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REVIEW

Chemical Biology in the Auxin Biosynthesis Pathway via Indole-3-Pyruvic Acid

Kazuo SOENO[1]*, Akiko SATO[2] and Yukihisa SHIMADA[2]

[1] Western Region Agricultural Research Center, National Agriculture and Food Research Organization, Zentsuji, Japan
[2] Yokohama City University, Kihara Institute for Biological Research, Yokohama, Japan

Abstract

Auxins are plant hormones that play key roles in almost all growth and developmental processes, such as cell division, elongation, differentiation, and environmental responses. However, biosynthetic pathways and regulatory mechanisms remain unclear. The indole-3-pyruvic acid (IPyA) pathway, from L-tryptophan (Trp) via IPyA, is the main biosynthetic pathway of the natural auxin indole-3-acetic acid (IAA). In this pathway, IAA is biosynthesized from Trp through two enzymatic reactions: aminotransferase (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 [TAA1]/ TRYPTOPHAN AMINOTRANSFERASE-RELATED [TARs]) and YUCCAs (YUCs), which are flavin-containing monooxygenases. We developed the inhibitors of TAA1/TARs and YUC and analyzed the physiological functions of the IPyA pathway using the biosynthesis inhibitors as chemical probes. This paper also describes the regulatory mechanism of the two-step enzymatic reactions in auxin biosynthesis, employing the novel IPyA analog compounds.

Discipline: Biotechnology
Additional key words: auxin, biosynthesis inhibitor, regulation of auxin biosynthesis, TAA1/TARs, YUCCA (YUC)

Introduction

Auxins are plant hormones that play key roles in regulating plant growth and development, including cell division, elongation, differentiation, and environmental responses (Teale et al. 2006, Woodward & Bartel 2005). Thus, auxin analogs have been used as agrochemicals in various applications, including herbicides, rooting compounds, and fruit set promotion, drop protection, and thinning. indole-3-acetic acid (IAA) is the most abundant, natural auxin. It was thought to be difficult to completely inhibit the biosynthesis of IAA with a single compound because of the complementary existence of the multibranched biosynthesis pathways.

In *Arabidopsis* plants, two types of IAA biosynthesis pathways had been proposed: one route is L-tryptophan (Trp) dependent, and the other is Trp independent (Fig. 1), comprising multiple pathways, such as the indole-3-pyruvic acid (IPyA), tryptamine (TAM), and indole-3-acetamide (IAM) pathways (Zhao 2010). In addition, the indole-3-acetaldoxime (IAOx) pathway is restricted to Brassicaceae plants (Sugawara et al. 2009). On the basis of the studies of biosynthesis-deficient *Arabidopsis* mutants, the IPyA pathway has been proposed as the main IAA biosynthesis pathway (Mashiguchi et al. 2011, Won et al. 2011).

In the IPyA pathway, IAA is biosynthesized from Trp in a two-step enzymatic reaction. The first enzymes are TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) and its close homologs, namely, TRYPTOPHAN AMINOTRANSFERASE-RELATED1 (TAR1) and 2 (TAR2), which catalyze the conversion of Trp to IPyA (Stepanova et al. 2008, Tao et al. 2008, Yamada et al. 2009, Zhou et al. 2011). The second step

*Corresponding author: soeno@afffrc.go.jp
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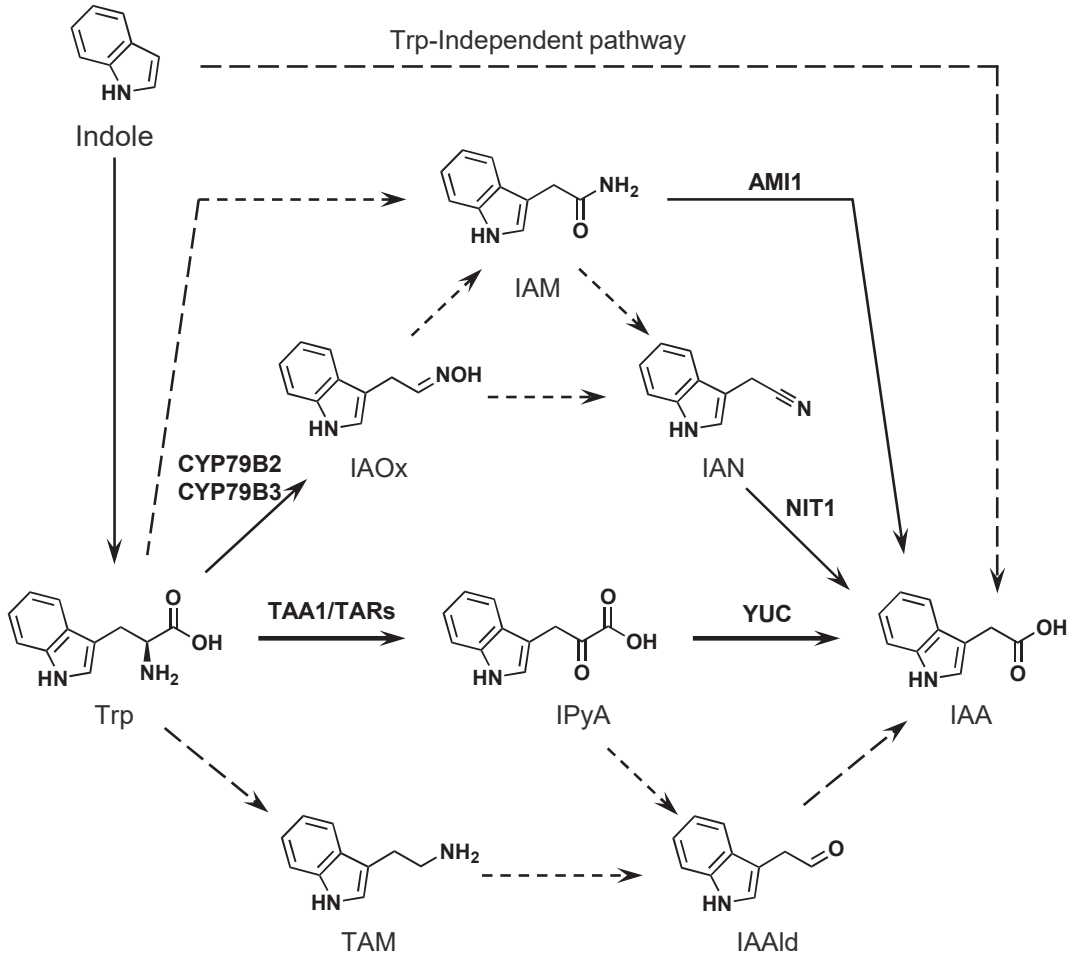

