The Arabidopsis YUCCA1 Flavin Monooxygenase Functions in the Indole-3-Pyruvic Acid Branch of Auxin Biosynthesis

Anna N. Stepanova, a Jeonga Yun, a Linda M. Robles, a Ondrej Novak, b, c Wenrong He, d Hongwei Guo, d Karin Ljung, b and Jose M. Alonso a, 1

a Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695
b Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden
c Laboratory of Growth Regulators, Faculty of Science, Palacky University and Institute of Experimental Botany Academy of Sciences of the Czech Republic, CZ–783 71 Olomouc, Czech Republic
d State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, China

The effects of auxins on plant growth and development have been known for more than 100 years, yet our understanding of how plants synthesize this essential plant hormone is still fragmentary at best. Gene loss- and gain-of-function studies have conclusively implicated three gene families, CYTOCHROME P450 79B2/B3 (CYP79B2/B3), YUCCA (YUC), and TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1)-RELATED (TAR), in the production of this hormone in the reference plant Arabidopsis thaliana. Each of these three gene families is believed to represent independent routes of auxin biosynthesis. Using a combination of pharmacological, genetic, and biochemical approaches, we examined the possible relationships between the auxin biosynthetic pathways defined by these three gene families. Our findings clearly indicate that TAA1/TARs and YUCs function in a common linear biosynthetic pathway that is genetically distinct from the CYP79B2/B3 route. In the redefined TAA1-YUC auxin biosynthetic pathway, TAA1/TARs are required for the production of indole-3-pyruvic acid (IPyA) from Trp, whereas YUCs are likely to function downstream. These results, together with the extensive genetic analysis of four pyruvate decarboxylases, the putative downstream components of the TAA1 pathway, strongly suggest that the enzymatic reactions involved in indole-3-acetic acid (IAA) production via IPyA are different than those previously postulated, and a new and testable model for how IAA is produced in plants is needed.

INTRODUCTION

Since the discovery of the auxin indole-3-acetic acid (IAA) in the 1930s (Thimann and Koepfli, 1935), the regulatory roles and molecular mechanisms of action of this key plant hormone have become the research focus for many plant biologists. Over the years, numerous reports of the critical involvement of this hormone in nearly every aspect of a plant’s life have flooded the scientific literature (reviewed in Bartel, 1997; Woodward and Bartel, 2005; Vanneste and Friml, 2009). Despite this massive research effort, our current understanding of how this hormone is made in plants remains, at best, fragmentary (Normanly, 2010; Zhao, 2010). Based primarily on biochemical experimental approaches, two general pathways for IAA production have been proposed, the Trp-dependent and the Trp-independent (Normanly et al., 1993). Very little is known about enzymatic reactions and metabolic intermediates implicated in the Trp-independent pathway (Östlin et al., 1999), and none of the genes involved have been characterized. It is, however, clear that this pool of auxin is derived from the Trp precursors indole and/or indole-3-glycerol phosphate (Ouyang et al., 2000). The situation is somewhat less dire for the Trp-dependent pathway, where four biosynthetic routes with candidate intermediates and potential enzymatic activities (responsible for the conversion of these intermediates into IAA) have been postulated (reviewed in Normanly, 2010; Zhao, 2010) (Figure 1). Each of these four putative biosynthetic routes is defined by the first metabolic intermediate generated from Trp, namely: i) indole-3-acetaldoxime (IAOx), ii) indole-3-pyruvic acid (IPyA), iii) indole-3-acetamide (IAM), or iv) tryptamine (TAM). Of these, the IAOx route is believed to be largely restricted to the Brassica genus. In Arabidopsis thaliana, IAOx is produced from Trp by a duo of highly related cytochrome P450s, CYP79B2 and CYP79B3 (Zhao et al., 2002; Sugawara et al., 2009). The bulk of IAOx produced is typically used in the synthesis of defense compounds, such as indole glucosinolates and camalexins (Glawischnig et al., 2004; Bender and Celenza, 2009). Under standard growth conditions, IAOx is believed to be a major source of IAA during normal plant development. On the other hand, high auxin levels can be reached when the conversion of IAOx into indole glucosinates is blocked by means of genetic mutations, such as superroot (sur1), sur2, or UDP-glucosyltransferase 74b1 (ugt74b1) (Bak et al., 2001; Grubb et al., 2004; Mikkelsen et al., 2004). In these...
mutants, large quantities of IAA can be produced via IAOx, but in this case, the potential metabolic steps between the precursor IAOx and the final product IAA have not been determined. Although several of the metabolic intermediates of the IAOx biosynthetic branch (such as IAM or IAN) are shared with the proposed IAM and TAM routes, the possible metabolic connections between these pathways are unknown.

Unlike the IAOx pathway, which is limited almost exclusively to *Brassica* spp., the IPyA, IAM, and TAM routes of Trp-dependent auxin biosynthesis are believed to be common to all plants, based on the ubiquitous distribution of at least some of the genes implicated in these pathways (De Smet et al., 2011). For example, *WEI8/TAAX1*, which encodes a Trp aminotransferase responsible for the conversion of Trp into IPyA, has homologous sequences across a wide array of evolutionarily diverse plant species, ranging from mosses (such as *Physcomitrella*) to monocots (such as maize [*Zea mays*] and rice [*Oryza sativa*]) and dicots (such as *Arabidopsis*) (Yamada et al., 2009). In this IPyA pathway, only the first biosynthetic step, the conversion of Trp into IPyA, has well characterized genetic results (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). By contrast, the two commonly postulated downstream steps, that is, the conversion of IPyA into indole-3-acetaldehyde and then into IAA by the consecutive action of a putative indole pyruvate decarboxylase (PDC) and an indole aldehyde oxidase (AAO), have very little experimental support in plants. Based on sequence similarities with the bacterial indole PDCs, in *Arabidopsis*, six putative PDCs can be grouped into two small families of four (PDC1, PDC2, PDC3, and PDC4) and two genes (PDC-Like2 [PDL2] and PDL1) each (see Supplemental Figure 1 online). One of the two genes of the latter family, PDL2/ALS/AHAS/CSR1/IMR1/TZP5, has been functionally characterized and shown to be involved in Val and Ile biosynthesis (Smith et al., 1989). Similarly, two of the four members of the PDC family, PDC1 and PDC2, have been implicated in ethanol fermentation (Ismond et al., 2003; Kürsteiner et al., 2003). There are also four predicted AAOs in *Arabidopsis*, AAO1, AAO2, AAO3, and AAO4. Although initial experiments showed that AAO1 is highly expressed in the *sur2* auxin-overproducing mutant (Seo et al., 1998), no additional evidence for a role of this gene in IAA biosynthesis was found. Moreover, several of the members of this small gene family have been shown to function in the biosynthesis of another plant hormone, abscisic acid, raising additional doubts about the role of AAOs in auxin production (Seo et al., 2000). Thus, in the absence of direct in planta evidence for the role of the predicted PDCs and AAOs in the IPyA pathway, the postulated current models should be considered working hypotheses.

