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

Satoko Sugawara^{a,b}, Shojiro Hishiyama^c, Yusuke Jikumaru^a, Atsushi Hanada^a, Takeshi Nishimura^b, Tomokazu Koshiba^b, Yunde Zhao^d, Yuji Kamiya^a, and Hiroyuki Kasahara^{a,1}

^aPlant Science Center, RIKEN, Yokohama, Kanagawa 230-0045, Japan; ^bDepartment of Biological Sciences, Tokyo Metropolitan University, Hachioji 192-0397, Japan; ^cForestry and Forest Products Research Institute, Ibaraki 305-8687, Japan; and ^dSection of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA 92093-0116

Edited by Joanne Chory, The Salk Institute for Biological Studies, La Jolla, CA, and approved February 6, 2009 (received for review November 7, 2008)

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 ¹³C₆-labeled IAOx was fed to *CYP79B*-deficient mutants, ¹³C₆ 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 (3–5).

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 monooxygenases, *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 *YUC*s 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 IAOx (5, 8, 10). It has also been proposed that IAOx is converted to IAA via several possible routes as shown in Fig. 1A (5). To test these ideas, we analyzed the relative contribution of the 2 pathways to the production of IAOx in *Arabidopsis* and other plants. We also analyzed the possible intermediates in the pathway converting IAOx to IAA. In this article, we show that

Author contributions: S.S. and H.K. designed research; S.S., Y.J., A.H., T.N., T.K., and H.K. performed research; S.H. contributed new reagents/analytic tools; S.S., Y.J., A.H., and H.K. analyzed data; and S.S., Y.Z., Y.K., and H.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. E-mail: kasahara@riken.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0811226106/DCSupplemental.

Fig. 1. Proposed IAA biosynthetic pathway in plants. (A) Previously proposed IAA biosynthetic pathway. The YUC and CYP79B pathways that lead to IAOx synthesis are illustrated by blue and red arrows, respectively. (B) The IAA biosynthetic pathway proposed in this study. Each number indicates a proposed pathway for biosynthesis of IAA from TRP. The bold arrows indicate novel pathways converting IAOx to IAA. Dotted square indicates the IAOx metabolic pathway in *Arabidopsis*. The IAOx-dependent IAA biosynthetic pathway is shown in the gray squares. Cloned genes that potentially encode enzymes for IAA, CL, and IG biosyntheses are shown in italics.

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 D₅-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 D₅-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 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 μ M) for 10 days, while $21.5 \pm 3.5\%$ ($n = 2$) of total IAA was ¹⁵N-labeled in the same

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

Plants	Intermediates (ng/gfw)			
	IAOx	IAN	IAM	IAA
<i>A. thaliana</i>	$1.7 \pm 0.1^*$	$9,720 \pm 2,120$	9.9 ± 2.1	11.2 ± 2.9
<i>cyp79b2-1 cyp79b3-1</i>	ND*	ND	0.5 ± 0.1	11.4 ± 1.7
<i>cyp79b2-2 cyp79b3-2</i>	ND*	ND	0.6 ± 0.1	11.5 ± 1.2
<i>cyp79b2-1 cyp79b3-3</i>	ND*	ND	0.4 ± 0.1	10.8 ± 3.4
<i>sur1-1</i>	4.2 ± 1.1	$9,230 \pm 2,130$	337 ± 4	235 ± 9
Rice	ND*	ND	3.2 ± 0.9	23.2 ± 5.7
Maize	ND*	ND	$11.2 \pm 0.1^*$	93.1 ± 22.8
Tobacco	ND*	ND	1.0 ± 0.5	11.3 ± 5.2

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

Table 2. The levels of IAA and its precursors in IAOx-treated *cyp79b2 cyp79b3* and WT seedlings

	IAOx (μ M)	Intermediates (ng/gfw)		
		IAN	IAM	IAA
<i>cyp79b2-2 cyp79b3-3</i>	3	779	4.0	21.1
	10	2,280	16.0	23.5
WT	3	8,630	12.8	35.9
	10	9,660	20.1	54.8

Seedlings of *cyp79b2-2 cyp79b3-3* and WT (10-day-old) were transferred to MS agar media IAOx containing, where they were incubated for 24 h (at 21°C) aseptically, before being subjected to analysis.