Fig. 1. Auxin biosynthesis pathways

Auxin (IAA) biosynthesis pathways with proposed enzymes and intermediates.

Plain text indicates intermediates: Trp, L-tryptophan; IPyA, indole-3-pyruvic acid; IAA, indole-3-acetic acid; IAM, indole-3-acetamide; IAOx indole-3-acetaldoxime; IAN, indole-3-acetonitrile; TAM, tryptamine; IAAld, indole-3-acetaldehyde.

Bold text indicates enzymes: TAA1, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1; TARs, TRYPTOPHAN AMINOTRANSFERASE RELATED; YUC, YUCCA, flavin-containing monooxygenase; AMI1, AMIDASE1; NIT, nitrilase; CYP79B2/B3, Cytochrome P450 79B2/B3.

enzymes are YUCCAs, which are flavin-containing monooxygenases (YUCs) that convert IPyA to IAA (Mashiguchi et al. 2011, Won et al. 2011).

To assess the regulatory mechanism of auxin biosynthesis and its physiological impacts by the chemical biology approach, we have developed auxin biosynthesis inhibitors targeting the IPyA pathway (Fig. 2, Table 1; Higashide et al. 2014, Kakei et al. 2015, Kakei et al. 2017, Narukawa-Nara et al. 2016, Sato et al. 2022, Soeno et al. 2010, Watanabe et al. 2021). This paper presents the results of our research to date. Using the chemical tools of the IPyA pathway, we herein demonstrate a new regulatory mechanism of TAA1/TAR enzyme reaction regarding the control of IAA synthesis in the IPyA pathway.

Discovery of auxin biosynthesis inhibitors and development of TAA1/TARs-specific inhibitors

The analysis of the *Arabidopsis* DNA microarray data revealed that the gene expression profiles in *Arabidopsis* seedlings treated with L-α-aminoethoxyvinylglycine (AVG) had a strong negative correlation with the expression profiles of auxin-treated seedlings (Fig. 3, Soeno et al. 2010). AVG is known to inhibit aminocyclopropane-1-carboxylic acid synthase (ACC synthase; ACS), a pyridoxal phosphate (PLP)-dependent enzyme in the ethylene biosynthesis pathway.

This suggests that AVG inhibits enzymes involved in PLP-dependent enzymes in the auxin biosynthesis pathway. Therefore, we screened candidate compounds for inhibitors of PLP-dependent enzymes, and we successfully identified L-aminooxyphenylpropionic acid (AOPP) as a candidate auxin biosynthesis inhibitor. *Arabidopsis* seedlings treated with AVG or AOPP showed reduced endogenous IAA levels in a dose-dependent manner, and AOPP inhibited primary root elongation in the *Arabidopsis* seedlings. AVG and AOPP also reduced endogenous IAA levels in the roots of rice and tomato seedlings. In an *in vitro* assay system using crude enzyme extracts of *Arabidopsis* and wheat, AVG and AOPP inhibited the conversion of Trp to IPyA in a dose-dependent manner. These results confirm that AVG and AOPP inhibit the aminotransferase step in the

