Redefining the roles of UDP-glycosyltransferases in auxin metabolism and homeostasis during plant development

Eduardo Mateo-Bonmatí1,2*, Rubén Casanova-Sáez1, Jan Šimura, and Karin Ljung*

Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden

ORCID (E.M.-B.): 0000-0002-2364-5173
ORCID (R.C.-S.): 0000-0001-5683-7051
ORCID (J.Š.): 0000-0002-1567-2278
ORCID (K.L.): 0000-0003-2901-189X

*Corresponding authors: Eduardo Mateo-Bonmatí (eduardo.mateo.bonmati@slu.se) and Karin Ljung (karin.ljung@slu.se)

1These authors equally contributed to this work.

2Current address: John Innes Centre, NR4 7UA, Norwich, United Kingdom

Running head: Redefining the roles of UGTs in IAA metabolism

Keywords: Auxin, IAA, UGT, IAA-glucose, oxIAA-glucose

Word count (total): 8183.
Word count breakdown:
Title page, 136; Abstract, 146; Introduction, 631; Results: 1814; Discussion, 1182; Methods, 1429; Figure legends, 735; Acknowledgments, 62; Author contributions, 52; References, 1738.

Figures: 6
Tables: 0
Supplemental Figures: 14
Supplemental Tables: 1
ABSTRACT

The levels of the important plant growth regulator indole-3-acetic acid (IAA) are tightly controlled within plant tissues to spatiotemporally orchestrate concentration gradients that drive plant growth and development. Metabolic inactivation of bioactive IAA is known to participate in the modulation of IAA maxima and minima. IAA can be irreversibly inactivated by oxidation and conjugation to Aspartate and Glutamate. Usually overlooked because its reversible nature, the most abundant inactive IAA form is the IAA-glucose (IAA-glc) conjugate. Glycosylation of IAA is reported to be carried out by the UDP-glycosyltransferase 84B1 (UGT84B1), while UGT74D1 has been implicated in the glycosylation of the irreversibly formed IAA catabolite oxIAA. Here we demonstrate that both UGT84B1 and UGT74D1 modulate IAA levels throughout plant development by dual IAA and oxIAA glycosylation. Moreover, we identify a novel UGT subfamily whose members modulate IAA homeostasis during skotomorphogenesis by redundantly mediating the glycosylation of oxIAA.
INTRODUCTION

Indole-3-acetic acid (IAA), the major natural auxin in plants, coordinates developmental programs throughout the plant's life cycle by integrating external and internal signals into regulated plant responses (Zažímalová et al., 2014; Casanova-Sáez et al., 2021). This is achieved by the formation of concentration gradients that establish auxin maxima and minima within plant tissues. In combination with inter- and intracellular transport, IAA metabolism modulates auxin gradients and is therefore critical for plant growth (Casanova-Sáez et al., 2021). In addition to the many layers of regulation of IAA biosynthesis, redundant mechanisms exist to keep about 75% of the pool of IAA molecules as either transient storage forms or catabolites, thus providing a robust and rapid-response system to fine-tune IAA levels (Ludwig-Muller, 2011). IAA is inactivated in Arabidopsis mainly by irreversible oxidation to oxIAA (2-oxoindole-3-acetic acid) facilitated by DIOXYGENASE FOR AUXIN OXIDATION 1 (DAO1) and DAO2 (Porco et al., 2016), while reversible conjugates can be formed via amide and ester bonds with amino acids and methyl groups by the action of GRETCHEH HAGEN 3 (GH3) and IAA carboxyl methyltransferase (IAMT) respectively (Qin et al., 2005; Staswick et al., 2005). The most abundant reversible IAA inactive forms, however, are IAA conjugates with sugars such as glucose, as observed by direct quantification of IAA metabolites in different plant species (Porco et al., 2016; Pěnčík et al., 2018; Brunoni et al., 2020).

Sugar conjugation confers higher stability and water solubility and has been considered to be a biological tagging mechanism controlling metabolite activity and compartmentalization (Jones & Vogt, 2001). UDP-glycosyltransferases (UGTs) catalyse the transfer of uridine-diphosphate-activated monosaccharides to a variety of compounds, including anthocyanins (Yonekura-Sakakibara et al., 2012), cell wall components (Lin et al., 2016), fatty acids (Rocha et al., 2016), flavonoids (Li et al., 2018), glucosinolates (Grubb et al., 2014) and phenylpropanoids (Sinlapadech et al., 2007). In Arabidopsis, UGTs comprise a gene superfamily of 115 members (Yu et al., 2017), clustered in 19 families (71-91) and 7 subfamilies (A-F), with up to 11 members per subfamily. Several UGTs have also been found to modulate the metabolism of different phytohormones by glycosylation of the bioactive form: UGT71B6 for abscisic acid (Priest et al., 2006); UGT73C5 and UGT73C6 for brassinosteroids (Poppenberger et al., 2005; Husar et al., 2011); UGT85A1, UGT76C1 and UGT76C2 for cytokinins (Smehilova et al., 2016); UGT76E1 for jasmonic acid (Haroth et al., 2019); and UGT89A2 and UGT76D1 for salicylic acid (Li et al., 2014; Chen & Li, 2017; Huang et al., 2018).
A group of UGTs have been suggested as playing a role in the reversible conversion of IAA to IAA-glucose (IAA-glc). Recombinant UGT84B1, UGT84B2, UGT75B1, UGT75B2, and UGT74D1 were found to be able to glycosylate IAA and other natural and synthetic auxins such as IPA (indole-3-propionic acid), IBA (indole-3-butyric acid), and NAA (naphthalene acetic acid) (Jackson et al., 2001; Jin et al., 2013) in vitro. UGT74D1 was further found to glycosylate oxIAA in vitro and it was suggested that it performs this function in vivo (Tanaka et al., 2014). However, the strongest IAA glycosyltransferase activity was observed with UGT84B1 (Jackson et al., 2001). Besides in vitro evidence, plants overexpressing UGT84B1 showed not only higher levels of IAA and IAA-glc, but also a wrinkled and curling leaf phenotype similar to those of other auxin-accumulating mutants (Jackson et al., 2002).

