Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in *Arabidopsis*

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Auxins are hormones that regulate many aspects of plant growth and development. The main plant auxin is indole-3-acetic acid (IAA), whose biosynthetic pathway is not fully understood. Indole-3-acetaldoxime (IAOx) has been proposed to be a key intermediate in the synthesis of IAA and several other indolic compounds. Genetic studies of IAA biosynthesis in *Arabidopsis* have suggested that 2 distinct pathways involving the CYP79B or YUCCA (YUC) genes may contribute to IAOx synthesis and that several pathways are also involved in the conversion of IAOx to IAA. Here we report the biochemical dissection of IAOx biosynthesis and metabolism in plants by analyzing IAA biosynthesis intermediates. We demonstrated that the majority of IAOx is produced by CYP79B genes in *Arabidopsis* because IAOx production was abolished in CYP79B-deficient mutants. IAOx was not detected from rice, maize, and tobacco, which do not have apparent CYP79B orthologues. IAOx levels were not significantly altered in the yuc1 yuc2 yuc4 yuc6 quadruple mutants, suggesting that the YUC gene family probably does not contribute to IAOx synthesis. We determined the pathway for conversion of IAOx to IAA by identifying 2 likely intermediates, indole-3-acetamide (IAM) and indole-3-acetonitrile (IAN), in *Arabidopsis*. When \(^{13}C_6\)-labeled IAOx was fed to CYP79B-deficient mutants, \(^{13}C_6\) atoms were efficiently incorporated to IAM, IAN, and IAA. This biochemical evidence indicates that IAOx-dependent IAA biosynthesis, which involves IAM and IAN as intermediates, is not a common but a species-specific pathway in plants; thus IAA biosynthesis may differ among plant species.

Indole-3-acetic acid | plant hormone

Plants produce small molecule hormones for intracellular and intercellular signal transduction in response to developmental and environmental cues. Auxins are 1 type of plant hormones that are involved in many developmental processes, including cell division, cell differentiation, phototropism, root gravitropism, apical dominance, and vascular differentiation (1). Although the structure of the predominant natural auxin, indole-3-acetic acid (IAA), has been known since the 1930s, elucidation of auxin biosynthesis has remained challenging probably because of the occurrence of multiple biosynthetic pathways (2–5). Both tryptophan (TRP)-dependent and independent IAA biosynthetic pathways have been proposed (2). Recently identified auxin biosynthetic genes all belong to the TRP-dependent pathway (2–5).

As shown in Fig. 1A, there are 4 proposed pathways for the biosynthesis of IAA from TRP in plants: (i) the CYP79B pathway, (ii) the YUCCA (YUC) pathway, (iii) the indole-3-pyruvic acid (IPA) pathway, and (iv) the indole-3-acetamide (IAM) pathway (5). In *Arabidopsis*, indole-3-acetaldoxime (IAOx) is known to be produced from the CYP79B pathway by the cytochrome P450 monoxygenases, CYP79B2 and CYP79B3 (6, 7). It has been suggested that IAOx is a common intermediate for the synthesis of IAA, 3-indolylmethyl-glucosinolate (IG), and camalexin (CL) in *Arabidopsis* (8–11). IG is a metabolite produced to deter herbivores (12, 13), and CL is a phytoalexin produced in *Arabidopsis* in response to pathogen infection (11, 14). The physiological importance of IAOx-dependent IAA biosynthesis has been demonstrated by analysis of cyp79b2 cyp79b3 double mutants, which have shorter hypocotyls and decreased IAA levels when grown at high temperatures (8). Disruption of IG biosynthesis resulted in auxin-overproduction phenotypes because of the presumed diversion of IAOx to IAA synthesis. Mutations in SUPERROOT1 (SUR1) and SUPERROOT2 (SUR2), which encode 2 enzymes in IG biosynthesis, cause the production of massive adventitious roots from hypocotyls (9, 10). However, the exact mechanisms by which IAOx is converted to IAA are not clear. Both indole-3-acetonitrile (IAN) and indole-3-acetaldehyde (IAAld) are suggested as potential intermediates (9, 15), but the biosynthetic routes and the genes involved have not been identified. Moreover, IAN has also been proposed as an intermediate in IAA biosynthesis via IG metabolism (5). Because the production of IAN from IG occurs in response to tissue damage, it is not clear whether IG metabolism contributes to IAA biosynthesis under normal growth conditions.

IAA biosynthesis via the YUC pathway is essential for many developmental processes including embryogenesis, seedling growth, vascular patterning, and flower development (16, 17). Overexpression of YUC genes leads to elongated hypocotyls and epinastic cotyledons, and inactivation of YUC genes causes severe developmental phenotypes that can be rescued by in situ production of IAA (17). YUC was proposed to catalyze the conversion of tryptamine (TAM) to N-hydroxy-tryptamine (HTAM), a rate-limiting step in a TRP-dependent IAA biosynthesis pathway (18). The roles of YUC genes in IAA biosynthesis and plant development have been extended to rice, maize, and petunia (19–21). Unlike the YUCs that exist throughout the plant kingdom, the CYP79B family has so far only been identified in *Arabidopsis*, Brassica napus, and Sinapis alba (22), suggesting that the YUC pathway is likely a universally used auxin biosynthesis pathway whereas IAA synthesis by the CYP79B family may have more specific roles in crucifers.

Genetic studies of IAA biosynthesis in *Arabidopsis* have suggested that the CYP79B and YUC pathways may converge at least in *Arabidopsis*. Disruption of IG biosynthesis resulted in auxin-overproduction pathways in *Arabidopsis*, Brassica napus, and Sinapis alba (22), suggesting that the YUC pathway is likely a universally used auxin biosynthesis pathway whereas IAA synthesis by the CYP79B family may have more specific roles in crucifers.

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the majority of IAOx is produced by the CYP79B family in Arabidopsis. The IAOx levels in the cyp79b2 cyp79b3 double mutants, and in rice, maize, and tobacco that do not have apparent CYP79B orthologues were below our detection limits. The YUC pathway probably does not contribute to IAOx synthesis, because IAOx levels were not significantly altered in the yuc quadruple mutants. We found that IAM and IAN are intermediates in IAOX-mediated IAA biosynthesis in Arabidopsis. This biochemical evidence indicates that IAOX-dependent IAA biosynthesis is not a common but a species-specific pathway in plants.

