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Original Paper

3-Phenyllactic acid is converted to phenylacetic acid and induces auxin-responsive root growth in Arabidopsis plants

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Abstract Many microorganisms have been reported to produce compounds that promote plant growth and are thought to be involved in the establishment and maintenance of symbiotic relationships. 3-Phenyllactic acid (PLA) produced by lactic acid bacteria was previously shown to promote root growth in adzuki cuttings. However, the mode of action of PLA as a root-promoting substance had not been clarified. The present study therefore investigated the relationship between PLA and auxin. PLA was found to inhibit primary root elongation and to increase lateral root density in wild-type Arabidopsis, but not in an auxin signaling mutant. In addition, PLA induced *IAA19* promoter fused *β-glucuronidase* gene expression, suggesting that PLA exhibits auxin-like activity. The inability of PLA to promote degradation of Auxin/Indole-3-Acetic Acid protein in a yeast heterologous reconstitution system indicated that PLA may not a ligand of auxin receptor. Using of a synthetic PLA labeled with stable isotope showed that exogenously applied PLA was converted to phenylacetic acid (PAA), an endogenous auxin, in both adzuki and Arabidopsis. Taken together, these results suggest that exogenous PLA promotes auxin signaling by conversion to PAA, thereby regulating root growth in plants.

Key words: Arabidopsis, auxin, lactic acid bacteria, phenylacetic acid, 3-phenyllactic acid.

Introduction

Many microorganisms, called plant growth-promoting bacteria (PGPB), have been reported to produce compounds that promote plant growth and are thought to be involved in the establishment and maintenance of symbiotic relationships (Glick 2012; Qin et al. 2011). These compounds are often plant hormones or their mimics, such as auxins, gibberellins, abscisic acid and cytokinin (Brader et al. 2014; Cohen et al. 2009). 3-Phenyllactic acid (PLA) was isolated from Bokashi fertilizer as a root-promoting substance (Maki et al. 2021). Bokashi fertilizer is a traditional type of fertilizer commonly used in Japan. This fertilizer is made by fermenting organic fertilizer with microorganisms such as lactic acid bacteria (LAB), filamentous fungi and yeast (Jaramillo-López et al. 2015; Nikitin et al. 2018). PLA, the catabolic product of phenylalanine through phenylpyruvic acid (PPA), is one of the major organic acids produced by many microorganisms, especially LAB (Cortés-Zavaleta et al. 2014; Vermeulen et al. 2006), and has been reported to be biologically active as a root promoter in plants (Adachi et al. 2010; Mikami et al. 1970; Tamura and Chang 1965). However, the molecular mechanisms by which PLA promotes root production have not been clarified.

Root development is regulated by complex mechanisms in response to multiple environmental stimuli and endogenous signaling molecules such as plant hormones. Auxins are versatile plant hormones that plays important roles in multiple physiological processes associated with plant development including root development (Abel and Theologis 2010; Woodward and Bartel 2005). Auxins regulate root system architecture by promoting the polarization, initiation, patterning, and emergence of lateral root founder cells (Du and Scheres 2018; Lavenus et al. 2013). Auxins are also the major hormones that promote the adventitious growth of plants (Lakehal and Bellini 2018; Li et al. 2009). Indole-3-acetic acid (IAA) is the major natural auxin, with other known auxins including 4-chloroindol-3-acetic acid (4-Cl-IAA), indole-3-butyric acid (IBA), and phenylacetic

Abbreviations: IAA, indole-3-acetic acid; ILA, indole-3-lactic acid; IPA, indole-3-pyruvic acid; PAA, phenylacetic acid; PLA, 3-phenyllactic acid.
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acid (PAA) (Simon and Petrášek 2011). In Arabidopsis, the auxin response is initiated when an auxin mediates the binding of TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFBs) proteins to AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors as a molecular glue leading to the proteasomal degradation of Aux/IAA (Dharmasiri et al. 2005; Parry et al. 2009). Many plants synthesize IAA from tryptophan by a two-step process. In Arabidopsis, TRYPTOPHAN AMINO TRANSFERASE 1 (TAA1) and its homologues TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4 (TAR1 to 4) convert L-tryptophan to indole-3-pyruvic acid (IPA), and YUCCA flavin-containing monooxygenases (YUC) convert IPA to IAA (Mashiguchi et al. 2011; Stepanova et al. 2008; Tao et al. 2008). PAA, which responds via TIR1/AFBs, is biosynthesized by TAA1/TARs and YUC and metabolized by GH3, similar to IAA (Sugawara et al. 2015).