Despite the biochemical evidence supporting a role for UGT84B1 in IAA glycosylation, it was recently suggested that UGT84B1 may also glycosylate oxIAA, on the basis of in bacteria assays (Brunoni et al., 2019). Here, we generate a CRISPR/Cas9-based knock-out allele of UGT84B1 and show, by tissue-specific auxin metabolite profiling and feeding experiments with isotope-labelled [13C6]IAA, that both UGT84B1 and UGT74D1 modulate IAA levels throughout the plant lifecycle by playing a dual role in IAA and oxIAA glycosylation. We additionally identify a new subfamily of UGTs that have a role in IAA homeostasis during skotomorphogenesis.

RESULTS

IAA-glucose accumulates in root tissues while UGT84B1 is mostly expressed during seed development

IAA-glc has been detected in many plant species, at particularly high abundance in seeds, and it is believed to constitute the main source of IAA during seedling germination and establishment (Ljung et al., 2001). However, the role of this conjugate at later developmental stages is unclear. To further understand the post-germination role of IAA-glc, we revisited datasets of tissue-specific quantification of IAA-glc levels in Arabidopsis (Porco et al., 2016), and observed that roots accumulate IAA-glc at more than 3-fold higher levels compared to other seedling tissues such as hypocotyls, cotyledons, first leaves and shoot apex (Figure 1a). Because the UDP-glycosyltransferase 84B1 (UGT84B1) is believed to account for IAA-glc formation in the plant (Jackson et al., 2001; Jackson et al., 2002), we explored whether the expression pattern of UGT84B1 parallels the abundance of IAA-glc in plant tissues. We retrieved expression profiling data for UGT84B1 from Genevestigator (Hruz et al., 2008) using only datasets corresponding to wild-type tissues. According to these data, transcripts
of UGT84B1 are present at very low levels during the vegetative phase, while they peak specifically in some reproductive structures such as endosperm, seeds and siliques (Figure 1b). To validate these data under our experimental conditions, we analysed the expression of UGT84B1 in young seedlings and siliques by qRT-PCR. Transcript levels of UGT84B1 were found to be more than 30-fold higher in siliques than in seedlings (Figure 1c), thus confirming the published findings.

**Tissue-specific auxin metabolite profiling of ugt84b1 mutants uncovers a role for UGT84B1 in oxIAA glycosylation throughout plant development**

To further understand the role of UGT84B1 in IAA metabolism during the Arabidopsis lifecycle, and because no loss-of-function allele was available at the time this study was initiated, we generated a CRISPR/Cas9-based ugt84b1 knockout mutant carrying two deletions in the protein sequence (amino acids 183-221 and 291-297; Figure 1d). In line with other recently reported UGT84B1 knockout alleles (Aoi et al., 2020), ugt84b1 mutant plants did not show any obvious developmental defect (Supplementary Figure 1).

To determine whether UGT84B1 contributes to IAA homeostasis in planta, we quantified the levels of IAA and oxIAA, their glycosylated forms IAA-glc and oxIAA-glc, and the irreversibly synthesized catabolites IAA-Asp and IAA-Glu in ugt84b1 plants. Given the differences in UGT84B1 expression and IAA-glc contents across plant tissues (Figure 1a), we decided to analyse three different tissues: roots and shoots from 7-days-old seedlings and young siliques from 39-day-old plants (Figure 2). In line with the reported role of UGT84B1 in IAA glycosylation (Jackson et al., 2001; Jackson et al., 2002; Aoi et al., 2020) and the specific expression of the gene in siliques (Figure 1c), we found decreased levels of IAA-glc in ugt84b1 siliques compared to Col-0 (Figure 2), while IAA-glc concentrations were higher in young roots and shoots of ugt84b1. Strikingly, the levels of oxIAA-glc were decreased in the ugt84b1 mutant in all tissues analysed (Figure 2). IAA homeostasis was significantly affected by the loss of UGT84B1 in all tissues, as indicated by the decreased IAA contents of the ugt84b1 mutant tissues (Figure 2) and by the altered contents of the catabolites oxIAA, IAA-Asp and IAA-Glu, notably in siliques (Supplemental Figure 2). Overall, these results suggest that UGT84B1 is additionally required for oxIAA glycosylation and auxin homeostasis during plant development, and strongly favours the hypothesis that other UGT members may function redundantly in IAA glycosylation at the seedling stage.

**Searching for new UGTs involved in IAA glycosylation**
In order to find additional members of the UGT superfamily with functions in IAA glycosylation and homeostasis, we performed an in silico search for IAA-inducible UGTs. We employed the dataset described in (Lewis et al., 2013) in which the authors exposed wild-type roots to 1 µM IAA, and retrieved for analysis those UGTs whose function had not yet been established (Figure 3a). Among all these UGTs, only UGT76E5 showed constant and strong induction upon IAA treatment, while UGT86A2 or UGT90A2 showed lesser induction (Figure 3a). We therefore focused on UGT76E5 for further experiments. To validate the microarray data under our experimental conditions, we used qRT-PCR to analyse the expression of UGT76E5 in 7-day-old seedlings treated with 1 µM of IAA. The relative expression of UGT76E5 after 4 hours of treatment was around 5-fold higher than in mock-treated plants (Figure 3b). Because UGT76E5 is a non-characterized member of this superfamily, we used the ATTEDII tool to analyse the UGT76E5 co-expression network. Based on array datasets, we found a gene involved in IAA inactivation, GH3.1 (At2g14960), within the co-expression map (Supplementary Figure 3a). Additional co-expression analyses based on RNA-seq datasets indicated that GH3.17 (At1g28130), SMALL AUXIN UP-REGULATED RNA-like 55 (SAUR-like 55; At5g50760), and SAUR21 (At5g01830) are also co-expressed with UGT76E5, thus supporting a relationship between UGT76E5 and IAA homeostasis (Supplementary Figure 3b).