Another proposed route for IAA production in plants is that defined by IAM. Although this pathway was originally believed to be present only in auxin-synthesizing bacteria, the identification of IAM in several plant species (Sugawara et al., 2009), as well as the characterization of tobacco (*Nicotiana tabacum*) and *Arabidopsis* IAM hydrolase enzymes capable of catalyzing the conversion of IAM into IAA, have opened the possibility of this pathway’s existence in plants (Pollmann et al., 2003; Nemoto et al., 2009). Nevertheless, to date there is no compelling evidence for a physiological role of this putative route of auxin biosynthesis in plants under normal growth conditions.

Finally, perhaps the most puzzling of all routes of auxin production is that defined by the YUC family of flavin monooxygenases, which in *Arabidopsis* is represented by 11 genes. This pathway is frequently referred to as the TAM route, because it was believed to start by the conversion of Trp into TAM (Zhao et al., 2001). TAM was then believed to be converted into N-hydroxyl TAM by the action of YUCs and then to IAOx (Zhao et al., 2001), an intermediate also shared by the CYP79B2/B3 branch of the auxin biosynthetic pathway. Recent findings have substantially weakened this model. Sugawara et al. (2009) showed that most, if not all, detectable IAOx in *Arabidopsis* is likely produced via CYP79B2/B3, suggesting that the YUC...
pathway does not converge on IAOx as previously believed. In addition, serious doubts about the involvement of YUC1 in the production of N-hydroxy TAM as well as about the nature of the in vivo substrate of this enzyme have recently been raised (Tivendale et al., 2010). Thus, YUCs, despite their undisputed central role in auxin production, have yet to be positioned in a defined Trp-dependent route of auxin biosynthesis. Based on the phenotypic similarities of many loss-of-function mutants of the YUC and TAA1 gene families, as well as the overlapping expression patterns of the corresponding genes, it was suggested that YUCs might function together with TAA1 in the IPyA route of IAA production (Strader and Bartel, 2008). Further support for this possibility was obtained by the less-than-the IPyA route of IAA production (Strader and Bartel, 2008).

The detailed quantification of several IAA intermediates clearly showed that IAN accumulates in Arabidopsis plants overexpressing YUC1 (Nonhebel et al., 2011). This result is quite surprising, because Sugawara et al. (2009) have found that most (if not all) of the IAN found in Arabidopsis is produced via CYP79B2/B3. Together, these findings suggest that the levels of IAN, an intermediate in the CYP79B2/B3 pathway, are directly or indirectly affected by YUC activity. Thus, to experimentally address the possibility of some type of interdependence between YUCs and CYP79B2/B3, we took two reciprocal genetic approaches. First, we crossed a well-characterized YUC1-overexpressing (ox) line (S5S:YUC1) (Zhao et al., 2001) into the cyp79b2/b3 double T-DNA knockout (KO) mutant. Unfortunately, this cross proved to be not informative, because we observed silencing of the YUC1ox transgene, possibly caused by the presence of multiple copies of the 3SS promoter in the cyp79b2/b3 T-DNA mutant plants. To bypass this problem, we used a transgenic construct, TAA1:YUC1, designed to confer the expression pattern of TAA1 to the YUC1 cDNA (see Methods). As shown in Figure 2, the expression of this construct in Columbia ecotype (Col-0) plants resulted in the characteristic high-auxin phenotypes (typically observed in the YUC1 overexpression lines) at all developmental stages examined, including dark-grown seedlings (Figure 2A), light-grown seedlings (Figure 2B), and adult plants (Figure 2C). Of note, the same high-auxin phenotypes were observed when this TAA1:YUC1 construct was introgressed into the double KO cyp79b2/b3 mutant plants (Figure 2). These results suggest that CYP79B2/B3 activity is not required for auxin production via YUC1.

To test further the relationship between the CYP79B2/B3 and YUC gene families, we examined the effects of activating both the CYP79B2/B3 and the YUC1 pathways simultaneously. To activate the CYP79B2/B3 route, we used a sur2 loss-of-function mutation. As shown in Figure 2, the high-auxin phenotypes of sur2 are completely suppressed by the cyp79b2/b3 mutations, indicating that the sur2 phenotype is the result of the CYP79B2/B3 pathway hyperactivation. If CYP79B2/B3 and YUCs function in parallel or independent pathways, the simultaneous activation of both routes is expected to result in additive phenotypes. The morphological defects of the sur2 YUCox line are in fact fully consistent with the additive model (see Supplemental Figure 2 online), providing additional support for CYP79B2/B3 and YUCs working in parallel/independent pathways.

Taken together, the aforementioned results clearly indicate that YUC1 does not function in the CYP79B2/B3 branch of auxin biosynthesis. Therefore, the previously reported accumulation of IAN in the YUC1ox plants (Nonhebel et al., 2011) is likely a secondary effect of altering the normal IAA homeostasis rather than a direct consequence of increasing the metabolic flow through the YUC pathway.

