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Role of Arabidopsis INDOLE-3-ACETIC ACID CARBOXYL METHYLTRANSFERASE 1 in auxin metabolism

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Abstract

The phytohormone auxin regulates a wide range of developmental processes in plants. Indole-3-acetic acid (IAA) is the main auxin that moves in a polar manner and forms concentration gradients, whereas phenylacetic acid (PAA), another natural auxin, does not exhibit polar movement. Although these auxins occur widely in plants, the differences between IAA and PAA metabolism remain largely unknown. In this study, we investigated the role of Arabidopsis IAA CARBOXYL METHYLTRANSFERASE 1 (IAMT1) in IAA and PAA metabolism. IAMT1 proteins expressed in *Escherichia coli* could convert both IAA and PAA to their respective methyl esters. Overexpression of *IAMT1* caused severe auxin-deficient phenotypes and reduced the levels of IAA, but not PAA, in the root tips of Arabidopsis, suggesting that IAMT1 exclusively metabolizes IAA *in vivo*. We generated *iamt1* null mutants via CRISPR/Cas9-mediated genome editing and found that the single knockout mutants had normal auxin levels and did not exhibit visibly altered phenotypes. These results suggest that other proteins, namely the IAMT1 homologs

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Author contributions

E.T. and H.K. conceived and designed research. E.T. and S.H. performed most of the research. M.K. performed GC-MS analysis. E.T. and Y.A. performed enzyme activity test. C.G., X.D., and Y.Z. generated *iamt1-c* mutants. K.H. generated *IAMT1ox* plants. K.F. and K.H. synthesized labeled compounds. E.T. and H.K. analyzed data and wrote the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

in the SABATH family of carboxyl methyltransferases, may also regulate IAA levels in Arabidopsis.

Keywords

auxin; indole-3-acetic acid; metabolism; methyltransferase; phenylacetic acid

1. Introduction

Auxin plays important roles in regulating plant development and environmental responses, including embryogenesis, organ formation, apical dominance, and tropism [1]. These regulations depend on the cellular concentration of auxin in specific tissues, which is determined by biosynthesis, inactivation, and transport, along with signal transduction [2]. Indole-3-acetic acid (IAA) is the most studied naturally occurring auxin, and its regulatory mechanism in plants has been elucidated. IAA is known to be directionally transported from cell to cell through membrane-localized proteins. Among membrane-localized proteins, PIN-FORMED efflux carrier proteins play a crucial role in polar auxin transport and form auxin concentration gradients [3]. In plants, the main biosynthesis pathway converts tryptophan to IAA via indole-3-pyruvate, involving TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) families [4–8]. Plants have various IAA inactivation pathways, including oxidation by DIOXYGENASE FOR AUXIN OXIDATION (DAO), amino acid conjugation by the GRETCHEN HAGEN 3 (GH3) family, glucosylation by UDP-GLUCOSYLTRANSFERASE 84B1 (UGT84B1), and methylation by IAA CARBOXYL METHYLTRANSFERASE 1 (IAMT1) [1,9]. Some inactivated forms, *e.g.*, IAA-Ala and -Leu, IAA-glucoside, and IAA-methyl ester (MeIAA) are thought to be storage forms with the potential to be reverted back into IAA for homeostatic regulation [10–12]. Among IAA metabolic enzymes, previous studies demonstrated the physiological importance of IAMT1 in plant growth and physiological responses to environmental cues [13–16]. Qin *et al.* (2005) demonstrated that the RNAi silencing mutants *iamt1* (*iamt1-RNAi*) showed high-auxin phenotypes. In contrast, the T-DNA insertion mutants of *iamt1* (*iamt1–1*, salk_072125) did not show obvious developmental phenotypes. Therefore, they assumed that products of a truncated transcript of *IAMT1* may still have methyltransferase activity [16]. Interestingly, recent studies demonstrated that *iamt1–1* mutants exhibited slightly faster opening of the apical hook, impaired gravitropism of hypocotyls, and tolerance to high-temperature male sterility [14,15], although no significant reduction in IAA levels was observed [15]. The reasons for the differences between the phenotype of *iamt1-RNAi* and *iamt1–1* are not clear. The dramatic high-auxin phenotypes of *iamt1-RNAi* might be caused by simultaneous repression of additional members of the SABATH family, a group of carboxyl methyltransferase genes including *IAMT1* [15].

