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Note

Comparison of indole derivatives as potential intermediates of auxin biosynthesis in *Arabidopsis*

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Abstract Biosynthesis of indole-3-acetic acid (IAA) is crucial for the regulation of plant growth and morphological changes. However, the pathways of IAA biosynthesis have not been completely understood. Although indole derivatives have been proposed to be intermediates of the pathway, it has not been thoroughly elucidated whether the proposed intermediates are indeed converted into an active auxin, IAA, or intermediate metabolites other than IAA. In the present work, we examined 11 indole derivatives whether they are indeed IAA intermediates. The indole derivatives were examined by measuring their effects on recovery from auxin-deficiency conditions caused by L-aminooxyphenylpropionic acid (AOPP), which we previously identified as an IAA biosynthesis inhibitor. We estimated auxin activity of each indole derivative by analyzing auxin-inducible marker gene expression and root morphology in *Arabidopsis*. We found that indole-3-pyruvic acid most effectively recovered seedlings from auxin deficiency among the indole derivatives examined, followed by *N*-hydroxyl tryptamine and indole-3-acetonitrile, with effective recovery from the auxin deficiency caused by AOPP. We also found that tryptophol recovered the root growth defects. Among the indole derivatives examined, only indole-3-acrylic acid did not show effective recovery from auxin deficiency. These results provide information on which compounds are reliable intermediates of IAA biosynthesis pathways in *Arabidopsis*.

Key words: Auxin biosynthesis inhibitor, auxin intermediates, L-aminooxyphenylpropionic acid.

Plants produce a variety of small-molecule compounds that function as plant hormones and regulate plant growth and development in response to environmental changes. Among plant hormones, auxin plays critical roles in many aspects of plant growth regulation. Indole-3-acetic acid (IAA) is the most abundant and indispensable natural auxin. So far, the biosynthesis of IAA is roughly classified into L-tryptophan (Trp)-dependent and -independent pathways. A number of genes and compounds involved in biosynthesis of IAA have been identified and several Trp-dependent pathways have been proposed, e.g., the tryptamine (TAM), indole-3-acetaldoxime (IAOx), indole-3-acetamide (IAM), and indole-3-pyruvic acid (IPyA) pathways (Figure 1; Mashiguchi et al. 2011; Normanly 2010; Won et al. 2011; Zhao 2010). However, it is still controversial as to which compounds are indeed functional intermediates in biosynthesis pathways of IAA, although multiple indole derivatives have been proposed as intermediates in the Trp-dependent pathways. To address this issue, it is important to analyze the indole derivatives to see whether they can recover plants from auxin deficiency.

Recently, aminoethoxyvinylglycine (AVG) and L-aminooxyphenylpropionic acid (AOPP) were identified as inhibitors of IAA biosynthesis (Soeno et al. 2010). They inhibit conversion from Trp to IPyA in both *Arabidopsis* and wheat enzyme extracts (Figure 1A), and the endogenous IAA level is reduced in the presence of these inhibitors (Soeno et al. 2010). In previous work, we induced auxin deficiency in *Arabidopsis* seedlings using these inhibitors, and analyzed several intermediates in a root growth assay as well as in an auxin-marker gene expression assay. In the auxin-deficient root growth assay, we used AOPP, in which main root elongation is inhibited and roots skew to the left when grown on vertical plate, while seedlings could

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Abbreviations: AOPP, L-aminooxyphenylpropionic acid; AVG, aminoethoxyvinylglycine; CinA, cinnamic acid; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAcA, indole-3-acrylic acid; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IPyA, indole-3-pyruvic acid; N-TAM, *N*-hydroxyl tryptamine; TAA, tryptophan aminotransferase of *Arabidopsis*; TAM, tryptamine; TOL, tryptophol; Trp, L-tryptophan; qRT-PCR, quantitative reverse transcription PCR.