In contrast to UGT84B1, UGT76E5 expression was detected in different vegetative organs, primarily in roots and hypocotyls (Figure 3e). To study the role of UGT76E5 in IAA metabolism, we obtained a T-DNA allele harbouring an insertion (SALK_006783) in the first exon of UGT76E5, which we named ugt76e5 (Figure 3c). We also generated transgenic plants expressing the UGT76E5 transcriptional unit under the control of a double 35S promoter (seven independent families with high UGT76E5 expression level were obtained; Supplementary Figure 4). Neither ugt76e5 nor 35Spro:UGT76E5 homozygous plants showed any noticeable morphological phenotype (Supplementary Figure 1). BLASTP searches allowed us to identify three close paralogs of UGT76E5 in the Arabidopsis genome, UGT76E3 (At3g46700), UGT76E4 (At3g46690), and UGT76E6 (At3g46680) (Figure 3d), whose redundancy might explain the lack of morphological defects in ugt76e5 plants. Like UGT76E5, these paralogs are expressed in vegetative organs and root tissues, with the exception of UGT76E6 whose expression is reminiscent of that of UGT84B1 and is largely restricted to the endosperm (Figure 3e). Phylogenetic analyses indicate that these four paralogs (hereafter UGT76E3456) fall into the same clade of the UGT6 family (Supplementary Figure 5) and multiple alignment of the UGT76E3456 protein sequences.
revealed a high degree of amino acid identity among them (Supplementary Figure 6). Finally, we found an IAA amido synthetase, GH3.17, and several SAUR and SAUR-like auxin responsive genes among the co-expression networks of UGT76E3, UGT76E4, and UGT76E6 (Supplementary Figure 3b). Taken together, the auxin-inducible expression of UGT76E5 along with the co-expression analyses of the UGT76E3456 family members suggest involvement of this subfamily in IAA homeostasis.

Feeding experiments with \([^{13}\text{C}_6]\text{IAA}\) reveal a dual role for UGT84B1 and UGT74D1 in IAA and oxIAA glycosylation, and a minor contribution by UGT76E3456

We next aimed to explore the role of UGT76E3456 in auxin metabolism in Arabidopsis seedlings. Because the four UGT76E genes are clustered together on chromosome 3 (Supplementary Figure 7a), we followed a CRISPR/Cas9-based approach to simultaneously inactivate the four paralogs. We found a genomic sequence with the potential to concurrently target UGT76E3, UGT76E4 and UGT76E5 (UGT76E345) (Supplementary Figure 8a). To generate the quadruple \(ugt76e3456\) knockout, we transformed the CRISPR/Cas9 construct into \(ugt76e6\) plants, which carry a T-DNA insertion (SALK_200519) in the first exon of UGT76E6 (Supplementary Figure 7b). After screening for edited plants, a single nucleotide insertion was found within the target region in UGT76E3 and UGT76E4 (Supplementary Figures 9 and 10) and a 66-nt deletion, encompassing part of the target region, in UGT76E5 (Supplementary Figure 11). These changes are predicted to truncate both UGT76E3 and UGT76E4 proteins and to generate a 22-amino acid deletion in UGT76E5 (Supplementary Figure 8b). We did not observe any noticeable morphological defect in plants from the quadruple \(ugt76e3456\) mutant under standard growth conditions (Supplementary Figure 1).

Besides UGT84B1, only one UGT family member has been previously reported to catalyse oxIAA-glc formation \textit{in vivo}, UGT74D1 (Tanaka \textit{et al.}, 2014). According to Genevestigator data, the expression pattern of UGT74D1 is opposite to that of UGT84B1, with expression being higher during vegetative stages, especially in roots, and lower in reproductive tissues (Supplementary Figure 12a). We obtained a T-DNA allele harbouring an insertion (SALK_004870) in the second exon of UGT74D1, which generates no wild-type transcript and we named \(ugt74d1\) (Supplementary Figure 12b-c; Tanaka \textit{et al.}, 2014). Like \(ugt84b1\) and \(ugt76e3456\), these plants did not show any morphological defect but a marginally early flowering (Supplementary Figure 1). To better understand the roles of UGT84B1, UGT74D1, and UGT76E3456 we quantified the levels of IAA and oxIAA, their glycosylated forms IAA-glc and oxIAA-glc, and the catabolites IAA-Asp and IAA-Glu in 7-day-
old seedlings of the wild-type Col-0, single mutants *ugt84b1* and *ugt74d1*, the quadruple mutant *ugt76e3456*, and the transgenic 35S*pro:UGT76E5* seedlings (Supplemental Figure 13). In parallel, we measured the levels of 13C6-isotope-labelled IAA metabolites in these plants after feeding with [13C6]IAA (Figure 4).

In line with our tissue-specific profiling of the *ugt84b1* mutant (Figure 2), lower steady-state levels of IAA and oxIAA-glc, but not IAA-glc, were detected in *ugt84b1* seedlings, again suggesting involvement of UGT84B1 in IAA homeostasis through the regulation of oxIAA-glc formation (Supplemental Figure 13). A similar trend was observed in *ugt74d1* (Supplemental Figure 13). However, the [13C6]IAA feeding experiment showed that the ability of the *ugt84b1* and *ugt74d1* mutants to synthesize de novo both IAA-glc and oxIAA-glc is severely impaired, while oxIAA, IAA-Asp and IAA-Glu formation occurred at a significantly higher rate (Figure 4). These results demonstrate a dual role for UGT84B1 and UGT74D1 in IAA homeostasis through IAA and oxIAA glycosylation in vivo.