Phenylacetic acid (PAA) is another naturally occurring auxin widely distributed in plants [17,18], but its physiological significance is yet to be demonstrated [18]. PAA is generally less effective than IAA except for the promotion of lateral root formation in peas [19], but endogenous amounts of PAA are higher than those of IAA in various plant species [17,20].

PAA and IAA regulate various genes via the same auxin signaling pathway and are assumed to share the same regulatory roles as auxins [17,21]. A distinctive difference is that PAA does not undergo polar transportation [17]. Based on this evidence, we recently proposed a model wherein IAA acts as a cell to cell communicator and PAA may play a role in the maintenance of steady-state auxin levels required for the preservation of cellular activity in plants [22]. More recently, we demonstrated that the GH3 family alters the ratio of IAA and PAA in Arabidopsis [23]. These observations suggest that various IAA metabolic enzymes may also regulate the endogenous levels of PAA in plants.

In this study, we investigated the role of Arabidopsis IAMT1 in auxin metabolism. We demonstrated that IAMT1 can convert not only IAA to MeIAA but also PAA to PAA-methyl ester (MePAA) *in vitro*. However, overexpression of *IAMT1* decreased the amounts of IAA, but not PAA, in root tips of Arabidopsis. We found that neither alteration of auxin levels nor obvious phenotypes were observed in the CRISPR/Cas9-based null mutants of *iamt1* under our growth conditions. Our results suggest that IAMT1 exclusively regulates the levels of IAA *in vivo* and that members of the SABATH family, a group of carboxyl methyltransferases, may also regulate IAA levels in Arabidopsis.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as the wild type (WT) plant. Arabidopsis seeds were stratified at 4°C for 2 d in the dark. Seedlings were grown vertically on Murashige and Skoog (MS) agar medium with 1% sucrose under 16 h light condition at 23°C. Transgenic Arabidopsis *pMDC7:IAMT1* were used for experiments. For β-estradiol (ER) treatment, seeds were germinated and grown on MS agar medium containing ER (2 μM) for 4 days. MS medium with 0.1% DMSO was used as a mock treatment. For quantitative RT-PCR (qRT-PCR) and auxin metabolite analysis, seedlings were grown vertically on MS agar medium for 10 days, transferred to MS media, underwent shaken cultured at 100 rpm for 2 d, and were then cultured for another 2 days in the presence of ER (2 μM final concentration) or the mock solution. After ER treatment, plants were collected, freeze-dried, and kept at −80°C until use. For the gravitropic response test, the WT and *iamt1-c* seeds were stratified at 4°C for 2 days in the dark, exposed to light for 6–8 h at 22°C, and were cultivated in the dark for 3 days and then for 12 h after rotating the plate 90°.

2.2. IAMT1 protein preparation in E. coli

DNA fragments of *IAMT1* and its homologs were amplified by PCR using genomic DNA from Arabidopsis seedlings as a template using gene-specific primer pairs listed in Table S1. A cDNA of the *IAMT1* gene was cloned into the *pCold-TF DNA* vector (Takara Bio, Kusatsu, Japan) using the In-Fusion system. The *E. coli* strain BL21 star (DE3) was transformed with the *pCold-TF:IAMT1* plasmid. The transformants were incubated at 37°C in TB medium with 50 μg/mL carbenicillin. Protein expression was induced by adding isopropyl β-D-l-thiogalactopyranoside at a final concentration of 1 mM. After incubation at 15°C for 24 h, the cells were collected by centrifugation. The cells were resuspended in sodium phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH

7.0) and sonicated to get the cell extract. The TF-fused IAMT1 proteins were purified from the cell extract using TALON metal affinity resin (Takara Bio, Kusatsu, Japan). The TF-fused proteins of IAMT1 homologs were also prepared in a similar manner. The trigger factor region was removed by the Factor-Xa following the manufacturer's instructions and purified proteins were condensed by ultrafiltration using an Amicon Ultra centrifugal unit (Merck Millipore, Burlington, MA).

