The main oxidative inactivation pathway of the plant hormone auxin

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Inactivation of the phytohormone auxin plays important roles in plant development, and several enzymes have been implicated in auxin inactivation. In this study, we show that the predominant natural auxin, indole-3-acetic acid (IAA), is mainly inactivated via the GH3-ILR1-DAO pathway. IAA is first converted to IAA-amino acid conjugates by GH3 IAA-amidosynthetases. The IAA-amino acid conjugates IAA-aspartate (IAA-Asp) and IAA-glutamate (IAA-Glu) are storage forms of IAA and can be converted back to IAA by ILR1/ILL amidohydrolases. We further show that DAO1 dioxygenase irreversibly oxidizes IAA-Asp and IAA-Glu into 2-oxindole-3-acetic acid-aspartate (oxIAA-Asp) and oxIAA-Glu, which are subsequently hydrolyzed by ILR1 to release inactive oxIAA. This work established a complete pathway for the oxidative inactivation of auxin and defines the roles played by auxin homeostasis in plant development.
Th e phytohormone auxin plays a pivotal role in almost every aspect of plant development. Auxin biosynthesis, polar transport, and inactivation pathways coordinate auxin homeostasis. Indole-3-acetic acid (IAA) is the predominant natural auxin and is synthesized from tryptophan via two consecutive enzymatic reactions in the indole-3-pyruvate pathway, which is widely conserved in land plants. Arabidopsis mutants exhibited auxin-accumulation phenotypes, but dao1-1 mutants exhibited only slightly impaired or normal phenotypes in auxin-regulated development processes, consistent with the redundant roles by these enzymes and pathways in maintaining IAA homeostasis.

IAA-Leu-Resistant1 (ILR1) was initially identified as an IAA-Leu amidohydrolase from a genetic screen for mutants resistant to IAA-Leu. The Arabidopsis ILR1-like (ILL) family consists of IRL1, ILL1, ILL2, ILL3, IAR3/ILL4, ILL6, and the pseudogene ILS5. ILR1/ILL enzymes convert various IAA-amino acid conjugates to IAA in vitro. However, whether the ILR1/ILL enzymes are involved in metabolizing IAA-Asp and IAA-Glu in plants has not been experimentally tested because both conjugates did not trigger auxin-phenotypes when added to plant growth media.

In this study, we demonstrate that IAA is mainly inactivated by GH3 enzymes and that DAO functions as an oxidase of IAA-amino acid conjugates to produce oxIAA-amino acid conjugates downstream of GH3. Using the membrane-permeable ester of IAA-amino acid conjugates, we demonstrate that IAA-Asp and IAA-Glu are reversible storage forms of IAA, contrary to the previously proposed function of the conjugates. ILR1/ILL enzymes revert IAA-Asp and IAA-Glu back to free IAA in plants. Unlike the widely accepted pathway, oxIAA is produced from oxIAA-amino acids by ILR1 but not by direct oxidation of IAA. Thus, we determine that IAA inactivation is coordinately regulated by GH3-ILR1-DAO-mediated IAA recycling and degradation to maintain auxin homeostasis. Our findings provide insights into the regulatory framework governing auxin homeostasis.

**Results**

GH3 pathway plays a central role in IAA inactivation. To determine the contributions of each IAA-inactivating enzyme to IAA homeostasis, we generated multiple null mutants by CRISPR-Cas9 gene editing technology. Arabidopsis dao dao2 double mutants and iamt1 ugt84b1 dao1 dao2 quadruple mutants showed primary root growth similar to that of wild-type (WT).
plants (Fig. 2a, b and Supplementary Fig. 1b). On the other hand, gh3.1 2 3 4 5 6 17 (gh3-sept) mutants exhibited very short roots and more adventitious roots, which are characteristic phenotypes of auxin overaccumulation. We generated a GH3 inhibitor designated kakeimide (KKI) that competitively inhibited IAA conjugation (Supplementary methods) and WT seedlings were incubated with IAA and KKI for 20 h. **c** [13C₆]IAA feeding experiment. [12C]endogenous and [13C₆]-labeled oxIAA and DioxIAA were measured using [13C₆]oxIAA and [13C₆]DioxIAA as internal standards after incubation of 7-d-old WT, daa1 daa2, and gh3-sept seedlings with 0.2 μM [13C₆]IAA for 20 h. The values are shown as the means ± SD (d, e, n = 4). The different letters represent statistical significance at η < 0.01 (Tukey’s HSD test).