Slightly lower de novo synthesis and steady-state levels of oxIAA-glc were also found in the *ugt76e3456* mutant (Figure 4 and Supplemental Figure 13), while no differences were seen in the 35S*pro:UGT76E5* transgenic overexpressing line compared to Col-0 at steady state (Supplemental Figure 13). However, 35S*pro:UGT76E5* plants fed with [13C6]IAA showed a roughly 2-fold higher IAA-glc formation rate compared to Col-0 and a reduced rate of synthesis of IAA-Asp and IAA-Glu, strongly suggesting that UGT76E5 possesses IAA glycosyltransferase activity in vivo and perturbs IAA homeostasis (Figure 4).

**UGT76E3456 control IAA homeostasis during skotomorphogenesis**

When kept in darkness, the seedling developmental program is set to a skotomorphogenic mode, in which resources are allocated to hypocotyl elongation in order to find the light (Josse & Halliday, 2008). Several groups have showed that mutations in genes involved in auxin homeostasis alter differential hypocotyl growth (Gray *et al.*, 1998; Sun *et al.*, 2012; Zheng *et al.*, 2016; Chen *et al.*, 2020). To determine whether mutations in any of the UGTs studied in this work affect hypocotyl growth, we measured the hypocotyl length in Col-0, *ugt84b1*, *ugt74d1*, and *ugt76e3456* plants under normal light conditions, shade and darkness. Although the length of light-grown hypocotyls was completely indistinguishable among all genetic backgrounds (Figure 5a), *ugt76e3456* mutant hypocotyls grown in either shade or darkness were found to be significantly longer than those of Col-0 (Figure 5b, 5c).

To further investigate whether the effect of UGT76E3456 on hypocotyl elongation is connected to modulation of auxin metabolism, we performed auxin metabolite profiling of...
dark-grown hypocotyls from Col-0 and the \textit{ugt76e3456} quadruple mutant (Figure 5d). In line with their longer hypocotyls, significantly higher levels of IAA were found in the hypocotyls of the \textit{ugt76e3456} quadruple mutant. Consistently with our observations from seedlings fed with labelled IAA (Figure 4), the quadruple mutant showed lower levels of oxIAA-gl c in the hypocotyls when grown in darkness (Figure 5d), thus opening up the possibility that these genes play a specific role in oxIAA glycosylation and IAA homeostasis during skotomorphogenesis.

**DISCUSSION**


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**A redefinition of the roles of UGT84B1 and UGT74D1 in IAA homeostasis**

Oxidation and conjugation have been shown to play important roles in the regulation of auxin levels for the control of plant growth and development (Casanova-Sáez \textit{et al.}, 2021). This complex and intertwined net of redundant pathways includes IAA glycosylation, a reversible reaction (through hydrolysis of IAA-gl c) that not only participates in temporary inactivation of IAA, but also creates a readily available source of energy and IAA without the need for \textit{de novo} synthesis to trigger fast auxin-mediated responses (Jones & Vogt, 2001).

IAA glycosylation is catalysed by of members of the \textit{UGT} multigene family, which are present from bacteria to humans (Mackenzie \textit{et al.}, 1997). The size of this family has evolved according to organism complexity in the plant kingdom; there are 3 UGTs in the unicellular microalga \textit{Chlamydomonas reinhardtii}, 21 in the moss \textit{Physcomitrella patens}, 115 in Arabidopsis and 200 in \textit{Oryza sativa} (Yu \textit{et al.}, 2017). Despite the challenging size of the family, genetic and biochemical approaches have revealed roles for specific family members in phytohormone glycosylation (Ostrowski & Jakubowska, 2014). \textit{In vitro} screens have uncovered UGT84B1 and UGT74D1 as two potentially major players in IAA glycosylation (Jackson \textit{et al.}, 2001; Jackson \textit{et al.}, 2002; Jin \textit{et al.}, 2013). UGT74D1 was later reported to participate in oxIAA glycosylation in the plant, as a T-DNA \textit{ugt74d1} insertional mutant was found to accumulate oxIAA, while having reduced oxIAA-gl c levels (Tanaka \textit{et al.}, 2014). The lack of loss-of-function mutants, however, has hampered a straightforward comparative analysis of the UGT84B1 function \textit{in vivo}. The function of UGT84B1 \textit{in vivo} was reported in overexpression lines nearly 20 years ago (Jackson \textit{et al.}, 2002), but it was not until very recently that the first \textit{ugt84b1} loss-of-function mutant was reported in Arabidopsis and shown to have decreased steady-state levels of IAA-gl c in seedlings (Aoi \textit{et al.}, 2020).

Here, we provide the first evidence of a dual role for UGT84B1 in both IAA and oxIAA glycosylation \textit{in vivo}, as demonstrated by the severely impaired \textit{de novo} formation of IAA-gl c
and oxIAA-glc in plants with our CRISPR/Cas9-based ugt84b1 knockout allele. Tissue-specific IAA metabolite profiling further revealed a stricter requirement for UGT84B1 for IAA glycosylation in siliques, while the loss of UGT84B1 function appears to be compensated by redundant UGT members in seedling roots and shoots. This agrees with the expression pattern of the UGT84B1 gene, which is preferentially transcribed in the endosperm. Remarkably, we found the levels of oxIAA-glc to be greatly reduced in seedling roots and shoots, and in siliques, of the ugt84b1 mutant, which points to UGT84B1 playing a predominant role in oxIAA glycosylation throughout plant development. The profound effects of UGT84B1 on IAA and oxIAA glycosylation in seedling tissues is in remarkable contrast with the relatively low expression of the gene at these stages. While further research will be needed to clarify the causes, that an enzyme mostly transcribed in reproductive tissues has a prevailing role during the vegetative phase represents an exciting challenge to be explored.