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 μ M and 10 μ M for 24 h can restore the levels of IAN in *cyp79b2 cyp79b3* mutants dose dependently to 8% and 23% of those in WT plants, respectively (Table 2). Moreover, endogenous IAN was completely $^{13}\text{C}_6$ -labeled by feeding $^{13}\text{C}_6$ -IAOx to *cyp79b2 cyp79b3* mutants under the same conditions (Fig. 2A). In addition, we fed $^{15}\text{N}_2$ -TAM to WT seedlings and analyzed $^{15}\text{N}_2$ -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 ^{15}N 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 ($16.7 \pm 2.2 \mu\text{g/gfw}$, $n = 3$) 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 IAN 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* 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 $^{13}\text{C}_6$ -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 $9.9 \pm 2.1 \text{ ng/gfw}$ ($n = 3$). 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 μ M) for 24 h. We found that IAM was $^{13}\text{C}_6$ -labeled (83.3%, $n = 2$) when $^{13}\text{C}_6$ -IAOx was fed to *cyp79b2 cyp79b3* seedlings under the same conditions (Fig. 2A). IAM was even more efficiently $^{13}\text{C}_6$ -labeled (97.5%, $n = 2$) when *cyp79b2 cyp79b3* seedlings were fed with 10 μ M of $^{13}\text{C}_6$ -IAOx (Fig. 2A). IAA 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 IAN is a main precursor of IAM, we performed an in vivo labeling experiment with WT plants. Since the IAN levels are nearly 3 orders of magnitude higher than those of IAM in WT plants (Table 1), we speculated that ^{13}C -enrichment of IAN and IAM in $^{13}\text{C}_6$ -IAOx-fed plants would be different if these intermediates are produced independently from IAOx. We found that the ^{13}C -enrichment of IAM and IAA was approximately 6-fold higher than that of IAN in the WT plants fed with $^{13}\text{C}_6$ -IAOx (Fig. 2B). The greater ^{13}C -enrichment of IAM relative to IAN suggests that IAN and IAM 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-

Fig. 2. In vivo feeding of $^{13}\text{C}_6$ -IAOx to *cyp79b2 cyp79b3* and WT seedlings. (A) Incorporation of $^{13}\text{C}_6$ into metabolites in *cyp79b2-2 cyp79b3-3* seedlings fed with 3 μ M and 10 μ M of $^{13}\text{C}_6$ -IAOx. (B) Incorporation of $^{13}\text{C}_6$ into metabolites in WT seedlings fed with 10 μ M of $^{13}\text{C}_6$ -IAOx. Ten-day-old seedlings were fed with $^{13}\text{C}_6$ -IAOx for 24 h at 21 °C. Data are means $\pm$ SD, $n = 2$.

Fig. 3. Effect of IAM on the growth of *cyp79b2 cyp79b3* at high temperatures. (A–C) Seven-day-old seedlings of WT, *cyp79b2-2 cyp79b3-2*, and 10 μ M IAM-fed *cyp79b2-2 cyp79b3-2*. Seedlings were germinated and grown at 26 °C. (D) Fresh weight of WT and *cyp79b2-2 cyp79b3-2* seedlings grown in the presence of 0, 1, 2, 5, and 10 μ M of IAM for 7 days. Data are means $\pm$ SD, $n = 16$. (Scale bar, 2 cm.)