Results

The Majority of IAOX Is Produced by CYP79B2 and CYP79B3 in Arabidopsis. We analyzed the IAOX levels in the cyp79b2 cyp79b3 double mutants to determine the contribution of CYP79B pathway to IAOX production in Arabidopsis (Fig. 1A). We used D2-IAOX as an internal standard and purified IAOX from plants using HPLC and solid phase extraction columns. From WT seedlings, we detected MS/MS ions ([M + H – 17]^+) corresponding to trans and cis forms of endogenous IAOX at m/z 158.1 and those of D2-IAOX at m/z 163.1; see supporting information (SI) Figs. S1A and S1B. The identity of IAOX was confirmed by in vivo feeding of a [13C8, 15N]indole to Arabidopsis where the MS/MS ions for endogenous IAOX showed an increase of 9 mass units (Fig. S2). As shown in Table 1, the level of IAOX in WT plants was 1.7 ± 0.1 ng/gram fresh weight (gfw) (n = 2) by liquid chromatography-electrospray ionization-mass/mass spectrometry (LC-ESI-MS/MS) analysis. The level of IAOX was increased in sur1–1 seedlings (2.5-fold) as compared to that in WT seedlings (Table 1). We measured IAOX levels in 3 T-DNA insertion lines of cyp79b2 cyp79b3 (Fig. S3). All of the 3 cyp79b2 cyp79b3 null lines did not show a visible phenotype on MS agar media under our growth conditions (Fig. S3), although cyp79b2 cyp79b3 previously showed subtle growth phenotypes in soil (8). Endogenous IAOX was not detected from these mutants (Table 1 and Figs. S1C and Figs. S1D). These results suggested that IAOX is mainly produced by CYP79B2 and CYP79B3 in Arabidopsis (Fig. 1B).

To investigate the contribution of the YUC genes to IAOX production in Arabidopsis, we analyzed the IAOX levels in yuc1 yuc2 yuc4 yuc6 quadruple mutants. The aerial parts of 4-week-old yuc1 yuc2 yuc4 yuc6 seedlings that showed growth defects in soil were used for IAOX analysis (Fig. S4). The IAOX level was not significantly altered in yuc1 yuc2 yuc4 yuc6 seedlings (2.9 ± 0.4 ng/gfw, n = 3) as compared to that in the WT plants (2.9 ± 0.7 ng/gfw, n = 3), suggesting that IAOX is not mainly produced through the YUC pathway in Arabidopsis. To further exclude a possibility that IAOX is involved in the YUC pathway, we fed 15N2-TAM to WT seedlings and analyzed 15N2-incorporation to IAOX. Arabidopsis WT plants produce TAM via the TRP metabolic pathway (Fig. S2) and possessed 209 ± 15 pg/gfw (n = 4) of this compound. However, 15N-incorporation into IAOX was not observed (<1%) even though seedlings were cultured in MS liquid media containing 15N2-TAM (100 μM) for 10 days, while 21.5 ± 3.5% (n = 2) of total IAA was 15N-labeled in the same

Table 1. The levels of IAA and its precursors in Arabidopsis, rice, maize, and tobacco

<table>
<thead>
<tr>
<th>Plants</th>
<th>Intermediate (ng/gfw)</th>
<th>IAOX</th>
<th>IAN</th>
<th>IAM</th>
<th>IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. thaliana</td>
<td></td>
<td>1.7 ± 0.1*</td>
<td>9,720 ± 2,120</td>
<td>9.9 ± 2.1</td>
<td>11.2 ± 2.9</td>
</tr>
<tr>
<td>cyp79b2–1</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>cyp79b2–2</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>cyp79b2–1</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>sur1–1</td>
<td>4.2 ± 1.1</td>
<td>9,230 ± 2,130</td>
<td>337 ± 4</td>
<td>235 ± 9</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Maize</td>
<td>ND*</td>
<td>ND</td>
<td>11.2 ± 0.1*</td>
<td>93.1 ± 22.8</td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td>ND*</td>
<td>ND</td>
<td>1.0 ± 0.5</td>
<td>11.3 ± 5.2</td>
<td></td>
</tr>
</tbody>
</table>

Seedlings of Arabidopsis (2-week-old), coleoptiles of rice (2-day-old), and maize (3-day-old), and apices of tobacco (2-month-old) were used for quantifying the levels of IAA and its precursors. Data are means ± SD (n = 3). *Quantification was performed in two independent experiments (data are means ± SD, n = 2). ND, not detected.

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plants. All of these results suggest that the YUC pathway may not produce IAOx in Arabidopsis (Fig. 1B).

We also analyzed the levels of IAOx in rice, maize, and tobacco, which are noncruciferous plants. As they do not have apparent orthologues of CYP79B genes, these plants can also be used to determine the contribution of CYP79B genes to IAOx production. As shown in Table 1, we detected IAA, but not IAOx from all of these plants, indicating that IAOx is most likely not a common but a species-specific metabolite of plants such as Arabidopsis (Fig. 1B).

IAN, IG, and CL Are Each Produced Independently from IAOx in Arabidopsis. IAN has been proposed as a common metabolite of both the CYP79B and YUC pathways as shown in Fig. 1A (3). We quantified IAN levels in 3 independent cyp79b2 cyp79b3 mutants by GC-MS to clarify the contribution of the CYP79B pathway to IAN production. As shown in Table 1, IAN was not detected in any of the cyp79b2 cyp79b3 mutant seedlings in contrast to a previous report (8). We found that treatment with IAOx at 3 μM and 10 μM for 24 h can restore the levels of IAN in cyp79b2 cyp79b3 mutants dose dependently to 8% and 23% of those in wild-type plants, respectively (Table 2). Moreover, endogenous IAN was completely 13C-labeled by feeding 13C6-IAOx to cyp79b2 cyp79b3 mutants under the same conditions (Fig. 2A). In addition, we fed 15N2-TAM to WT seedlings and analyzed 15N2-incorporation to IAN to elucidate the contribution of YUC genes to IAN synthesis. As in the case of IAOx, we could not observe the enrichment of 15N atoms in IAN (<1%). These results indicate that the majority of IAN in Arabidopsis is derived from the CYP79B pathway but not from the YUC pathway (Fig. 1B).

