Dioxygenase-encoding AtDAO1 gene controls IAA oxidation and homeostasis in Arabidopsis

Silvana Porco1,2, Aleš Pěnčík3,4,5, Afaf Rashed6, Ute Voß6,7, Rubén Casanova-Sáez2,1, Anthony Bishopp8, Agata Golebiowska9,9, Rahul Bhosale2, Ranjan Swarup3, Kamal Swarup9, Paulina Peňáková2,6, Ondrej Novák2,8,9, Paul Stawski2, Peter Hedden5, Andrew L. Phillips9, Kris Vissenberg5, Malcolm J. Bennett3,4, and Karin Ljung3,4

1Centre for Plant Integrative Biology, Plant and Crop Science Division, School of Biosciences, University of Nottingham, Loughborough LE12 9SR, United Kingdom; 2Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden; 3Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany Academy of Sciences of the Czech Republic (AS CR), CZ-78371 Olomouc, Czech Republic; 4Faculty of Science, Palacky University, CZ-78371 Olomouc, Czech Republic; 5Integrated Molecular Plant Physiology Research, Biology Department, Antwerp University, 2020 Antwerp, Belgium; 6Department of Agronomy and Horticulture, University of Nebraska, NE 68803-0915; and 7Department of Plant Biology and Crop Science, Rothamsted Research, Hertfordshire ALS 2QJ, United Kingdom

Edited by Mark Estelle, University of California at San Diego, La Jolla, CA, and approved July 26, 2016 (received for review March 22, 2016)

Auxin represents a key signal in plants, regulating almost every aspect of their growth and development. Major breakthroughs have been made dissecting the molecular basis of auxin transport, perception, and response. In contrast, how plants control the metabolism and homeostasis of the major form of auxin in plants, indole-3-acetic acid (IAA), remains unclear. In this paper, we initially describe the function of the Arabidopsis thaliana gene DIOXYGENASE FOR AUXIN OXIDATION 1 (AtDAO1). Transcriptional and translational reporter lines revealed that AtDAO1 encodes a highly root-expressed, cytoplasmically localized IAA oxidase. Stable isotope-labeled IAA feeding studies of loss and gain of function AtDAO1 lines showed that this oxidase feedse the major regulator of auxin degradation to 2-oxoindole-3-acetic acid (oxIAA) in Arabidopsis. Surprisingly, AtDAO1 loss and gain of function lines exhibited relatively subtle auxin-related phenotypes, such as altered root hair length. Metabolite profiling of mutant lines revealed that disrupting AtDAO1 regulation resulted in major changes in steady-state levels of oxIAA and IAA conjugates but not IAA. Hence, IAA conjugation and catabolism seem to regulate auxin levels in Arabidopsis in a highly redundant manner. We observed that transcripts of AtDAO1 AIA oxidase and GH3 IAA-conjugating enzymes are auxin-inducible, providing a molecular basis for their observed functional redundancy. We conclude that the AtDAO1 gene plays a key role regulating auxin homeostasis in Arabidopsis, acting in concert with GH3 genes, to maintain auxin concentration at optimal levels for plant growth and development.

Arabidopsis thaliana | IAA degradation | oxidase | dioxygenase | root hair elongation

Distinct indole-3-acetic acid (IAA) conjugation and degradation pathways operate to maintain optimal auxin concentrations for plant growth and development processes. There are three major forms of auxin conjugates identified in diverse plants: ester-linked IAA-sugar conjugates, amide-linked IAA-amino acid conjugates, and amide-linked IAA peptide and protein conjugates (reviewed in ref. 1). In Arabidopsis thaliana, Group II of the Gretchen Hagen3 (GH3) family of auxin-inducible asial amid synthetases converts IAA to IAA-amino acids (2, 3). Most amino acid IAA conjugates are believed to be inactive; however, there are enzymes that can hydrolyze some of the IAA-amino acid conjugates to free IAA (reviewed in ref. 1). Some of the IAA-amino acid conjugates, such as indole-3-acetic acid aspartic acid (IAA-Asp) and indole-3-acetic acid glutamic acid (IAA-Glu), can also be further metabolized (4–6). The conversion of IAA to indole-3-acetic acid (IAA-glc) is catalyzed by the UDP glucosyltransferase UGT84B1 (7).

