. 3). The levels of OxIAA-Glc were nearly two orders of magnitude higher than that of IAA. No significant phenotype or change in IAA concentration was observed in *ugt74d1* (Jin et al. 2013; Fig. 3A, B). However, the endogenous level of OxIAA in the *ugt74d1* mutant was 2.5-fold higher than that in the wild-type (Fig. 3C). Moreover, the level of OxIAA-Glc decreased by 85% in *ugt74d1* when compared with that in the wild type (Fig. 3D). These results indicate that UGT74D1 mainly catalyzes the formation of OxIAA-Glc from OxIAA in *Arabidopsis*. To examine whether *UGT74D1*-related genes contribute to OxIAA-Glc formation, we further analyzed the endogenous levels of OxIAA and OxIAA-Glc in the knockout mutants of *UGT74D1* homologs, *ugt74e1* (SALK\_045974) (Supplementary Fig. S3) and *ugt74e2* (SALK\_016116) (Tognetti et al. 2010). Our data showed that no significant change in the levels of OxIAA and OxIAA-Glc occurred in these mutants (Fig. 3B, C, D). From these results, we conclude that a large fraction of OxIAA-Glc is produced by UGT74D1, but other UGTs may play an auxiliary role in the formation of OxIAA-Glc in *Arabidopsis*.

### Induction of UGT74D1 reduces the root gravitropism in *Arabidopsis*

Previous studies clearly showed that the endogenous levels of IAA-Glc and IBA-Glc significantly increased in transgenic plants overexpressing *UGT84B1* and *UGT74E2*, respectively (Jackson et al. 2002, Tognetti et al. 2010). To demonstrate further the biological function of UGT74D1 as an OxIAA glucosyltransferase *in vivo*, we generated *Arabidopsis* plants transformed with  $\beta$ -estradiol (EST)-inducible *UGT74D1* (*UGT74D1ox*) (Fig. 4A). As shown in Fig. 4B, the expression of *UGT74D1* was markedly increased in EST-treated *UGT74D1ox* plants when compared with non-EST-treated (mock) or vector control plants (*pER8*).



**Fig. 3** Analysis of the OxIAA pathway in *ugt74d1*. (A) Phenotypes of the wild-type (WT), *ugt74d1*, *ugt74e1* and *ugt74e2*. Bars indicate 5 mm. (B and C) Endogenous concentration of IAA, OxIAA and OxIAA-Glc in the WT, *ugt74d1*, *ugt74e1* and *ugt74e2*. Values are mean  $\pm$  SD ( $n = 3$ ). \*Differences between the WT and mutant are statistically significant at  $P < 0.05$  (Student's  $t$ -test).