2.3. Enzyme assays

Enzymatical assays with IAMT1 and homolog proteins were performed in a 50 μL volume containing 10 μg purified recombinant protein, 50 mM Tris-HCl at pH 7.5, 1 mM IAA or PAA, and 1 mM *S*-adenosyl-L-methionine (SAM). Enzymes were boiled at 100°C and used as controls. The assays were initiated by the addition of SAM, maintained at 25°C for 4 h, and stopped by the addition of 80 μL of *n*-hexane. MeIAA and MePAA were extracted by mixing the liquid and the upper layers were collected. These samples were analyzed using the LECO Pegasus HT gas chromatography (GC)-mass spectrum system (LECO, St. Joseph, MI). An SGE BPX35 column (Shimazu, Kyoto, Japan) was used with helium as the carrier gas at a flow rate of 1 mL min^{-1}. The GC program was as follows: 50°C for 2 min, ramp to 320°C at 30°C min^{-1}, followed by a 3 min hold at 320°C. Reaction products were determined by comparison of GC retention time and mass spectrum patterns with those of authentic standards. For the calculation of specific activities, 10 μM of IAMT1 recombinant protein was reacted with 100 μM IAA or PAA, 1 mM SAM, and 50 mM Tris-HCl, at pH 7.5 and 40°C for 30 min at a 50 μL volume. The reactions were initiated by the addition of SAM and stopped by the addition of 50 μL acetonitrile. The reaction products were extracted with 100 μL of *n*-hexane containing 1 ng/μL ethyl PAA (EtPAA) as an internal standard and were analyzed by GC-MS.

2.4. Generation of IAMT1ox plants

The full-length *IAMT1* cDNA was provided by RIKEN BioResource Research Center, Japan. The ORF of *IAMT1* was amplified by adapter PCR from the RAFL cDNA clone using gene-specific primers (IAMT1-F and IAMT1-R) and adaptor attB primers (adaptor attB1 and adaptor attB2). The *IAMT1* fragment was inserted into the *pDONR/Zeo* vector using the BP clonase II reaction and then this entry clone was transferred into the *pMDC7* vector [24] by the LR clonase II reaction (Invitrogen, Waltham, MA). Arabidopsis WT plants were transformed with the resulting construct, *pMDC7:IAMT1,* using the floral dip method with the *Agrobacterium tumefaciens* GV3101 pMP90 strain.

2.5. Generation of CRISPR/Cas9-based iamt1 mutants

Knockout mutants for the *IAMT1* gene were generated using CRISPR/Cas9-based genome editing methods previously described [25,26]. We designed two guide RNAs to target the *IAMT1* gene. The target sequences were GACTTCTCAGCATGAAAGGTGG and CCGAGCCACTAGCCATGCCAAA. The *iamt1-c1* and *iamt1-c2* mutants were genotyped using the gene-specific primers, IAMT1-GT1 and IAMT1-GT2. The *iamt1* mutants would generate a PCR fragment approximately 620 bp when the primer pair IAMT1-GT1/GT2 was used, whereas using the same primers, WT plants produced a 3.7 kb fragment, which often failed to amplify. To determine zygosity, we used IAMT1-GT1 and IAMT1-GT3 primers for

PCR reactions. WT plants and heterozygous plants produced a 620 bp fragment, whereas *iamt1* mutants could not produce any PCR fragments.

2.6. qRT-PCR analysis

Total RNA was isolated from *pER8*, *IAMT1ox*, *iamt1-c1*, and *iamt1-c2* plants using RNeasy (Qiagen, Venlo, Netherlands). The PrimeScriptTM RT reagent kit with a gDNA eraser (Takara Bio, Kusatsu, Japan) was used to generate first-strand cDNA. qRT-PCR was performed on a G8830A AriaMx Real-time PCR system (Agilent Technologies, Santa Clara, CA) using a THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) and specific primers are listed in Table S1.

2.7. LC-MS/MS analysis of auxin metabolites

LC-ESI-MS/MS analysis of IAA, oxIAA, PAA, PAA-Asp, and PAA-Glu was performed as previously described [22].

3. Results

3.1. IAMT1 can catalyze the conversion of PAA to MePAA in vitro

Because of the structural similarity of PAA to IAA, we hypothesized that IAMT1 may convert PAA to its methyl ester (Fig. 1). To investigate this hypothesis, we expressed the trigger factor (TF)-fused IAMT1 proteins in *E. coli* using the pCold-TF vector. After removal of the TF region, recombinant IAMT1 proteins were reacted with IAA or PAA in the presence of the methyl group donor SAM. After the reaction, we analyzed MeIAA and MePAA using gas chromatography-mass spectrometry (GC-MS). MeIAA was identified using a molecular ion at *m/z* 189 and a base ion at *m/z* 130 (Fig. 1C, D). Similarly, MePAA was identified using a molecular ion at *m/z* 150 and a base ion at *m/z* 91 (Fig. G, H). As shown in Fig. 1E and F, IAMT1 proteins expressed in *E. coli* formed MeIAA from IAA, as previously reported [27]. We found that IAMT1 proteins also exhibited the carboxyl methyltransferase activity against PAA and formed MePAA (Fig. 1I, J). Furthermore, MePAA was not detected from the reaction mixture with denatured IAMT1 protein. IAMT1 displayed similar specific activity as IAA (0.64 nmol min^{-1} mg^{-1} protein) and PAA (0.65 nmol min^{-1} mg^{-1} protein), respectively.