Simultaneous Knockdown of Several TAA1/TAR and YUC Family Members Has Less-Than-Additive Effects

Based on the similarities of the loss-of-function phenotypes of TAA1/TARs and of multiple YUCs, it has been suggested that the YUC and TAA1/TAR gene families may function in the same branch of auxin biosynthesis rather than in the two independent

RESULTS

The CYP79B2/B3 Function Is Not Required for YUC1-Mediated Auxin Production

To examine the relationship between the different auxin biosynthetic routes genetically defined in Arabidopsis, we first considered the possible points of action for the YUC1 monooxygenase. Theoretically, the YUC activity may be required in the CYP79B2/B3 route, in the TAA1/TAR route, or in a branch of auxin biosynthesis that is independent of either of these two gene families. To discriminate between these three alternatives, we first examined the possibility that YUCs and CYP79B2/B3 function in the same pathway. A priori, this seemed a very unlikely scenario, because the CYP79B2/B3 pathway is known to operate mainly in Brassica spp. (Bender and Celenza, 2009; Sugawara et al., 2009), whereas auxin biosynthesis via YUCs has also been demonstrated in species beyond this genus (Tobeña-Santamaría et al., 2002; Gallavotti et al., 2008). Nevertheless, the relationship between these two pathways may not be as clear as one may have
pathways proposed by the current models (Strader and Bartel, 2008). A much stronger argument in favor of such possibility was recently obtained upon the analysis of double loss-of-function mutants in the maize orthologs of YUC1 (spi1) and TAA1 (vt2), which were found to display a less-than-additive phenotype (Phillips et al., 2011). Thus, for our first approach to test the interaction between these two gene families in Arabidopsis, we used a similar experimental strategy. One important difference, however, is that in contrast with the situation in maize, where spi1 and vt2 single mutants show dramatic developmental defects, the high degree of functional redundancy among TAA1/TAR as well as YUCs in Arabidopsis made the generation of higher-order mutant combinations necessary. Starting with a wei8 tar2 double mutant, where the IPyA branch of IAA biosynthesis is critically impaired (Stepanova et al., 2008), higher-order mutants with up to four additional yuc KOs were generated. The expectation was that if TAA1/TARs and YUCs work in the same pathway, knocking out one or more YUC genes in the wei8-1 tar2-1 genetic background would have little, if any, effect. On the other hand, if TAA1/TARs and YUCs work in parallel pathways, even a minor reduction in the YUC pathway activity would produce strong effects in a plant with a compromised TAA1/TAR pathway. As shown in Figure 3, loss of up to four YUC genes (YUC1, YUC2, YUC4, and YUC6) in the wei8-1 tar2-1 double mutant background had no additional phenotypic effects beyond those of the corresponding parental lines. It is important to point out that, although the contribution of these four YUC genes to the overall auxin production may not be very large in 3-d-old seedlings (Figure 3A), in older plants the role of YUC1, YUC2, YUC4, and YUC6 in synthesizing auxin is significant, as demonstrated both by the phenotypic analysis (Figure 3B) and the quantification of IAA and IAA metabolites (see below) in the yuc1/2/4/6 quadruple mutant. Thus, in agreement with the similar findings in maize (Phillips et al., 2011), Arabidopsis loss-of-function studies support (but do not necessarily prove) the idea that TAA1/TARs and YUCs work in the same biosynthetic pathway.

Figure 2. The cyp79b2/b3 Double Mutant Can Suppress the High-Auxin Phenotypes of sur2, but Not That of TAA1:YUC1.

Col, Col TAA1:YUC1, sur2, cyp79b2/b3, TAA1:YUC1 cyp79b2/b3, and sur2 cyp79b2/b3 were grown in plates for 3 d in the dark (A), 3 d in the dark followed by 4 d in constant light (B), or for 3 weeks in soil under a 16-h light/8-h dark cycle (C). Representative plants of each genotype are shown.

Pharmacological Inhibition of TAA1/TAR Activity Suppresses the High-Auxin Phenotypes of YUC1 Overexpression

To test further the hypothesis of a linear TAA1-YUC1 pathway model, we decided to take advantage of kynurenine (Kyn), a potent inhibitor of in vivo TAA1/TAR activity (He et al., 2011). The rationale for these experiments was that if TAA1/TARs and YUCs work together to produce IAA, blocking the in vivo activity of TAA1/TARs with Kyn would suppress the high-auxin phenotypes of YUC1ox plants. To achieve high levels of YUC1 activity in plants, we used both the YUC1ox (35S:YUC1) and the TAA1:YUC1 constructs. In contrast with what we observed in the cyp79b2/b3 T-DNA KO lines, no silencing of the YUC1ox construct was observed in the Col-0 wild-type background. As shown in Figure 4, both YUC1 constructs result in plants with the characteristic high-auxin phenotypes both in dark-grown (Figure 4A) and light-grown (Figure 4B) plants. It is important to point out that the plants expressing the YUC1 cDNA under the control of the TAA1 regulatory sequences displayed a much stronger root phenotype, characterized by an extremely short root length both
in light- and dark-grown seedlings (Figure 4). On the other hand, the YUC1ox dark-grown seedlings displayed a hookless phenotype that was not observed in the TAA1::YUC1 plants. Remarkably, Kyn treatment blocked all of the high-auxin phenotypes of both types of YUC1ox lines, namely the hookless phenotype of the dark-grown YUC1ox seedlings, the short root of both YUC1ox and TAA1::YUC1 in dark- and light-grown seedlings, as well as the dark-green epinastic cotyledons in the light-grown plants (Figure 4). Different concentrations of Kyn were required to suppress these different phenotypes. For example, the short root was completely suppressed at Kyn concentrations as low as 1 μM, whereas much higher concentrations were required to suppress the epinastic cotyledons. Interestingly, at high Kyn concentrations, both the wild-type and TAA1::YUC1 dark-grown seedlings become hookless, whereas the hook curvature was partially restored in the YUC1ox plants. As a control for these experiments, we used sur2, an auxin-overproducing mutant that (as was shown in the previous section) works in a parallel and independent route of auxin production. As shown in Figure 4, Kyn had little effect on the high-auxin defects of this mutant at the concentrations tested. Taken together, the results presented...
indicate that the high-auxin phenotypes of both YUC1ox and TAA1:YUC1 require the function of TAA1/TARs and, therefore, support the linear action model for TAA1/TARs and YUCs.