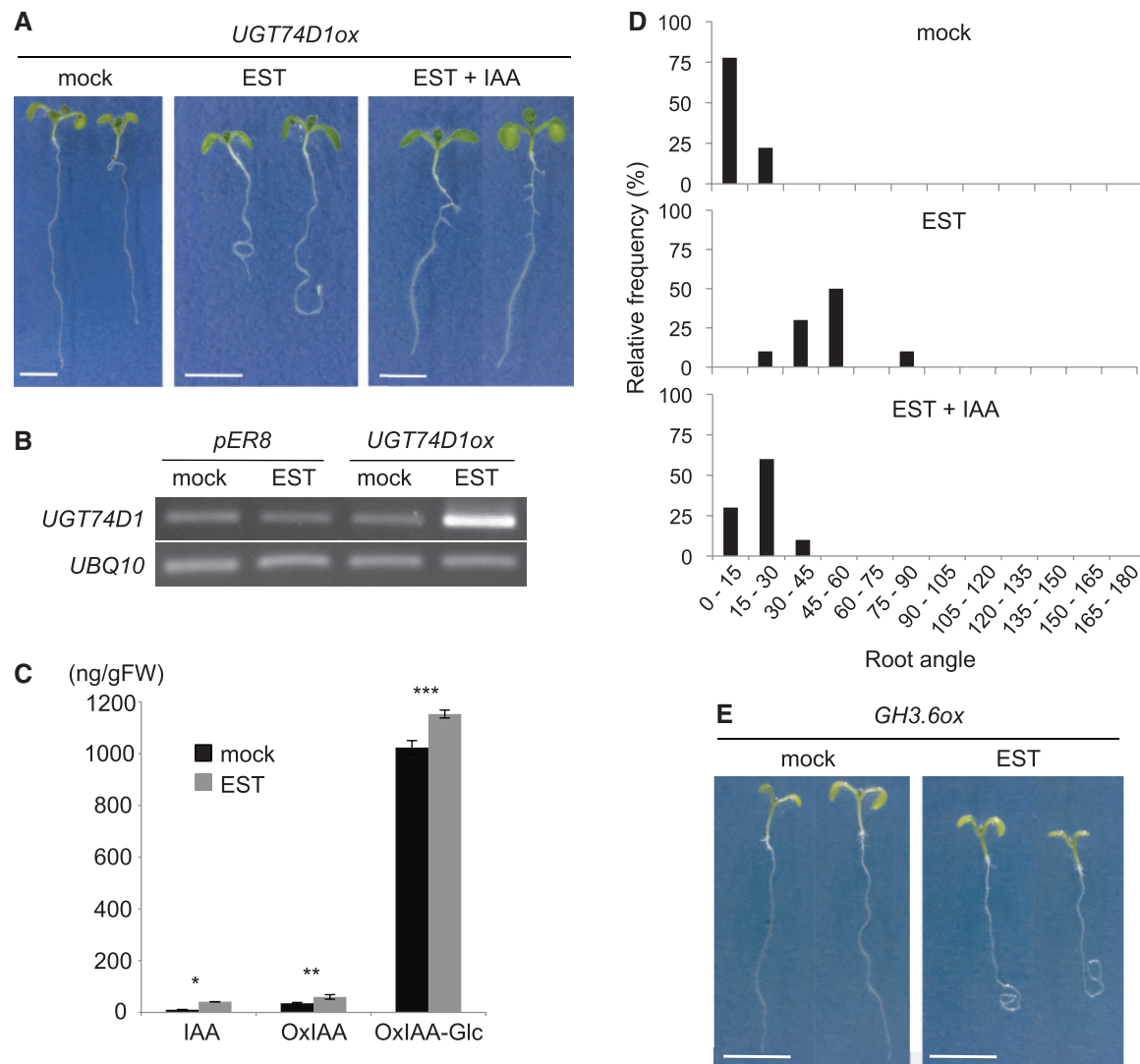
We found that the endogenous levels of OxIAA-Glc increased by 13% in *UGT74D1ox* treated with EST (Fig. 4C), supporting that *UGT74D1* mediates the formation of OxIAA-Glc in *Arabidopsis*. We also noted that the levels of IAA and OxIAA increased in *UGT74D1ox* plants (Fig. 4C), suggesting that enhanced glucosylation of OxIAA promotes the formation of IAA and OxIAA.

Intriguingly, the induction of *UGT74D1* remarkably reduced root gravitropism in *Arabidopsis* seedlings (Fig. 4A, D), indicating that *UGT74D1* catalyzes a committed step in the OxIAA pathway. We note that the loss of root gravitropism in *UGT74D1ox* plants was similar to that observed in the EST-inducible *GH3.6* (*GH3.6ox*) plants (Fig. 4E). *GH3.6* encodes IAA-amino acid conjugate synthase, which catalyzes a committed step in the GH3 pathway, and ectopic expression of *GH3.6* displays the low-auxin phenotype in *Arabidopsis* (Staswick et al. 2005). To examine if the decrease in auxin level led to loss of root gravitropism, we grew the *UGT74D1ox* plants in Murashige and Skoog (MS) agar medium containing 20 nM IAA. We found

that root gravitropism in *UGT74D1ox* plants was recovered by supplementation with IAA (Fig. 4A, D). These results demonstrate that *UGT74D1* catalyzes the glucosylation of OxIAA.