IG metabolism has been proposed as a major pathway for IAN synthesis (Fig. 1A), while myrosinase (MYR) generates IAN from IG in response to tissue damage (5, 12, 13). To investigate this notion, we analyzed IAN levels in sur1–1 null mutants, which do not produce IG (10). We found that IAN levels were not significantly reduced in sur1–1 mutants in comparison to those in the WT plants (Table 1), suggesting that IG metabolism is not a major pathway for IAN synthesis under normal growth conditions. To test if increasing the metabolic flux from IAOx to IAA can affect the level of IAN in sur1–1 mutants, we applied IAOx to WT plants and quantified IAN levels. As shown in Table 2, application of IAOx increased IAA levels, but did not significantly alter the level of IAN in WT plants. Arabidopsis also produces IAN from IAOx by CYP71A13 for the synthesis of CL in response to pathogen attack (14). Consistent with this idea, IAN levels were not decreased in cyp71a13 knockout mutants (16.7 ± 2.2 μg/gfw, n = 3) without pathogen attack. These results suggest that IAN, IG, and CL are independently produced from IAOx in Arabidopsis under normal growth conditions (Fig. 1B).

We also analyzed the levels of IAN in rice, maize, and tobacco. As shown in Table 1, we detected IAA, but not IAN from all 3 plant species we investigated, suggesting that IAN is not a common intermediate in IAA biosynthesis. However, as IAN was previously detected in maize coleoptiles (23) and cyp79b2 cyp79b3 double mutants (8), we cannot completely exclude the possibility that IAN may be a common intermediate in IAA biosynthesis. IAN may be detected if we increase the amount of plant material used for IAN analysis, use plants grown under different conditions, or use previously reported methods (23, 24).

IAM Is Involved in IAA Biosynthesis via IAOx. Arabidopsis nitrilases (NIT1, NIT2, and NIT3) can convert IAN to IAA and IAM in vitro (25). Since our results indicated that IAM is mainly produced from IAOx, we hypothesized that IAM is a possible intermediate in the pathway converting IAOx to IAA (Fig. 1B). If that is the case, we expect IAM levels to be reduced in cyp79b2 cyp79b3 mutants and increased in sur1–1 mutants. We detected a MS/MS ion ([M + H – 45]–) for endogenous IAM at m/z 130.1 from WT seedlings along with a MS/MS ion for 13C6-IAM at m/z 136.1 as an internal control peak (Fig. S5). As shown in Table 1, the IAM level in WT plants was 9.9 ± 2.1 ng/gfw (n = 3). We found that IAM levels were drastically decreased to approximately 0.4–0.6 ng/gfw in all cyp79b2 cyp79b3 mutants, but substantially increased (more than 34-fold) in sur1–1 seedlings. These results suggest that the majority of IAM in Arabidopsis is produced from IAOx.

Synthesis of IAM from IAOx was directly demonstrated by in vivo labeling experiments. First, we examined the restoration of IAM levels in cyp79b2 cyp79b3 mutants by application of IAOx. As shown in Table 2, the IAM level was moderately restored in cyp79b2 cyp79b3 seedlings (40.4%) by applying IAOx (3 μM) for 24 h. We found that IAM was 13C6-labeled (83.3%, n = 2) when 13C6-IAOx was fed to cyp79b2 cyp79b3 seedlings under the same conditions (Fig. 2A). IAM was even more efficiently 13C6-labeled (97.5%, n = 2) when cyp79b2 cyp79b3 seedlings were fed with 10 μM of 13C6-IAOx (Fig. 2A). IAM was moderately labeled from IAOx in these conditions (Fig. 2A). These results provided further evidence indicating that IAM is mainly produced from IAOx in Arabidopsis (Fig. 1B).

To determine if IAM is a main precursor of IAN, we performed an in vivo labeling experiment with WT plants. Since the IAM levels are nearly 3 orders of magnitude higher than those of IAN in WT plants (Table 1), we speculated that 13C-enrichment of IAM and IAA in 13C6-IAOx-fed plants would be different if these intermediates are produced independently from IAOx. We found that the 13C-enrichment of IAM and IAA was approximately 6-fold higher than that of IAN in the WT plants fed with 13C6-IAOx (Fig. 2B). The greater 13C-enrichment of IAM relative to IAN suggests that IAM and IAA are produced independently from IAOx in Arabidopsis (Fig. 1B).

It was previously demonstrated that cyp79b2 cyp79b3 double mutants show growth defects and reduced IAA levels as com-
pared to WT plants at 26 °C (8). We therefore examined the phenotypic complementation of cyp79b2 cyp79b3 mutants by applying IAM. As shown in Fig. 3 A and B, cyp79b2 cyp79b3 mutants displayed the growth defects at 26 °C under our growth conditions. We found that the phenotype of cyp79b2 cyp79b3 mutants can be restored to WT levels by treatment with 10 μM of IAM for 7 days at 26 °C (Fig. 3 C and D). The level of IAA in cyp79b2 cyp79b3 mutants was 20% lower than that in WT plants at 26 °C, but it was also 30% increased by application of 10 μM IAM. IAM rescue of cyp79b2 cyp79b3 mutants depends on IAA production because auxin resistance 1–3 (axr1–3), in which the auxin signal pathway is disrupted, can suppress IAM-dependent hypocotyl elongation (Fig. S6). In contrast, application of IAA or IAOx to cyp79b2 cyp79b3 mutants resulted in the inhibition of root growth and did not complement the phenotype under the same conditions (data not shown), presumably because of the difference in diffusion and/or metabolic rate between IAM and these compounds in plants as suggested previously (26). Together, all of these results indicated that IAM is an intermediate of IAOx-dependent IAA biosynthesis in Arabidopsis (Fig. 1B).

We analyzed IAM levels in rice, maize, and tobacco by LC-ESI-MS/MS. As shown in Table 1, we have detected IAM from all of these plants. Because these plants do not have apparent CYP79B orthologues and are devoid of IAOx, our data suggest that IAM is also produced in the IAOx-independent pathway, but the mechanisms are still unknown (Fig. 1B).