**Gene Loss- and Gain-of-Function Studies Suggest That YUC1 and TAA1/TARs Function in the Same Biosynthetic Pathway**

To test further the linear model hypothesis for the action of TAA1/TARs and YUCs, we took a genetic approach. We reasoned that if the proposed linear model was correct, then the TAA1/TAR and YUC activities should coexist in the same cells and at the same time. Although previous studies based on gene expression suggest a good deal of overlap between these two gene families (Cheng et al., 2006; Cheng et al., 2007; Stepanova et al., 2008; Tao et al., 2008), the presence of 11 YUCs and three TAA1/TARs makes the direct approach to examine the coexpression of these genes' products challenging and possibly inconclusive. To bypass this potential problem, we decided to generate transgenic plants in which a YUC1 cDNA is driven by the regulatory sequences of TAA1 (i.e., the TAA1:YUC1 construct), thereby ensuring that the cells expressing TAA1 also express YUC1. The expectation is that this construct would result in high levels of auxin only if the cells that normally express TAA1 also had all of the enzymes required to produce IAA via the YUC1 pathway. By contrast, no additional auxin would be produced if, in the cells expressing TAA1 (and, therefore, the TAA1:YUC1 construct), one or more of the components of the YUC pathway were missing. As shown in Figure 5, Col-0 plants that express YUC1 cDNA under the control of the regulatory sequences of TAA1 show high-auxin phenotypes very similar to those of YUC1ox plants at all developmental stages examined (Figures 2, 4, and 5) (Zhao et al., 2001). The shorter roots and normal hooks observed in the TAA1:YUC1 dark-grown seedlings (as compared with those of the YUC1ox transgenics) can be explained by the specific and highly localized expression pattern of TAA1 in the root meristem and the apical hook (Stepanova et al., 2008). Importantly, these results suggest that TAA1/TARs and YUCs coexist in the same cells and at the same time, which is a prerequisite for the linear model being tested herein.

Next, we examined whether or not the high-auxin phenotypes observed in the TAA1:YUC1 plants require functional TAA1 and TARs. To this end, we introgressed the TAA1:YUC1 transgene into the following mutant backgrounds: wei8-2, wei8-2 tar1-1, wei8-2 tar2-1, wei8-2 tar1-1 tar2-1, and yuc1/2/4/6. Interestingly, the single wei8-2 mutant could fully suppress the short root phenotype of TAA1:YUC1 (Figures 5A and 5B), whereas the epinastic cotyledons of light-grown seedlings and the general morphology of the TAA1:YUC1 adult plants were suppressed only in the wei8-2 tar2-1 double and wei8-2 tar1-1 tar2-1 triple mutant backgrounds (Figure 5). Although these results strongly support the idea that functional TAA1 and TARs are required for the auxin biosynthetic activity of TAA1:YUC1, it is formally possible that TAA1:YUC1 can confer a high auxin biosynthetic activity in the wei8-2 tar2-1 mutants, but this additional auxin is not sufficient to reach a certain threshold required for rescuing the low-auxin phenotypes of wei8-2 tar2-1. To rule out this possibility, we used two different approaches. First, we introgressed the TAA1:YUC1 transgene into the quadruple mutant yuc1/2/4/6, a yuc mutant combination with auxin-deficient phenotypes very similar to those of the wei8 tar2 doubles (Figure 5C; see Supplemental Figure 3 online) and, as shown below, with similar levels of free IAA. As an additional control, we also generated double wei8-2 tar2-1 mutants harboring a TAA1:iaaM transgene, in which the expression of the bacterial auxin biosynthetic gene iaaM is under the control of the same TAA1 regulatory sequences that drive the expression of YUC1 in the TAA1:YUC1 construct. Importantly, the TAA1:YUC1 transgene was able to revert the auxin-deficient phenotypes of the quadruple yuc mutant, resulting in plants phenotypically similar to Col TAA1:YUC1 (Figure 5; see Supplemental Figure 3 online). Furthermore, the TAA1:iaaM construct effectively complemented the wei8-2 tar2-1 auxin defects (see Supplemental Figure 4 online). These results provide strong genetic support to the idea that auxin production via YUC1 requires the activity of the TAA1/TAR gene family or, in other words, that TAA1/TARs and YUCs function in the same genetic pathway. In addition, the different degrees by which the wei8-2 single mutant suppresses TAA1:YUC1 in the various tissues examined could be indicative of the different levels of functional overlap between TAA1 and other family members in these tissues. Thus, for example, if most of the cells that express TAA1 in roots do not express any of the other family members, it would be expected that the expression of YUC1 in these TAA1-specific tissues will render no additional IAA in the wei8 mutant background. On the other hand, if the cells that express TAA1 in the cotyledons also express TARs, the expression of YUC1 in these cells would still produce significant amounts of IAA even in the wei8-2 mutant.

**Quantification of Free IAA and Several IAA Metabolites Indicates That the Function of TAA1 and TARs Is Required for IAA Production via YUCs**

Taken together, the pharmacological and genetic data presented above strongly support the idea that TAA1/TARs and YUCs function in a common branch of the auxin biosynthetic pathway. These types of experiments, although highly informative, have an important caveat. They rely on the use of morphological changes as an indirect readout of the IAA biosynthetic activity (the primary effect of the imposed genetic or pharmacological conditions). Furthermore, the approaches described thus far cannot clarify the relative position of TAA1/TARs and YUCs in the biosynthetic pathway. Therefore, we examined the levels of free IAA and IAA metabolites in several TAA1/TAR and YUC mutants with or without the TAA1:YUC1 transgene.

In agreement with previous studies (Stepanova et al., 2008; Tao et al., 2008), significantly lower levels of free IAA were detected in the single wei8-2 as well as in the double wei8-2 tar2-1 mutants (Figure 6A). Importantly, the levels of free IAA in the quadruple yuc1/2/4/6 were also much lower compared with those in the wild-type plants. In fact, the IAA levels in the yuc quadruple mutant were nearly as low as in wei8-2 tar2-1 (Figure 6A). It is important to point out that these auxin measurements were done using 2-week-old plants. At this developmental stage, both wei8-2 tar2-1 and yuc1/2/4/6 show clear auxin-deficient phenotypes. We also measured free IAA in 3-d-old dark-grown
seedlings, but in that case, no reduction in the levels of free IAA was detected in the quadruple yuc1/2/4/6 mutant, consistent with the lack of obvious auxin-deficient phenotypes at this developmental stage (Figure 5A). In addition to free IAA, we also measured the most abundant IAA conjugates and catabolites in Arabidopsis, namely 2-oxindole-3-acetic acid (oxIAA), indole-3-acetyl Asp (IAAsp) and indole-3-acetyl Glu (IAAGlu) (Östlin et al., 1998; Kowalczyk and Sandberg, 2001). All three IAA metabolites were reduced in the auxin-depleted genotypes wei8-2, wei8-2 tar2-1, and yuc1/2/4/6 (Figures 6B to 6D), as would be expected of plants impaired in the production of IAA.