### Responsiveness of *UGT74D1* and *GH3* genes to auxin

We analyzed the regulation of *UGT74D1* by auxin in *Arabidopsis* through applying IAA. We treated 1-week-old seedlings of *Arabidopsis* with 1  $\mu$ M IAA for from 1 to 48 h and analyzed the expression levels of *UGT74D1* by reverse transcription-PCR (RT-PCR). We also analyzed the expression levels of *GH3.2*, *GH3.3* and *GH3.5* as early auxin-responsive genes (Staswick et al. 2005). As shown in Fig. 5, the expression of *GH3* genes was strongly induced by IAA treatment within 1 h. The expression of *UGT74D1* is different from that of *GH3* in that it showed a slight increase only after 3 h of IAA treatment. This result demonstrates that both *UGT74D1* and *GH3* genes are implicated in IAA metabolism, but they are likely to be regulated by distinct mechanisms in *Arabidopsis*.



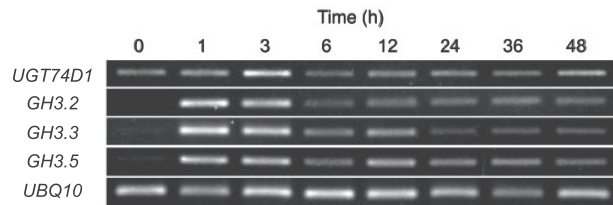
**Fig. 4** Analysis of the OxIAA pathway in *UGT74D1ox*. (A) Phenotypes of *UGT74D1ox* without EST (mock), with 10  $\mu$ M EST (EST) and with 10  $\mu$ M EST and 20 nM IAA (EST + IAA). Bars indicate 5 mm. (B) Semi-quantitative RT-PCR analysis of *UGT74D1* expression in the vector control (*pER8*) and *UGT74D1ox* without EST (mock) or with 10  $\mu$ M EST. (C) IAA, OxIAA and OxIAA-Glc concentrations in *UGT74D1ox* without (mock) or with 10  $\mu$ M EST. Values are mean  $\pm$  SD ( $n = 3$ ). \* \*\*Differences between *UGT74D1ox* with and without EST are statistically significant at  $P < 0.01$  (Student's *t*-test). \*\*Differences between *UGT74D1ox* with and without EST are statistically significant at  $P < 0.05$  (Student's *t*-test). (D) Histogram displaying the range of the root angles from the gravity vector for *UGT74D1ox* plants without EST (mock), with 10  $\mu$ M EST and with 10  $\mu$ M EST and 20 nM IAA (EST + IAA) are shown ( $n = 9-10$ ). (E) Phenotypes of *GH3.6ox* without EST (mock) or with 10  $\mu$ M EST. Bars indicate 5 mm.

## Discussion

### Biological function of UGT74D1

Herein, we provide genetic, biochemical and metabolite-based evidence that UGT74D1 plays a major role in the conversion of OxIAA to OxIAA-Glc in *Arabidopsis*. Recently, Jin et al. (2013) showed that UGT74D1 catalyzes the glucosylation of various auxin-related compounds in the following order of substrate preference: IBA, indole-3-propionic acid, IAA, NAA, 2,4-D and indole-3-carboxylic acid. In the present study, we demonstrated that UGT74D1 catalyzes the glucosylation of OxIAA and IAA to

their corresponding glucosides (Table 2). These results taken together indicate that UGT74D1 has low substrate specificity towards auxin-related compounds. However, several lines of evidence indicate that UGT74D1 may actually have relatively high substrate specificity towards OxIAA in plants. First, the  $K_m$  value of UGT74D1 for OxIAA was 5.5 times lower than that for IAA and the catalytic efficiency ( $k_{cat}/K_m$ ) value of the enzyme for OxIAA was 6.6 times higher than that for IAA, indicating that OxIAA is a more preferred substrate *in vitro* (Table 2). Secondly, the endogenous concentration of OxIAA-Glc in plants varies with the expression level of *UGT74D1*; the OxIAA-Glc level decreased by 85% in the *ugt74d1* mutant