3.2. Overexpression of IAMT1 results in the reduction of IAA levels but not PAA levels

A previous study showed that the constitutive overexpression of *IAMT1* (*35S-IAMT1*) results in a series of reduced-auxin phenotypes in Arabidopsis [16]. However, it has not been demonstrated whether the endogenous levels of auxins were altered by the overexpression of *IAMT1*. The results from our biochemical characterization of *IAMT1* suggest that endogenous levels of both IAA and PAA may be reduced by overexpression of *IAMT1* in plants. To investigate the role of IAMT1 in auxin metabolism *in vivo*, we generated transgenic plants harboring the *IAMT1* gene in an ER-inducible expression system using a *pMDC7* vector (*IAMT1ox*) (Fig. 2A). Similar to *35S-IAMT1* [16], two independent lines of *IAMT1ox1* and *IAMT1ox2* exhibited severe auxin-deficient phenotypes, including agravitropic root growth and reduced root length, upon induction of the overexpression of *IAMT1* by ER treatment (Fig. 2A, B and Fig. S1). For auxin analysis, we used *IAMT1ox1*

plants, in which the levels of *IAMT1* transcripts were increased by 750-fold in MS medium containing ER (Fig. 2C). We analyzed the endogenous IAA and PAA levels in whole seedlings using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Interestingly, no significant changes in IAA and PAA levels were observed in whole seedlings of *IAMT1* overexpressing plants (Fig. 2D). Given that the overexpression of *IAMT1* results in an agravitropic root phenotype, we assumed that the auxin levels might be reduced in root apexes, which accumulate relatively higher levels of IAA. To test this hypothesis, we collected root tips of *IAMT1ox1* seedings and analyzed the levels of auxins. The IAA levels were reduced by 70% in the root tips of *IAMT1* overexpressing plants compared to the levels in mock-treated plants, whereas the PAA levels were not changed (Fig. 2E). Similarly, the levels of 2-oxindole-3-acetic acid (oxIAA), one of the major IAA metabolites in Arabidopsis, were reduced by 65% in the root tips of *IAMT1* overexpressing plants (Fig. 2E). These results suggest that IAMT1 selectively metabolizes IAA in Arabidopsis.

We attempted to analyze the levels of MeIAA and MePAA in the WT and *IAMT1ox1* plants using GC-MS, but we were not able to quantify these metabolites in this study.

3.3. CRISPR/Cas9-based iamt1 knockout mutants do not exhibit obvious phenotypes

To further investigate a role of *IAMT1* in auxin homeostasis, we generated null mutants of *IAMT1* (*iamt1-c1* and *-c2*) using a CRISPR/Cas9-based genome editing approach (Fig. 3) [25]. Using genomic DNA sequence analysis, we confirmed that more than 90% of the coding regions of *IAMT1* were deleted in the *iamt1-c1* and *-c2* lines, respectively (Fig. 3 and Fig. S2A). The genotypic analysis further confirmed that *iamt1-c1* and *-c2* lines are null mutants (Fig. S2B). Although *iamt1-RNAi* mutants reportedly showed high-auxin phenotypes [16], no obvious phenotypes were observed in our *iamt1-c1* and *-c2* lines compared to that of WT plants under normal growth conditions (Fig. 3B–D). We analyzed the levels of IAA and PAA in *iamt1-c1* seedlings, but these auxin levels were comparable with that of WT plants (Fig. 3E). No increase of oxIAA levels was observed in the *iamt1-c1* seedlings either (Fig. 3E). These results suggested that disruption of *IAMT1* does not affect auxin levels in Arabidopsis. A previous study demonstrated that the hypocotyls of T-DNA-inserted *iamt1–1* mutants showed an abnormal gravitropic response [15]. To test this, we analyzed the gravitropic response of *iamt1-c1* and *-c2* under dark conditions. However, these mutants showed normal gravitropism of hypocotyls (Fig. S3), suggesting that the *iamt1* single knockout mutants did not display obvious phenotypes.