Simultaneous Application of IAM and IAN to WT Plants Phenocopies sur1 Mutants. Although IAM and IAN are identified as metabolites of IAOx, it remains unclear how IAA is overproduced in sur1 mutants. Because IAM levels are greatly increased in sur1–1 mutants (Table 1), we investigated if WT plants show sur1-like phenotype by application of IAM. Application of IAM (60 μM) triggered elongation of hypocotyls and petioles of WT plants, but did not cause formation of massive adventitious roots like that observed in sur1–1 (Fig. 4 A–C). In contrast, application of 30 μM IAN to WT plants results in the massive adventitious roots and root growth inhibition (Fig. 4D) as reported previously (27). We found that simultaneous application of IAM and IAN to WT plants phenocopied sur1–1 mutants (Fig. 4E), suggesting that both IAM and IAN may be overproduced in sur1–1. Since the level of IAN was not increased in sur1–1 and IAOx-treated WT plants (Tables 1 and 2), excessive IAN may be promptly converted to IAM and IAA in Arabidopsis. To test this idea, we applied D2-IAN (30 μM) to WT plants and analyzed D2-enrichment in IAM and IAA by LC-ESI-MS/MS. We found that 69.9% and 34.8% of total IAM and IAA, respectively, were labeled with D2 atoms. These results indicate that IAA is likely overproduced in sur1 mutant by conversion of IAOx to both IAN and IAM (Fig. 1B).

Discussion

In this work we demonstrated that the majority of IAOx is produced from the CYP79B pathway because cyp79b2 cyp79b3 mutants and noncruciferous plants such as rice, maize, and tobacco did not produce IAOx. We also showed that the YUC pathway probably does not contribute to IAOx synthesis, because the IAOx levels were not significantly altered in yuc quadruple mutants and 15N2-incorporation to IAOx was not observed after feeding 15N2-TAM to Arabidopsis. Furthermore, we showed that IAOx is converted into IAM and IAA, the 2 likely intermediates in a pathway that converts IAOx into IAA.

Biosynthesis of IAOx. Genetic studies have identified several genes in IAA biosynthesis, but it has been difficult to determine whether both the CYP79B and YUC pathways contribute to IAOx synthesis. Analysis of IAOx levels in CYP79B-deficient and YUC-deficient mutants is critical for biochemical dissection of these pathways. It has been assumed that oximes are unstable compounds that do not accumulate in the cell, and the low Km of SUR2 for IAOx would prevent accumulation of IAOx in plants (9). The analysis of IAOx in Chinese cabbage was previously performed using GC-MS and proved difficult, because the full-mass spectrum of endogenous IAOx was not completely identical with that of authentic IAOx because of the presence of contaminant ions (28). Here we demonstrated that IAOx is detectable using LC-ESI-MS/MS coupled with a rapid IAOx purification from plants under neutral conditions. The
analysis of IAOx in WT, cyp79b2 cyp79b3 and yue quadruple mutants of Arabidopsis indicate that the majority of IAOx is produced by CYP79B enzymes. Our results from in vivo feeding experiments suggest that TAM, a proposed substrate of YUC, may not be a precursor of IAOx in Arabidopsis.

It is well established that IAOx can be produced from TRP by CYP79B2 and CYP79B3, but it was not clear whether other pathways also contribute to the production of IAOx. We demonstrated that IAOx was not detectable in rice, maize, and tobacco that presumably do not have CYP79B genes. Similar to CYP79B2, Arabidopsis CYP79A2 catalyzes the conversion of L-phenylalanine to phenylacetaldoxime (29). Rice, which does not have the CYP79B family, possesses the CYP79A family (30). Although CYP79A2 catalyzes a similar enzyme reaction, our results suggested that the CYP79A family does not contribute to the production of IAOx in Arabidopsis and rice. These results suggest that IAOx is produced from the CYP79B pathway but not from other pathways in the model plants we investigated.

Conversion of IAOx to IAA. Genetic evidence demonstrated that IAOx can be converted to IAA, but the exact mechanisms are not known. We have shown that IAOx is converted to IAN in Arabidopsis plants (Fig. 2). It is known that IAN has IAA-like effects (27), and presumably IAN is converted to IAA in vivo. We also discovered that IAOx is converted to IAM (Fig. 2), which is a well-known IAM biosynthesis intermediate. It appears that IAM and IAN are independently produced from IAOx, because 13C-enrichment of IAM from 13C2-IAOx was much lower than that of IAM in WT plants (Fig. 2B). It is known from animal systems that oximes can be converted to amides by a Beckmann-rearrangement-type enzymatic reaction requiring NADPH (31). Therefore, a similar enzymatic reaction might be involved in the formation of IAM from IAOx in Arabidopsis. However, we do not exclude the possibility that IAM is a direct precursor of IAM in Arabidopsis (Fig. S7), because it has previously been proposed that the metabolic pool of IAN relevant for IAA biosynthesis is separated from the larger second pool by strict compartmentation or as a consequence of metabolite channeling (32). This was suggested by the fact that IAN was labeled from D2-TRP in WT plants, but its isotopic abundance was lower than that of IAA. It will be important to elucidate whether the multiple knockout mutants of nitrilases (NIT1, NIT2, and NIT3) show reduced IAM levels under normal conditions, and growth defects under high temperature conditions like those of the cyp79b2 cyp79b3 mutants.

The Role of IAOx in IAA Biosynthesis. It is evident that IAM cannot be made from several TRP-dependent pathways (Fig. 1) (2–5). IAM was the only IAox-producing pathway. CYP79B3 caused subtle developmental defects and only a slight decrease in IAA levels (8). cyp79b2 cyp79b3 double mutants did not show a visible phenotype or a decrease in IAA levels under our standard growth conditions (Table 1 and Fig. S3). Furthermore, CYP79B genes appear not to exist universally throughout the plant kingdom. Our biochemical analysis of IAOx levels in cyp79b2 cyp79b3 indicated that IAOx-mediated IAA biosynthesis is probably not responsible for the production of the bulk of IAA in Arabidopsis. The CYP79B pathway does play a role in IAA production when Arabidopsis plants are grown at higher temperatures (8). In plants grown at 26 °C, we observed apparent growth defects and decreased IAA levels in cyp79b2 cyp79b3 mutants in comparison to WT plants, and the phenotype and IAA levels could be rescued by treatment with IAM (Fig. 3).

Genetic studies on the YUC pathway and the IPA pathway suggest that the 2 pathways play a more prominent role in plant growth and development than IAOx-mediated IAA biosynthesis. The YUC genes have been shown to be essential for embryogenesis, seedling growth, vascular patterning, and flower development (16, 17). The IPA pathway also plays a key role in embryogenesis and shade avoidance (34, 35). Unlike the CYP79B genes, the YUC and TAA1 gene families appear to be widely distributed in plants, suggesting that the YUC and IPA pathways are universally conserved for IAA biosynthesis. Our data suggest that IAOx-dependent IAA biosynthesis is probably limited to Arabidopsis and other crucifers and plays a more restricted role in plant growth and development. Further analysis of IAOx, as well as CYP79B, in other plant species may allow us to understand the evolution of IAOx-dependent IAA biosynthesis and whether CYP79B is the only IAOx-producing pathway.