**Fig. 5** Auxin responsiveness of *UGT74D1* and *GH3* genes in *Arabidopsis*. Semi-quantitative RT-PCR analysis of *UGT74D1*, *GH3.2*, *GH3.3* and *GH3.5* expression levels in *Arabidopsis* treated with 1  $\mu\text{M}$  IAA for 1–48 h. *UBQ10*, ubiquitin control.

and increased by 13% in the *UGT74D1ox* plants (Figs. 3D, 4C). Moreover, previous studies have demonstrated that *UGT84B1* and *UGT74E2* catalyze the glucosylation of IAA and IBA, respectively, and ectopic expression of these genes in *Arabidopsis* results in similar phenotypes (e.g. compressed rosette, dwarf stature and a loss of apical dominance) (Jackson et al. 2002, Tognetti et al. 2010). On the other hand, *UGT74D1* catalyzes the glucosylation of both IAA and IBA in vitro, but ectopic expression of *UGT74D1* results in a curling leaf phenotype in *Arabidopsis* (Jin et al. 2013). In summary, these results suggest that the biological function of *UGT74D1* is distinct from those of *UGT84B1* and *UGT74E2* in plants. However, we cannot exclude the possibility that *UGT74D1* plays an auxiliary role in the glucosylation of IAA and IBA.

### Metabolic regulation of IAA concentration

OxIAA-Glc is one of the major IAA metabolites in terms of endogenous concentration, and the level of OxIAA-Glc was nearly two orders of magnitude higher than that of IAA, IAA-Asp, IAA-Glu, IAA-Glc and OxIAA in *Arabidopsis* (Kai et al. 2007). Herein, we showed that the OxIAA-Glc level is reduced by 85% in *ugt74d1* mutants (Fig. 3D). However, the *ugt74d1* mutant still accumulates a wild-type level of IAA and does not show any IAA-related morphological phenotype (Fig. 3A, B). This result suggests that the IAA concentration in *ugt74d1* is probably compensated to wild-type levels by the regulation of IAA biosynthesis and/or metabolism. Detailed analysis of the expression levels for the IAA biosynthetic genes (e.g. *TAA*, *YUC* and *CYP79B* genes) and IAA metabolic genes (e.g. *GH3* genes, *UGT84B1* and *IAMT1*) in the *ugt74d1* mutant may shed light on understanding the mechanisms regulating IAA homeostasis in plants.

Although previous studies suggest that the OxIAA pathway operates in plants, the rate-limiting step of this pathway remains unknown. Herein, we demonstrated that the induction of *UGT74D1* resulted in the loss of root gravitropism in *Arabidopsis* (Fig. 4A, D), indicating that *UGT74D1* catalyzes a committed step in the OxIAA pathway and that an increase in the glucosylation of OxIAA may promote the conversion of IAA to OxIAA. In agreement with this, an increase in OxIAA level was observed in the *UGT74D1ox* plants (Fig. 4C). Furthermore, treatment of *UGT74D1ox* plants with IAA led to recovery of

root gravitropism (Fig. 4A, D), suggesting that the level of IAA in the root apex of *UGT74D1ox* plants decreased below the level required for gravitropic response. Intriguingly, the IAA level did not decrease but rather slightly increased in the *UGT74D1ox* plants when the whole plant was used for IAA analysis (Fig. 4C). A similar phenomenon was observed in *Arabidopsis* plants overexpressing *UGT84B1* (Jackson et al. 2002), in which the overexpression of *UGT84B1* resulted in the low-auxin phenotype, although an increase in IAA level was evident. Likewise, an increase in IBA level was observed in *Arabidopsis* plants overexpressing *UGT74E2* (Tognetti et al. 2010). These results suggest that plants might overcompensate the decreases in IAA and OxIAA levels due to overexpression of *UGT74D1* by enhancing their formation to maintain cellular homeostasis (Tognetti et al. 2010).