Consistent with the high-auxin phenotypes, endogenous IAA levels were elevated, and IAA-Asp and IAA-Glu were undetected, in the gh3-sept mutants (Fig. 2c and Supplementary Fig. 3a). KKI caused a significant accumulation of IAA by inhibiting the conversion of IAA to IAA-Asp and IAA-Glu in WT (Fig. 2d and Supplementary Fig. 3d). Furthermore, KKI also led to a high accumulation of IAA and repressed the conversion of IAA to auxin-overproduction plants (Supplementary Fig. 3d). Endogenous IAA levels did not differ significantly between iamt1 ugt84b1 daa1 daa2 mutants and WT plants (Fig. 2c). These results...
DAO functions as an IAA-amino acid oxidase. The previous model suggests that AtDAO1 is involved in basal IAA inactivation. When IAA levels are elevated, GH3s convert IAA to IAA-Asp and IAA-Glu to maintain IAA homeostasis (Fig. 1b). GH3 expression is induced in the dao1-1 loss-of-function mutant in response to reduced IAA degradation, resulting in extreme accumulation of the two conjugates in dao1-1 (Supplementary Fig. 3a, d)10. Thus, we expected that oxIAA would accumulate to high levels in gh3-sept mutants. Surprisingly, oxIAA was significantly decreased in gh3-sept mutants (Fig. 2c), and KKI repressed oxIAA production from IAA in YUC2-overexpressing and WT plants (Supplementary Fig. 3d and Fig. 2d). It is known that dioxIAA (3-hydroxy-oxIAA) is an oxidized product of oxIAA24. KKI also inhibited dioxIAA production from IAA in WT (Fig. 2d). DioxIAA was significantly decreased in both the dao1 dao2 and gh3-sept mutants (Supplementary Fig. 3c). Feeding experiments using [13C6]-labeled IAA confirmed that exogenous [13C6]IAA was not converted to [13C6]oxIAA and [13C6]dioxIAA in dao1 dao2 and gh3-sept mutants, but was efficiently converted in WT plants (Fig. 2e). These results indicate that the production of oxIAA depends on the presence of GH3 enzymes and that AtDAO1 likely functions downstream of GH3 in the same pathway (Fig. 1c).

To assess the substrate specificity of DAO enzymes, we studied the enzymatic activity of recombinant Arabidopsis AtDAO1 and rice OsDAO. AtDAO1 efficiently oxidized IAA-Asp, IAA-Glu, IAA-alanine (IAA-Ala), and IAA-leucine (IAA-Leu) to the corresponding oxIAA-amino acid conjugates. The Km values of DAO functions as an IAA-amino acid oxidase. The previous model suggests that AtDAO1 is involved in basal IAA inactivation10. When IAA levels are elevated, GH3s convert IAA to IAA-Asp and IAA-Glu to maintain IAA homeostasis (Fig. 1b). GH3 expression is induced in the dao1-1 loss-of-function mutant in response to reduced IAA degradation, resulting in extreme accumulation of the two conjugates in dao1-1 (Supplementary Fig. 3a, d)10. Thus, we expected that oxIAA would accumulate to high levels in gh3-sept mutants. Surprisingly, oxIAA was significantly decreased in gh3-sept mutants (Fig. 2c), and KKI repressed oxIAA production from IAA in YUC2-overexpressing and WT plants (Supplementary Fig. 3d and Fig. 2d). It is known that dioxIAA (3-hydroxy-oxIAA) is an oxidized product of oxIAA24. KKI also inhibited dioxIAA production from IAA in WT (Fig. 2d). DioxIAA was significantly decreased in both the dao1 dao2 and gh3-sept mutants (Supplementary Fig. 3c). Feeding experiments using [13C6]-labeled IAA confirmed that exogenous [13C6]IAA was not converted to [13C6]oxIAA and [13C6]dioxIAA in dao1 dao2 and gh3-sept mutants, but was efficiently converted in WT plants (Fig. 2e). These results indicate that the production of oxIAA depends on the presence of GH3 enzymes and that AtDAO1 likely functions downstream of GH3 in the same pathway (Fig. 1c).