Materials and Methods

Plant Materials and Growth Conditions. All mutants are in the Arabidopsis thaliana Col-0 background. sur1–1, cyp79b2–1, and cyp79b2–2 mutant seeds were obtained from the Arabidopsis Biological Resource Center. cyp79b3–2 and cyp79b3–3 mutant seeds were from the Nottingham Arabidopsis Stock Centre. cyp79b2 cyp79b3 double mutants were generated by crossing the corresponding single mutants as described in SI Text Materials and Methods. Plants were grown at 21 or 26 °C under continuous light, 30–50 μmol of photons per m² per second, with cool-white illumination. Plants were germinated and grown in Murashige-Skoog (MS) agar media (pH 5.7) supplemented with thiamin hydrochloride (3 μg/mL), nicotinic acid (5 μg/mL), pyridoxin hydrochloride (0.5 μg/mL), myo-inositol (100 μg/mL) and 1% (wt/vol) sucrose. Rice (Oryza sativa) seeds were germinated in H2O, and grown at 30 °C on agar media in the dark. Two days after germination, coleoptiles were excised from the seedlings with a razor blade and kept at 80 °C until use. Coleoptile tips from Nicotiana tabacum were excised as described in SI Text Materials and Methods. WT tobacco (Nicotiana tabacum) plants were grown on soil in a growth room at 25 °C with 16 h light for 2 months. Shoot apices were excised from the adult plants with a razor blade and kept at −80 °C until use.

Chemical Synthesis, LC-ESI-MS/MS, GC-MS and Labeling Experiments. IAOx, D2-IAOx, 13C2-IAOx, 13C2-IAM, D2-IAN, and 15N2-TAM were synthesized as described in SI Text Materials and Methods. Arabidopsis, rice, and tobacco were grown in soil in a growth room at 25 °C with 16 h light for 2 months. Arabidopsis mutants were supported in part by grants from the MEXT of Japan (19780090 to H.K.) and from National Institutes of Health (R01GM68631 to Y.Z.).


Supporting Information

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SI Materials and Methods

**Generation of cyp79b2 cyp79b3 Double Mutants.** The T-DNA muta-
tants were genotyped as described in Zhao et al. (1). Primer 79B2-5P (5'-ATGAAACACTTCTACCTCAAAC-3') and LBa1 primer (5'-TGGTTTCAGTAGGCGCCATCG-3') were used to identify the T-DNA insertion at 925 bp (cyp79b2-2) after the ATG of the CYP79B2 gene. Primer 79B3-2-5P (5'-ATGGACCATTTGAGTTCCAAAC-3') and pAC161-LB primer (5'-GGGCTACAGTAAATTTGAGTC-3') were used to identify the T-DNA insertion at 1781 bp (cyp79b3-3) after the start codon of the CYP79B3 gene. The primer 79B3-3-5P (5'-TCACCTCACCATTGGGTAAAGG-3') and pAC161-LB primer were used to identify the T-DNA insertion at 1962 bp (cyp79b3-4) after the start codon of the CYP79B3 gene. The insertions were confirmed by DNA sequencing of the PCR fragments generated with LBa1 primer, pAC161-LB primer, and gene-specific primers. cyp79b2-2, cyp79b3-3 and cyp79b2-1 cyp79b3-3 double mutants were generated by crossing the corresponding single mutants.

**Synthesis of Chemicals**

**General Experimental Conditions.** 1H and 13C NMR spectra were recorded on a JNM Lambda 400 NMR spectrometer (JEOL). Chemical shifts are shown as δ-values from TMS as the internal reference. Peak multiplicities are quoted in Hertz (Hz). Mass spectra were measured on a JMS-700 spectrometer (JEOL). Column chromatography was carried out on columns of silica gel 60 (230–400 mesh, Merck). All chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Synthesis of IAOx, D2-IAOx, and 13C6-IAOx. Oximes were synthesized as described by Rausch et al. (2) and Glawischnig et al. (3).

**Indole-3-ethanol.** IAA (100 mg, 0.57 mmol) was added to a suspension of lithium aluminum hydride (LAH, 50 mg, 1.32 mmol) in dry tetrahydrofuran (10 mL) under N2 at room temperature. After stirring for 2 h, excess LAH was quenched by addition of 1N-HCl solution. The whole mixture was extracted with ether. The ether layer was dried over MgSO4. Removal of the solvent in vacuo afforded a residue, which was purified by column chromatography eluted with hexane-EtOAc (1:1) to afford indole-3-ethanol (86 mg, 94%) as a white solid.

1H-NMR (CDCl3, 400 MHz) δ ppm: 1.60 (1H, bs), 3.03 (2H, t, J = 6.3 Hz), 3.90 (2H, t, J = 6.3 Hz), 7.05 (1H, d, J = 2.2 Hz), 7.13 (1H, bt, J = 7.4 Hz), 7.21 (1H, bt, J = 7.5 Hz), 7.35 (1H, bd, J = 8.0 Hz), 7.62 (1H, bd, J = 7.6 Hz), and 8.08 (1H, bs).

13C-NMR (CDCl3, 400 MHz) δ ppm: 28.7, 62.6, 111.2, 112.2, 118.8, 119.4, 122.5, 127.4, and 136.4.

**IAAld and IAOx.** Sulfur trioxide pyridine complex (SO3/Py, 1.1 g, 6.9 mmol) was added to a stirred solution of indole-3-ethanol (370 mg, 2.3 mmol), DMSO (3.2 mL) and triethylamine (Et3N) (1.6 ml, 11.5 mmol) in dry dichloroethane (DCE, 12 mL) at 0 °C under N2. After stirring at 0 °C for 30 min, the reaction mixture was directly subjected to a short column of silica gel chromatography to afford crude IAAld as a yellow-brown oil, which was used for the next step without further purification. Hydroxylamine hydrochloride (319 mg, 4.6 mmol) and sodium acetate (377 mg, 4.6 mmol) were added to a stirred solution of the crude IAAld in ethanol (15 mL) at room temperature. After stirring overnight, the reaction mixture was directly purified by column chromatography eluted with hexane-EtOAc (3:2) to give IAOx (230 mg, mixture of cis-trans form, 58%, 2 steps) as a yellow solid. The NMR spectral data were consistent with those reported in the literature (3).