Östin et al. (1998) previously demonstrated that IAA was mainly metabolized to OxIAA and OxIAA-Glc and only a minor portion was conjugated with aspartate or glutamate when a relatively low level of IAA (0.5  $\mu\text{M}$ ) was applied to *Arabidopsis*. In contrast, IAA was mainly metabolized to IAA-Asp and IAA-Glu when a relatively high concentration of IAA (5  $\mu\text{M}$ ) was used (Östin et al. 1998). The levels of OxIAA (approximately 60  $\text{ng g}^{-1}$  FW) and OxIAA-Glc (approximately 1,000  $\text{ng g}^{-1}$  FW) were relatively higher than that of IAA (approximately 20  $\text{ng g}^{-1}$  FW) in *Arabidopsis* seedlings (Fig. 3B–D). On the other hand, the levels of IAA-Asp and IAA-Glu (<10  $\text{ng g}^{-1}$  FW) were much lower than that of IAA, but the co-induction of *TAA1* and *YUC* genes in the IPA-dependent IAA biosynthesis pathway remarkably increased the levels of these metabolites (approximately 600–6,000  $\text{ng g}^{-1}$  FW) (Mashiguchi et al. 2011). In accordance with these results, our RT-PCR data demonstrated that *UGT74D1* was constitutively expressed and not markedly affected by the application of IAA, whereas *GH3* genes were induced rapidly and strongly (Fig. 5). These results suggest that the OxIAA and *GH3* pathways are major pathways that may have distinct roles in IAA homeostasis. The OxIAA pathway may function constitutively to maintain the basal levels of IAA concentration in plants. In contrast, the *GH3* pathway may play an important role in cases where plant cells rapidly reduce a relatively large amount of IAA in response to developmental and environmental changes. Identification of the IAA oxidase that catalyzes the conversion of IAA to OxIAA is critical to elucidate further the role of the OxIAA pathway in the metabolism of IAA in *Arabidopsis*.

## Materials and Methods

### Plant materials

*Arabidopsis thaliana* ecotype Columbia-0 was used as the wild type in this study. Seeds of *ugt74d1* (SALK\_004870), *ugt74e1* (SALK\_045974) and *ugt74e2* (SALK\_016116) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Sterilized seeds were germinated on MS agar medium (pH 5.7) supplemented with thiamine hydrochloride (3  $\mu\text{g ml}^{-1}$ ),

nicotinic acid ( $5 \mu\text{g ml}^{-1}$ ), pyridoxine hydrochloride ( $0.5 \mu\text{g ml}^{-1}$ ), *myo*-inositol ( $100 \mu\text{g ml}^{-1}$ ) and sucrose (1%, w/v) after imbibition at  $4^\circ\text{C}$ . Seedlings were grown at  $21^\circ\text{C}$  under continuous white light ( $30\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). For RT-PCR and LC-ESI-MS/MS analyses, 7-day-old seedlings grown on MS medium were used. For EST treatment, 4-day-old seedlings of *pER8*, *UGT74D1ox* and *GH3.6ox* were transferred to MS medium containing  $10 \mu\text{M}$  EST and grown for a further 3 d. For recovery of root gravitropism in *UGT74D1ox* by IAA, 4-day-old seedlings were transferred to MS medium containing  $10 \mu\text{M}$  EST or  $10 \mu\text{M}$  EST with  $20 \text{ nM}$  IAA and grown for 1–3 d.