The major IAA catabolite in Arabidopsis is 2-oxoindole-3-acetic acid (oxIAA), the oxidized form of IAA (4, 6, 8). oxIAA can be further metabolized by conjugation to glucose (4, 5), and it was recently shown that the UDP glucosyltransferase UGT74D1 is able to catalyze the glucosylation of oxIAA to 2-oxoindole-3-acetic acid glucose (oxIAA-gluc (9)). IAA catabolism has been shown to be an irreversible step (4, 10), and the oxIAA product has very little biological activity and is not transported via the polar auxin transport system (6, 8).

Although many metabolites in the conjugation and catabolic pathways have been identified in Arabidopsis (4–7, 9–11), it has proved difficult to identify and characterize the genes and enzymes involved. We have now identified two closely related components of the IAA degradation machinery in Arabidopsis, DIOXYGENASE FOR AUXIN OXIDATION 1 (AtDAO1) and AtDAO2 (AtIg14120), which are closely related to genes described in apple [Adventitious Rooting Related Oxygenase 1 (12)] and rice [DAO (13)]. The AtDAO1 and AtDAO2 genes belong to a distinct clade of the 2-oxogluturic acid-dependent dioxygenase family and are related to enzymes involved in gibberellin biosynthesis and inactivation. We report that the AtDAO1 gene plays a key role regulating auxin homeostasis in Arabidopsis, acting in concert with GH3 genes, to maintain auxin concentration at optimal levels for plant growth and development. As a result, plants lacking AtDAO1 activity led to


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freesly available online through the PNAS open access option.

See Commentary on page 10742.

2. Present address: Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037.
3. M.J.B. and K.L. contributed equally to this work.
4. To whom correspondence may be addressed. Email: malcolm.bennett@nottingham.ac.uk or karin.ljung@slu.se.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1604375113/-/DCSupplemental.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freesly available online through the PNAS open access option.

See Commentary on page 10742.

2. Present address: Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037.
3. M.J.B. and K.L. contributed equally to this work.
4. To whom correspondence may be addressed. Email: malcolm.bennett@nottingham.ac.uk or karin.ljung@slu.se.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1604375113/-/DCSupplemental.
major changes in conjugated forms of the hormone, but auxin level remains within a normal range, helping explain why the mutant exhibited subtle phenotypic changes. We conclude that IAA catabolism and conjugation regulate auxin homeostasis in Arabidopsis in a highly redundant manner.

Results

IAA Oxidation in Arabidopsis Is Controlled by the Dioxygenase Gene AtDAO1. To study the in vivo formation of IAA metabolites, a feeding experiment was performed using \([^{13}C_6]IAA\) with WT Arabidopsis seedlings. We observed rapid labeling of the pools of the major IAA conjugates IAA-Asp, IAA-Glu, and IAA-glc as well as the catabolites oxIAA and oxIAA-glc within 3 h after treatment began, revealing that IAA is rapidly oxidized in Arabidopsis seedlings (Fig. 1A).

A rice DAO gene that encodes a 2-oxoglutarate–dependent Fe (II) dioxygenase-like enzyme has recently been reported to mediate IAA oxidation (13). Phylogenetic analyses revealed that the Arabidopsis genome contains two genes related to OsDAO1 termed At1g14130 (AtDAO1) and At1g14120 (AtDAO2). AtDAO1 and AtDAO2 belong to the 2-oxoglutarate–dependent Fe (II) dioxygenase gene family, and their sequences are related to enzymes involved in gibberelin biosynthesis and inactivation (Fig. S1A). A BLAST search of the sequenced plant genomes at Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) identified sequences closely related to AtDAO1 and AtDAO2 in a range of species, with amino acid identities ranging from 47 to 93% (Fig. S1B).