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IAA-Asp and IAA-Glu function as IAA storage forms in vivo. IAA-Asp and IAA-Glu have long been suggested to be irreversible metabolites in plants because these two conjugates lack auxin activity when applied to Arabidopsis plants. However, both IAA-Asp and IAA-Glu are hydrophilic IAA conjugates with dicarboxylic acids and thus might not be incorporated into cells due to poor membrane permeability. To test this hypothesis, we synthesized membrane-permeable prohormone IAA-Asp diesters as the prodrug form of IAA-Asp. We found that diesters acted as prodrugs of IAA-Asp and IAA-Glu to release free IAA, thereby exhibiting auxin activities in planta (Supplementary Fig. 7a, b). On the other hand, a monester of IAA-Asp showed weak auxin activity, perhaps due to a lower membrane permeability. To confirm that the IAA-amino acid dimethyl esters are metabolically converted into IAA, we analyzed the metabolites from [2H3]-labeled IAA-Asp-dimethyl ester ([D2]IAA-Asp-DM: 3H2-methylene group in IAA moiety) in Arabidopsis plants. The unmodified [D2]IAA-Asp was detected in WT plants after treatment with [D2]IAA-Asp-DM (Fig. 3b), [D2]IAA appeared in WT plants, but D2-IAA was not detectable in ilr1 ill2 iar3 ilr3 mutants after incubation with D2-IAA-Asp-DM (Fig. 3b), indicating that ILR1/ILL hydrolases converted IAA-amino acid to free IAA in plants. These results illustrated that plasma membrane-permeable forms of IAA conjugates, IAA-Asp-DM and IAA-Glu dimethyl ester (IAA-Glu-DM), were metabolically converted to the unmodified IAA-Asp and IAA-Glu, and then ILR1/ILL enzymes hydrolyze the two conjugates to release free IAA. Consequently, the released IAA elicited typical auxin responses, such as root growth inhibition and lateral root promotion (Fig. 3c and Supplementary Fig. 7–9). IAA-Asp-DM and IAA-Glu-DM induced the expression of auxin-responsive DR5 reporter gene (Supplementary Fig. 7c). Auxin-resistant signaling mutants were insensitive to IAA-amino acid diesters (Supplementary Fig. 7d). Furthermore, IAA-Asp-DM and IAA-Glu-DM induced root growth in the monocot plants Oryza sativa (rice) and Brachypodium distachyon. (Supplementary Fig. 8). These results demonstrate that IAA-Asp and IAA-Glu function as IAA storage forms in planta and are the substrates of the ILR1/ILL hydrolases. Other synthetic methyl esters of IAA-Leu, IAA-Ala, IAA-Phe, and IAA-Met also function as prodrugs of IAA-amino acid conjugates that were converted to free IAA in Arabidopsis plants (Supplementary Fig. 10a). On the other hands, endogenous IAA-Ala and IAA-Leu were not detected in the dao1-1 mutant and WT plants in our measurements (Supplementary Fig. 10b), suggesting that these conjugates are minor metabolites in the IAA catabolic pathway.

DAO oxidizes IAA-Asp and IAA-Glu in planta. The AtDAO1-overexpressing lines (35 S::AtDAO1) were highly resistant to IAA-amino acids and their esters, including IAA-Asp-DM and IAA-Glu-DM (Fig. 3c and Supplementary Fig. 9a, c, d and 10a, c, d), but showed the same response to IAA as WT (Supplementary Fig. 9e), suggesting that DAO1 rapidly converts IAA-Asp and IAA-Glu to inactive oxIAA-amino acids but does not efficiently oxidize IAA in planta. In contrast, the dao1-1 mutant was hypersensitive to IAA-amino acid methyl ester (Fig. 3c and, Supplementary Fig. 9a, c, d and 10a, c, d). Additionally, unmodified IAA-Glu and IAA-Asp showed auxin activity in root growth and DR5 transgene induction in dao1 mutant (Fig. 3d and Supplementary Fig. 7c). Furthermore, the estradiol-inducible AtDAO1 transgene (pMDC7::AtDAO1) complemented the high sensitivity to IAA-Asp-DM exhibited by the dao1-1 mutant (Supplementary Fig. 9b). Overexpression of native and GFP-fused O. sativa OsDAO and B. distachyon BdDAO decreased the sensitivity to IAA-amino acid diesters in both WT (Fig. 4a, b) and dao1-1 mutants (Supplementary Fig. 11a). The dao1-1 mutants showed impaired fertility and shorter siliques with an irregular pattern in the primary inflorescence10,11. This impaired phenotype in dao1-1 was complemented by 35 S::GFP-AtDAO1 and 35 S::GFP-OsDAO (Fig. 4c). On the other hand, overexpression of GFP-AtDAO2 and the loss-of-function dao2-1 mutation did not affect the sensitivity to IAA-Glu-DM (Fig. 4a, b and Supplementary Fig. 11b). Consistent with phenotypic and biochemical studies, oxIAA-Asp and oxIAA-Glu were significantly decreased in the dao1 mutants (Fig. 3e and Supplementary Fig. 3b). The 35 S::GFP-AtDAO1-overexpressing lines in dao1-1 mutants increased
production of oxIAA, dioxIAA and their conjugates. The oxIAA metabolites accumulated after IAA-amino acid diester treatment in the WT and AtDAO1 overexpression lines but not in the dao1-1 mutant (Fig. 3c and Supplementary Fig. 12). As observed for the dao1 mutants, the gh3-sept mutants also did not produce oxIAA-Asp and oxIAA-Glu in WT plants (Fig. 2d). Similarly, oxIAA conjugates and dioxIAA were highly accumulated in both 6-d-old WT and ilr1 ill2 ill3 ilr1/ill quadruple mutants with 1μM [D2]IAA-Asp-DM for 6 h. The values shown are the means ± SD (**P < 0.01, ns: not significant, two-tailed Student’s t test, n = 4). e Phenotypes of dao1-1 and AtDAO1-overexpressing (35S::AtDAO1) plants grown for 6 days with 1μM IAA-Asp-DM. Scale bar, 10 mm. d Effects of IAA-Glu on root growth in dao1-1, dao1 dao2, 35S::AtDAO1, iar3 dao1-1, and ilr1 dao1-1 mutants. Root length (6-d-old) is shown as the percentage of that in mock-treated plants (100%). The different letters represent statistical significance at P < 0.001 (Tukey’s HSD test, n = 20). e The levels of IAA metabolites after treatment with IAA-amino acid diesters. WT, dao1-1, and GFP-AtDAO1-overexpressing dao1-1 mutants (7-d-old) were incubated for 20 h with the compounds. The values shown are the means ± SD (**P < 0.01, two-tailed Student’s t test, n = 3).