### Generation of transgenic plants

A cDNA of *UGT74D1* in the pDONR207 vector was transferred into the pMDC7 vector by an LR recombination reaction using LR clonase II<sup>TM</sup> (Invitrogen). A cDNA of *GH3.6* was transferred into the pMDC7 vector from the entry vector pENTR/D-TOPO (Invitrogen) by an LR recombination reaction using LR clonase II<sup>TM</sup> (Invitrogen). *Arabidopsis* wild-type plants were transformed with the resulting construct *pMDC7::UGT74D1* or *pMDC7::GH3.6* by the floral dip method using the *Agrobacterium tumefaciens* GV3101 strain (Clough and Bent 1998).

### Expression analysis

Total RNA was isolated from plants using the RNeasy Plant Mini Kit (Qiagen). The PrimeScript RT reagent Kit with gDNA Eraser (Takara) was used for the generation of first-strand cDNA. PCR was carried out with the primers listed in [Supplementary Table S2](#). The cDNA ( $1 \mu\text{l}$ ) was amplified by PCR using the *UGT74D1* or *GH3* gene-specific primers. Ubiquitin was used as a reference gene.

### Chemicals

[phenyl- $^{13}\text{C}_6$ ]OxIAA, [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc, OxIAA, OxIAA-Glc and IAA-Glc were synthesized as described in the [Supplementary Methods](#).

### LC-ESI-MS/MS analysis of IAA and its metabolites

IAA and OxIAA were analyzed as described in the [Supplementary Methods](#). For analysis of OxIAA-Glc in *Arabidopsis* seedlings, 30–70 mg of fresh plants were quickly weighed, frozen with liquid nitrogen and stored at  $-80^\circ\text{C}$ . Plant material was homogenized in 80% acetone/ $\text{H}_2\text{O}$  (0.2–1 ml) containing 20–40 ng of [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc with ceramic beads (3 mm) using a Tissue Lyser (Qiagen) for 3 min. The supernatants were centrifuged at  $15,000\times g$  for 3 min at  $4^\circ\text{C}$  and transferred to test tubes. The extraction was repeated twice using 80% acetone/ $\text{H}_2\text{O}$  (0.2–1 ml) without the internal standard. The supernatants were combined and evaporated by nitrogen gas and centrifuged at  $15,000\times g$  for 5 min after the volume was decreased to  $<200 \mu\text{l}$ . The supernatant was applied to a  $5 \mu\text{m}$ ,  $4.6 \times 150 \text{ mm}$  Symmetry shield  $\text{C}_{18}$  column (Waters) coupled to a  $5 \mu\text{m}$ ,  $4.6 \times 10 \text{ mm}$   $\text{C}_{18}$  guard column (Senshu