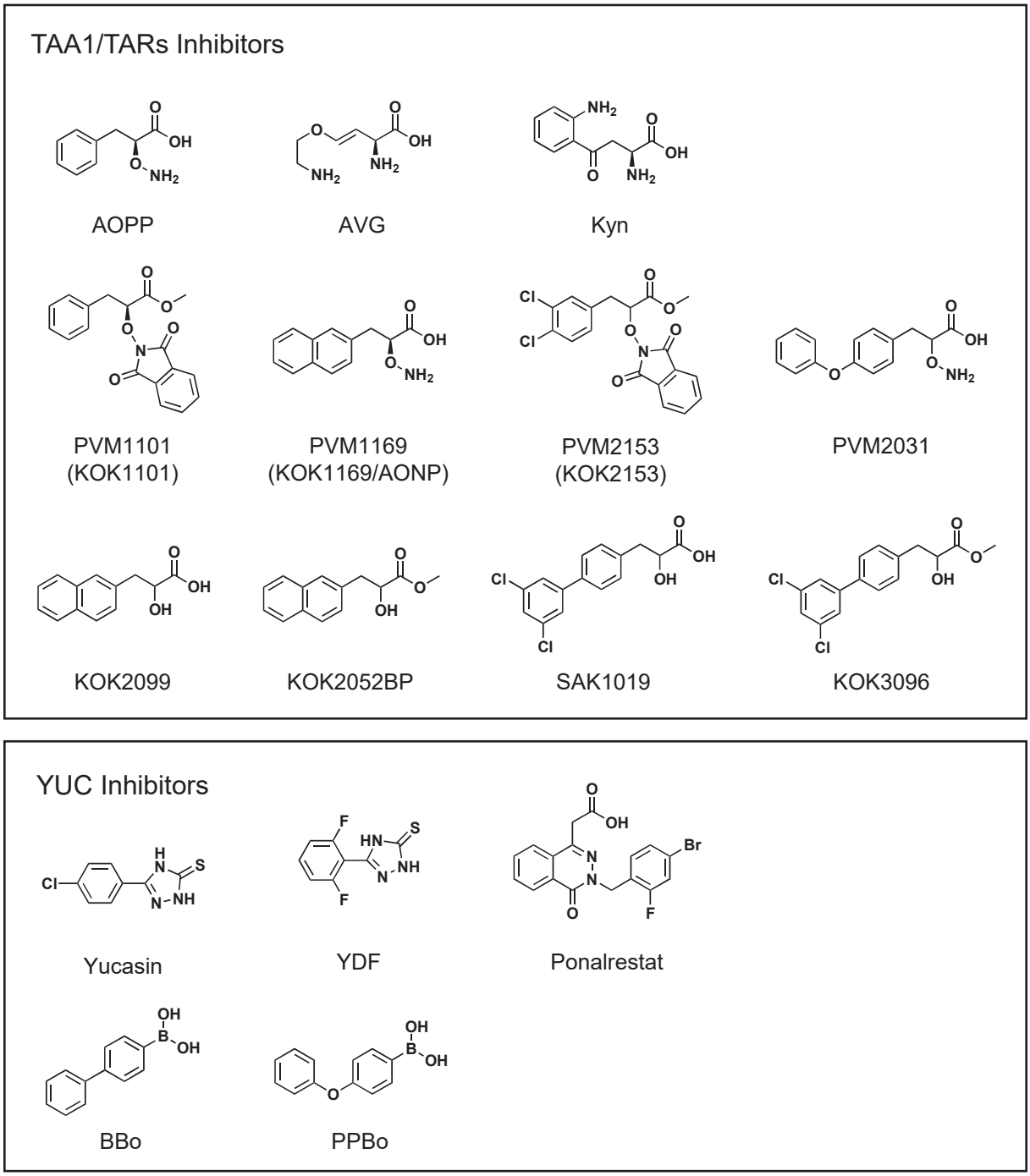

Fig. 2. Structures of biosynthesis inhibitors in the IPyA pathway of auxin biosynthesis