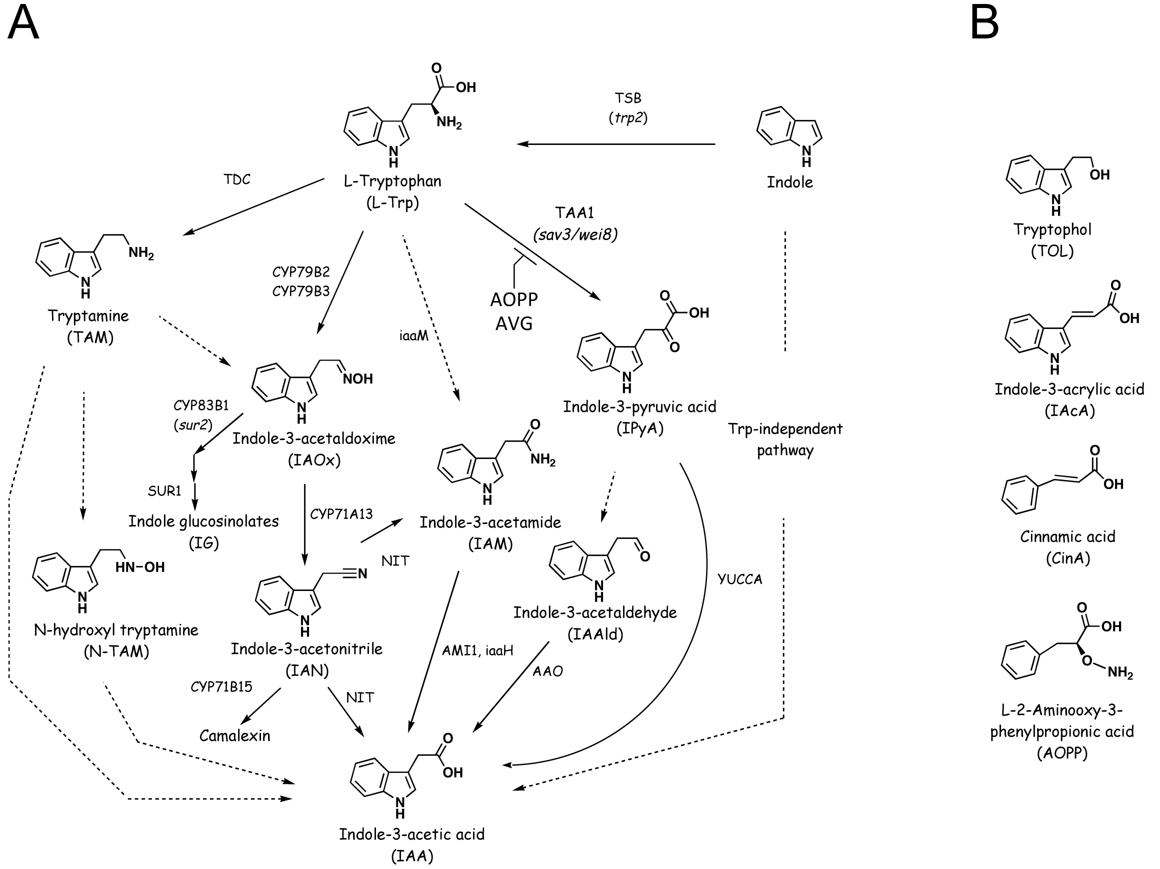

Figure 1. Biosynthesis pathways of IAA in *Arabidopsis*. Solid arrows represent steps with identified genes coding enzymes responsible for the conversion of IAA intermediates (TAA: tryptophan aminotransferase of *Arabidopsis*; NIT: nitrilase; AAO: *Arabidopsis* aldehyde oxidase; AMI: indole-3-acetamide amidohydrolase). Dashed arrows are steps that have been proposed in *Arabidopsis* (A). CinA, TOL, IAcA, and AOPP were used in this study as putative IAA intermediates or biosynthesis inhibitors (B).

recover from the growth inhibition in the presence of IAA or an IAA intermediate. In the previous report, only seven conjectured intermediates (indole, Trp, TAM, IAM, indole-3-acetonitrile [IAN], IPyA, and indole-3-acetaldehyde [IAAld]) were analyzed in a root growth assay of *Arabidopsis* among the IAA intermediates shown in Figure 1A (Soeno et al. 2010). Among the analyzed compounds, IPyA was the most effective for root growth recovery. However, other IAA intermediate compounds such as *N*-hydroxyl tryptamine (N-TAM) and IAOx remain to be assayed. In the auxin-marker gene assay, we performed 24-h treatment of AVG to examine recovery using six conjugated intermediates (indole, Trp, TAM, IAN, IAM, and IPyA), but AOPP remained to be analyzed, even though AOPP was shown to be a more specific inhibitor of IAA biosynthesis.