Pak) connected to an HPLC system equipped with a 2475 multi  $\lambda$ -fluorescence detector (Waters). The samples were eluted at a flow rate of  $1 \text{ ml min}^{-1}$  with 0.01 M ammonium acetate (solvent A) and 100% methanol (solvent B) by using 10% solvent B for 1 min and a gradient of 10–50% solvent B over 30 min. HPLC fractions eluted at the retention time of OxIAA-Glc (16.5–17.5 min) were collected and evaporated using a SpeedVac (Thermo). The dried OxIAA-Glc fraction was redissolved with 1% acetic acid/ $\text{H}_2\text{O}$  (1 ml) and applied to an Oasis HLB column (Waters). The column was washed with 10% methanol/ $\text{H}_2\text{O}$  containing 1% acetic acid (1 ml) before eluting the OxIAA-Glc with 60% methanol/ $\text{H}_2\text{O}$  containing 1% acetic acid (1 ml). The eluate was evaporated to dryness using a SpeedVac. OxIAA-Glc was analyzed by an ACQUITY Ultra Performance Liquid Chromatography (UPLC)-MS/MS Q-ToF-premier (Waters). The chromatography was performed on an ACQUITY UPLC BEH  $\text{C}_{18}$   $1.7 \mu\text{m}$ ,  $2.1 \times 50 \text{ mm}$  column (Waters). The OxIAA-Glc fraction was redissolved in 50% acetonitrile/ $\text{H}_2\text{O}$  ( $20 \mu\text{l}$ ) and injected into the UPLC column. Elution of the samples was carried out with 0.05% acetic acid (solvent A2) and acetonitrile with 0.05% acetic acid (solvent B2) using 2% solvent B2 for 0.1 min and a gradient ranging from 2% to 30% of solvent B2 for 7.4 min at a flow rate of  $0.2 \text{ ml min}^{-1}$ . The temperature of the UPLC column was  $40^\circ\text{C}$ . The retention time of OxIAA-Glc and [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc was 3.14 min. OxIAA-Glc and [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc were analyzed in the negative ion mode. MS/MS analysis conditions were as follows: capillary = 2.65 kV, source temperature =  $100^\circ\text{C}$ , desolvation temperature =  $400^\circ\text{C}$ , collision energy = 9.4 V, sampling cone voltage = 28 V, scan time = 0.6 s per scan (delay = 0.05 s) and the MS/MS transition ( $m/z$ ) = 352.1/190.1 for unlabeled OxIAA-Glc and 358.1/196.1 for [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc. Quantification was carried out using the extracted ion chromatogram of OxIAA-Glc and [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc. A standard curve was generated using OxIAA-Glc and [phenyl- $^{13}\text{C}_6$ ]OxIAA-Glc as described above, except for the omission of the HPLC purification step.

### Isolation of OxIAA glucosyltransferase using budding yeast

Full-length cDNAs of UGTs were amplified by PCR with the primers listed in [Supplementary Table S2](#) using RIKEN *Arabidopsis* full-length clones as templates ([Supplementary Table S1](#)). To express the UGT proteins in yeast, *UGT* genes were subcloned into the pDONR207 vector by the BP recombination reaction using BP clonase II<sup>TM</sup> (Invitrogen) and transferred into the modified pYES-DEST52 vector described in Kanno et al. (2012) by an LR recombination reaction using LR clonase II<sup>TM</sup> (Invitrogen). The primer design and recombination reactions were performed following the manufacturer's instructions. The *S. cerevisiae* w303-1a strain was transformed and incubated in medium for 16 h at  $30^\circ\text{C}$ . The culture was added to the medium when the  $\text{OD}_{600}$  was 0.1. After incubation with  $10 \mu\text{M}$  OxIAA for 24 h at  $30^\circ\text{C}$ , the cell culture was



centrifuged at 3,000×g for 5 min. The supernatant was filtered by a 0.22 µm filter and fractionated by HPLC. The OxIAA-Glc fraction was applied to an Oasis HLB cartridge and analyzed by LC-ESI-MS/MS.

### Preparation of recombinant GST-UGT74D1

To express UGT74D1 as a GST-fused protein (GST-UGT74D1), a cDNA of *UGT74D1* was amplified using UGT74D1\_F and UGT74D1\_R primers (Supplementary Table S2), cloned into the pDONR207 vector and transferred into the pDEST15 vector (Invitrogen) by an LR recombination reaction. The *E. coli* BL21 Star (DE3) strain transformed with the resulting construct *pDEST15::UGT74D1* was incubated at 18°C in Terrific-broth medium containing 100 µg ml<sup>-1</sup> carbenicillin. For induction of GST-UGT74D1 expression, Isopropyl β-D-1-thiogalactopyranoside (1 mM, final concentration) was added to the culture when the OD<sub>600</sub> was approximately 0.6. After incubation of cells for 2 d at 18°C, the GST-UGT74D1 protein was purified using glutathione-Sepharose 4B (GE Healthcare) following the manufacturer's instructions. The purified proteins were kept at -80°C with 20% (w/v) glycerol until enzyme assays.