The present study was designed to determine the molecular mechanism underlying PLA-responsive root growth. PLA was shown to be converted to PAA in Arabidopsis plants and to induce auxin-responsive root growth.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) ecotype was used as wild type in this study. The *Arabidopsis thaliana* mutant *tir1-1 afb2* (Columbia background), and the mutant *tar2-1 WEI8-/wei8-1* (CS16414, Columbia background) was from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH, USA). Seeds were surface-sterilized and placed on half-strength MS medium supplemented with 1% sucrose with or without added PLA. After cold treatment for 2 days to synchronize germination, seeds were incubated at 22°C under a 16/8 h light/dark cycle and grown vertically. After 14 days, the number of emerged lateral roots was counted using a stereoscopic microscope and the length of each primary root was measured. The homoheterozygous test for *wei8-1* was performed by PCR as described (Stepanova et al. 2008). The *pIAA19:GUS* reporter Arabidopsis line has been described previously (Tatematsu et al. 2004).

Seeds of adzuki bean (*Vigna angularis* (wild.) Ohowi and Ohashi) cv. Erimoshouzu were sown in vermiculite and grown under continuous light (44 μmol m^{-2} s^{-1}) at 25°C (Mitsuhashi et al. 1969). PAA was quantified in these seedlings, and PLA was labeled for feeding analyses.

GUS staining

The expression of beta-glucuronidase (GUS) was assessed by incubating seedlings at 25°C for 30 min in 100 mM sodium phosphate, pH 7.0, containing 0.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4FE(CN)_6$, 1 mM EDTA and 0.1% Tween-20 (Tatematsu et al. 2004) .

Yeast heterologous reconstitution system

Auxin activity of PLA was investigated by yeast heterologous reconstitution system as described (Shimizu-Mitao and Kakimoto 2014) with several modifications. Mated yeast strains obtained from Dr. Kakimoto were cultured in 3 ml YPAD at 30°C for 24 h and diluted with YPAD to an OD_{600} of 0.6. A 50 μl aliquot of this suspension was added to each well of a 96-well plate, followed by the addition to each well of 50 μl of a solution of 2 mM each of the respiratory inhibitor potassium cyanide and sodium azide and 400 μM luciferine in YPAD. After stabilization of the luciferase signal, 10 μl of IAA or 1 mM of D, L-PLA in 0.1% DMSO were added to each well. The luciferase signal was measured with a luminometer (Atto, AB-2350 PHELIOS) for 2 h. Each sample was tested in duplicate, and the average of the two tests was calculated. Results were standardized relative to the value immediately before addition of compounds and the value following addition of 0.1% DMSO.