3.4. Screening of auxin carboxyl methyltransferases in the SABATH family

There are 24 members in the Arabidopsis SABATH family of carboxyl methyltransferases [15,28]. The results of our *iamt1-c* and previous *RNAi-iamt1* mutant analysis suggest that other auxin carboxyl methyltransferases may play a redundant role in Arabidopsis [16]. Based on the phylogenetic analysis of the Arabidopsis SABATH family [15], we noticed that members of the family are divided into three groups (Table S2). IAMT1 belongs to the same clade as benzoate/salicylate carboxyl methyltransferase (BSMT1), which is different from that of the other two groups with gibberellin methyltransferase (GAMT) or farnesoic acid carboxyl methyltransferase (FAMT) [15]. To explore novel auxin carboxyl

methyltransferases, we investigated IAMT1 homologs in the BSMT1 clade (Table S2). Among 15 members in the BSMT1 clade, we successfully expressed six IAMT1 homologs as the TF-fused proteins in *E. coli* and performed the enzyme activity test using IAA and PAA as substrates (Table S2). However, none of these proteins, including BSMT1, showed methyltransferase activity, whereas IAMT1 catalyzed the methylation of IAA and PAA (Table S2).

4. Discussion

Despite the severe auxin-deficient phenotypes of *IAMT1ox* plants, significant changes in auxin levels have not been demonstrated. In this study, we found that IAA levels were reduced in root tip tissues, but not in the entire seedling (Fig. 2D). Although IAMT1 can convert PAA to MePAA *in vitro*, the levels of PAA were not altered in *IAMT1ox* plants (Fig. 2D). These results suggest that IAMT1 exclusively metabolizes IAA in Arabidopsis. Therefore, we conclude that auxin-deficient phenotypes of *IAMT1ox* plants are attributed to a reduction in IAA. Regarding the discrepancy of auxin-deficient phenotypes and normal IAA levels in the entire seedling of *IAMT1ox* plants, this may be caused by the IAA homeostasis by the Arabidopsis MeIAA esterase (AtMES) family [11]. Yang *et al.* (2008) demonstrated that members of the AtMES family, including AtMES17, showed esterase activity and converted MeIAA to IAA *in vitro*. Given that the AtMES family consists of 20 homologs, various AtMES proteins may hydrolyze MeIAA to strictly maintain IAA levels in *IAMT1ox* plants. We assume that the esterase activity would be relatively low in root tips because a significant reduction of IAA levels was observed in these tissues of *IAMT1ox* plants. Investigation of the role of *AtMES* family genes in *IAMT1ox* plants is important to elucidate metabolic regulation of MeIAA in Arabidopsis.

Here, we demonstrated that CRISPR/Cas9-based *iamt1-c1* and *-c2* null mutants neither show obvious phenotypes, nor an increase in IAA levels. The reasons for the differences between the phenotype of our CRISPR/Cas9-based *iamt1-c* mutants and previously described *iamt1-RNAi* and T-DNA inserted *iamt1–1* lines are not clear. A previous study showed that *iamt1–1* mutants still produce the same levels of IAA and reduced levels of MeIAA compared to that of WT plants [15]. *iamt1-RNAi* plants might display high-auxin phenotypes because of the simultaneous repression of an additional member of the SABATH family [16]. Combined with the fact that *iamt1-c1* and *-c2* do not show obvious phenotypes, we assumed that other auxin carboxyl methyltransferase genes may exert a redundant property in Arabidopsis. We tested the enzyme activity of six IAMT1 homologs in the SABATH family in this study, but no proteins were shown to be auxin carboxyl methyltransferase other than IAMT1 (Table S2). Further biochemical analysis of members in the SABATH family is crucial to understanding IAA homeostasis in Arabidopsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

gFW	gram fresh weight
IAA	indole-3-acetic acid
IAMT1	IAA CARBOXYL METHYLTRANSFERASE 1
MeIAA	IAA-methyl ester
MePAA	PAA-methyl ester
PAA	phenylacetic acid

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Highlights

- Arabidopsis IAMT1 protein can catalyze the methylation of both IAA and PAA *in vitro*.
- Overexpression of *IAMT1* reduces IAA levels and results in auxin-deficient phenotypes.
- No reduction in PAA levels was observed following overexpression of *IAMT1*.
- Knockout of *IAMT1* did not affect auxin levels in Arabidopsis.
- IAMT1 homologs may also participate in Arabidopsis IAA homeostasis.