### Assay for glucosyltransferase activity

OxIAA glucosyltransferase activity was determined by measuring OxIAA-Glc formation. Each assay consisted of HEPES buffer (50 mM, pH 7.5), UDP-glucose (1 mM) and OxIAA (100 µM) in a total volume of 100 µl. The enzyme reaction was initiated by the addition of the GST-UGT74D1 protein (1 µg). The reactions were carried out at 30°C for 4 min. The reactions were terminated by adding 100 µl of acetonitrile. After centrifugation at 15,000×g, the supernatant was diluted 10-fold with 50% acetonitrile/H<sub>2</sub>O and quickly injected into the LC-ESI-MS/MS apparatus for OxIAA-Glc analysis.

IAA glucosyltransferase activity was determined by measuring IAA-Glc formation. Each assay consisted of HEPES buffer (50 mM, pH 7.5), UDP-glucose (1 mM) and IAA (100 µM) in a total volume of 100 µl. The enzyme reaction was initiated by the addition of the GST-UGT74D1 protein (1 µg). The reactions were carried out at 30°C for 8 min. The reactions were terminated by adding 100 µl of acetonitrile. After centrifugation at 15,000×g, the supernatant was injected into the HPLC system. IAA-Glc was applied to a Symmetry shield C<sub>18</sub> column coupled to a C<sub>18</sub> guard column connected to an HPLC system and detected by a fluorescence detector. The samples were eluted at a flow rate of 1 ml min<sup>-1</sup> with 0.01 M ammonium acetate (solvent A) and 100% methanol (solvent B) using 10% solvent B for 1 min and a gradient ranging from 10% to 50% of solvent B over 30 min.

### Steady-state kinetic parameters

The OxIAA glucosyltransferase assay based on the formation of OxIAA-Glc was employed for the steady-state kinetic studies of UGT74D1. Each assay consisted of HEPES buffer (50 mM, pH 7.5), UDP-glucose (2.5 mM) and OxIAA in a total volume of

50 µl. The concentration of OxIAA varied (2.5, 5, 7.5, 10, 20, 30 and 40 µM). The enzyme reaction was initiated by the addition of the GST-UGT74D1 protein. The reactions were carried out at 30°C for 4 min. The reactions were terminated by adding 50 µl of acetonitrile containing [phenyl-<sup>13</sup>C<sub>6</sub>]OxIAA-Glc. The reaction mixtures were centrifuged at 15,000×g for 10 min at 4°C. The supernatants were diluted 10-fold with 50% acetonitrile/H<sub>2</sub>O and quickly injected into the LC-ESI-MS/MS system for OxIAA-Glc analysis. The initial velocity was determined from the slope of a plot of OxIAA-Glc concentration vs. incubation time. Steady-state kinetic parameters were calculated from a Lineweaver-Burk plot.

An IAA glucosyltransferase assay based on the formation of IAA-Glc was employed for the steady-state kinetic studies of UGT74D1. Each assay consisted of HEPES buffer (50 mM, pH 7.5), UDP-glucose (2.5 mM) and IAA in a total volume of 50 µl. The concentration of IAA varied (10, 20, 30, 40, 60, 80, 100, 150 and 200 µM). The enzyme reaction was initiated by the addition of the GST-UGT74D1 protein, and the reaction was carried out at 30°C for 8 min. The reaction was terminated by adding 50 µl of acetonitrile. The reaction mixtures were centrifuged at 15,000×g for 10 min at 4°C. The supernatant was injected into the HPLC system for IAA-Glc analysis. Initial velocity was determined from the slope of a plot of the concentration of IAA-Glc vs. incubation time. Steady-state kinetic parameters were calculated from a Lineweaver-Burk plot.

### Supplementary data

Supplementary data are available at PCP online.

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### Disclosures

The authors have no conflicts of interest to declare.

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