In this study, we synthesized N-TAM and IAOx as intermediate candidates of auxin biosynthesis. For preparation of the compounds, IAOx was synthesized as previously described (Sugawara et al. 2009), and synthesis of N-TAM was done as described in a previous report (Hino et al. 1990) with modifications (Fernandes et al. 2008). The purity of synthesized compound was confirmed by NMR spectroscopy (data not shown). The other chemicals analyzed were purchased from commercial sources, and IAAld was prepared as described in our previous report (Soeno et al. 2010). Then, AOPP was used to induce auxin deficiency in *Arabidopsis* seedlings to analyze the 11 potential intermediates shown in Figure 1, both in short-term (1 h) and long-term (4 days) auxin-related responses.

In the short-term assay, we analyzed the auxin-marker gene expression. Wild-type *Arabidopsis* (ecotype Columbia-0) seeds were sterilized and sown in half-strength Murashige–Skoog (1/2MS) liquid medium containing 1% sucrose, cultured for 7 days. The cultured seedlings were pretreated with 60 μM AOPP for 2 h, and then incubated with IAA or its putative 11 intermediate (10 μM) for 1 h. RNA isolation and qRT-PCR of *Aux/IAA1* and *Aux/IAA19* genes was conducted as described in our previous report (Soeno et al. 2010).

In the long-term root growth assay, we tested 5 compounds, cinnamic acid (CinA), indole-3-acrylic acid (IAcA), tryptophol (TOL), N-TAM, and IAOx, which were absent from the previous report. It has been reported that TOL is converted into IAA and is a side-branch product of IAAld in pea and *Pinus* (Quittenden et al. 2009; Sandberg 1984). It has been previously reported

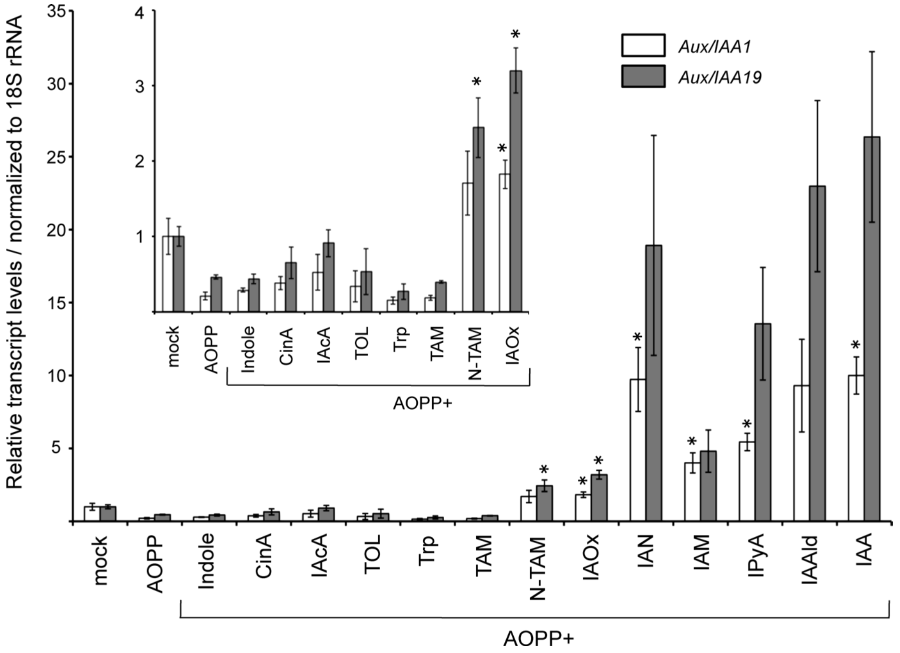

Figure 2. Recovery of the expression of *Aux/IAA* genes. Seven-day-old seedlings were pretreated with 60 *µ*M AOPP for 2 h, and IAA or its putative intermediates were then added and cultured for an additional 1 h. qRT-PCR for 18S ribosomal RNA (as endogenous control) and *Aux/IAA* genes was performed. Bars represent the means±SE ($n=3$). Statistical significance (Student's *t*-test $p<0.05$) compared to the AOPP treatment is indicated with asterisks.