Once we had established that the levels of free IAA and IAA conjugates were similarly reduced in both the wei8-2 tar2-1 double mutant and in the yuc1/2/4/6 quadruple mutant, we examined the effects of expressing the TAA1:YUC1 transgene in different genetic backgrounds. First, we observed a small increase in the levels of free IAA in Col-0 plants expressing TAA1:YUC1 (Figure 6A). Although this increase was not significant, we found a fivefold to 10-fold increase in the levels of IAA metabolites in this line (Figures 6B to 6D). These results clearly indicate that TAA1:YUC1 enhances the IAA biosynthetic activity in the otherwise wild-type control plants and that IAA catabolism and conjugation are induced to maintain IAA homeostasis. These effects of TAA1:YUC1 on the levels of IAA and IAA metabolites were somewhat reduced in the wei8-2 mutant background and were nearly completely obliterated in the wei8-2 tar2-1 double mutants (Figure 6). By contrast, the quadruple yuc mutant had only negligible effects on the enhanced auxin biosynthetic activity conferred by the TAA1:YUC1 transgene (Figure 6). The simplest interpretation of the overall results is that the auxin biosynthetic activity of YUCs requires functional TAA1/TARs. Therefore, our findings firmly position the two gene families in the same branch of the auxin biosynthesis pathway. These results, however, do not provide conclusive information on the relative position of TAA1/TARs with respect to YUCs.

Gene Loss- and Gain-of-Function Analyses Suggest That None of the Annotated Arabidopsis PDCs Are Involved in Auxin Biosynthesis

The results described in previous sections show that YUC1, a flavin monooxygenase, functions downstream of TAA1/TARs in the conversion of IPyA into IAA. To narrow down the number of putative YUC1 substrates, we examined whether or not any of the four predicted Arabidopsis PDCs were involved in auxin production. Again, we took a genetic approach and generated loss- and gain-of-function alleles for the four predicted Arabidopsis PDCs. We have obtained homozygous T-DNA mutants in all four PDC genes, PDC1, PDC2, PDC3, and PDC4. T-DNA insertion sites were identified by sequencing of the PCR-amplified T-DNA/genomic DNA junction fragments. pdc1-10, pdc2-10, pdc3-10, and pdc4-11 harbor T-DNAs in exons, whereas pdc4-10 has a T-DNA insertion within the coding region.
in the intron (see Supplemental Figures 1A to 1C and Supplemental Data Set 1 online). Phenotypic analysis of dark- and light-grown seedlings in plates and of soil-grown adult plants failed to uncover any auxin-related phenotypes. Similarly, the very sensitive root elongation assay in response to ethylene (which can pick up even very mild auxin deficiency) was unsuccessful at detecting any phenotypic abnormalities. To deal with the possible functional redundancy problem, we generated and characterized several double and triple pdc mutant combinations. Again, no auxin-related phenotypes were observed (see Supplemental Figure 1D online). To test further the potential role of these genes in auxin biosynthesis, we constructed four different pdc triple mutant combinations in the sensitized wei8-1 mutant background, but again failed to observe any auxin-related defects beyond those of wei8 alone (see Supplemental Figure 1D online). Finally, we also took a complementary approach and overexpressed all four Arabidopsis PDCs in the wild-type plants using the strong constitutive 35S promoter. Again, we were unable to detect any auxin-related phenotypes in any of these lines (see Supplemental Figure 1D online).

Consistent with our findings, PDC1 and PDC2 were previously implicated in ethanolic fermentation: A KO allele of PDC1 was shown to be hypersensitive to anoxia (Kürsteiner et al., 2003), whereas overexpression of either PDC1 or PDC2 resulted in increased resistance to low oxygen levels (Ismond et al., 2003). These data, together with the lack of auxin-related defects in multiple pdc mutants and the inability of any of the PDCs to use IPyA as a substrate in vitro (Ye and Cohen, 2009), suggest that the PDC gene family may not be involved in auxin biosynthesis in plants. Therefore, a completely revised working model of how IPyA is converted into IAA is required.

DISCUSSION

To date, none of the four predicted routes of auxin biosynthesis in plants have been experimentally resolved in full. The current models of the auxin biosynthetic pathway, although obviously very useful, should be considered as “working hypotheses.”
rather than well established and solidly grounded facts. Indeed, recent studies have raised some legitimate concerns about the validity of several aspects of these models, primarily regarding the position of the YUC genes in auxin biosynthesis. This work was aimed at specifically addressing this uncertainty and providing a solid foundation for the future elucidation of the complete IPyA route of IAA biosynthesis. Our genetic, pharmacological, and biochemical data provide compelling evidence for YUCs and TAA1/TARs working in the same Trp-dependent branch of the auxin biosynthetic pathway. Although previous reports have suggested this possibility, to our knowledge, no direct proof had been presented until now. Based on the fact that Trp is the substrate for TAA1 and that YUC1 is unlikely to be involved in Trp production, the simplest model would position YUCs downstream of TAA1/TARs. On the other hand, serious doubts about the role of PDCs downstream of the IPyA route in the IAA biosynthetic pathway have been raised herein and in Ye and Cohen (2009). Thus, in contrast with auxin-synthesizing bacteria, plants may produce IAA from IPyA using a different enzymatic mechanism that involves the catalytic activity of YUCs. In fact, based on examples from the literature (Lockridge et al., 1972), we speculate that flavin monoxygenases, like YUCs, may catalyze the conversion of IPyA into IAA.