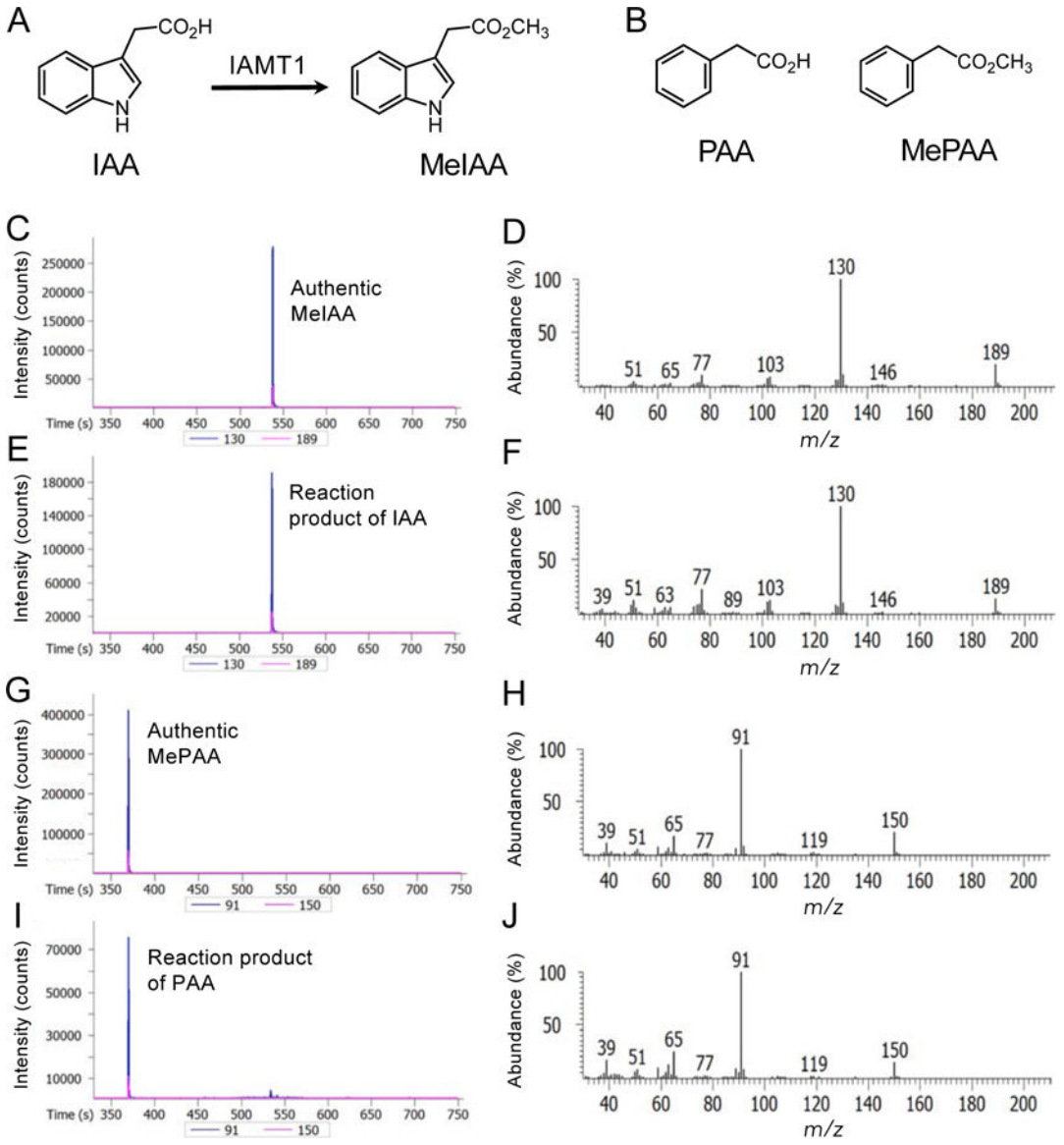

Fig. 1.

Conversion of IAA and PAA to their methyl esters by IAMT1 *in vitro*.