To address the putative IAA oxidase role of AtDAO1 in Arabidopsis, we identified several Transfer-DNA (T-DNA) insertion lines (Fig. 1B). The T-DNA for the Salk 093162 line (dao1-1) is inserted in the first exon of At1g14130 311 bp downstream of the start codon. Sail line 349_D02 (dao1-2D) has a T-DNA insertion in the intergenic region between the AtDAO1 and AtDAO2 genomic sequences 542 bp upstream of the AtDAO2 start codon. T-DNA insertions in Salk_082522 (dao1-3) and Salk_095931 (dao1-4D) lines are positioned upstream of the start codon of AtDAO1 (591 and 789 bp, respectively). Quantitative RT-PCR (qRT-PCR) was performed to measure transcript levels of DAO1 and DAO2 in the T-DNA lines (Fig. S2A). DAO1 mRNA was strongly reduced in dao1-1 and dao1-3 lines, suggesting that they represent DAO1 overexpressing lines. No significant changes were detected in the expression level of DAO2 in any of these lines compared with the WT (in Fig. S2). The \([^{13}C_6]IAA\) feeding experiments were also performed using seedlings from dao1-1 (AtDAO1 mutant) and dao1-2D (AtDAO1 overexpressing) lines, revealing that (unlike the WT and dao1-2D) dao1-1 seedlings failed to oxidize \([^{13}C_6]IAA\) to \([^{13}C_6]oxIAA\) (Fig. 1A). Formation of \([^{13}C_6]oxIAA\)-glc was also abolished in the dao1-1 mutant line. Instead, \([^{13}C_6]\)-labeled IAA was converted to IAA conjugates (\([^{13}C_6]I\)IAA-Asp and \([^{13}C_6]I\)IAA-Glu) at a higher rate than in WT seedlings. Conversely, the dao1-2D line oxidized \([^{13}C_6]I\)IAA to \([^{13}C_6]oxIAA\) and \([^{13}C_6]oxIAA\)-glc more rapidly than the WT, and the \([^{13}C_6]I\)IAA conjugate levels were diminished in this line. Labeling of IAA-glc is similar in all lines, suggesting that the formation of this conjugate is not altered in AtDAO1 mutant and overexpressing lines (Fig. 1A). Hence, our \([^{13}C_6]IAA\) feeding experiments revealed that AtDAO1 plays an essential role regulating IAA oxidation in Arabidopsis seedlings.

Mutants Disrupting AtDAO1 Activity Exhibit Subtle Changes in Shoot and Root Phenotypes. We next characterized the phenotypes of the dao1 mutants (Fig. 2). Given the apparent importance of AtDAO1 mediating IAA oxidation in Arabidopsis seedlings (Fig. 1A), we expected mutant and overexpressing plants to exhibit multiple IAA-related developmental defects. Surprisingly, AtDAO1 mutant and overexpressing lines exhibited only mild defects in plant growth and development (Fig. 2). Compared with WT seedlings, AtDAO1 mutant lines exhibit a slight reduction in primary root length and increase in lateral root density (Fig. 2A and Fig. S3 A and B). The root gravitropic response was faster in loss of function dao1-1 and dao1-3 mutants compared with in dao1-2D and dao1-4D, with response that was similar to that in the WT (Fig. S3C). In shoot tissues, increased branching and shorter siliques were observed in dao1-1, whereas dao1-2D adult plants exhibited a growth phenotype closer to the WT (Fig. 2 B and C).