Because the levels of oxIAA-glc, but not IAA-glc, were previously reported for a ugt74d1 insertional mutant (Tanaka et al., 2014), we also aimed to explore a potential dual role for UGT74D1 in IAA and oxIAA glycosylation. The metabolic behaviour of ugt74d1 was very similar to that of ugt84b1, with a dramatic impairment of de novo synthesis of IAA-glc and oxIAA-glc. This dual role for UGT74D1 was recently suggested by experiments using an heterologous approach in E. coli (Brunoni et al., 2019). Our results demonstrate that UGT74D1 modulates IAA levels by IAA and oxIAA glycosylation in vivo.

Our tissue-specific metabolite profiling supports the conclusion that both UGT84B1 and UGT74D1 modulate IAA levels during plant development. However, given that glycosylation of IAA and oxIAA involves, respectively, transient and catabolic inactivation of auxin it is surprising that IAA levels are reduced rather than increased in tissues of the ugt84b1 and ugt74d1 mutants. It seems plausible, nevertheless, that such regulation of IAA levels reflects a specific compensatory mechanism operating in plants, as overexpression of UGT84B1 has been shown to result in increased IAA levels (Jackson et al., 2002; Aoi et al., 2020). This increase in free IAA levels has also been reported in Arabidopsis plants overexpressing an IAA glycosyltransferase from Maize (Ludwig-Muller et al., 2005). This strongly suggests that the hydrolysable nature of the IAA-glc conjugates, together with the redundant action of additional UGT members, underlie the observed regulation of IAA homeostasis.

A new set of redundant players locally controlling IAA homeostasis

Large gene families such as the one consisting of UGTs arose from genome duplications (Soltis et al., 2009). After duplication of the ancestral gene, new copies may acquire
divergent functions (neofunctionalization); alternatively they can maintain the ancestral
function but specialize with respect to the time or the tissue in which they act
(subfunctionalization). Considering the short genetic distance between UGT76E3, UGT76E4,
UGT76E5, and UGT76E6, it is likely that they all derive from recent duplication events and
act in a redundant manner. Our data indeed suggest that the four paralogs of the
UGT76E3456 subfamily play redundant roles in oxIAA glycosylation and IAA homeostasis,
which is particularly required for regulating the skotomorphogenic response. Considering the
multifunctionality of these enzymes, we cannot completely exclude the possibility that the
metabolic and phenotypic effects presented here arise from altering the metabolism of other
phytohormones or secondary metabolites. However, our experiments performed both in
seedlings and in etiolated hypocotyls consistently showed altered levels of oxIAA-gluc in a
quadruple ugt76e3456 mutant. Moreover, the longer dark-grown hypocotyls in the quadruple
ugt76e3456 mutant correlate with decreased oxIAA-gluc and increased IAA levels in their
hypocotyls. This degree of tissue- and process-specialization has already been reported for
GH3.17, a member of the GH3 family of IAA amidosynthetases. Mutations in GH3.17 block
the IAA-Glu pathway, provoking an increment in the IAA content and the length of the
hypocotyls (Zheng et al., 2016). More recently, a member of the UGTs (UGT76F1) was
found to modulate IAA biosynthesis by glycosylating the major auxin precursor indole-3-
pyruvic acid specifically in hypocotyls (Chen et al., 2020). While the connection between the
glycosylation of oxIAA, the major IAA catabolite, and the modulation of IAA levels is still
unknown, the mutants presented here represent a valuable tool with which to explore this
area of research.

It is worth noting that our data on de novo formation of isotope-labelled IAA metabolites
do not fully exclude a role for UGT76E5 in IAA glycosylation, as 35Spro:UGT76E5 plants were
able to produce a 2-fold higher IAA-gluc level compared to Col-0. At the same time, the similar
steady-state IAA-gluc levels found in ugt84b1, ugt74d1 and ugt76e3456 suggest that other
IAA-glycosyltransferases participate redundantly in IAA homeostasis.

Overall, this work supports differential and developmental stage-specific contributions of
the UGT84B1, UGT74D1 and UGT76E3456 glycosyltransferases to IAA homeostasis by
mediating IAA and oxIAA glycosylation (Figure 6). Our data indicate that IAA homeostasis is
redundantly controlled by UGT84B1, UGT74D1 and UGT76E3456 through IAA and oxIAA
glycosylation. The identification of putative additional IAA and oxIAA glycosyltransferases,
along with the genetic and biochemical analysis of multiple mutants, will advance our
understanding of the contribution of UGTs to IAA homeostasis and plant development.
MATERIALS AND METHODS

Plant material, culture conditions and IAA treatments

All *Arabidopsis thaliana* plants studied in this work were homozygous for the mutations indicated. The Nottingham Arabidopsis Stock Centre provided seeds for the wild-type accession Col-0 (N1092), as well as seeds of the *ugt76e5* (SALK_006783; N25012), *ugt76e6* (SALK_200519; N687668), and *ugt74d1* (SALK_004870; N504870) mutants. The presence and positions of all T-DNA insertions were confirmed by PCR amplification using gene-specific primers and the LbB1.3 primer (Supplemental Table 1).

Seeds were surface sterilized with bleach solution (40% vol/vol commercial bleach in dH₂O and 0.002% Triton X-100) for 8 min and then washed four times with sterile deionized water. Seeds were stratified for 3 days and sown under sterile conditions on petri dishes containing half-strength Murashige & Skoog salt mixture (Duchefa, M0221), 1% sucrose, 0.05% MES hydrate (Sigma, M2933) and 0.8% plant agar (Duchefa, P1001), at pH 5.7.

Flowering plants were grown in pots containing a 3:1 mixture of organic soil and vermiculite.

All plants were grown in long-day conditions (16h light, 8h dark) at 22 ± 1°C under cool white fluorescent light (150 μmol photons m⁻² s⁻¹). IAA treatments and feeding experiments with 

₁³C₆-labelled IAA were performed on 7-day-old seedlings. Seedlings were incubated in half-strength liquid MS media with and without 1 μM of IAA for 1, 2 and 4 hours, or with and without 1 μM of [¹³C₆]IAA for 12 hours, under gentle shaking and in darkness. The 12-hour timepoint was chosen because we had previously observed a peak of IAA de novo glycosylation in Col-0 seedlings after 12 hours of feeding with [¹³C₆]IAA (Porco *et al*., 2016).