Fig. 3 DAO1 irreversibly oxidized IAA-amino acid conjugates in a plant. a Michaelis–Menten plot and kinetics parameters of the AtDAO1 enzyme for IAA-Glu (n = 3). Error bars, SE. b in vivo conversion of IAA-Asp-DM to IAA. Endogenous and [D2]IAA-Asp and [D2]IAA were measured after incubation of 8-d-old WT and ilr1 ill2 ill3 (ilr1/ill) quadruple mutants with 1μM [D2]IAA-Asp-DM for 6 h. The values shown are the means ± SD (**P < 0.01, ns: not significant, two-tailed Student’s t test, n = 4). c Interactions between DAO1 proteins and the IAA-Glu conjugates. Scale bar, 10 mm. d Effects of IAA-Glu on root growth in dao1-1, dao1 dao2, 35S::AtDAO1, iar3 dao1-1, and ilr1 dao1-1 mutants. Root length (6-d-old) is shown as the percentage of that in mock-treated plants (100%). The different letters represent statistical significance at P < 0.001 (Tukey’s HSD test, n = 20). e The levels of IAA metabolites after treatment with IAA-amino acid diesters. WT, dao1-1, and GFP-AtDAO1-overexpressing dao1-1 mutants (7-d-old) were incubated for 20 h with the compounds. The values shown are the means ± SD (**P < 0.01, two-tailed Student’s t test, n = 3).

ILR1/ILL regulate IAA homeostasis in planta. To investigate the physiological role of the ILR1/ ILL genes (Supplementary Fig. 1b), the sensitivity of ilr1 ill2 mutants to IAA-amino acid diesters was examined. Among the single mutants, only ilr1 showed resistance to IAA-Asp-DM and IAA-Glu-DM in primary root growth and lateral root promotion (Fig. 5a and Supplementary Fig. 14, 15d). The ilr1 iar3 double mutants were more resistant to these conjugate esters, and the ilr1 ill2 iar3 ill3 quadruple mutants were insensitive to these conjugate esters at the tested concentrations (Supplementary Fig. 15). We also analyzed the expression of ILR1-, ILL2- and IAR3-GFP/GUS fusion proteins under their native promoters (Fig. 5b and Supplementary Fig. 16). These fusion proteins complemented the insensitivity of the ilr1 and iar3 mutants to IAA-amino acid diesters (Supplementary Fig. 16a, b). The ILR1-GFP/GUS and IAR3-GFP/GUS fusion proteins were expressed at the hypocotyl, lateral root tip, and meristem zone of the root and shoot, where IAA is constitutively synthesized and metabolized. The ILL2-GUS protein was also detected at the shoot meristem (Supplementary Fig. 16j). These findings further indicate that AtDAO1 and its DAO1 orthologs primarily function as IAA-amino acid oxidases in vivo.
slightly auxin-deficient phenotypes, such as short hypocotyls and fewer lateral roots, as previously reported in the Ws background. The ilr1 ili2 iar3 ili3 quadruple mutants in the Col background exhibited more severe auxin-deficient phenotypes in hypocotyl elongation and lateral root formation (Fig. 5c, d). These data are in agreement with the idea that ILR1/ILL genes are involved in IAA release from the IAA storage forms IAA-Asp and IAA-Glu in planta.