that N-TAM is catalyzed from tryptamine (TAM) by YUCCA in vitro, thus YUCCA pathway was formerly proposed as the TAM pathway (Expósito-Rodríguez et al. 2007; Zhao et al. 2001). Recent reports showed that YUCCA mediates the conversion of IPyA to IAA in *Arabidopsis*; hence YUCCA pathway was amended as shown in Figure 1A (Mashiguchi et al. 2011; Won et al. 2011). CYP79B2 and B3 produce IAOx, and IAOx is converted into both IAN and IAM (Zhao et al. 2002). It has been reported that AOPP inhibits phenylalanine ammonia lyase (PAL), which converts phenylalanine into CinA (Amrhein and Gerhardt 1979). Therefore, we examined recovery by CinA from repression of *AUX/IAA* genes and root growth deficiency caused by AOPP, as a negative control, to confirm that the auxin defects are not due to its inhibition of PAL. IAcA is not proposed as an IAA intermediate. If *Arabidopsis* has an ammonia lyase that converts Trp to IAcA, IAcA could be an intermediate of IAA biosynthesis. We also measured recovery effect by IAcA as a potential intermediate of IAA biosynthesis. The recovery effect of the 5 compounds was analyzed by measuring root angle and root length of *Arabidopsis* seedlings. Wild-type *Arabidopsis* seeds were sterilized and grown on agar plates containing 1/2MS, 0.8% agar, and 1% sucrose for 4 days, and then the seedlings were transferred and grown for 4 days on agar plates containing 0.8% agar and 1% sucrose, supplemented with 50 *µ*M AOPP and various concentrations of putative IAA intermediates. The photo images were analyzed using ImageJ image-analysis software (NIH, Bethesda, MD, USA).

As a short-term analysis (1 h) of auxin intermediate activities, we measured expression of auxin-marker genes using qRT-PCR in 7-day-old *Arabidopsis* seedlings. Because auxin induces the expression of *Aux/IAA1* and *Aux/IAA19* within 1 h (Abel et al. 1995; Nakamura et al. 2003), *Aux/IAA1* and *Aux/IAA19* were selected as auxin-marker genes in short-term analysis. Since we analyzed auxin intermediates at 10 *µ*M in our previous report (Soeno et al. 2010), we applied the putative intermediate compounds at the same concentration. Expression of the *Aux/IAA1* and *Aux/IAA19* genes were repressed via 3-h treatment with 60 *µ*M AOPP (Figure 2). Application of indole, CinA, IAcA, TOL, Trp, and TAM (10 *µ*M, 1 h, with 2-h 60 *µ*M AOPP pretreatment) did not significantly alter expression levels of the *Aux/IAA* genes compared to AOPP treatment (Figure 2). In contrast, N-TAM, IAOx, IAN, IAM, IPyA, IAAld, and IAA treatment (10 *µ*M, 1 h, with 2-h 60 *µ*M AOPP pretreatment) induced expression of the *Aux/IAA* genes more than the mock treatment (relative transcription levels >1). Induction of *Aux/IAA1* was significant in plants treated with IAOx, IAN, IAM, IPyA, and IAA (10 *µ*M, 1 h, with 2-h 60 *µ*M AOPP pretreatment), as was *Aux/IAA19* in N-TAM and IAOx samples (10 *µ*M, 1 h, with 2-h 60 *µ*M AOPP pretreatment) compared to the AOPP treatment (60 *µ*M, 3 h, Student's *t*-test $p<0.05$; Figure 2).