Plant phenotyping

For root phenotyping, plants were grown vertically in square petri dishes and plates were imaged using Epson Perfection V600 Photo scanners. For skotomorphogenesis experiments, seeds were stratified at 4°C for 3 days, and then transferred to light at 22 ± 1°C for 8 hours to induce germination. Plates were then transferred to darkness and incubated at 22 ± 1°C for five days. To simulate shade conditions, plants were grown normally for four days and then transferred to darkness for five days. Lengths of the roots and hypocotyls were measured using FIJI software (Schindelin *et al*., 2012).

Plasmid construction and plant transformation

The CRISPR/Cas9-based vector for the knockout of the *UGT84B1* gene was constructed
using the GreenGate system (Lampropoulos et al., 2013) as described in (Capovilla et al., 2017). Four guide RNAs (gRNAs) targeting the UGT84B1 coding sequence (Table S1 and Supplemental Figure 14) were designed using CRISPR-P (http://crispr.hzau.edu.cn/CRISPR/). Two supermodules were first created by assembling GreenGate modules into the intermediate plasmid vectors pGGM000 and pGGN000. The M intermediate vector resulted from assembly of the modules A: EC1.2enhancer-EC1.1promoter; B: Arabidopsis thaliana codon-optimized Cas9; C: rbcs terminator; D: gRNA sg84b1.4; E: gRNA sg84b1.1; and FH-adapter (pGGG001). The N intermediate vector resulted from assembly of the HA-adapter (pGGG002); A: UBQ10 promoter (pGGA006); B: mCherry; C: rbcs terminator; D: gRNA sg84b1.2; E: gRNA sg84b1.3; F: hygromycin B phosphotransferase (pGGF005). The M and N supermodules were then combined into the destination vector pGGZ003 to create the final construct. The gRNAs were generated using the primers listed in Supplemental Table 1 and cloned into the D and E modules by digestion-ligation (Lampropoulos et al., 2013). The mCherry sequence was amplified from the pGGC015 plasmid using the mCherry-BasI primers (Supplemental Table 1) and cloned into a B module by digestion-ligation (Lampropoulos et al., 2013). The pGGA006, pGGC015, pGGG001, pGGG002, pGGM000 and pGGN000 plasmids were purchased from Addgene. The B:Cas9, C:rbcs terminator, pGGF005 and pGGZ003 were kindly provided by Prof. Markus Schmid. The A module containing the previously reported EC1.2enhancer-EC1.1promoter construct (Wang et al., 2015) was kindly provided by Dr. Wei Wang. The integrity of the insert sequences in all modules was confirmed by Sanger sequencing. The correct assembly of the supermodules in the intermediate and destination vectors was confirmed by restriction analysis.

To construct the 35Spro:UGT76E5, the UGT76E5 transcription unit, from the ATG to the stop codon, was amplified from Col-0 cDNA using Q5 High-Fidelity DNA Polymerase (NEB), as recommended by the manufacturer. The oligonucleotides attB_UGT76E5_F and attB_UGT76E5_R, which contained attB sites at their 5' ends, were used (Supplemental Table 1). PCR product of the expected size was purified from the agarose gel after electrophoresis using the Monarch DNA Gel Extraction Kit (NEB), and cloned into the pDONR207 vector (Invitrogen) by following the Gateway BP Clonase II Enzyme Mix (Thermo Fisher) protocol. Chemically competent Escherichia coli DH5α cells were transformed by the heat-shock method (Dagert & Ehrlich, 1979), and the sequence integrity of the insert carried by transformants was verified by Sanger sequencing. The insert in pDONR207 was subcloned into the pMDC32 Gateway-compatible destination vector (Curtis & Grossniklaus,
2003) via an LR Clonase II (Thermo Fisher) reaction. Seven independent transgenic lines were obtained without any morphological phenotype and high UGT76E5 expression levels (Supplementary Figure 4). Line #1 was chosen for the metabolic analysis.

To create the CRISPR/Cas9 construct to knock out UGT76E3, UGT76E4 and UGT76E5, the pKl1.1R plasmid was used following the protocol described in Tsutsui and Higashiyama (2017). Briefly, the circular pKl1.1R plasmid was linearized by incubating 1.5 µg of the purified plasmid with the AarI restriction enzyme for 10 hours, and then dephosphorylated using FastAP (Thermo Fisher). A target-specific gRNA was designed using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/). Oligonucleotides harbouring the gRNA target (sgRNA_UGT76E345_F and sgRNA_UGT76E345_R; Supplementary Table 1) were hybridized by slow cooling from 95-25ºC and then phosphorylated using T4 Polynucleotide Kinase (NEB). The digested plasmid and the hybridized oligonucleotides were ligated using T4 Ligase (NEB) and then transformed into Escherichia coli DH5α competent cells. The sequence integrity of inserts carried by transformants was verified by Sanger sequencing.

All constructs were mobilized into Agrobacterium tumefaciens GV3101 (C58C1 RifR) cells. The CRISPR-UGT84B1 and the 35Spro:UGT76E5 constructs were used to transform Col-0 and CRISPR-UGT76E345 was used to transform ugt76e6 plants by the floral dip method (Clogh and Bent, 1998). T1 transgenic plants were selected on plates supplemented with 15 mg/L hygromycin B (Invitrogen).