A) Conversion of IAA to MeIAA by IAMT1. B) Structures of PAA and MePAA. C) GC-MS chromatogram of authentic MeIAA (*m/z* 130 and 189). D) Full mass spectra of authentic MeIAA. E) GC-MS chromatogram of the reaction product of IAMT1 with IAA (*m/z* 130 and 189). F) Full mass spectra of the reaction product of IAMT1 with IAA. G) GC-MS chromatogram of authentic MePAA (*m/z* 91 and 150). H) Full mass spectra of authentic MePAA. I) GC-MS chromatogram of the reaction product of IAMT1 with PAA (*m/z* 91 and 150). J) Full mass spectra of the reaction product of IAMT1 with PAA.

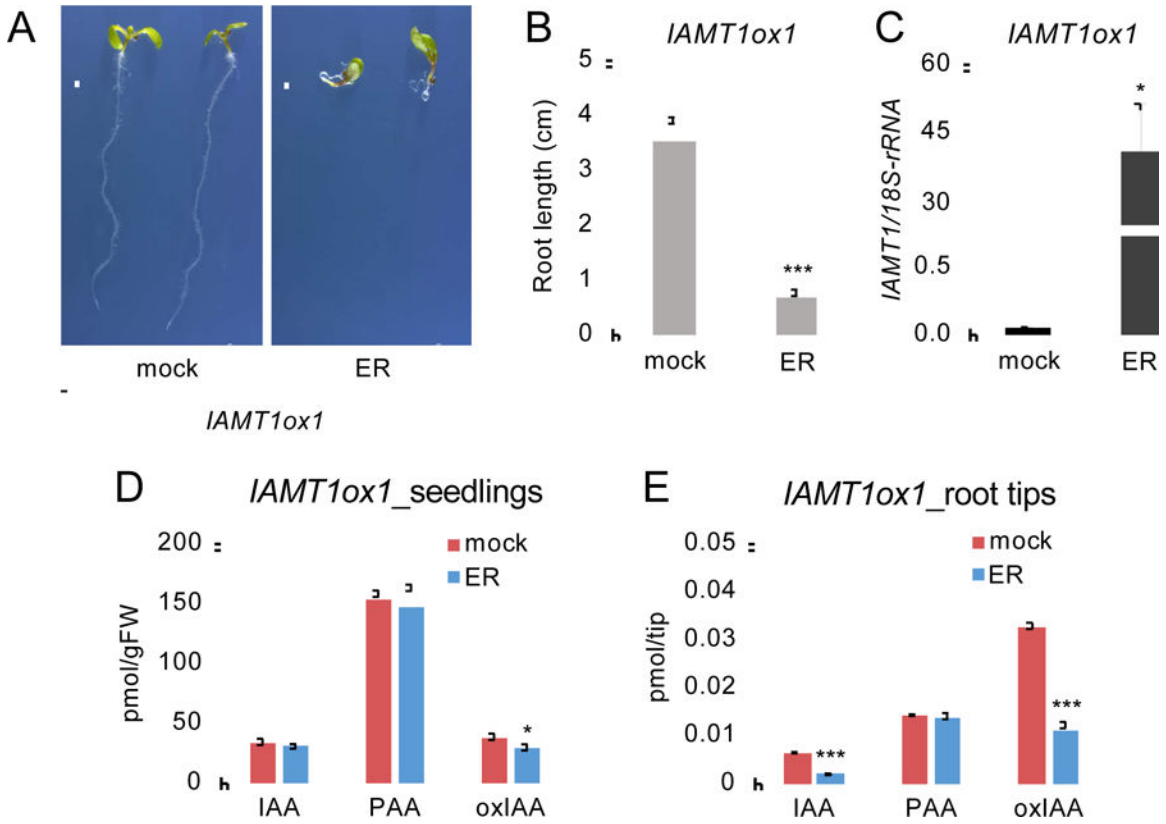

Fig. 2.

Analysis of *IAMT1ox1* plants.

A) Phenotypes of *IAMT1ox1* plants. Plants were treated with mock or ER (2 μM) for 4 days. Scale bars = 1 cm. B) Primary root length of *IAMT1ox1* plants. Plants were treated with mock or ER for 5 days. Differences between mock- and ER-treated plants are statistically significant at $P < 0.05$ (***$P < 0.001$, Student's *t*-test). C) Expression levels of *IAMT1* in mock- and ER-treated *IAMT1ox1* plants. Differences between mock- and ER-treated plants are statistically significant at $P < 0.05$ (*$P < 0.05$, Student's *t*-test). D) Auxin levels in whole seedlings of mock- and ER-treated *IAMT1ox* plants. Differences between mock- and ER-treated plants are statistically significant at $P < 0.05$ (*$P < 0.05$, Student's *t*-test). E) Auxin levels in root tips of mock- and ER-treated *IAMT1ox* plants. Differences between mock- and ER-treated plants are statistically significant at $P < 0.05$ (***$P < 0.001$, Student's *t*-test).