Our root hair (RH) measurements revealed that their length increased ~80% in dao1-1 and dao1-3 seedlings compared with the WT (Fig. 2 D and E). RH determination and elongation in Arabidopsis is controlled (in part) by a small family of basic Helix-Loop-Helix transcription factors that includes the auxin-inducible gene member RSL4 (14–16). Datta et al. (17) have recently shown that RH length is proportional to RSL4 mRNA abundance. Significantly, qRT-PCR analysis revealed that dao1-1 and dao1-3 seedlings contained an ~80% increase in RSL4 transcript levels (Fig. 2F), which is directly proportional to their altered RH length. Interestingly, multicellular root modeling predicts an accumulation of IAA in epidermal tissues in the dao1-1 mutant (18), and the RH phenotype in dao1-1 and dao1-3 (Fig. 2 D and E) supports the model. We conclude that, despite the importance of AtDAO1 controlling auxin oxidation (Fig. 1A), loss and gain of function lines exhibit surprisingly weak auxin-related phenotypic alterations (Fig. 2). Nevertheless, dao1-1 primary root length, lateral root density, and lateral root length were all more sensitive to external IAA treatments than the WT or dao1-2D (Fig. S3 D–F), consistent with the dao1-1 mutant having a reduced ability to break down auxin.

AtDAO1 Encodes a Root-Expressed Cytoplasmically Localized IAA Oxidase. To determine whether the mild dao1 RH phenotype (Fig. 2 D–F) could be caused by an RH-specific expression.

Fig. 1. IAA oxidation in Arabidopsis is regulated by the dioxygenase gene family member At1g14130 (AtDAO1). (A) IAA feeding experiment. Formation of labeled IAA conjugates and catabolites after incubation of 7-d-old Arabidopsis Col-0, dao1-1, and dao1-2D seedlings with \([^{13}C_6]IAA\). Samples were analyzed in three independent biological replicates. Error bars represent SD. FW, fresh weight. (B) Structure of the Arabidopsis DAO1 gene with indication of the position of the T-DNA insertions (triangles) in the different dao1 alleles studied.

Porco et al.
pattern, we created a transcrip

**IAA Metabolite Profiling Reveals That AtDAO1 Regulates oxIAA Levels in Arabidopsis.** To study the effect of the AtDAO1 gene on auxin homeostasis, we performed IAA metabolite profiling of dao1-1 mutant and dao1-2D overexpressing lines. Using liquid chromatography-tandem MS (LC-MS/MS) analysis (11), we quantified free IAA and the major IAA precursors, catabolites, and conjugates in 7-d-old seedlings of WT Columbia-0 (Col-0), dao1-1, and dao1-2D.

In dao1-1 and dao1-2D, the concentrations of IAA and the IAA precursors indole-3-acetamide, indole-3-pyruvic acid, and indole-3-acetonitrile were significantly reduced in these lines compared with those in the WT (Fig. S5). However, the IAA precursors indole-3-acetamide, indole-3-pyruvic acid, and indole-3-acetonitrile were not significantly affected (Fig. S5). As a control, we also quantified IAA conjugates and catabolites in dao1-1 and dao1-2D seedlings and observed a significant reduction of oxIAA and oxIAA-glue levels in dao1-1 as well as a significant increase in oxIAA and oxIAA-glue levels in dao1-2D, confirming that dao1-1 and dao1-2D behave as KO and overexpressing lines, respectively (Fig. 4). In contrast, the levels of IAA-glue were not changed in these lines. In a second experiment, we performed IAA metabolite profiling of confocal images of the pDAO1::DAO1-GFP expressing root cells revealed that the IAA oxidase is localized in the cytoplasm and probably excluded from nuclei and central vacuole compartments (Fig. 3 C–E).

**IAA Metabolite Profiling Reveals That AtDAO1 Regulates oxIAA Levels in Arabidopsis.** To study the effect of the AtDAO1 gene on auxin homeostasis, we performed IAA metabolite profiling of dao1-1 mutant and dao1-2D overexpressing lines. Using liquid chromatography-tandem MS (LC-MS/MS) analysis (11), we quantified free IAA and the major IAA precursors, catabolites, and conjugates in 7-d-old seedlings of WT Columbia-0 (Col-0), dao1-1, and dao1-2D.