**RNA isolation, cDNA synthesis, and qRT-PCR**

For qRT-PCR, total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen). DNA was removed using a TURBO DNA-free Kit (Invitrogen). First-strand cDNA synthesis was performed using an iScript cDNA Synthesis Kit (BioRad) following the manufacturer’s instructions. ACTIN2 was used as an internal control in relative expression analyses. Three biological replicates were analysed in triplicate. qPCR reactions were performed in a 10-µl volume containing 5 µl of LightCycler 480 SYBR Green I Master (Roche), 4 µl of the corresponding primer pair (1.5 µM each), and 1 µl of cDNA template. Quantification of relative gene expression was performed using the comparative C_\(T\) method (2^{\Delta\Delta C_T}) (Schmittgen & Livak, 2008) on a CFX96 Real-Time System (BioRad). Primers used are listed in Supplemental Table 1.

**Bioinformatics analyses**

To identify UGT76E5 paralogs, BLASTP searches (Altschul et al., 1997) were performed...
at NCBI using the refseq_protein database and default parameters. Proteins with a percentage of sequence identity higher than 70% were selected, aligned using ClustalW (Thompson et al., 1994), and shaded with BOXSHADE3.21 (https://embnet.vital-it.ch/software/BOX_form.html). A phylogenetic tree was constructed by the neighbour-joining clustering method, inferred from 1000 replicates, with MEGA X (Kumar et al., 2018) using default parameters (model: Poisson; rates among sites: uniform rates; gaps/missing data treatment: pairwise deletion). Auxin-related genes co-expressed with UGT76E3, UGT76E4, UGT76E5, and UGT76E6 were retrieved from the ATTED-II database (Obayashi et al., 2018).

IAA metabolite profiling

Extraction and purification of the targeted compounds (IAA, oxIAA, IAA-Asp, IAA-Glu IAA-Glc, oxIAA-Glc, both unlabelled and [13C6] labelled compounds) were performed according to (Novák et al., 2012), with slight modifications. Briefly, 10 mg of frozen material per sample was homogenized using a bead mill (27 Hz, 10 min, 4°C; MixerMill, Retsch GmbH, Haan, Germany) and extracted in 1 ml of 50 mM sodium phosphate buffer containing 1% sodium diethylthiocarbamate and a mixture of deuterium/nitrogen isotopically labelled internal standards ([2H5]IAA, ([2H4]oxIAA, [15N,2H5]IAA-Asp, [15N,2H5]IAA-Glu, Olchemim, Olomouc, Czech Republic). After centrifugation (20 000 g, 15 min, 4°C), the supernatant was transferred into new Eppendorf tubes. The pH was then adjusted to 2.5 with 1 M HCl and samples were immediately applied to preconditioned solid-phase extraction columns (Oasis HLB, 30 mg of 1 ml; Waters Inc., Milford, MA, USA). After sample application, each column was rinsed with 2 ml 5% methanol. Compounds of interest were then eluted with 2 ml 80% methanol. UHPLC-MS/MS analysis was performed according to the method described in (Pěnčík et al., 2018), using an LC-MS/MS system consisting of a 1290 Infinity Binary LC System coupled to a 6490 Triple Quad LC/MS System with Jet Stream and Dual Ion Funnel technologies (Agilent Technologies, Santa Clara, CA, USA).

Accession Numbers

ACTIN2 (At3g18780), UGT74D1 (At2g31750), UGT76E3 (At3g46700), UGT76E4 (At3g46690), UGT76E5 (At3g46720), UGT76E6 (At3g46680), UGT84B1 (At2g23260).
Figure 1. IAA-glc contents do not correlate with UGT84B1 expression in vegetative tissues.
(a) IAA-glucose levels in different tissues of Col-0 plants. Data retrieved from Porco et al. 2016.
(b) Expression pattern of the UGT84B1 gene in different tissues and organs. Transcriptomic data were obtained from Genevestigator datasets corresponding to wild-type tissues. Color scale indicates percentage of expression potential.
(c) Relative expression of the UGT84B1 gene in wild-type seedlings and young siliques. Bars indicate relative expression of UGT84B1 in 7-day-old Col-0 seedlings and in young siliques from 39-day-old Col-0 plants. Error bars indicate the interval delimited by 2^\Delta\Delta CT±SD.
(d) Structure of the UGT84B1 gene. Open and black boxes represent untranslated and translated regions respectively. Red horizontal lines represent the positions of the nucleotide sequences (not drawn to scale) used to design the guide RNAs for CRISPR/Cas9-based gene editing. ugt84b1 plants carry two nucleotide deletions (positions 547-663 and 870-890) that generate two protein deletions (positions 183-221 and 291-297; highlighted in red). Scale bar indicates 100 bp.

Figure 2. Tissue-specific profiling of IAA metabolites in the ugt84b1 mutant. IAA and the IAA metabolites IAA-glucose (IAA-glc) and oxIAA-glucose (oxIAA-glc) were quantified in roots (green outline) and shoots (purple outline) from 7-day-old seedlings, and in young siliques (red outline) from 39-day-old plants. The concentrations of all metabolites are given in picomoles per gram of fresh weight. Samples were analysed with ten independent biological replicates, and error bars represent the standard deviation (SD). Asterisks indicate statistically significant differences from Col-0 (**p < 0.01, ***p < 0.001, and ****p < 0.0001; Student’s t test).

Figure 3. A non-characterized UGT family member is upregulated by auxin in roots.
(a) Transcriptional responses of all uncharacterized Arabidopsis UGT genes to 1 µM IAA in root tissues. Transcriptomic data were obtained from the GSE42007 dataset at Genevestigator.
(b) Relative expression analysis of UGT76E5 in response to 1 µM IAA treatments. Bars indicate relative expression of UGT76E5 in 7-day-old Col-0 seedlings after mock (black) and IAA (grey) treatments at different times. Error bars indicate the interval delimited by 2^\Delta\Delta CT±SE. Asterisks indicate ΔCT values significantly different from those of the mock treatment in a Mann-Whitney U test (*p<0.001; n=9).
(c) Structure of the UGT76E5 gene indicating the position of the ugt76e5 mutation. Boxes and lines represent exons and introns respectively. Open and black boxes represent untranslated and translated regions respectively. The triangle indicates a T-DNA insertion. Horizontal arrows represent the oligonucleotides q_UGT76E5_F (q5-F) and q_UGT76E5_R (q5-R), not drawn to scale, used as primers in (b).
(d) Neighbour-joining tree of proteins of the UGT76E subfamily. The tree was constructed with MEGAX (see methods) and inferred from 1000 replicates.
(e) Expression patterns of the UGT76E3, UGT76E4, UGT76E5, and UGT76E6 genes in different tissues. Transcriptomic data were obtained from Genevestigator datasets corresponding to wild-type tissues. Scale bars indicate (c) 0.5 kilobases and (d) 5% amino acid sequence changes according to the Poisson correction method (Nei and Kumar, 2000). Genevestigator colour scales indicate (a) log2-ratio values from -2.5 to 2.5 and (e) percentage of expression potential.