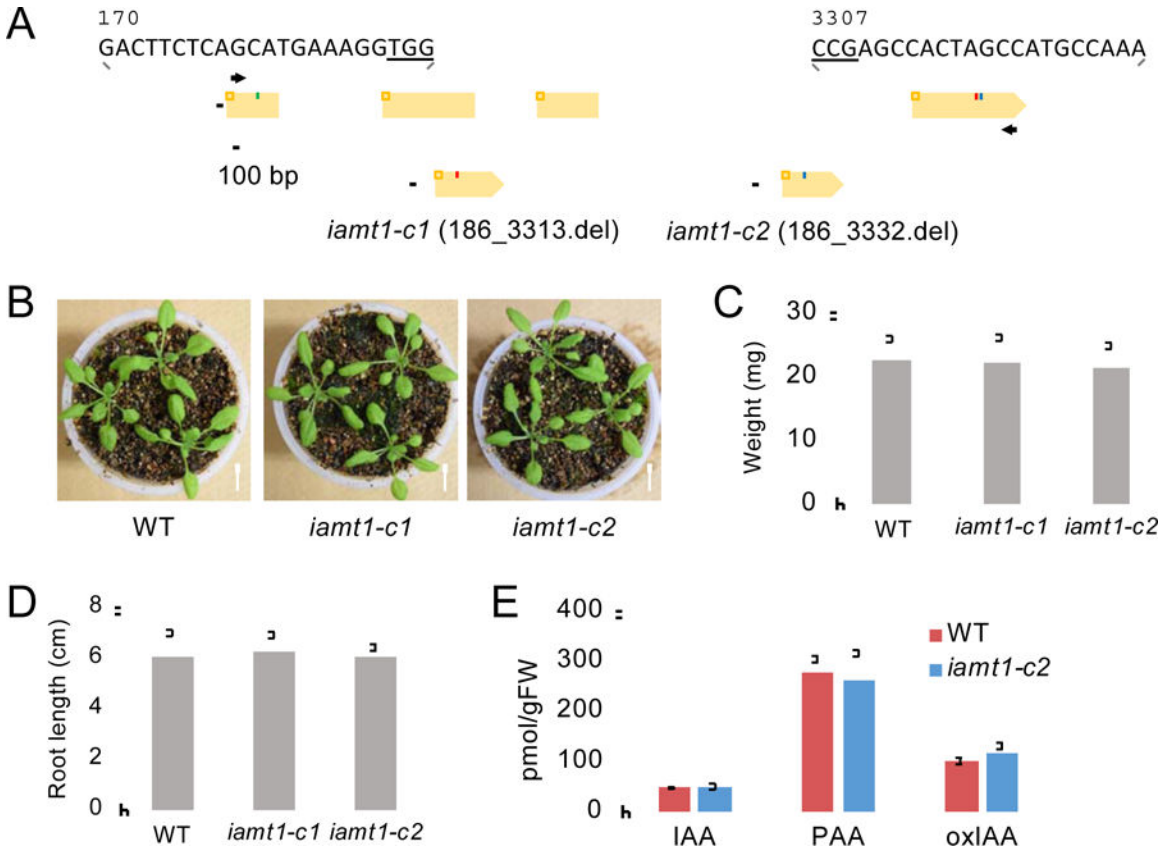

Fig. 3.

Characterization of the *iamt1-c* mutants.

A) Mutations in *iamt1-c1* and -*c2* mutants. Orange boxes represent the exons and black lines represent the introns in the *IAMT1* gene. Arrows indicate gene-specific primers used for genotyping. B) Phenotype of 17 d-old *iamt1-c* seedlings. Scale bars = 1 cm. C) Weight of shoot tissues of 12 d-old *iamt1-c* seedlings ($n = 30$). D) Primary root length of 8 d-old *iamt1-c* seedlings ($n = 30$). E) Auxin metabolite levels in whole seedlings of 8 d-old WT and *iamt1-c2* mutants ($n = 4$).