Table 1. List of biosynthesis inhibitors in the auxin IPyA biosynthesis pathway

Compound name	CAS registry number[a]	Target Protein(s)	Mode of action[b]	K_i value	Reference	Commercial source[c]	Application in auxin related study
AOPP	42990-62-5	TAA1/ TARs	CI	350 n mol/L (TAA1) 3.9 n mol/L (AtPAL2) 848 n mol/L. (AtACS8)	Soeno K. et al. (2010)	A	Miki S. et al. (2015) Tamaki H. et al. (2015) Xing X. et al. (2016) Ishibashi M. et al. (2017) Li Z. et al. (2018) Zhang C. et al. (2018) Pan R. et al. (2020) Singh R.R. et al. (2020) Yan L. et al. (2021) Wang Y. et al. (2022)[d]
AVG	49669-74-1	TAA1/ TARs	CI	8.6 μ mol/L (TAA1) N.D. (AtPAL2) 37.6 nmol/L (AtACS8)	Soeno K. et al. (2010)	A	L.C. et al. (2010) Yin C. et al. (2010) Tsang D.L. et al., (2011) Du H. et al. (2012) Lemaire L. et al. (2013) Böttcher C. et al. (2013) Zheng Q. et al. (2013) Bhattacharyyaa D. et al. (2015) Wang Z. et al. (2015) Tsugama D. et al. (2016) Xing X. et al. (2016) Bhattacharyyaa D. & Lee Y.H., (2017) Cunha C.P. et al. (2017) Li X. et al. (2017) Park S-H. et al. (2017) Malheiros R.S.P. et al. (2019) Schuetz M. et al. (2019) Talosawa et al. (2019) Cui S. et al., (2020)
Kyn	2922-83-0	TAA1/ TARs	AS/CI	11.52 μ mol/L (TAA1)	He W. et al. (2011)	A	Nishimura, T. et al. (2014) Suzuki M. et al. (2015) Inaji A. et al. (2020) Koike I. et al. (2020) Xi et al. (2021) Ishida S. et al. (2022) Ohbayashi I. et al. (2022)
PVM1101 (KOK1101)	1394950-57-2	TAA1/ TARs	CI		Higashide, T. et al. (2014)	N	
PVM1169 (KOK1169)/AONP	1394949-95-1	TAA1/ TARs	CI	76.8 n mol/L (TAA1)	Narukawa-Nara M. et.al. (2016)	B	
PVM 2153 (KOK2153)	1394950-62-9	TAA1/ TARs	CI		Narukawa-Nara M. et.al. (2016)	N	Du M. et al., (2022)[d] Tillmann M. et al. (2021) Tillmann M. et al. (2022)
PVM 2031	1394950-16-3	TAA1/ TARs	CI	276 n mol/L (OsTAR1)	Kakei Y. et al. (2017)	N	
KOK2099	917247-86-0	TAA1/ TARs	CI		Sato A. et al. (2022)	B	
KOK2052BP	1365548-59-9	TAA1/ TARs	CI		Sato A. et al. (2022)	B	
SAK1019	unregistered	TAA1/ TARs	CI		Sato A. et al. (2022)	N	
KOK3096	1615702-65-2	TAA1/ TARs	CI		Sato A. et al. (2022)	N	
Yucasin	26028-65-9	YUC	CI		Nishimura, T. et al. (2014)	A	Ohtaka K. et al. (2017) Ishida S. et al. (2022)
BBo	5122-94-1	YUC	CI	67 n mol/L (AtYUC2)	Kakei Y. et al. (2015)	A	Ohtaka K. et al. (2017) Kaneko S. et al. (2020) Lin W-J. et al. (2020)

(Continued on next page)

Table 1. List of biosynthesis inhibitors in the auxin IPyA biosynthesis pathway (Continued)

PPBo	51067-38-0	YUC	CI	57 n mol/L (AtYUC2)	Kakei Y. et al. (2015)	A	Hirano K. et al. (2017) Michaud O. et al. (2017) Ohtaka K. et al. (2017) Demecsová L. et al. (2020) Inaji A. et al. (2020) Koike I. et al. (2020) Jia Z. et al. (2021) Ohishi N. et al. (2021) Watanabe M. et al. (2021) Xi Y. et al. (2021) Ishida S. et al. (2022) Ohbayashi I. et al. (2022)
YDF	1094690-87-5	YUC	CI		Tsugafune S. et al. (2017)	B	Tillmann M. et al. (2022)
Ponalrestat	72702-95-5	YUC	SA		Zhu Y. et al. (2019)	B	

[a]The CAS registration number (CAS RN®) is a unique identification number assigned by the Chemical Abstracts Service to individual chemical substances used worldwide. Registration number as of February 2023.

[b]Type of mode of action; AS:alternate substrate, CI: competitive inhibitor, SA: substrate antagonist.

[c]As of February 2023 on the CAS search service SciFinder[n], (A) is commercially available from several suppliers, (B) is commercially available from a few suppliers, and (N) is not commercially available.

[d]Applicated in supplementary.

Compound name: AVG, L-α-aminoethoxyvinylglycine; AOPP, L-aminooxyphenylpropionic acid; Kyn, L-kynurenine ; BBo, 4-biphenylboronic acid; PPBo, 4-phenoxyphenylboronic acid; YDF, Yucasin DF.

Protein name: TAA1, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1; TAR, TRYPTOPHAN AMINOTRANSFERASE-RELATEDs; YUC, flavin-containing monooxygenases (YUCCA); AtPAL2, phenylalanine ammonia lyase 2 from *Arabidopsis thaliana*; AtACS8, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) 8 from *Arabidopsis thaliana*; OsTAR1, rice TRYPTOPHAN AMINOTRANSFERASE-RELATED1; AtYUC2, YUC 2 from *Arabidopsis thaliana*.