For long-term (4 days) analysis, we estimated the recovery effect of the putative auxin intermediates by measuring the root angle and root length of *Arabidopsis*

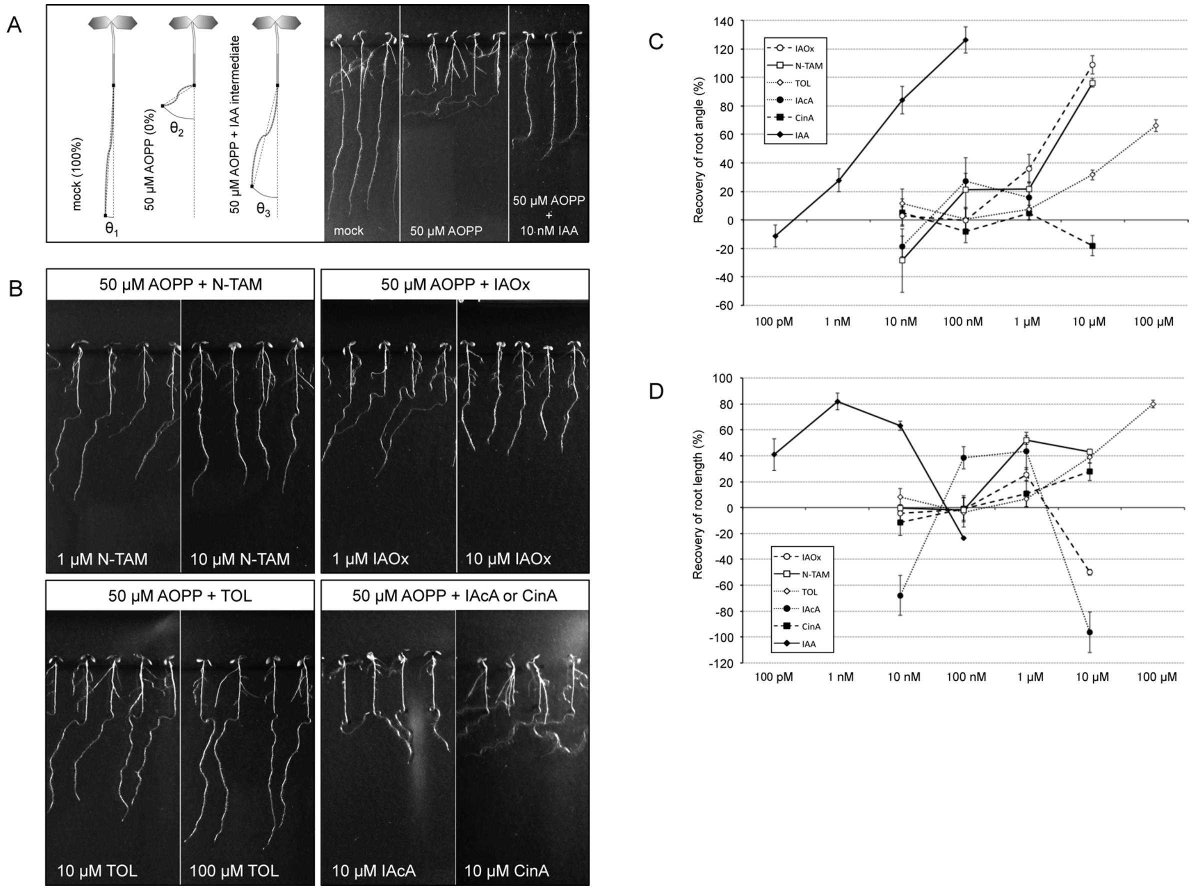

Figure 3. Recovery of root angle and length by putative IAA intermediates. (A) Scheme (left panel) and picture (right panel) of AOPP-induced root growth defects and recovery with IAA intermediates. Wild-type (Col-0) seedlings were grown on agar plates containing 1/2MS, 0.8% agar, and 1% sucrose (1/2MS agar) for 4 days, and then the seedlings were transferred and grown for 4 days on 1/2MS agar supplemented with 50 μM AOPP with or without IAA. (B) Root growth recovery with various IAA intermediates. Seedlings were grown as described in (A) but in the presence or absence of various concentrations of putative IAA intermediates. Dose-dependent recovery of root growth by putative IAA intermediates is analyzed for the angle (C) and length (D). Recovery of root angle was defined as $(\theta_2-\theta_3)/(\theta_2-\theta_1)\times 100$ (%) (A). Roots of mock-treated (0.1% dimethyl sulfoxide; DMSO) and AOPP-treated seedlings are configured as 100% and 0%, respectively. Data represent the means ±SE (n=15).