In addition to shedding new light on the relationship between the different branches of auxin biosynthesis, our results also raise several obvious questions that should become the subject of future investigations. Perhaps the most important of these issues is how IPyA, a labile molecule (Badenoch-Jones et al., 1984; Tam and Normanly, 1998), is converted into IAA via one or more enzymatic reactions that comprise (possibly, among others) the monoxygenase activity of the YUC enzyme. Although at this point we do not have experimental evidence to show how this is accomplished in plants, we can make several observations that may guide us in this future endeavor. For example, the known chemical instability of IPyA, the high affinity of Trp-aminotransferases for IPyA, and the cytoplasmic localization of both TAA1 and YUCs may be indicative of the existence of a multiprotein functional complex implicated in the catalysis of two or more sequential enzymatic reactions, including those of TAA1 and YUCs. Although merely speculative at this point for the TAA1-YUC case, this idea of “metabolic channeling” is by no means new, and there are many examples, including the Trp biosynthetic pathway itself, where the product of one enzyme is directly fed to the next enzyme in the pathway (Miles, 2001).

Another interesting observation came from the comparison of the morphological phenotypes and IAA levels across the different mutants examined. Thus, for example, it was surprising to see that the strong high-auxin phenotypes of the TAA1:YUC1 lines did not correspond to a statistically significant increase in the levels of free IAA. By contrast, a similar degree of reduction in the levels of free IAA in the 2-week-old double we8-2 tar2-1 and quadruple yuc1/2/4/6 mutants resulted in phenotypic alterations of different severity (although at later developmental stages, the two genotypes become nearly indistinguishable). In theory, these results could be indicative, as has been suggested before for the YUC genes (Tobena-Santamaria et al., 2002), of these genes being involved in the production of a bioactive compound other than IAA. We, however, disfavor this idea for several reasons. First, both exogenous auxin treatment (see Supplemental Figure 5 online) (Stepanova et al., 2008) and stimulation of endogenous auxin production by means of sur2 (Stepanova et al., 2008) or iaaM (Cheng et al., 2006; Tao et al., 2008) complement most of the phenotypic defects of the loss-of-function mutants. Furthermore, the dramatic increase in the levels of the major IAA catabolite oxiIAA and of the IAA conjugates IAAsp and IAGlu in the TAA1:YUC1 lines suggests that the apparent lack of correlation between the IAA levels and the phenotypic alterations observed is caused by a very effective homeostasis mechanism that regulates the levels of free IAA.

Finally, our results also show that the expression of YUC1 under either a constitutive promoter or the regulatory sequences of TAA1 results in very similar but not identical high-auxin phenotypes. This suggests that nearly every cell that is capable of producing IAA via YUC also expresses TAA1, and the difference between the constitutive YUC1ox (35S:YUC1) and the TAA1:YUC1-ox overexpressing lines is caused by the few cells or developmental stages in which either TAR1 or TAR2 are expressed but TAA1 is not. This hypothesis is also consistent with the lack of phenotypes in the tar1 or tar2 single or tar1 tar2 double mutants and with the fact that the phenotypic consequences of the loss of function of these two genes can be observed only in the absence of functional TAA1 (Stepanova et al., 2008). An alternative explanation would be that the YUC1ox and the TAA1:YUC1-ox overexpressing lines produce high levels of auxin in very different places, but the auxin transport and catabolism machineries mask these differences. In either case, however, the few but clear phenotypic differences observed between the YUC1ox and the TAA1:YUC1-ox lines (i.e., the shorter root and normal apical hook curvature of the TAA1:YUC1 lines compared with the YUC1ox) indicate that not only the overall levels but also the patterns of auxin biosynthesis play important roles, at least in processes such as root elongation and differential cell growth in the apical hook. These findings provide further evidence for the key role of local auxin biosynthesis in plant development.

Taken together, the results reported here represent one additional step toward the construction of a revised model of the IPyA branch of the auxin biosynthetic pathway. The future research in this area is likely to focus on determining how IPyA is converted to IAA in plants and on establishing the metabolic intermediates involved and the enzymes responsible for each step. These studies should clarify the biochemical role of YUCs in auxin production. Having a complete view of the major TAA1/YUC-mediated route of auxin biosynthesis will greatly advance our understanding of the role of auxin biosynthesis in plant growth and development and will open new avenues to manipulating the levels and distribution of this essential plant hormone for the benefit of mankind.

METHODS

Strains, Constructs, Plant Transformation, and Genotyping

All Arabidopsis thaliana mutant strains and transgenic lines reported herein are in Col background: we8-1, we8-2, tar1-1, tar2-1, tar2-2, and tar2-3 were described in Stepanova et al. (2008), sur2 was reported in Stepanova et al. (2006). The cyp79b2/b3 double mutant (Zhao et al., 2002) was provided by Dr. J. Celenza. The yuc1/2/4/6 quadruple mutant (Cheng et al.,...
Genotype of the TAA1 locus in various weil-2-containing mutant combinations was determined by sequencing.

Extraction of plant genomic DNA for genotyping purposes was performed in cetyltrimethylammonium bromide (CTAB) buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8, 3% CTAB [Calbiochem]) either individually or in a 96-well format. For individual sample extraction, fresh or frozen tissues (typically one average-size adult leaf or five to 20 seedlings) were ground dry in microfuge tubes for 6 to 15 s in the presence of ~100 µL of 1-mm glass beads in a Silamat SS shaker (Ivoclar Vivadent), followed by 6 to 15 s with 250 µL of CTAB buffer. For high-throughput extraction, tissues were collected into the individual wells of 96-well plates, frozen at −80°C, ground dry on a Qiagen shaker with 4.5-mm steel zinc-plated beads, and resuspended in 250 µL of CTAB buffer. Samples were incubated at 65°C for 30 min, extracted once with 1 volume of chloroform, and precipitated with 1 volume of isopropanol alcohol. DNA-containing pellets were washed once with 70% ethanol, were air-dried, and were resuspended in 100 to 300 µL deionized water.