Figure 4. De novo synthesis of IAA metabolites in ugt84b1, ugt74d1, ugt74d1 ugt84b1, ugt76e3456 and 35S pro:UGT76E5 plants. Formation of IAA metabolites in 7-day-old seedlings of the genotypes indicated after incubation with [13C6]IAA for 12 hours. Bars indicate the concentrations (picomoles / g of fresh weight) ± SD of all metabolites. Samples
were analysed with five independent biological replicates. Statistically significant differences from Col-0 (*\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), and ****\(p < 0.0001\); Student’s \(t\) test).

**Figure 5.** \textit{ugt76e3456} plants show enhanced shade- and darkness-induced hypocotyl elongation. (a-c) Hypocotyl length of Col-0, \textit{ugt84b1}, \textit{ugt74d1}, and \textit{ugt76e3456} seedlings grown in (a) light, (b) shade, and (c) darkness. (d) IAA and IAA metabolite quantification performed in dark-grown hypocotyls. Bars indicate (a-c) length means ± SE (\(n \geq 30\)), (d) concentration (picomoles / g of fresh weight) means ± SD (\(n = 4\)). Statistically significant differences from Col-0 (*\(p < 0.05\), **\(p < 0.01\), and ****\(p < 0.0001\); Student’s \(t\) test).

**Figure 6.** Proposed model of action of UGTs in IAA metabolism at different developmental stages. Simplified IAA inactivation pathways in etiolated seedlings (a), light-grown seedlings (b), and siliques (c). The major players in IAA and oxIAA glycosylation at each stage are indicated by coloured boxes. Smaller boxes indicate likely minor contributions by the UGTs indicated. DAO: Dioxygenase for Auxin Oxidation.

**SUPPLEMENTARY MATERIAL**

Supplementary Figure 1. Morphological phenotypes of \textit{ugt84b1}, \textit{ugt74d1} \textit{ugt76e5}, and \textit{ugt76e3456} plants.

Supplementary Figure 2. Tissue-specific profiling of IAA catabolites in the \textit{ugt84b1} mutant.

Supplementary Figure 3. \textit{UGT76E3}, \textit{UGT76E4}, \textit{UGT76E5}, and \textit{UGT76E6} are co-expressed with auxin-related genes.

Supplementary Figure 4. qRT-PCR analysis of the relative expression of \textit{UGT76E5} in seven independent transformants carrying the \textit{35S_{pro:UGT76E5}} transgene.

Supplementary Figure 5. Phylogenetic analysis of the UGT76 family.

Supplementary Figure 6. Alignment of the amino acid sequences of the Arabidopsis proteins \textit{UGT76E3}, \textit{UGT76E4}, \textit{UGT76E5} and \textit{UGT76E6}.

Supplementary Figure 7. The \textit{UGT76E3}, \textit{UGT76E4}, \textit{UGT76E5}, and \textit{UGT76E6} genes are clustered together on Arabidopsis chromosome 3.

Supplementary Figure 8. CRISPR-based approach to knock out the entire \textit{UGT76E3456} subfamily.

Supplementary Figure 9. Detailed information about the \textit{UGT76E3} gene editing.

Supplementary Figure 10. Detailed information about the \textit{UGT76E4} gene editing.

Supplementary Figure 11. Detailed information about the \textit{UGT76E5} gene editing.

Supplementary Figure 12. \textit{UGT74D1} expression pattern and molecular characterization of the \textit{ugt74d1} mutant.

Supplementary Figure 13. Steady-state levels of IAA metabolites in \textit{ugt84b1}, \textit{ugt74d1}, \textit{ugt76e3456} and \textit{35S_{pro:UGT76E5}} plants.

Supplementary Figure 14. Sequence information for the \textit{UGT84B1} gene editing.

**Supplementary Table 1.** Primer sets used in this work.
ACKNOWLEDGMENTS

Research in the laboratory of Karin Ljung is supported by grants from the Swedish Foundation for Strategic Research (Vinnova), the Knut and Alice Wallenberg Foundation (KAW), the Swedish research councils VR and Formas, and Carl Tryggers Stiftelse för Vetenskaplig Forskning. E.M.-B. (JCK-1811) and R.C.-S. (JCK-1111) held postdoctoral fellowships from Kempeföreningen. We also acknowledge the Swedish Metabolomics Centre (http://www.swedishmetabolomicscentre.se/) for access to instrumentation.

AUTHOR CONTRIBUTIONS

E.M.-B., R.C.-S. and K.L. conceived and designed the research; E.M.-B. and R.C.-S. performed most of the experiments; J.Š. performed the hormone analyses. E.M.-B. wrote the manuscript draft and prepared the figures and tables; E.M.-B. and R.C.-S. wrote the manuscript with input from all authors. This research was supported by funds to K.L.
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Mateo-Bonmatí et al. Figure 2

Roots

- IAA
- IAA-glc
- oxtIAA-glc

Shoot

- IAA
- IAA-glc
- oxtIAA-glc

Young siliques

- IAA
- IAA-glc
- oxtIAA-glc

Col-0 vs. ugt84b1