seedlings. In the presence of AOPP, roots of *Arabidopsis* seedlings were skewed to the left and elongation was inhibited (Figure 3A). The root skewing and inhibition of root elongation were recovered almost completely to the level of control plants in the presence of 1 nM and 10 nM IAA, respectively, as found in our previous study (Soeno et al. 2010; Figures 3A, 3C and 3D). Here, we analyzed, IAcA, TOL, N-TAM, and IAOx as candidate intermediates of the IAA biosynthesis pathway and CinA as a negative control. At 100 nM or less, the newly examined compounds showed no effective recovery for either root angle or elongation (less than 50% of mock samples; Figures 3C and 3D). At 1 μM, N-TAM did not recover the root angle but showed effective (>50%) recovery of inhibition of root elongation (Figures 3B, 3C and 3D). At this concentration, recovery by IAOx, IAcA, TOL, and CinA was not effective for either root angle or elongation (Figures 3C and 3D). At 10 μM, N-TAM and IAOx showed very effective (>75%) recovery for root angle (Figure 3C), but recovery of root length was less than 50% for N-TAM or was inhibited by IAOx treatment (Figure 3D). As for TOL and CinA, the recovery rate was less than 50% for both root angle and elongation (Figures 3C and 3D). We could not measure the recovery of root angle when seedlings were treated with 10 μM IAcA, because the roots were rotated (>360°; Figures 3B and 3C) and root elongation was inhibited (Figures 3B and 3D). At 100 μM, only TOL showed effective recovery for root angle and was very effective for recovery of inhibition of root elongation (Figures 3B, 3C and 3D). Because CinA, IAOx, N-TAM, and IAcA strongly inhibited root elongation at 100 μM, we could not determine the recovery effects.

In this study, we estimated the activities of putative auxin intermediates using a root growth assay and qRT-PCR of auxin-marker genes, to see whether these compounds could recover auxin deficiency in *Arabidopsis* seedlings. Because IAA recovers the inhibition of root elongation caused by AOPP, indole derivatives that recover auxin deficiency of plants were assumed

Table 1. Summary of recovery rates of analyzed compounds.

		IAA*	IPyA*	N-TAM	IAN*	IAAld*, IAOx	IAM*	Indole*	TOL	Trp*	TAM*	IAcA, CinA
Short term	*Aux/IAA*	effective						ineffective				
Long term	angle	++^{b}	++^{b}	++^{c}	++^{c}	++^{c}	−	++^{d}	+^{d}	+^{d}	−	−
	length	++^{a}	++^{a}	+^{b}	+^{c}	−	+^{c}	+^{c}	++^{d}	+^{c}	+^{c}	−

Recovery rate of root growth by the compounds are indicated as ++: >75%, +: >50%, −: less than 50%. The most effective concentrations for recovery are indicated with a: 100 nM or less (best), with b: 1 μM, with c: 10 μM or with d: 100 μM (worst). Results not included in this work for the long-term analysis were adapted from those presented in our previous report, which are indicated with asterisk (Soeno et al. 2010).

to be practical intermediates of the IAA biosynthesis pathway. This is the first such comprehensive analysis. We previously performed root recovery assays for IAAld, IPyA, IAN, IAM, Trp, and indole (Soeno et al. 2010). The results of that study are combined with those obtained in the present work and are summarized in Table 1. In short-term experiments, compounds could be classified into effective and ineffective groups. Because AOPP is assumed to inhibit auxin biosynthesis at the step from Trp to IPyA, intermediates downstream of IPyA in the IPyA pathway or intermediates of IPyA-independent pathways could recover the auxin response of seedlings having auxin deficiency. In contrast, the intermediates upstream of IPyA could not recover the seedlings. The ineffective group contained indole and Trp, and is consistent with our finding that the most probable site of AOPP action is conversion from Trp to IPyA (Soeno et al. 2010). Although in the experiments, we could not evaluate the stability of IPyA, taking the long-term and short-term auxin-related responses together, IPyA was as effective as IAA intermediate for auxin-deficient *Arabidopsis* seedlings grown in the presence of AOPP (Table 1).