The ilr1 mutation dramatically decreased the sensitivity of the dao1-1 mutant to IAA-Glu and its methyl ester (Fig. 3d and Supplementary Fig. 17a). The ilr1 iar3 dao1-1 and ilr1 ili2 iar3 dao1-1 mutants showed lower sensitivity to IAA-amino acid methyl esters than did dao1-1 mutants in lateral root formation (Supplementary Fig. 17b). In contrast, overexpression of ILL1-GFP in the dao1 dao2 mutant increased its sensitivity to unmodified IAA-Asp and IAA-Glu (Supplementary Fig. 17c, d). The ilr1 iar3 dao1-1 triple mutants completely ameliorated the high-auxin phenotypes of long root hairs, many lateral roots, and large cotyledons observed in dao1-1 mutants (Fig. 5e and Supplementary Fig. 18a, b). The impaired fertility of the primary inflorescence in dao1-1 was also recovered in ilr1 dao1-1, ilr1 iar3 dao1-1 and ilr1 ili2 iar3 dao1-1 mutants (Supplementary Fig. 18c). This evidence indicates that high-auxin phenotype of dao1-1 mutants are the consequence of the IAA release from IAA-Asp and IAA-Glu by ILL1/ILLS.

ILR1 produces oxIAA from oxIAA-Asp and oxIAA-Glu. The ilr1 mutants showed higher resistance to IAA-Glu-DM than IAA-Asp-DM (Supplementary Fig. 14b, c). Our kinetic experiments revealed that recombinant GST-ILR1 enzyme preferred IAA-Glu (Kcat/Km: 344 M$^{-1}$ s$^{-1}$) to IAA-Asp (Kcat/Km: 7.4 M$^{-1}$ s$^{-1}$) (Supplementary Fig. 19a-e). Interestingly, GST-ILR1 enzyme also efficiently hydrolyzed oxIAA-Glu (Kcat/Km: 3300 M$^{-1}$ s$^{-1}$) and oxIAA-Asp (Kcat/Km: 28 M$^{-1}$ s$^{-1}$) to produce oxIAA (Fig. 6a, b and Supplementary Fig. 19f, g), implying that ILR1 hydrolyzes oxIAA-Glu and oxIAA-Asp to produce oxIAA in planta.

Consistent with ILR1 enzyme activities for hydrolyzing oxIAA conjugates, our metabolite analysis demonstrated that the levels of endogenous oxIAA and dioxIAA dramatically decreased, and whereas oxIAA-Glu greatly accumulated in the two ilr1 mutants (Fig. 6c and Supplementary Fig. 20a). The complemented line, pILR1::ILR1-GFP in ilr1, showed the same oxIAA and oxIAA-Glu levels as WT. In the feeding experiment, the oxIAA and dioxIAA levels were elevated in WT after treatment with IAA, IAA-Asp-DM, and IAA-Glu-DM (Fig. 6c). Additionally, oxIAA-Glu levels were enormously elevated in ilr1 plants after IAA or IAA-Glu-DM treatment (Fig. 6d and Supplementary Fig. 20). In contrast, oxIAA and dioxIAA were not produced in the ilr1 mutant after incubation with IAA, IAA-Asp-DM, and IAA-Glu-DM (Fig. 6c, d and Supplementary Fig. 20a, b), indicating that oxIAA-Asp and oxIAA-Glu were converted to oxIAA by ILR1. In contrast to the significant accumulation of oxIAA-Glu in ilr1 mutants, the oxIAA-Asp level was slightly elevated in ilr1 mutants even after IAA and IAA-Asp-DM treatments (Fig. 6d and Supplementary Fig. 20d), suggesting that oxIAA-Asp is metabolized by other enzymes in addition to ILR1.

**Discussion**

Inactivation of auxin plays a key role in maintaining auxin homeostasis in vivo, but the previously proposed molecular mechanisms governing auxin inactivation are fragmented and lack a clear picture. Several enzymes have previously been implicated in IAA inactivation by amino acid conjugation, methylation, glycosylation, and oxidation, and the IAA-
inactivating enzymes appear to participate in different catabolic routes. In this article, we established a complete pathway for oxidative inactivation of IAA, and we were able to arrange the previously described auxin-inactivating enzymes in the same metabolic pathway, thereby providing a clear picture of auxin inactivation. The GH3-ILR1-DAO pathway clearly defines the roles of three enzymes in storing auxin, reactivating stored auxin, and irreversibly deactivating auxin (Fig. 1).