In dao1-1 and dao1-2D, the concentrations of IAA and the IAA precursors indole-3-acetamide, indole-3-pyruvic acid, and indole-3-acetonitrile were significantly reduced in these lines compared with the WT (Fig. S5). However, the IAA precursors indole-3-acetamide, indole-3-pyruvic acid, and indole-3-acetonitrile were not significantly affected (Fig. S5). As a control, we also quantified IAA conjugates and catabolites in dao1-1 and dao1-2D seedlings and observed a significant reduction of oxIAA and oxIAA-glue levels in dao1-1 as well as a significant increase in oxIAA and oxIAA-glue levels in dao1-2D, confirming that dao1-1 and dao1-2D behave as KO and overexpressing lines, respectively (Fig. 4). In contrast, the levels of IAA-glue were not changed in these lines.
all DAO1 mutant and overexpressing lines compared with the Col-0 WT. The profiles were very similar between the two mutant (dao1-1 and dao1-3) as well as between the two overexpressing (dao1-2D and dao1-4D) lines (Fig. S2B).

Strikingly, a major up-regulation of the IAA amino acid conjugates IAA-Asp and IAA-Glu was observed in dao1-1 compared with the WT (around 280- and 46-fold, respectively) (Fig. 4). In contrast, the oxidase overexpressing line dao1-2D exhibited significantly reduced levels of IAA-Glu and IAA-Asp (Fig. 4). This discovery prompted us to quantify IAA metabolites in a mutant line disrupting IAA amino acid conjugation. The GH3 sextuple mutant gh3.1,2,3,4,5,6, which comprises six of eight IAA-amino acid-conjugating enzyme genes, exhibited a significant increase in IAA and its precursor TRP (Fig. S5). However, levels of oxIAA, oxIAA-glc, and IAA-glc were not affected compared with Col-0, but the levels of IAA-Asp were below the detection limit, whereas the IAA-Glu levels were significantly elevated (Fig. 4). This increase is most likely caused by the IAA-conjugating activity of GH3.17, which prefers Glu over other amino acids, whereas GH3 1–6 exhibits activity with Asp (2, 3). The gh3.1,2,3,4,5,6 mutant line also has a stronger auxin overproduction phenotype compared with the wild auxin overproduction phenotype in dao1-1, with increased lateral root formation and elongated petioles (Fig. S6).

IAA metabolite profiling different tissues of 10-d-old seedlings revealed similar changes in metabolite distribution in every WT and dao1-1 tissue analyzed (Fig. S7). We conclude that disrupting AtDAO1 caused major changes in steady-state levels of oxIAA, oxIAA-Glc, and IAA conjugates but only minor changes in IAA levels (Fig. 4 and Figs. S2B and S7).

**IAA Conjugation and Catabolism Redundantly Regulate IAA Homeostasis.**

To monitor the rates of de novo synthesis of these metabolites, a feeding experiment was performed using [15N]lindole as IAA precursor. IAA as well as IAA conjugates and catabolites were rapidly labeled after incubation (Fig. S8). The gh3.1,2,3,4,5,6 line exhibited the most rapid labeling of IAA, consistent with a higher IAA synthesis rate and/or lower turnover via conjugation. Labeling of oxIAA and oxIAA-glc was highest in dao1-2D and lowest in dao1-1 as expected in IAA oxidase overexpressing and IAA oxidase mutant lines, respectively. In contrast, extremely rapid labeling of IAA-Asp and IAA-Glu was observed in dao1-1, indicating that the high endogenous IAA conjugate levels observed (Fig. 4 and Figs. S2B and S7) originate from de novo synthesis.

We also quantified IAA metabolites in two 35S:DAO1 lines (Fig. S9). In these constitutive IAA oxidase overexpressing lines, we could not observe any significant changes in IAA and oxIAA levels compared with in the WT. However, a significant up-regulation of both oxIAA-glc and IAA-glc was detected in one of the 35S:DAO1 lines, suggesting that glucosylation pathways were active, whereas the levels of IAA-Asp and IAA-Glu were strongly down-regulated in both 35S:DAO1 lines (Fig. S9). Hence, IAA metabolite profiling has revealed that IAA conjugation and catabolism regulate IAA homeostasis redundantly in Arabidopsis seedlings.