Genotyping of DNA samples extracted from various T-DNA mutants and/or their derivatives was performed by PCR. The origin of all mutant alleles used in this study and their respective genotyping primers are listed in Supplemental Tables 1 and 2 online. The typical 10-µL PCR reaction contained 1 µL of genomic DNA, 1 µL of 10× PCR buffer, 0.25 µL of 2 mM deoxynucleotides triphosphate, 0.25 µL of homemade Taq polymerase, 0.25 µL of 20 µM forward primer, 0.25 µL of 20 µM reverse primer, and 7 µL of H2O. A typical PCR program was (30 s at 94°C, 30 s at 56°C, 1 to 3 min at 72°C [1 min per kb]) times 40 cycles. PCR products were separated on 1× Tris-acetate-EDTA 1 to 2% agarose gels, and the gel images were photographed and scored.

The YUC1 cDNA and iaaM genomic clone were provided by Drs. Y. Zhao and G. Jurgens, respectively. TAA1:YUC1 and TAA1:iaaM constructs were generated by bacterial recombineering (Zhou et al., 2011) in the backbone of a transformation-ready BAC clone JAtY73L21. The TAA1 gene (nucleotides 221 to 2256) was replaced by the open reading frame of YUC1 or iaaM using Galk selection/counterselection (Warming et al., 2005), and the recombinated BAC clones were transformed into the weil-2 (for TAA1:YUC1) or weil-2 tar2-1/+ (for TAA1:iaaM) background by a modified Agrobacterium-mediated “flower-drop” method (Zhou et al., 2011). Selection of the T1 generation was performed on AT plates (1× Murashige and Skoog salts, 1% Suc, pH 6.0, with 1 M KOH, 0.7% Bacto Agar) supplemented with 20 µg/mL phosphinothricin (Basta). For TAA1: YUC1, one representative line (of more than 12 that displayed characteristic high-auxin phenotypes, including epinastic cotyledons and leaves) was crossed to Col, cyp79b2/b3, weil-2 tar2-1, weil-8 tar2-1 tar2-2, and yuc1/2/4/6, and the progeny of the crosses was genotyped in F2, F3, and/or F4. sur2 cyp79b2/b3 triple mutant was generated by crossing sur2 with the cyp79b2/b3 double mutant. Various pdc mutant combinations (with and without weil-8) were generated by sequential crossing of single and then higher-order mutants and by genotyping of the progeny in F2 and F3 generations of each cross. PDC overexpression constructs were generated by Gateway technology (Invitrogen) in the pGWB2 vector (Nakagawa et al., 2007). A PDC3 cDNA clone in the pENTR/D-TOPO vector was obtained from ABRC (G22953) and was subcloned into pGWB2 by LR Clonase. Genomic DNA fragments of PDC1, PDC2, and PDC4 (from the start codons to the ends of the open reading frames minus stop codons) were PCR-amplified by Pfx50 polymerase using BAC DNA minipers (clones JA-Y57K14, JA-Y68117, and JA-Y54M22, respectively) as templates. PCR fragments were subcloned into pENTR/D-Topo, sequence-verified, and then transferred to pGWB2 by LR recombination. All four 3SS:PDC constructs were introduced into Col plants by the Agrobacterium-mediated transformation using the flower-drop method (Clough and Bent, 1998). Transformants (20 or more per gene) were selected on AT plates supplemented with 100 µg/mL kanamycin and analyzed in T1 and T2.

Multiple weil-8 tar2-1 yuc combinations were obtained by crossing weil-1 tar2-1/+ yuc1/+ yuc4ycz6+/+ mutant (because homozygous versions of these mutant combinations are sterile/lethal). Ten out of 60 F1 plants tested had inherited the mutant versions of tar2-1, yuc1, and yuc6. A total of 248 individual F2 plants from the progeny of one of these 10 plants (number 79) were genotyped for all seven segregating loci. A single F2 plant, number 79-114, with the genotype weil-1 12-1/+ tar1-1/+ yuc1 yuc2/+ yuc4/+ yuc6/+ was selected for further analysis, because it had undergone recombination between the closely linked tar2 and yuc1 loci. Then, 47 individual F3 progeny of number 79-114 were genotyped for tar2-1, yuc2, yuc4, and yuc6 mutations. Two of these F3 progeny, numbers 79-114-44 and 79-114-62, were shown to be genotypically weil-8-1 12-1/+ yuc1 yuc2/+ yuc4/+ yuc6. F4 progeny of these two lines were planted on AT plates supplemented with 10 µM 1-amino-cyclopropane-1-carboxylate (ACC), phenotypically preselected in the triple response assay for the characteristic weil-8-1 tar2-1 double mutant-like defects (lack of apical hook, long agravitropic or missing root, and/or missing hypocotyls, and/or monopetalous-like defects) in 3-d-old etiolated seedlings, and photographed individually. A total of 164 preselected F4s were grown for an additional 15 d in constant light, were photographed, and were then harvested for DNA extraction and genotyping. Three out of 164 F4s were found to be homozygous sextuple weil-8-1 12-1 yuc1 yuc2 yuc4 yuc6 mutants, which corresponds to ~1/55 instead of the expected 1/16 sextuple mutants among the tar2-1 preselected plants.

Physiological Assays

Seeds were surface-sterilized with 50% bleach (spiked with approximately five drops of Triton-X100 per liter to avoid seed clumping) for ~10 min, washed three or more times with sterile dH2O, resuspended in melted precocooned 0.7% low-melting-point agarose in water, and planted on the surface of AT plates (see above) supplemented or not with ACC or Kyn. Plates with seeds were kept at 4°C for 2 to 4 d, light-treated for 1 to 2 h to equalize germination, wrapped in aluminum foil, and placed horizontally in a 22°C dark incubator for 3 d. Phenotypes of etiolated seedlings were scored, and their photographs were taken at 72 h of age.

For the light assay, plates with dark-grown 3-d-old seedlings were transferred to constant light and were incubated for an additional 4 to 15 d, at which point pictures were taken, phenotypes were scored, and/or tissues were collected for IAA level quantification. For soil-grown plants, seeds were germinated on plates and then transplanted to soil (50:50 mix of Fafard 4P [Fafard] and Sunshine Professional Growing Mix [Sun Gro Horticulture]) and grown under a 16-h light/8-h dark cycle.

For the triple response assay and Kyn treatment, seeds were germinated for 3 d in the dark on horizontal AT plates supplemented with 10 µM ACC or with the indicated concentrations of Kyn, 50 µM ACC (Calbiochem) stocks were made in dH2O, whereas stocks of 50 mM Kyn (Sigma-Aldrich) were prepared in 0.1 N HCl in water.