**D2-IAOx.** [indole-D2]IAOx (56 mg, cis/trans = 1.81:1, 97% D2-labeled) was synthesized from [indole-D2]IAA (100 mg, 97% D2-labeled, Cambridge Isotope Laboratories) as described above for IAOx. 1H-NMR (CD3OD, 400 MHz) δ ppm: cis form, 3.59 (2H, d, J = 6.5 Hz), 7.48 (1H, t, J = 6.5 Hz); trans form, 3.79 (2H, d, J = 5.4 Hz), 6.79 (1H, t, J = 5.4 Hz).

**13C6-IAOx.** [phenyl-13C6]IAOx (4 mg, 98% 13C6-labeled) was prepared from [phenyl-13C6]IAA (20 mg, 98% 13C6-labeled, Cambridge Isotope Laboratories) as described above for IAOx. 1H-NMR (CD3OD, 400 MHz) δ ppm: cis form, 3.60 (2H, dd, J = 6.4, 3.9 Hz), 6.7–7.8 (6H, m); trans form, 3.79 (2H, d, J = 5.2, 4.1 Hz), 6.7–7.8 (6H, m).

**Synthesis of 13C6-IAAM.** [phenyl-13C6]IAAM (10 mg, 0.06 mmol, 98% 13C6-labeled), N-ethoxy-carbonyl-2-ethoxo-, 2-dihydroquinone (EEDQ, 15.5 mg, 0.06 mmol) (4), and ammonium hydrogencarbonate (NH4HCO3, 13.5 mg, 0.17 mmol) were suspended in CHCl3 (4 mL), and the mixture was stirred overnight at room temperature. The mixture was then subjected to silica gel column chromatography eluted with EtOAc-acetone (20:1) to afford 13C6-IAAM (8 mg, 80% yield, 98% 13C6-labeled) as a white solid.

**Synthesis of D2-IAN.** 1.8-Diazabicyclo[5.4.0]undec-7-ene was added to a stirred solution of IAN in 50% DMF in D2O (vol/vol, 2 mL, 98% D2-labeled) at room temperature under argon. The mixture was heated to 80 °C for 3 h. After cooling, saturated NaCl solution was added and the resulting mixture was extracted with ether. The ether layer was dried over MgSO4. Removal of the solvent in vacuo afforded a residue, which was purified by column chromatography eluted with hexane-EtOAc (3:1) to give [2-D2]IAAN (202 mg, 95% D2-labeled) as yellow oil.

**Synthesis of trans15N2-TAM.** [15N2]TAM (60 mg, 98% 15N2-labeled) was synthesized as described by Barton et al. (5) using [15N2]TRP (100 mg, 98% 15N2-labeled, Cambridge Isotope Laboratories). The structure of [15N2]TAM was confirmed as described above, LC-ESI-MS/MS analysis of TAM.

**Analysis of IAOx.** For analysis of IAOx, ~0.5 g of fresh Arabidopsis tissue were weighed quickly, frozen with liquid N2 and lyophilized overnight. The dried plant material was pulverized with liquid N2-chilled ceramic beads (10 mm) using a vortex for 2 min followed by extraction with 80% acetone (2.5 mL) containing 0.5 ng of D2-IAOx (97% D2-labeled) at 4 °C for 30 min with gradual shaking. The extracts were centrifuged at 3,000 × g for 10 min, and the supernatant was removed. The extraction was repeated with 80% acetone and the supernatants were pooled and evaporated with a Speed Vac (Thermo) at 35 °C. The dried extract was resuspended with 200 μL of DMSO, and centrifuged at
15,000 x g for 5 min. The supernatant was applied to a 5-μm, 4.6 x 150-mm, Symmetry shield C18 column (Waters) coupled to a 5-μm, 4.6 x 10-mm, C18 guard column (Senshu Pak) connected to a HPLC system equipped with a Waters 2475 multi-

λ-fluorescence detector. The samples were eluted at a flow rate of 1 mL/min with 0.01 M ammonium acetate (solvent A) and 100% methanol (solvent B) using 10% solvent B for 1 min and a gradient from 10 to 50% solvent B over 30 min. During elution, the samples were monitored continuously using a fluorescent detector (280 nm excitation and 355 nm emission). Fractions eluting at the retention time of trans and cis forms of IAOx (28.6 and 31.1 min) were collected and evaporated immediately using a Speed Vac. The dried IAOx fraction was redissolved with H2O (2 mL) and applied to an Oasis HLB column (Waters). The column was washed with H2O (3 mL) before eluting the IAOx with methanol (3 mL). The methanolic eluate was then evaporated to dryness using a Speed Vac.

IAOx was analyzed by an ACQUITY Ultra Performance Liquid Chromatography (UPLC)-MS/MS Q-ToF-premier (Waters). The chromatography was performed on an ACQUITY UPLC BEH C18 1.7-μm, 2.1 x 50-mm column (Waters). The IAOx fraction was redissolved in 50% methanol/H2O containing 1% acetic acid (20 μL) and injected to UPLC. Elution of the samples was carried out with H2O (solvent A) and acetonitrile with 0.05% acetic acid (solvent B) using 3% solvent B for 0.1 min, and a gradient from 3 to 30% solvent B over 10 min, at a flow rate of 0.2 mL/min. The retention time (trans/cis form) of IAOx and D5-IAOx was 8.84/9.39 min and 8.2/9.38 min, respectively.

For analysis of IAOx in rice coleoptiles (0.2 g), maize coleoptiles (50 mg), and tobacco apices (0.5 g), each sample was extracted with 80% acetone (0.25–2.5 mL) containing 0.2 ng of D5-IAOx, purified with HPLC and a HLB column, and analyzed by LC-ESI-MS/MS as described above.