Previously described components of the IAA homeostasis machinery, such as GH3 genes, have been described to be rapidly auxin inducible (1). Transcript profiling of auxin-treated root apical tissues revealed that, like GH3 genes, AtDAO1 was auxin inducible, albeit with a lower level of induction and on a longer timescale >4 h (18). IAA metabolite profiling of AtIDAO1 and GH3 mutant lines has revealed that IAA conjugation and catabolism regulate auxin homeostasis redundantly (Fig. 4). Given that IAA-Asp and IAA-Glu were present at high levels in the dao1-1 mutant, we measured the expression of GH3.2 and GH3.3 genes in the dao1-1 and dao1-2D mutant backgrounds (Fig. S10). A 1.8-fold increase in gene expression was detected for GH3.3 in the dao1-1 mutant compared with in the WT. In contrast, GH3.3 expression was down-regulated in the dao1-2D overexpressor line (Fig. S10). We conclude that auxin-inducible expression of both AtDAO1 and GH3 genes could provide a molecular mechanism to explain the observed functional redundancy in IAA catabolism and conjugation that regulates auxin homeostasis.

**Discussion**

Maintaining auxin homeostasis is critical for growth and developmental processes in plants. Several mechanisms have been proposed to control auxin homeostasis, including IAA metabolism (biosynthesis, degradation, and conjugation), transport, and compartmentation (reviewed in ref. 19). In this paper, we initially describe the key gene regulating IAA oxidation in Arabidopsis, AtDAO1. In parallel, Zhang et al. (20) have shown that the recombinant AtDAO1 protein (and the closely related AtDAO2 sequence) has IAA oxidase activity in vitro. Confocal imaging of GFP-tagged AtDAO1-expressing transgenic plants suggests that the IAA oxidase is localized cytoplasmically (Fig. 3) and based on subcellular fractionation studies (20), behaves as a soluble protein.

In planta stable isotope-labeled auxin feeding studies have revealed the critical importance of AtDAO1 converting IAA to oxIAA in Arabidopsis seedlings (Fig. 1). In contrast, the in planta role of AtDAO2 is currently less clear given that oxidation of [15C]IAA to [15C]oxIAA and [15C]oxIAA-glc was completely blocked in the dao1-1 loss of function mutant (Fig. 4A). A clue comes from transcript profiling results by Zhang et al. (20), which reported that AtDAO1 is widely expressed in root and shoot tissues at much higher levels than AtDAO2. In addition, transcriptomic profiling revealed that AtDAO1 and AtDAO2 exhibit auxin and circadian regulated patterns of expression, respectively (18,21). Hence, the Arabidopsis AtDAO1 and AtDAO2 sequences seem to have subfunctionalized since their tandem duplication event, with AtDAO1 functioning as the major IAA oxidase at the seedling stage of development.

Given how widely the IAA oxidase AtDAO1 is expressed across root and shoot tissues, loss of function dao1 mutant alleles exhibit surprisingly subtle auxin-related developmental defects (Fig. 2 and Fig. S3) (20). This observation can be explained in part, following metabolite profiling and modeling studies (Fig. 4).
expression leads to up-regulation of IAA degradation and conjugation regulate IAA homeostasis in Arabidopsis (S6). T-DNA insertion mutants were obtained from Porco et al. Arabidopsis Seeds were surface sterilized with 50% (vol/vol) hypo-
chlorous acid for 5 min and then, washed three times with sterile deionized water. For IAA metabolite profiling experiments, plant seeds were plated in Murashige and Skoog medium (4.4 g salts per 1 L), including 1% sucrose and 0.5 g/L MES monohydrate, at pH 5.7 and solidified with 0.8% Plant Agar (Duchefa). Seeds were stratified at 4 °C in the dark to synchronize germination and then, incubated vertically in a culture room under long-day (LD) conditions (16 h light at 22 °C and 8 h dark at 18 °C; 150 μmol m−2 s−1) and at a relative humidity of 67%. For the rest of the experiments, seeds were plated on 1/2 Murashige and Skoog medium (2.17 g salts per 1 L) without sucrose, stratified at 4 °C for 2–3 d, and then, incubated vertically in a culture room at 22 °C under continuous light (120–150 μmol m−2 s−1).