It has been reported that YUCCA catalyzes the conversion of TAM to N-TAM *in vitro* (Zhao et al. 2001). TAM has been suggested to be a major intermediate of auxin biosynthesis in *Arabidopsis*. Sugawara et al. (2009) reported that ^{15}N-labeled TAM is incorporated into IAA in *Arabidopsis* seedlings cultured for 10 days, and Mashiguchi et al. (2011) and Won et al. (2011) showed that YUCCA utilizes IPyA as substrate rather than TAM for IAA biosynthesis. In our experiments, TAM was ineffective in short-term experiments (Figure 2) but recovered root elongation in long-term experiments (Soeno et al. 2010; Table 1). It remains to be studied whether or not feeding of N-TAM increases endogenous IAA content. Recently, Tivendale et al. (2010) reported that there is insufficient evidence to consider N-TAM an intermediate for IAA biosynthesis in pea. In contrast, in our experiments with *Arabidopsis*, N-TAM effectively recovered the root skewing (Figure 3C) and expression of the *Aux/IAA* genes (Figure 2), but was less effective for root elongation (Figure 3D and Table 1).

Although IAcA and TOL did not recover *Aux/IAA* gene repression caused by AOPP in the short-term experiment, their recovery effects for root growth defects in the long-term experiment differed. Recovery effects with TOL were rather similar to those with indole. Both TOL and indole do not recover the *Aux/IAA* genes in the short-term experiment, but effectively recovered root growth defects in the long-term experiment (Soeno et al. 2010; Figures 3C and 3D), probably because TOL is converted to IAA via some minor pathway in the biosynthesis pathway. Although no gene related to the conversion of TOL into IAA has been identified, it is presumed that TOL is converted into IAAld in the plant, and consequently converted into IAA (Quittenden et al. 2009). However, because IAcA did not recover either *Aux/IAA* gene repression (Figure 2) or root growth defects (Figure 3C and 3D), IAcA is likely the least reliable intermediate in *Arabidopsis* among the indole derivatives examined.

It has been reported that IAOx is produced by CYP79B2 and B3 as another major intermediate in the auxin biosynthesis pathway, and *cyp79b2cyp79b3* double mutants show growth defects at high temperature by causing auxin deficiency (Sugawara et al, 2009; Zhao et al, 2002). In our results, IAOx recovered expression levels of the *Aux/IAA* genes (Figure 2), and also recovered root skewing (Figure 3C). However, it was less effective in the root elongation assay (Figure 3D) when compared to the results for Trp and indole (Soeno et al. 2010). From these observations, it still remains controversial whether or not IAOx is a major intermediate of auxin biosynthesis.

In both the short-term and long-term assays, IAN treatment resulted in recovery of auxin deficiency and the effects were similar to those of N-TAM and IPyA (Table 1). We should note that IAN did not recover the expression of *Aux/IAA* genes in plants treated with AVG in our previous study, but did so in plants treated with AOPP (Figure 2). There were several differences among the experimental conditions of the two studies, including the duration of auxin or inhibitor treatment; therefore, the two experiments cannot be directly compared. Further studies are required to confirm our results.

Among the indole derivatives analyzed as putative IAA intermediates, each of Trp, TAM, IAOx, IAM, IAN, IPyA and IAAld was detected in *Arabidopsis* (Novák et al. 2012). Our report has shown that indole, N-TAM and TOL recovered auxin deficiency (Figure 3C and D; Soeno et al. 2010). In contrast, detection of indole, N-TAM and TOL in *Arabidopsis* has not been reported. Consequently, it is important to analyze endogenous content of indole, N-TAM and TOL in *Arabidopsis*.

In summary, the results of the auxin-responsive genes and root recovery assays performed in the present study provide information on which compounds are reliable intermediates of IAA biosynthesis pathways in *Arabidopsis*.

Acknowledgments

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