Our genetic and metabolic analyses demonstrate that GH3 enzymes play a major role in auxin inactivation. IAMT1 and UGT84B1 did not redundantly compensate for GH3-mediated IAA inactivation (Fig. 2 and Supplementary Fig. 2), suggesting that IAMT1 and UGT84B1 likely play only a minor role in IAA inactivation. DAO-mediated oxidative IAA inactivation is believed to be irreversible degradation, thereby regulating the basal level of IAA. However, we clearly demonstrate that AtDAO1 irreversibly oxidizes IAA-Asp and IAA-Glu, the IAA storage forms, to oxIAA-Asp and oxIAA-Glu, respectively, thereby modulating the levels of the storage forms in Arabidopsis plants (Fig. 3 and Supplementary Fig. 4). The metabolic analysis of ilr1 mutants and kinetic study for ILR1 enzyme revealed that ILR1 converts oxIAA-Asp and oxIAA-Glu to oxIAA in Arabidopsis plants (Fig. 6 and Supplementary Fig. 19, 20). Our feeding experiments showed that oxIAA and dioxIAA levels in ilr1 mutants remain unchanged even after IAA treatment (Fig. 6d and Supplementary Fig. 20b). Similarly, previous work demonstrated that oxIAA was not produced from exogenous IAA in dao1-1 mutants. These metabolite analyses firmly established that ILR1 is essential for oxIAA production and that DAO1 did not directly convert IAA to oxIAA. This evidence suggests that most of oxIAA is produced from IAA-amino acid conjugates by DAO1 and ILR1 enzymes, but not by enzymatic or non-enzymatic direct oxidation of IAA. Although the feeding experiment showed that [13C6] oxIAA is not synthesized from [13C6]-labeled IAA in tobacco BY-2 cultured cells and Arabidopsis dao1-1 mutants, the endogenous oxIAA (30–40% amounts in WT plants) that were higher than the levels in the dao1 dao2 and ilr1 mutants (Fig. 2c). At a high level of IAA in gh3-sept mutants, the group III GH3 enzymes, such as AtGH3.15 might produce IAA-amino acid conjugates to compensate for an impaired IAA inactivation in gh3-sept mutants, thereby accumulating small amounts of oxIAA. Recent work on IAA metabolic analysis indicated that oxIAA-Asp did not accumulate in DAO1 knockout tobacco BY-2 cultured cells and Arabidopsis dao1-1 mutants, suggesting that DAO1 oxidizes IAA-Asp in planta.

In contrast to the previous model that IAA-Asp and IAA-Glu are not a storage form of auxin, our chemical biology study demonstrates that the two IAA conjugates actually serve as the main IAA storage forms that are readily converted back to free IAA by ILR1/ILL hydrolases in planta (Fig. 1c). IAA-Asp and IAA-Glu accumulate as major metabolites after exogenous IAA treatments. Furthermore, IAA-Asp and IAA-Glu strongly accumulate in the dao1-1 mutant, IAA-Ala and IAA-Leu have been previously thought to be IAA storage forms. However, the two conjugates have not been detected as endogenous metabolites in the WT and dao1-1 mutant, although dao1-1 is hypersensitive to endogenous IAA-Ala and IAA-Leu (Supplementary Fig. 10c, d). IAA-Ala and IAA-Leu were also not detected in rice. Our in vitro
**Fig. 6** ILR1 hydrolyzed IAA amino acid conjugates to produce oxIAA. **a** HPLC chromatogram of the reaction products of the recombinant GST-ILR1 enzyme. **b** Lineweaver-Burk plot and kinetics parameters of GST-ILR1 for oxIAA-Glu as the substrate \((n = 4)\). **c** The endogenous levels of oxIAA and oxIAA-Glu in WT, ilr1-1 (Ws, EMS mutant), ilr1 (Col, SAIL_631_F01) and pILR1::ILR1-GFP in ilr1 (Col) complementation lines. Seven-day-old plants were treated with 0.5 μM IAA-Asp-DM or IAA-Glu-DM for 20 h. The values shown are the means ± SD \((n = 3)\). The different letters represent statistical significance at \(P < 0.001\) (Tukey’s HSD test). **d** The endogenous levels of oxIAA-Asp, oxIAA-Glu, and oxIAA in WT and ilr1 (Col) seedlings. Seven-day-old plants were treated with 0.5 μM IAA for 24 h. The values shown are the means ± SD (two-tailed Student’s \(t\) test, \(n = 3\)).
enzymatic assays for OsDAO demonstrate that OsDAO recognizes IAA-Asp and IAA-Glu as substrates but not IAA-Ala and IAA-Leu (Supplementary Fig. 6). Thus, IAA-Ala and IAA-Leu are unlikely to function as IAA storage forms in Arabidopsis and rice. In monocots, O. sativa and B. distachyon, both IAA-Asp and IAA-Glu also function as storage forms, and the two IAA conjugates are eventually oxidized to dioxygenA via oxIAA conjugates as intermediates (Supplementary Fig. 8, 13). Notably, dioxygenA accumulates 50–200 times more than oxIAA in these monocots, suggesting that oxIAA is readily oxidized to dioxygenA (Supplementary Fig. 13). Similar to B. distachyon, the gymnosperm Picea abies permoxIAA-glucoside as an abundant metabolite32. OxIAA displays (Supplementary Fig. 13). Similar to GH3 natively, auxin-inducible IAA to IAA-Glu to maintain basal IAA inactivation. Alter-