Materials and Methods

Growth Conditions. Seeds were surface sterilized with 50% (vol/vol) hypochlorous acid for 5 min and then, washed three times with sterile deionized water. For IAA metabolite profiling experiments, plant seeds were plated in Murashige and Skoog medium (4.4 g salts per 1 L), including 1% sucrose and 0.5 g/L MES monohydrate, at pH 5.7 and solidified with 0.8% Plant Agar (Duchefa). Seeds were stratified at 4 °C for 72 h in the dark to synchronize germination and then, incubated vertically in a culture room under long-day (LD) conditions (16 h light at 22 °C and 8 h dark at 18 °C; 150 μmol m−2 s−1) and at a relative humidity of 67%. For the rest of the experiments, seeds were plated on 1/2 Murashige and Skoog medium (2.17 g salts per 1 L) without sucrose, stratified at 4 °C for 2–3 d, and then, incubated vertically in a culture room at 22 °C under continuous light (120–150 μmol m−2 s−1).

Plant Material. The Arabidopsis ecotype Columbia was used as the WT in all experiments. The dao1 T-DNA insertion mutants were obtained from Nottingham Arabidopsis Stock Centre, and the insertion was confirmed by PCR using forward and reverse primers in combination with LB1.3 (for dao1-1, dao1-3, and dao1-4D) or LB1 (for dao1-2D) (Table S1). The gh3 sextuple mutant (gh3.1,2,3,4,5,6) was assembled by repeated rounds of crossing between the single-gene insertion mutants or their double- or triple-mutant derivatives. Mutants gh3.1 and gh3.2 are in the Landsberg erecta background, and primers for their genotyping were previously described (21). All other mutants were in the Col-0 background; gh3.5 was described previously as wes1 (22), and gh3.4 and gh3.6 are T-DNA insertion lines (Salk_002549 and Salk_013458, respectively), whereas gh3.1 is a transposon line (SM_37350). Genotyping of gh3 insertions was done with forward and reverse primers along with Lba1 (for gh3.4, gh3.5, and gh3.6) or 3’Spm (for gh3.3) (Table S1). Each single mutant was verified to be essentially a complete KO by Northern blot hybridizations.

Root Phenotyping. The number of lateral roots per seedling was counted using a stereo microscope, and digital images were taken. For RH measurements, seedlings were imaged with a camera mounted to a Leica stereomicroscope. The digital images were used for RH and primary root length measurement with Fiji software. RHs –3 mm from the root tip were measured (10 RHs per seedling). For hormone treatment, the indicated concentration of IAA was added to 1/2 Murashige and Skoog medium before it solidified. For gravitropic response assays, plates containing 6-d-old seedlings grown on a 12-h/12-h light/dark period were turned 90°, and the root angle was imaged every 60 min for 8 h using an automated image acquisition system (23) and quantified using Fiji software.

Cloning of At1g14130 and Arabidopsis Transformation. The transcriptional and translational fusions as the overexpression lines of At1g14130 were generated in gateway-compatible vectors. Genomic DNA from Col-0 was used to amplify 847 bp upstream of the At1g14130 translational start codon by using the At1g14130pro forward and reverse primers (Table S1). The PCR product was cloned into pENTR-o-Topo entry vector (Invitrogen) and recombined by LR re-action into the pGW323 destination binary vector to create a transcriptional GUS fusion. For the translational fusion, a fragment of 1,846 bp, containing the At1g14130 promoter and coding sequence region without the stop codon, was amplified with At1g14130pfpf primers (Table S1). The PCR product was cloned into pENTR-o-Topo entry vector (Invitrogen) and recombined by LR reaction into the pGW84 (24) destination binary vector to create a translational GFP fusion. To create an overexpression line, the coding sequence of At1g14130 was amplified using the At1g14130cds primers (Table S1). The PCR product was cloned into pENTR-o-Topo entry vector (Invitrogen) and recombined by LR reaction into the pK2GW7 (24) destination binary vector. The authenticity of all constructs was verified by sequencing before transformation. A. thaliana (ecotype Columbia) plants were transformed with Arabidopsis tumefaciens (strain GV3101) using the floral dipping method (25). Screening of T3 seeds for 100% antibiotic resistance identified homozygous plants for the transgene.