**Analysis of IAM.** For analysis of IAM, 0.1–0.2 g of fresh plant tissues were quickly weighed, chilled with liquid N2, and freeze-dried overnight. Plant materials were homogenized in 80% methanol/H2O (0.1–0.25 mL) containing 0.1–0.25 ng of D2-IAA (97% D2-labeled, Sigma-Aldrich) with ceramic beads (3 mm) containing Tissue Lyser for 3 min. The extracts were centrifuged at 15,000 x g for 5 min, and the supernatant was transferred to a new 1.5-mL tube. This extraction was repeated without D2-IAA and the supernatants were combined and evaporated using a Speed Vac at 35 °C. The residue was suspended in 1 mL of H2O and adjusted to pH 2–3 with 1N HCl, and IAM was eluted with 100–200 mg of Sigma-Aldrich XAD7 (Amberlite XAD7HP) for 30 min at 4 °C. The aqueous solution was removed, and the XAD7 was washed with 1% acetic acid/H2O (1 mL x 3) and 15% B (acetonitrile with 0.05% acetic acid). The retention time of IAM and 13C6-IAM was 6.69 min. MS/MS conditions were as follows: collision energy, 10 and MS/MS transition (m/z): 175.2/130.1 for unlabeled IAM and 176.2/130.1 for 13C6-IAM, respectively. Quantification was carried out using the extracted ion chromatogram of IAM (m/z 130.1) and 13C6-IAM (m/z 136.1). The linear dynamic range was determined by injecting a standard mixture of IAM and 13C6-IAM in a wide concentration range (1 pg/μL to 100 pg/μL).

**Analysis of TAM.** For analysis of TAM, 0.2–0.5 g of fresh plant tissue were quickly weighed, chilled with liquid N2, and freeze-dried overnight. Plant material was homogenized in 80% methanol/H2O (0.1–0.25 mL) containing 0.1–0.25 ng of D2-TAM (97% D2-labeled, Sigma-Aldrich) with ceramic beads (3 mm) containing Tissue Lyser for 3 min. The extracts were centrifuged at 15,000 x g for 5 min, and the supernatant was transferred to a new 1.5-mL tube. This extraction was repeated without D2-TAM and the supernatants were combined and evaporated using a Speed Vac at 35 °C. The residue was resuspended in 1 mL of H2O and adjusted to pH 2–3 with 1N HCl, and TAM was absorbed with 100–200 mg of Sigma-Aldrich XAD7 (Amberlite XAD7HP) for 30 min at 4 °C. The aqueous solution was removed, and the XAD7 was washed with 1% acetic acid/H2O (1 mL x 3). During elution, the samples were monitored continuously using a fluorescent detector (370 nm excitation and 460 nm emission). Fractions eluting at the retention time of TAM and 13C6-TAM was 7.15 min. MS/MS conditions were as follows: collision energy, 9 and MS/MS transition (m/z): 175.1/130.1 for unlabeled IAM and 181.1/136.1 for 13C6-IAM, respectively. Quantification was carried out using the extracted ion chromatogram of TAM (m/z 130.1) and 13C6-TAM (m/z 136.1). The linear dynamic range was determined by injecting a standard mixture of IAM and 13C6-IAM in a wide concentration range (1 pg/μL to 100 pg/μL).
MS. Elution of the samples was carried out with gradient solutions A2 and B of 0–3 min 3% B and 0.1–10 min 3–30% B, at a flow rate of 0.2 mL/min. The retention times of TAM and 15N2-TAM were 5.67 min and 5.69 min, respectively. MS/MS conditions were as follows: collision energy, 6 and MS/MS transition (m/z): 161.2/144.1 for TAM and 163.2/145.1 for 15N2-TAM. Quantification was carried out using the extracted ion chromatogram of TAM (m/z 144.1) and 14N2-TAM (m/z 145.1). The linear dynamic range was determined by injecting a standard mixture of TAM and 15N2-TAM in a wide concentration range (2 pg/μL to 100 pg/μL).

**GC-MS Analysis of IAN.** For analysis of IAN in cyp79b2 cyp79b3 plants, ~1 g of plant material was extracted with 80% acetone/H2O (5 mL) containing 5 ng of D2-IAOx as described above. The IAN fraction was collected by HPLC (retention time of IAN = 30.5 min) and evaporated using a Speed Vac. The IAN fraction was redissolved with methanol (2 mL) and applied to an Oasis MCX column. The flow-through was collected and the solvents were dried with a Speed Vac. The IAN fraction was redissolved in 20 μL of 1% trimethylchlorosilane/N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) solution (PIERCE) and heated at 70 °C for 10 min. IAN was analyzed as trimethylsilyl (TMS)-IAN by GC-MS with an Agilent 6890 series GC system connected to a JEOL GC-mate II mass spectrometer (source conditions: electric ionization mode and 70 eV at 250 °C) with a He gas flow rate of 1 mL/min. A DB-1 capillary column (J & W Scientific, 0.25 mm 15 m, 0.25 μm film thickness) was used. The oven temperature program was 80 °C for 1 min, 80–245 °C at 30 °C/min, 245–280 °C at 5 °C/min, and 280 °C for 1 min. The retention times of TMS-IAN and TMS-D2-IAN were 5.22 and 5.23 min, respectively. The molecular ions for TMS-IAN and TMS-D2-IAN were m/z 228 and 234, respectively. Quantification was carried out using the extracted ion chromatogram of TMS-IAN (m/z 228) and TMS-D2-IAN (m/z 234). The linear dynamic range was determined by injecting a standard mixture of TMS-IAN and TMS-D2-IAN in a wide concentration range (100 pg/μL to 2 ng/μL).

For analysis of IAN in WT and sur1 mutants, ~10 mg of plant materials were homogenized in 80% acetone/H2O (200 μL) containing 100 ng D2-IAOx with ceramic beads (3 mm) using Tissue Lyser for 3 min. The extracts were centrifuged at 15,000 g for 5 min, and the supernatant was transferred to a new 1.5 mL tube. This extraction was repeated without D2-IAOx and the supernatants were combined and evaporated using a Speed Vac at 40 °C. The residue was redissolved with methanol (2 mL) and applied to an Oasis MCX column. The flow-through was collected and the aliquot (200 μL) was dried using a Speed Vac. TMS-derivatization of IAN and GC-MS analysis of TMS-IAN were performed as described above.

For analysis of IAN in rice coleoptiles (~20 mg), maize coleoptiles (~20 mg), and apexes of tobacco (~50 mg), each plant material was extracted in 80% acetone/H2O (200 μL) containing 2 ng D2-IAOx, separated with HPLC (retention time, 30.5 min) and purified with an Oasis MCX column as described above. Each IAN fraction was redissolved in 10 μL of MSTFA solution, heated at 70 °C (10 min), and injected (5 μL) to GC-MS. The detection limit of our IAN analysis: 100 pg.