2 GH3.17 IAA-Glu (Supplementary Fig. 19). IAA-Glu in IAA homeostasis in Arabidopsis. IAA-Glu at an elevated IAA level, the released IAA from IAA-

that IAA is more rapidly converted to IAA-Asp rather than to amount of IAA-Asp under high levels of endogenous IAA. Given

development.

The

further research seeking to elucidate the dynamics of auxin gra-

homeostasis can be maintained by the balance among three

IAA metabolites, and GH3 inhibitor (kakeimide) were synthesized as described in Supplementary Methods. GH3-12345617 were previously described40,41. The

The

ilr1/ill

do double mutants (Col background) were generated by ordinary crossing. Homozygous mutants were identified by PCR genotyping and DNA sequencing using the primers described in Supplementary Table 1. Sterile seeds were placed on GM medium (one-half-strength Murashige and Skoog salts, 1.2% [w/v] Suc, and 0.5 g/L MES, pH 5.8, containing 85 vitamins and 0.4% [w/v] agar for horizontal culture or 0.6% [w/v] gellan gum (FUJIFILM Wako Pure Chemical, Japan) for vertical growth unless otherwise stated. The seeds were

were germinated for 2–3 days at 4 °C. Seedlings were grown at 23 °C in a growth chamber (MLR-352, Plymouth, Japan). Rice (Oryza sativa) Japonica Group cv. Nipponbare seeds were sterilized with 10% (v/v) bleach for 30 min and washed thoroughly with sterilized water. The seeds were germinated in the dark for 28 days. The germinated seeds were placed on 0.4% (w/v, sterilized water) agar containing the test compounds in a plastic culture dish and cultured at 25 °C for 3 days. The germinated seeds, Brachypodium distachyon B21 seeds were sterilized with 10% (v/v) bleach for 20 min and washed with sterilized water. The seeds were kept at 4 °C for 3 days and then cultured on 0.4% (w/v, water) agar at 24 °C for 2 days under continuous light. The germinated seeds were transferred to GM medium (0.4% w/v agar) containing the test compounds and incubated at 24 °C for another 6 days under continuous light.

Measurements of the growth of hypocotyls, primary and lateral roots, root hairs, and cotyledon area. For primary root elongation assays, Arabidopsis seedlings were grown on GM plates (6 g/L gellan gum for the vertical plate culture and 4 g/L agar for horizontal plate culture) containing the indicated concentrations of chemicals for 6–8 days under continuous light at 24 °C. The lengths of the hypocotyl and primary root were recorded with a digital camera. For lateral root measurements, Arabidopsis seedlings were vertically grown for 6 days at 24 °C in continuous light on GM plates (6 g/L gellan gum). The seedlings were transferred to a horizontal GM plate (2 g/L gellan gum) containing the indicated concentrations of chemicals. The seedlings were cultivated under continuous light for another 2 days at 24 °C. The primary root length and the number of lateral roots were measured. The cotyledon area were expressed by scion. The cotyledons were recorded with a digital camera. The image was analyzed by NIH Image J software.

Chemical synthesis of IAA metabolites, and GH3 inhibitor. Enzyme substrate, IAA metabolites, and GH3 inhibitor (kakeimide) were synthesized as described in Supplementary Methods.

Auxin metabolite measurements. For feeding experiments, Arabidopsis seedlings were grown vertically on GM agar plates (14 g/L agar) and then transferred into liquid GM media with or without chemicals. The seedlings were incubated for the indicated times under continuous light. The rice and B. distachyon B21 seedlings were incubated in 300-ml culture vials containing 40 mL of water with or without chemicals under continuous light. The roots were excised and stored in a deep freezer until use after extensive washing with distilled water.