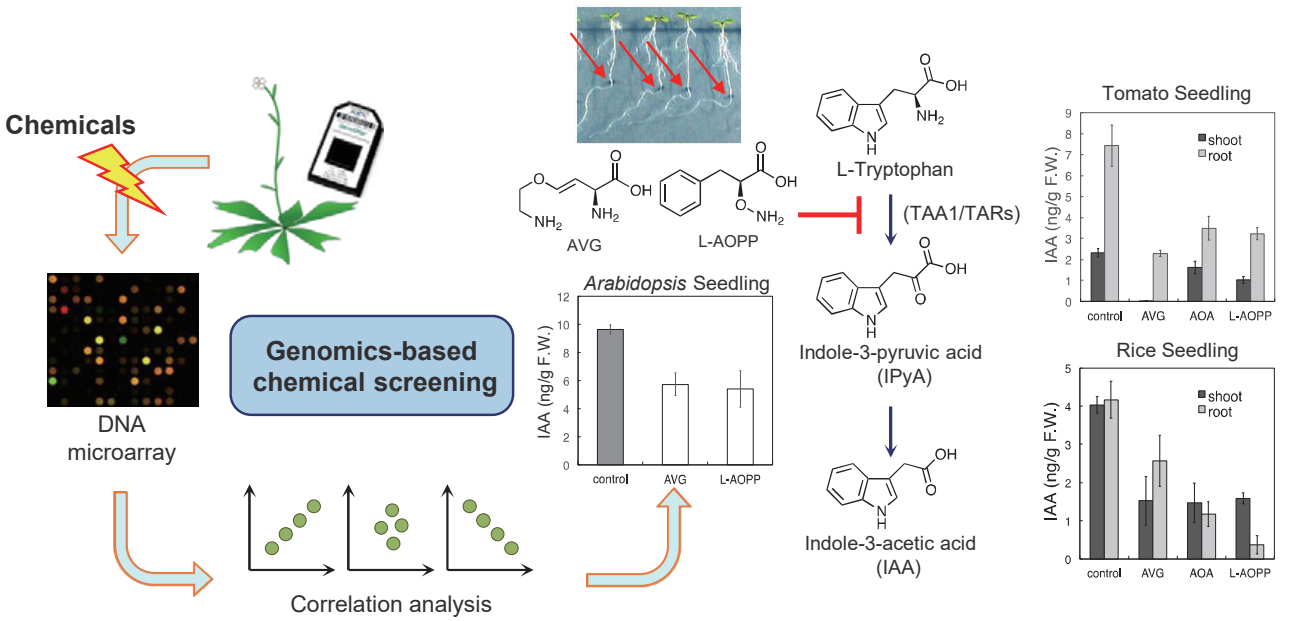

Fig. 3. Scheme of the discovery of the first auxin biosynthesis inhibitors, AVG and AOPP, through genomic approaches

IPyA pathway. AVG and AOPP have been used as ACS inhibitors in ethylene biosynthesis and phenylalanine (Phe) ammonia lyase (PAL), respectively (Fig. 4), and their specificities for TAA1/TARs were low. The inhibition constant (K_i) of AVG for *Arabidopsis* TAA1 was 229-fold higher than that of AVG for *Arabidopsis* ACS, and that of AOPP for TAA1 was 90-fold higher than that of AOPP for *Arabidopsis* PAL (Table 1; Narukawa-Nara et al. 2016).

These side effects are problematic for their use as specific auxin biosynthesis inhibitors. Therefore, novel AOPP derivatives were designed and synthesized, and