**In Vivo Labeling Experiments.** For 15N2-TAM feeding experiments, 7-day-old WT seedlings were transferred to MS liquid media (15 mL) containing 100 μM of 15N2-TAM, and incubated aseptically for 10 days with shaking at 100 rpm. Total IAOx and IAA were purified from 15N2-TAM fed plants (ca 0.5 g) and analyzed by LC-ESI-MS/MS as described above except for the addition of internal standards. For 15N2-IAOx analysis, MS/MS transition was set to m/z 177.2/160.2. The 15N-incorporation rate into IAOx was calculated using the extracted ion chromatogram of IAOx (m/z 158.2) and 14N2-IAOx (m/z 160.2). For 15N-IAA analysis, MS/MS transition was set to m/z 177.2/131.1. 15N-Incorporation rate into IAA was calculated using the extracted ion chromatogram of IAA (m/z 130.1) and 14N-IAA (m/z 131.1). Total IAN was purified from seedlings (~0.05 g) and analyzed by GC-MS as described above without addition of the internal standard. The 15N-incorporation rate into IAN was calculated using the extracted ion chromatogram of IAN (m/z 228) and 15N2-IAOx (m/z 230).

For the feeding experiment with 13C6-IAOx, 10-day-old cyp79b2 cyp79b3 WT seedlings were transferred to MS agar media containing 13C6-IAOx (3 μM and 10 μM) and incubated aseptically for 24 h. For analysis of 13C6-iaox into products, total IAM, IAA, and IAN were purified from seedlings (~0.1 g) and analyzed by LC-ESI-MS/MS and GC-MS, respectively, as described above (without addition of internal standards). The 13C6-incorporation rate into IAOx was calculated using the extracted ion chromatogram of IAOx (m/z 130.1) and 14C6-IAOx (m/z 136.1). Similarly, the 13C6-incorporation rate into IAA was calculated using the extracted ion chromatogram of IAA (m/z 130.1) and 13C6-IAA (m/z 136.1). The 13C6-incorporation rate into IAN was calculated using the extracted ion chromatogram of IAN (m/z 228) and 15N2-IAOx (m/z 230), respectively.

For feeding of D2-IAOx to WT plants, 7-day-old seedlings were transferred to MS agar media containing D2-IAOx (30 μM) and incubated aseptically for 4 days. Total IAN and IAA were purified from seedlings (~0.1 g) using HPLC as described above without addition of the internal standard and analyzed by LC-ESI-MS/MS. For D2-IAOx analysis, MS/MS transition was set to m/z 177.1/132.1. The D2-incorporation rate was calculated using the extracted ion chromatogram of IAOx (m/z 130.1) and D2-IAOx (m/z 132.1). For D2-IAA analysis, MS/MS transition was set to m/z 178.1/132.1. The D2-incorporation rate was calculated using the extracted ion chromatogram of IAA (m/z 130.1) and D2-IAA (m/z 132.1).

**Fig. S1.** LC-ESI-MS/MS analysis of IAOx. (A and B) The MS/MS chromatogram ([M + H − 17]⁺) for trans and cis IAOx (m/z 158.1, 9.05 and 9.60 min) and trans and cis D5-IAOx (m/z 163.1, 8.95 and 9.55 min) in WT seedlings. (C and D) The MS/MS chromatogram ([M + H − 17]⁺) for the IAOx fraction (m/z 158.1) and trans and cis D5-IAOx (m/z 163.1, 8.95 and 9.55 min) in cyp79b2 cyp79b3 seedlings.
Fig. S2. In vivo labeling of IAOx and TAM from $[^{13}\text{C}_8,^{15}\text{N}]$indole in Arabidopsis. The TRP-auxotroph trp1–1 homozygotes were selected from the progeny of TRP1–1/trp1–1 plants after incubation on MS agar plates for 7 days. Five trp1–1 seedlings were transferred to MS liquid media (15 mL) containing $[^{13}\text{C}_8,^{15}\text{N}]$indole (IND) (100 μM) in a 100-mL flask. Seedlings were incubated for 10 days at 100 rpm until the phenotype of the trp1–1 seedlings were nearly identical to WT. IAOx and TAM were purified from plants and analyzed by LC-ESI-MS/MS. (A and B) WT and trp1–1 seedlings (3-week-old). (C) Scheme for the in vivo labeling experiment using trp1–1 mutants. (D) The MS/MS chromatogram ([M + H − 17]$^-$) for IAOx and $[^{13}\text{C}_8,^{15}\text{N}]$IAOx. The chromatograms for IAOx and $[^{13}\text{C}_8,^{15}\text{N}]$IAOx are shown by blue and red lines, respectively. (E) The MS/MS chromatogram ([M + H − 17]$^-$) for TAM and $[^{13}\text{C}_8,^{15}\text{N}]$TAM. The chromatogram for TAM and $[^{13}\text{C}_8,^{15}\text{N}]$TAM are shown by blue and red lines, respectively.
Fig. S3. Analysis of cyp79b2 cyp79b3 double mutants. (A) Identification of T-DNA insertion mutants for the CYP79B2 and CYP79B3 genes. The T-DNA insertion sites are schematically indicated and the exact insertion sites shown as the distance from the ATG codon in the genomic sequence. (B) Two-week-old seedlings grown at 21 °C. The T-DNA insertion sites for cyp79b2–1 cyp79b3–1 mutants were previously reported by Zhao et al. (1). (Scale bar, 2 cm.)

Fig. S4. Phenotype of 4-week-old yuc1 yuc2 yuc4 yuc6 quadruple mutants. Seeds were germinated and grown on MS agar plates for 2 weeks, then transferred to soil and grown for another 2 weeks. Plants were collected before bolting for LC-ESI-MS/MS analysis. (Scale bar, 1 cm.)
**Fig. S5.** LC-ESI-MS/MS analysis of IAM. The MS/MS chromatogram ([M + H – 45]⁺) for IAM (m/z 130.1, 5.25 min) and ¹³C₆-IAM (m/z 136.1, 5.25 min) from WT seedlings.
Fig. S6. Effect of IAM on WT and axr1–3 seedlings. Seeds of WT and axr1–3 were germinated and grown for 5 days on MS agar media containing IAA (1 μM) or IAM (10 μM). (Scale bar, 4 mm.)
Fig. S7. Alternative model for IAOx-dependent IAA biosynthesis in Arabidopsis. Dotted square indicates the IAOx metabolic pathway in Arabidopsis. The alternative pathway for IAOx-dependent IAA biosynthesis is shown in the gray square. Cloned genes that potentially encode enzymes for IAA, CL, and IG biosyntheses are shown in italics.