IAA metabolites were measured by LC-MS/MS analysis5,6. For analysis of IAA, oxIAA, IAA-Asp, IAA-Glu, IAA-Ala, IAA-Leu, oxIAA-Asp, oxIAA-Glu and DHAIA, frozen plant materials (30–50 mg) were prepared in three to four replicates and homogenized with zirconia beads (3 mm) in 0.3 mL of 80% acetonitrile/1% acetic acid/H2O containing [phenyl-13C]IAA, [phenyl-13C]IAA,

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oxIAA, IAA-[13C5, 15N]Asp, IAA-[15N]Glu, [2H2]IAA-Ala, [2H2]IAA-Leu, [2H2]oxIAA-Asp, [2H2]oxIAA-Glu and [2H2]DioxIAA using a Beads Crusader μT-12 (TAKARA Bio, Japan) for 2 min. The extracts were centrifuged at 13,000 × g for 5 min at 4 °C, and the supernatant was collected. Extraction was repeated twice without internal standards. The extracts were combined and evaporated to dryness using a CVE-3000 centrifugal evaporator (EYELA, Japan). Each extract was redissolved in 1% acetic acid/H2O (1 mL) and loaded onto an Oasis HLB column (1 mL; Waters, USA). The column was washed with 1% acetic acid/H2O (1 mL), IAA, oxIAA and DioxIAA were eluted with 80% acetonitrile/1% acetic acid/H2O (2 mL). The eluted fractions were evaporated to dryness using a CVE-3000 centrifugal evaporator. Each eluted fraction was redissolved in 1% acetic acid/H2O (1 mL) and loaded onto an Oasis WAX column (1 mL; Waters, USA). A 30-μL aliquot was mixed with 400 μL of methanol containing 50 mM phosphate acid in a 1.5-mL microtube to terminate the reaction. After centrifugation at 12,000 × g for 10 min at 2 °C, an aliquot of the supernatant was immediately analyzed by an HPLC system (EXTREMA, JASCO Japan) as described in Supplementary Methods. For the OsDAO reaction for IAA, oxIAA-amino acids were added to the reaction mixture and incubated at 30 °C for 200 min. After the enzyme reaction, 100 µL of the reaction mixture was mixed with 400 µL of methanol containing 50 mM phosphate acid in a 1.5-mL microtube to terminate the reaction. After centrifugation at 12,000 × g for 10 min at 2 °C, the supernatant was immediately analyzed by an HPLC system (EXTREMA, JASCO Japan) as described in Supplementary Methods.

Enzyme activity of GST-ILR1. For the GST-ILR1 enzymatic reactions for IAA-amino acids and oxIAA-amino acids, the reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM diethiothreitol, 1 mM MnCl2, and the substrates (50–1000 µM for IAA-Asp, 20–1000 µM for IAA-Glu, 10–200 µM for oxIAA-Asp and 1–50 µM for oxIAA-Glu) and 40–200 ng boiled or unboiled GST-ILR1 enzymes (2–10 ng/µL final concentration) for 20 min. The reaction mixture was incubated at 32 °C for 6 h with a thermal cycler (MJ Mini, Bio-Rad). After the enzyme reaction, 20 µL of methanol containing 50 mM phosphate acid was added to the reaction mixture to terminate the reaction. After centrifugation, 5 µL of the supernatant was immediately analyzed by an HPLC system (EXTREMA, JASCO Japan) as described in Supplementary Methods.

Microscopy. Photographic images were taken with a SXZ16 microscope (Olympus, Japan), and fluorescent images were collected with an FV-3000 laser scanning confocal microscope (Olympus, Japan) using 488-nm light combined with 500–550-nm filters for GFP.

Statistical analysis. Statistical analysis was performed by One-way ANOVA Tukey’s post hoc test and two-tailed t-test. Box plots in all figures show the median as lines in the box, the interquartile range beyond the box, and the end caps represent the minimum and maximum. Box plot overlaid with beeswarm plot was drawn with RSstudio software. For LC-MS/MS analysis, three or four biological replicates in an independent experiment were analyzed, and the means of the replicates were indicated.

Data availability. All data are available in the main text or the supplementary materials. The clones and plant materials generated during this study are available from the corresponding authors upon request. Source data are provided with this paper.

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Author contributions

K.H. and K.F. conceived and designed the experiments. K.H. wrote the paper with help from K.F., H.K. and Y.Z. K.A., Y.T., K.F., Y.A., H.H., H.K., R.G., Y.H., C.G., Y.Z. and K.H. conducted the experiments and contributed to the study design. H.K. and K.H. analyzed the data.

Competing interests

The authors declare no competing interests.

Additional information

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