Quantification of Free IAA and IAA Metabolites

Col, Col TAA1:YUC1 (hemizygous), weil-8-2, weil-8-2 TAA1:YUC1 (homozygous), weil-8-2 tar2-1/+ yuc1 yuc2 yuc4 yuc6 mutant (because homozygous versions of these mutant combinations are sterile/lethal) were used for quantitative analysis. Extracts of etiolated seedlings were prepared as previously described (Zhang et al., 2009). Freeze-dried seedling material (~25 mg) was extracted in 0.6 M HClO4 for 1 h, centrifuged, and the supernatant was neutralized and analyzed for IAA and IAA metabolites as previously described by Hedin and Korhonen (1999).
independent biological replicates per genotype, with each replicate originating from a different seed batch) were collected, weighed, and frozen in liquid nitrogen. Phenotype selection of the segregating lines was performed in 3-d-old etiolated seedlings in the following way. Col TAA1: YUC1 lines were selected for two copies of the TAA1:YUC1 transgene based on the very short root phenotype (Col plants hemizygous for this transgene show intermediate root length). Col tar2 double mutants, yuc1/2/4/6 and yuc1/2/4/6 TAA1:YUC1 (hemizygous for the transgene) were selected from the yuc1/2/4/6 TAR1:YUC1 hemizygous populations based on their long versus very short root phenotype, respectively (hemizygous seedlings show an intermediate phenotype and were discarded). Correlations between the root length and the copy number of the TAA1:YUC1 transgene were observed in multiple transgenic lines (in Col and yuc1/2/4/6 background) across several generations. Genotype of the preselected we8-2 tar2-1 homozygotes was confirmed by the characteristic phenotypes of 2-week-old plants and/or by PCR.

For quantification of IAA and its metabolites, whole plants were collected and purified in triplicates (20 mg fresh weight/sample). Extraction and purification were done according to Alenda et al. (1995) with minor modifications. The frozen samples were homogenized, extracted, and purified with the addition of the following [13C6]-labeled internal standards: [indole-13C6]-IAA (10 pmol), [indole-13C6]-oxIAA (10 pmol), [indole-13C6]-IAAsp (5 pmol), and [indole-13C6]-IAGlu (5 pmol) (Kowalczyk and Sandberg, 2001). All samples were analyzed by liquid chromatography–multiple reaction monitoring–mass spectrometry. The eluates were evaporated to dryness and dissolved in 20 μL of mobile phase before mass analysis using a 1290 Infinity LC system and a 6460 Triple Quad LC/MS system (Agilent Technologies). A total of 10 μL of each sample was injected onto a reversed-phase column (Kinetex C18 100A, 50 × 2.1 mm, 1.7 μm; Phenomenex) and eluted with 10-min gradient (0 to 10 min, 10/90 to 50/50% A/B; flow rate, 0.2 mL min−1; column temperature, 30°C, where A was 0.1% acetic acid in methanol and B was 0.1% acetic acid in water) (see Supplemental Figure 6 online). At the end of the gradient, the column was washed and equilibrated to initial conditions for 6 min. Quantification was obtained by monitoring the precursor (M+H)+ and the appropriate product ions. The multiple reaction monitoring transitions 182.2>136.1, 198.2>152.1, 297.2>136.1, and 311.1>136.1 were used for labeled IAA, oxIAA, IAAsp, and IAGlu, respectively. Tandem mass spectrometry conditions were as follows: drying/sheath gas temperature, 250/200°C; drying/sheath gas flow, 10/12 L min−1; nebulizer pressure, 60 psig; capillary voltage, 2250 V; fragmentor, 65 to 85 V; collision energy, 9 to 22 V. The limits of detection (signal-to-noise ratio, 1:3) were close to 1 pg for IAA and its metabolites. The linear range was established to be 0.01 to 100 ng per injection with a correlation coefficient of 0.9987 to 0.9998. Chromatograms were analyzed using the MassHunter software (Agilent Technologies), and the compounds were quantified by standard isotope dilution analysis (Rittenberg and Foster, 1940).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At4g33200 (PDC1), At5g54980 (PDC2), At5g01330 (PDC3), At5g01320 (PDC4), At5g18580 (PDL1), At5g17380 (PDL2), At5g20960 (AAO1), At3g43600 (AAO2), At2g27150 (AAO3), At1g04580 (AAO4), At1g17050 (TAA1), At1g23320 (TAR1), At4g24670 (TAR2), At4g39950 (CYP79B2), At4g32330 (CYP79B3), At4g31500 (SUR2), At4g23540 (YUC1), At4g13280 (YUC2), At5g11320 (YUC4), and At5g25620 (YUC6).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Loss- and Gain-of-Function Analysis of the Putative PDC Gene Family in Arabidopsis.

Supplemental Figure 2. Simultaneous Activation of the CYP79B2/B3 and YUC Pathways in the sur2 YUC1ox lines Leads to Additive Phenotypic Defects.

Supplemental Figure 3. TAA1:YUC1 Can Suppress the Flower Defects of yuc1/2/4/6, but Not of we8-2 tar2-1.

Supplemental Figure 4. Functional TAA1 and TARs Are Not Required for iaaM-Mediated Auxin Biosynthesis.

Supplemental Figure 5. Exogenous IAA Can Restore Some but Not All of the we8 tar2 Mutant Phenotypes.

Supplemental Figure 6. Representative Chromatograms of Free IAA and IAA Catabolites/Conjugates.

Supplemental Table 1. Primers Used to Genotype the Different Mutants Used in This Study

Supplemental Table 2. Sequences of the Primers Used in This Study

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis in Supplemental Figure 1A.

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AUTHOR CONTRIBUTIONS

A.N.S. and J.M.A. designed experiments and performed research, analyzed data, and wrote the article. J.Y. and L.M.R. performed research. O.N. and K.L. performed research, analyzed data, and contributed new analytic tools. W.H. and H.G. contributed information about a new analytic tool. J.M.A., K.L., O.N., A.N.S., and J.Y. contributed in writing the article.

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