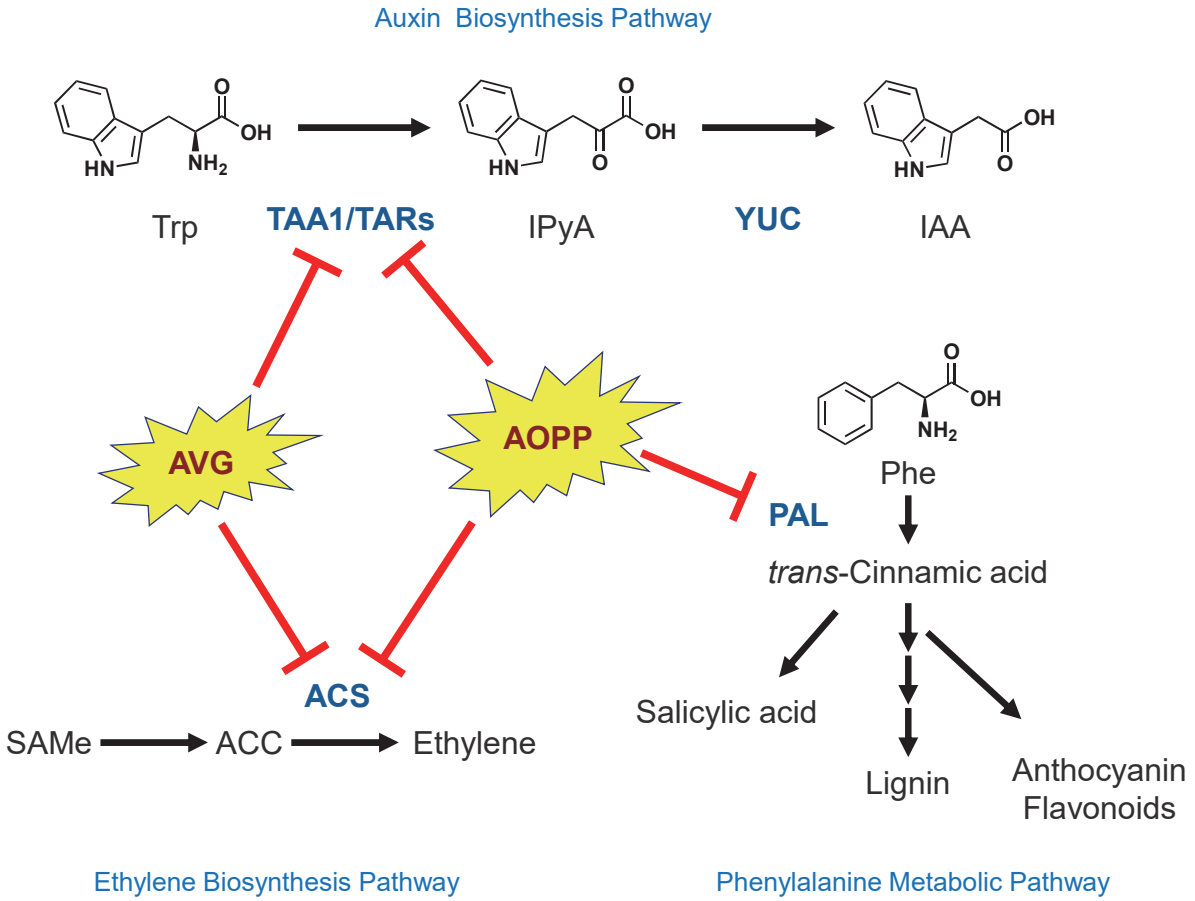

Fig. 4. AVG and AOPP are known as inhibitors of PLP-dependent enzymes (ACS/PAL)
Blue text indicates enzymes: ACS, ACC synthase; PAL, phenylalanine ammonia lyase.
Black text indicates intermediates: Phe, phenylalanine; SAMe, S-adenosylmethionine; ACC, aminocyclopropane-1-carboxylic acid.
Dark red text indicates inhibitors: AVG, aminoethoxyvinylglycine; AOPP, L -aminooxyphenylpropionic acid.
Red lines indicate inhibition.

the specific inhibitory activities of AOPP analogs on TAA1 were examined. The newly developed compounds, "pyruvamine (PVM)," are a class of specific inhibitors of TAA1/TARs in the IPyA pathway, and PVM has an aminooxy group and its derivatives in the side chain. Among them, 2-aminooxy-3-naphthalene-2-yl-propanoic acid (PVM1169/AONP; the name in the cited reference is KOK1169; Fig. 2) had a lower K_i value for TAA1 in *Arabidopsis in vitro* but a higher K_i value for PAL or ACS than AOPP, indicating that PVM1169 is a more specific inhibitor of auxin biosynthesis than AOPP *in vitro* (Table 1). In *Arabidopsis*, PVM1169 reduced endogenous IAA, with no effect on Phe accumulation compared with AOPP and a lesser effect on ethylene production compared with AVG. However, when the inhibitory activities of PVM compounds were analyzed in recombinant enzyme reactions of a rice TAR homolog, OsTAR1, the compound 2-aminooxy-3-(4-phenoxyphenyl)-propanoic acid (PVM2031; Fig. 2, Table 1) showed higher inhibitory activity than PVM1169 (Kakei et al. 2017). PVM2031-treated rice seedlings exhibited reduced seminal root length and lateral root density, which recovered by exogenous IAA treatment. PVM2031 reduced endogenous IPyA and IAA contents in rice root. These results indicate that the optimal structure of TAA1/TARs inhibitors depends on the enzyme structure of each plant species. In addition to PVM, L-kynurenine (Kyn; Fig. 2) has been found in commercial chemical libraries as a TAA1/TARs inhibitor (He et al. 2011), with a high K_i value against TAA1 (Table 1).

Development of YUC-specific inhibitors

In the second step of the IPyA pathway, YUCs catalyze the conversion of IPyA to IAA. Two types of

YUC inhibitors have been identified to date. Yucasin was identified as a competitive inhibitor through commercial chemical library screen maize coleoptiles by other research groups (Fig. 2, Table 1; Nishimura et al. 2014). Yucasin restored the phenotype of *Arabidopsis YUC* overexpression mutants, but it did not induce auxin-deficient phenotypes in the wild types. Therefore, on the basis of the structure–activity relationships, yucasin DF (YDF) was identified as a potent YUC inhibitor (Fig. 2, Table 1; Tsugafune et al. 2017). Moreover, ponalrestat, a known aldose reductase inhibitor, has been reported as a YUC substrate antagonist (Fig. 2, Table 1; Zhu et al. 2019).

We also found that phenylboronic acid was a lead compound of YUC inhibitor using *Arabidopsis* YUC2 recombinant enzyme, and 4-biphenylboronic acid (BBo) and 4-phenoxyphenylboronic acid (PPBo) were selected as YUC-specific inhibitors by screening using *YUC1* overexpressing transgenic plants (Fig. 5; Kakei et al. 2015). BBo and PPBo treatment of wild-type *Arabidopsis* seedlings reduced endogenous IAA content and induced auxin-deficient phenotypes, including the inhibition of primary root elongation and lateral root formation. Exogenous IAA restored auxin-deficient phenotypes caused by these boronic acids. These compounds inhibited recombinant YUC activity *in vitro* as competitive inhibitors of the substrate IPyA, and the K_i values of BBo and PPBo were 67 and 56 nmol/L, respectively (Table 1). BBo and PPBo also inhibited growth and reduced endogenous IAA content in the monocot *Brachypodium* model plant (Kakei et al. 2015) and rice (Watanabe et al. 2021).