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 μ M 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 μ M 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 μ M) triggered elongation of hypocotyls and petioles of WT plants, but

Fig. 4. Effect of IAM and IAN on the growth of *Arabidopsis* seedlings. (A–E) Two-week-old seedlings of WT, *sur1-1*, WT treated with 60 μ M IAM, WT treated with 30 μ M IAN, and WT treated with 60 μ M IAM and 30 μ M IAN. Arrows indicate outgrowth of numerous adventitious roots. (Scale bar, 3 mm.)

did not cause formation of massive adventitious roots like that observed in *sur1-1* (Fig. 4 A–C). In contrast, application of 30 μ M 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 μ M) 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 IAN and IAM, 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 *K_s* and *K_m* 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 *yuc* 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 do 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 IAA biosynthesis intermediate. It appears that IAM and IAN are independently produced from IAOx, because ^{13}C -enrichment of IAN from $^{13}\text{C}_6$ -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 IAN 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 D_5 -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 IAA can be made from several TRP-dependent pathways (Fig. 1) (2–5). IAOx was proposed as a key intermediate in IAA biosynthesis on the basis of several observations. (i) Overexpression of CYP79B2 leads to high auxin phenotypes such as elongated hypocotyls similar to known auxin overproduction lines such as *yuc1D* and *iaaM* overexpression lines (8, 33). (ii) Inactivation of *SUR1* or *SUR2* blocks IG biosynthesis, and leads to auxin overproduction phenotypes because of the diversion of IAOx to IAA synthesis (9, 10). (iii) Overexpression of *SUR2* results in a bushy phenotype resembling multiple *YUC* knockout mutants (9, 17). It was unclear whether IAOx-mediated IAA biosynthesis plays an essential role in plant growth and development for 2 reasons. First, inactivation of CYP79B2 and

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 m^2 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 $\mu\text{g}/\text{mL}$), nicotinic acid (5 $\mu\text{g}/\text{mL}$), pyridoxin hydrochloride (0.5 $\mu\text{g}/\text{mL}$), myo-inositol (100 $\mu\text{g}/\text{mL}$) and 1% (wt/vol) sucrose.

Rice (*Oryza sativa*) seeds were germinated in H_2O , 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 of maize (*Zea mays*) were prepared as previously reported (36). 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, D_5 -IAOx, $^{13}\text{C}_6$ -IAOx, $^{13}\text{C}_6$ -IAM, D_2 -IAN, and $^{15}\text{N}_2$ -TAM were synthesized as described in *SI Text Materials and Methods*. LC-ESI-MS/MS and GC-MS analyses of IAA and IAA intermediates and in vivo labeling experiments were performed as described in *SI Text Materials and Methods*.

ACKNOWLEDGMENTS. We thank Drs. Belay T. Ayele, Shinjiro Yamaguchi, Yuichiro Tsuchiya, and Steve Swain for helpful comments on the manuscript. We thank Dr. Jane Glazebrook for providing the *cyp71a13* seeds and Dr. Tatsuya Sakai for *axr1-3* seeds. We are grateful to Ms. Akiko Onozuka for assistance in preparing plant materials and genotyping *cyp79b2* and *cyp79b3* mutants. This work was supported in part by grants from the MEXT of Japan (19780090 to H.K.) and from National Institutes of Health (R01GM68631 to Y.Z.).

1. Davies PJ (2004) in Plant Hormones, ed Davies PJ (Kluwer Academic Publishers, Dordrecht), pp 1–15.
2. Cohen JD, Slovin JP, Hendrickson AM (2003) Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis. *Trends Plant Sci* 8:197–199.
3. Ljung K, et al. (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol Biol* 49:249–272.

4. Pollmann S, Muller A, Weiler EW (2006) Many roads lead to “auxin”: of nitrilases, synthases, and amidases. *Plant Biol (Stuttg)* 8:326–333.
5. Strader LC, Bartel B (2008) A new path to auxin. *Nat Chem Biol* 4:337–339.
6. Hull AK, Vij R, Celenza JL (2000) *Arabidopsis* cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *Proc Natl Acad Sci USA* 97:2379–2384.

7. Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79B2 from *Arabidopsis* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. *J Biol Chem* 275:33712–33717.
8. Zhao Y, et al. (2002) Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. *Genes Dev* 16:3100–3112.
9. Bak S, et al. (2001) CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in *Arabidopsis*. *Plant Cell* 13:101–111.
10. Mikkelsen MD, Naur P, Halkier BA (2004) *Arabidopsis* mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. *Plant J* 37:770–777.
11. Glawischnig E, Hansen BG, Olsen CE, Halkier BA (2004) Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in *Arabidopsis*. *Proc Natl Acad Sci USA* 101:8245–8250.
12. Grubb CD, Abel S (2006) Glucosinolate metabolism and its control. *Trends Plant Sci* 11:89–100.
13. Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol* 57:303–333.
14. Nafisi M, et al. (2007) *Arabidopsis* cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *Plant Cell* 19:2039–2052.
15. Barlier I, et al. (2000) The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. *Proc Natl Acad Sci USA* 97:14819–14824.
16. Cheng Y, Dai X, Zhao Y (2007) Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. *Plant Cell* 19:2430–2439.
17. Cheng Y, Dai X, Zhao Y (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. *Genes Dev* 20:1790–1799.
18. Zhao Y, et al. (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* 291:306–309.
19. Yamamoto Y, et al. (2007) Auxin biosynthesis by the YUCCA genes in rice. *Plant Physiol* 143:1362–1371.
20. Gallavotti A, et al. (2008) *sparse inflorescence1* encodes a monocot-specific YUCCA-like gene required for vegetative and reproductive development in maize. *Proc Natl Acad Sci USA* 105:15196–15201.
21. Tobena-Santamaria R, et al. (2002) *FLOOZY* of petunia is a flavin mono-oxygenase-like protein required for the specification of leaf and flower architecture. *Genes Dev* 16:753–763.
22. Bak S, Nielsen HL, Halkier BA (1998) The presence of CYP79 homologues in glucosinolate-producing plants shows evolutionary conservation of the enzymes in the conversion of amino acid to aldoxime in the biosynthesis of cyanogenic glucosides and glucosinolates. *Plant Mol Biol* 38:725–734.
23. Park WJ, et al. (2003) The nitrilase ZmNIT2 converts indole-3-acetonitrile to indole-3-acetic acid. *Plant Physiol* 133:794–802.
24. Llic N, Normanly J, Cohen JD (1996) Quantification of free plus conjugated indoleacetic acid in *Arabidopsis* requires correction for the nonenzymatic conversion of indolic nitriles. *Plant Physiol* 111:781–788.
25. Pollmann S, Muller A, Piotrowski M, Weiler EW (2002) Occurrence and formation of indole-3-acetamide in *Arabidopsis thaliana*. *Planta* 216:155–161.
26. Savaldi-Goldstein S, et al. (2008) New auxin analogs with growth-promoting effects in intact plants reveal a chemical strategy to improve hormone delivery. *Proc Natl Acad Sci USA* 105:15190–15195.
27. Normanly J, Grisafi P, Fink GR, Bartel B (1997) *Arabidopsis* mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the *NIT1* gene. *Plant Cell* 9:1781–1790.
28. Ludwig-Muller J, Hilgenberg W (1988) A plasma membrane-bound enzyme oxidizes L-tryptophan to indole-3-acetaldoxime. *Physiol Plant* 74:240–250.
29. Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79A2 from *Arabidopsis thaliana* L. catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. *J Biol Chem* 275:14659–14666.
30. Nelson DR, et al. (2004) Comparative genomics of rice and *Arabidopsis*. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. *Plant Physiol* 135:756–772.
31. Golander Y, Sternson LA (1979) Metabolic conversion of fluorenone oxime to phenanthridinone by hepatic enzymes. *J Pharm Sci* 68:820–822.
32. Muller A, Hillebrand H, Weiler EW (1998) Indole-3-acetic acid is synthesized from L-tryptophan in roots of *Arabidopsis thaliana*. *Planta* 206:362–369.
33. Romano CP, et al. (1995) Transgene-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy6-1* hypocotyl elongation and *axr1* auxin-resistant mutants. *Plant Mol Biol* 27:1071–1083.
34. Tao Y, et al. (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell* 133:164–176.
35. Stepanova AN, et al. (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell* 133:177–191.
36. Nishimura T, et al. (2006) Red light causes a reduction in IAA levels at the apical tip by inhibiting de novo biosynthesis from tryptophan in maize coleoptiles. *Planta* 224:1427–1435.