IAA Metabolite Profiling. A. thaliana WT Col-0 as well as gh3.1,2,3,4,5,6 and dao1 mutant lines were grown under LD conditions for 7 or 10 d. Whole seedlings or dissected tissues were collected in five replicates (20 mg tissue per sample). Sample purification and quantification of IAA metabolites were performed as described in ref. 11.

Feeding Experiments with Labeled IAA and Indole. Seven-day-old Arabidopsis seedlings (Col-0, gh3.1,2,3,4,5,6, and dao1 mutant lines) were incubated with liquid medium containing 0.5 μM [13C2]IAA or 10 μM [15N]Jindole for 0, 3, 6, 12, and 24 h under gentle shaking and in darkness. For each time point, 20 mg whole seedlings were collected in three replicates. The samples were extracted and purified by solid-phase extraction as described in ref. 11. Incorporation of the label into IAA metabolites was measured using LC-MS/MS, with the Multiple Reaction Monitoring transitions corresponding to endogenous and labeled compounds. De novo synthesis of IAA metabolites was expressed as the concentration of labeled metabolites at individual time points after correction for natural isotope abundances (26).

Microscopy. Confocal microscopy was performed using a Leica SPS Confocal Laser-Scanning Microscope (Leica Microsystems). Cell walls were stained using propidium iodide (10 μg/mL, Sigma). Scanning settings used for one experiment were optimized and kept unchanged throughout the experiment. Images were processed using the Leica SPS Image Analysis software. Images for Fig. 2 were processed with Adobe Photoshop only for adjustment of brightness and contrast.

Histochemical Analysis. The GUS gene activity was revealed by incubating seedlings at 37° for 1 h in a phosphate buffer (500 mM; pH 7) containing...
0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1 mM EDTA (pH 8), 0.5% (vol/vol) Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Sigma). The X-Gluc was initially dissolved in dimethylformamide to reach a final concentration of 0.5%. After GUS staining, seedlings were cleared and mounted in 50% (wt/vol) glycerol.

qRT-PCR. Real-time qRT-PCR reactions were performed on a LightCycler 480 apparatus (Roche) using SYBR Green Master Mix (Bioline). A standard re-action mixture (12 μL) contained 5 μL cDNA template, 6 μL 2× SYBR Green I Master Mix (Roche), 0.1 μL 100 μM forward and reverse primers, and 0.8 μL H2O. All of the specific primer pairs (Table S1) were designed with the Universal Probe Library Assay Design Center (Roche; lifescience.roche.com/shop/homet) and used to quantify the gene expression levels. Arabidopsis ACT2 gene (At3g18780) was used as a constitutive internal standard, which showed no clear changes in Cycle threshold values, to normalize the obtained gene expression results. All individual reactions were done in triplicates.


ACKNOWLEDGMENTS. We thank the Swedish Metabolomics Centre for the use of instrumentation. This work was funded by the Swedish Foundation for Strategic Research, the National Swedish Research Council, Carl Tryggers Stiftelse for Vetenskaplig Forskning, and Kempestiftelsen. A.G. and K.V. were supported by Research Foundation Flanders Grants G009412N, G.0.602.11.N.10, and 1.5.091.11.N.00 and University of Antwerp Grant BOF-DOPCR04. K.S. and M.J.B. acknowledge support of the European Research Council FUTUREROOTS Project. P.H. and A.L.P. are funded by the 20:20 Wheat Institute Strategic Programme Grant from the Biotechnology and Biological Sciences Research Council of the United Kingdom.