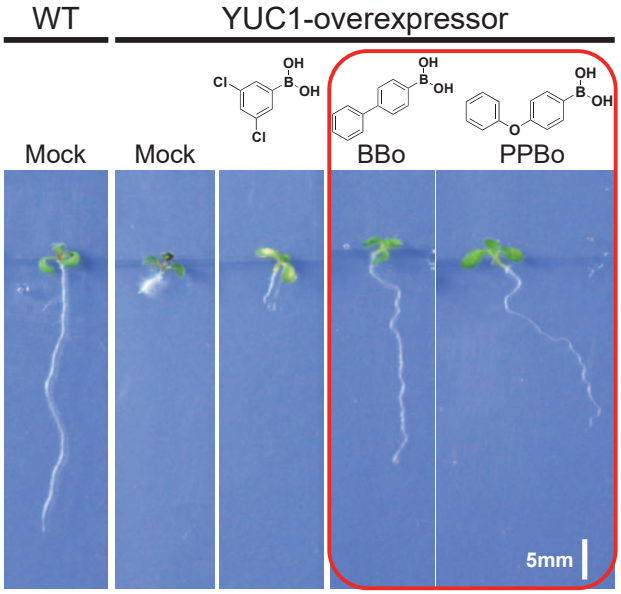

Fig. 5. Screening of YUC inhibitors

Arabidopsis YUC1-overexpressing transgenic plants were placed on 1/2 MS agar medium containing YUC inhibitor candidate compounds and cultured for seven days. On the far left is the wild-type of *Arabidopsis* (no inhibitor treatment; Mock). The untreated *YUC1*-overexpressing transgenic plant (second from left; Mock) shows an auxin-overexpressing phenotype due to IAA overproduction; but when grown on medium containing BBo and PPBo, IAA overproduction is suppressed and the phenotype is rescued.
Scale bar: 5 mm (Kakei et al. 2015).

Effects of IPyA analogs on the regulation of TAA1/TARs activity

IAA levels are regulated by the biosynthesis and catabolism of IAA. Transcriptional and enzymatic regulation has been reported in the IAA catabolic pathway. For the regulation of IAA biosynthesis, the YUC enzyme catalyzes the rate-limiting step of the IPyA pathway (Zhao 2014). Transcriptional feedback regulation of *YUC* genes in response to endogenous auxin level has been reported, but *TAA1*/*TARs* genes are less regulated in this manner (Suzuki et al. 2015, Takato et al. 2017). Given that IPyA is unstable and can be degraded nonenzymatically to other compounds, including IAA, the overaccumulation of IPyA may increase the IAA levels in plants. Therefore, IPyA accumulation in plants must be prevented. However, in vigorously growing organs involving fruit development and seed germination, a sufficient amount of IPyA is required because a large amount of IAA is synthesized to support rapid growth in these developmental stages. It remains unclear how plants ensure a sufficient IPyA quantity while maintaining it at a low level. In addition, the overexpression of the *TAA1* gene in transgenic plants does not induce an overaccumulation of IAA (Mashiguchi et al. 2011, Stepanova et al. 2008, Tao et al. 2008), but overexpression of *YUC* genes results in the overproduction of IAA (Zhao 2014).

The regulatory mechanism of the two-step enzymatic reaction (TAA/TARs and YUC), which is the main auxin biosynthesis pathway (Sato et al. 2022), was addressed. On the basis of the previous findings, it was expected that IPyA itself, a product of TAA1/TAR, has some regulatory function in auxin biosynthesis. Therefore, IPyA analogs (KOK2099 and KOK2052BP) were developed, and their activities were investigated. These analogs inhibited the TAA1 enzyme activity competitively. Further analysis of the sequential reaction of TAA1 and YUC with IPyA and IPyA analogs showed that TAA1 was negatively regulated by its product, IPyA. In addition, TAA1 had reverse enzymatic activity

to convert IPyA to Trp (Fig. 6). IPyA and its analogs also inhibited TAA1/TARs enzymes from rice and tomato, indicating that the negative feedback regulation of IAA biosynthesis by IPyA is a common regulatory mechanism of auxin biosynthesis in plants. Thus, the negative feedback regulation of TAA1/TARs activity by IPyA is achieved by the reversibility of Trp aminotransferase activity and competitive inhibition of TAA1/TARs enzyme activity by IPyA. The K_m values of IPyA and Trp for the TAA1 enzyme were 0.7 and 43.6 µmol/L, respectively. These enzyme kinetic data indicated that IPyA is maintained at low levels *in vivo* by the reverse reaction (IPyA to Trp) of TAA1/TARs. Appropriate IPyA levels are maintained by the push

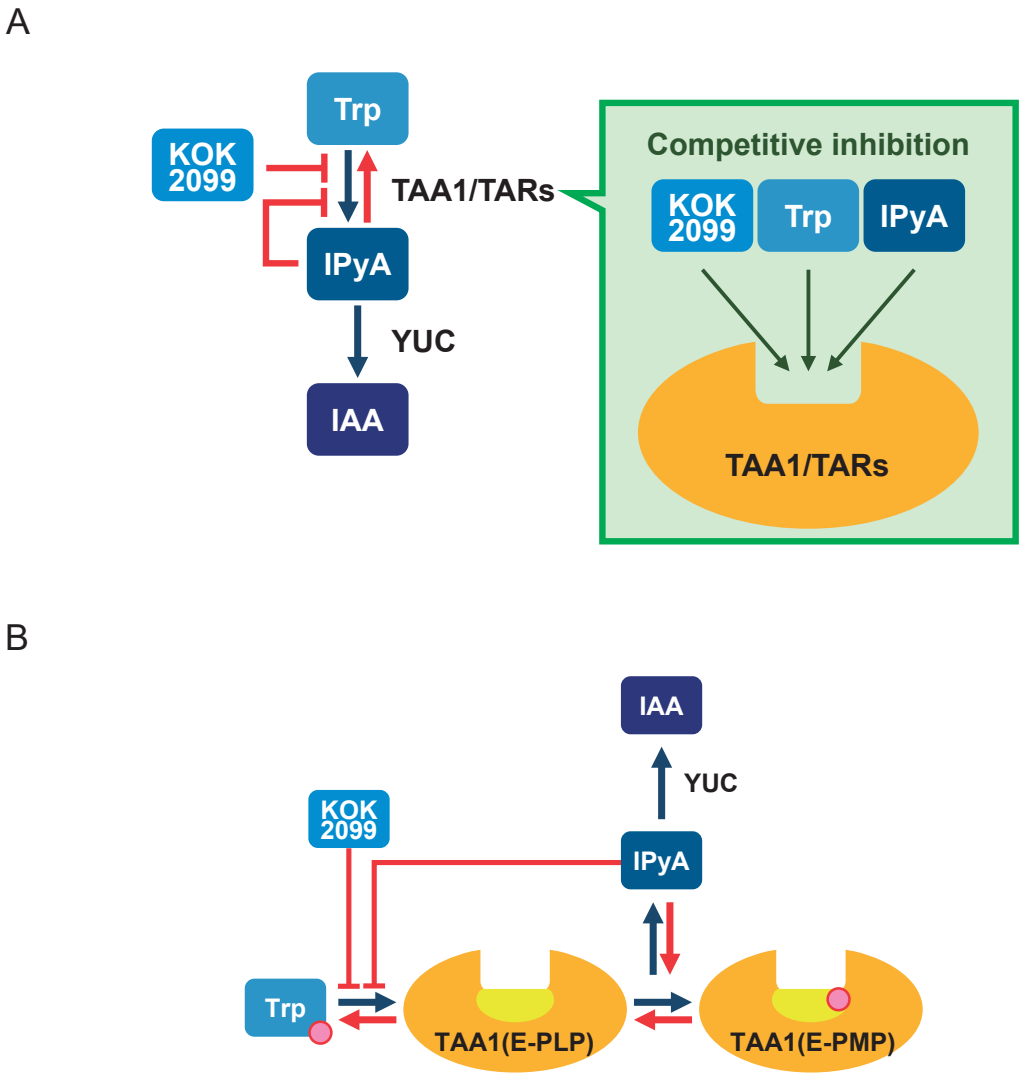

Fig. 6. Regulatory mechanism of a two-step auxin biosynthesis pathway and its inhibitors

(a) IAA is biosynthesized from Trp via IPyA in a two-step enzymatic reaction (TAA1/TARs and YUC). TAA1/TARs exhibit reverse reaction activity to convert IPyA to Trp, whereas IPyA and KOK2099 act as competitive inhibitors of Trp. Appropriate IPyA levels are maintained by the push (TAA1/TARs) and pull (YUC) of the two biosynthesis enzymes, in which TAA1 plays a key role in preventing the over- or underaccumulation of IPyA as coordinating the two steps of auxin biosynthesis.

(b) Scheme of aminotransferase reaction of TAA1. Aminotransferases perform two reactions; amino groups (pink circle) from amino acids and converting them to 2-oxoacids, and transferring amino groups in the enzyme to 2-oxoacids and converting them to amino acids. TAA1 (internal aldimine; E-PLP) removes the Trp amino group and converts them to IPyA and TAA1 (pyridoxamine phosphate; E-PMP). Given that this reaction is reversible and occurs at a common site on the enzyme molecule, IPyA and its analogs act as competitive inhibitors against Trp.

(TAA1/TARs) and pull (YUC) of the two biosynthetic enzymes, in which TAA1 plays a key role in preventing the over- or underaccumulation of IPyA to coordinate the two steps of auxin biosynthesis.

Future for practical use in agricultural fields

In addition to the auxin biosynthesis inhibitors, small-molecule compounds that inhibit auxin transport and signal transduction are widely utilized as chemical probes in auxin biology (Hayashi 2021). As shown in Table 1, inhibitors targeting the IPyA pathway have been widely used to elucidate auxin actions.

In the test for practical use in the agricultural field, treatment of tomato seedlings grown in pots with KOK1101, a PVM compound, showed inhibitory activity regarding aboveground dry matter weight, stem length, leaf area, and aboveground dry matter content in field application studies (Fig. 2, Table 1; Higashide et al. 2014). However, its use as a practical plant growth regulator requires further investigation. For the practical utilization of these auxin chemical probes in agricultural fields, further structural optimization and derivatization of lead chemical probes would be essential to improve the chemical and metabolic stability and the membrane permeability of lead compound. Establishing a technology to control auxin action using chemical probes may lead to the development of highly selective and/or low-dose herbicides, which will also be useful in developing agricultural technologies to achieve high yields and/or high-quality agricultural products.

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