Quantification of PAA and IAA

Sixty-five 12-day-old adzuki seedlings, were cut at 3 cm above the soil surface and their apical buds were removed, were dipped in test solution for 24 h. 6–7 g of 7-cm stems, which of basal one cm-length were removed, were ground to a powder with liquid nitrogen, followed by extractions with 10 ml of 80% acetonitrile containing 0.1% acetic acid twice. To each preparation was added 10 ng of $IAA^{13}C_6$ (Cambridge Isotope Laboratories, Inc.) as an internal standard for IAA quantification. Following centrifugation at 15,000 rpm for 10 min at 4°C, the supernatant was dried and dissolved in 10 ml of 5% sodium bicarbonate aqueous solution. The solution was washed three times wash with ethyl acetate and the aqueous phase was extracted with ethyl acetate at pH 2.5. The organic phase containing PAA and IAA was dried and dissolved in 5 ml of 40% methanol and purified with a Sep-Pak® Plus tC18 Environmental cartridge (Waters) by collecting the fraction that passed through the cartridge following acclimation with 40% methanol. Samples for LC-ESI-MS/MS were dried and dissolved in 10 ml 80% acetonitrile containing 0.1% acetic acid, and 1 μl of each sample was injected into an LC-ESI-MS/MS system (Shimadzu, QP8050), multiple reaction monitoring (MRM) mode. LC was performed with 0.01% acetic acid (solvent A) and acetonitrile (solvent B) using a gradient from 5% to 95% for 15 min at a flow rate of 0.2 ml min^{-1}. The temperature of the UPLC column (L-Colulmn2 ODS, φ2.1×100 mm, Chemicals Evaluation and Research Institute, Japan) was set at 40°C. The MS/MS analysis conditions were: ESI negative ion mode, probe voltage=3.0 kV, nebulizing gas flow=2 l min^{-1}, drying gas flow=10 l min^{-1}, DL temperature=250°C, block heater temperature=400°C, collision energy and MS/MS transition (m/z)=9.0 V, 135.3/91.2 for PAA, 15.0 V, 176.0/130.0 for IAA, and 16.0 V, 182.0/136.1 for $IAA^{13}C_6$. PAA was analyzed by the external standard method and IAA by the internal standard method.

Synthesis of labeled PLA

One g of D,L-phenyl-d5-alanine-2,3,3-d3 (Cambridge Isotope Laboratories, Inc.) was dissolved in 20 ml of 0.5 M sulfuric acid, to which was added 20 ml of a solution containing 1.2 g sodium nitrite was added over 5 min at room temperature. The reaction mixture was stirred overnight and extracted with ethyl acetate. The ethyl acetate phase was washed with 1 M HCl three times and evaporated, followed by recrystallization in acetone and the molecular weight was confirmed by LC/MS Q3 scan mode (ESI-). It was confirmed to be the target compound by compared with the fragmentation of the PLA standard by product ion scan mode with a collision energy 17.0 V.

Detection of labeled PAA

Fifty 12-day-old adzuki seedlings, cut as described above, were dipped in test solution for 24 h followed by dipping in distilled water for 48 h. Extraction and purification were performed as described above.

Arabidopsis thaliana Col-0 seeds were surface-sterilized and placed on half-strength MS medium supplemented with 1% sucrose with or without added labeled PLA. After cold treatment for 2 days to synchronize germination, the seeds were incubated at 22°C under a 16/8 h light/dark cycle and grown vertically. After 14 days, the roots of 50 seedlings were removed, ground in liquid nitrogen and extracted twice with 20 ml 80% acetonitrile containing 0.1% acetic acid. The protocols for extraction and analysis were identical to those for the adzuki seedlings.

A 10 μl aliquot of each sample was injected into an LC-ESI-MS/MS system (Shimadzu, QP8050), as above. Labeled PAA was detected by selected ion monitoring mode for m/z 140–142. And detected peaks identified by product ion scan mode with a collision energy of 9.0 V

Adzuki root-promoting assay for ILA

Root-promoting assay for ILA was performed as described (Maki et al. 2021) with some modifications. Twelve-day-old seedlings of adzuki were cut at 3 cm above the soil surface, and their apical buds were removed. The basal parts of the cuttings were dipped in an ILA solution adjusted to pH 7.0 for 72 h, and the cuttings were transferred to distilled water and grown under continuous light for 4 days. The number of emerged roots was counted 7 days after treatment.

Results

PLA possessed auxin-like activity in Arabidopsis roots

To investigate the effect of PLA on Arabidopsis root growth, seedlings were grown on medium containing D-PLA or L-PLA. Wild type Arabidopsis showed short primary roots and high lateral root density in a concentration-dependent manner (Figure 1A, B, C). These phenotypes were similar to reported responses to IAA (Normanly et al. 1997). To investigate the relationship between PLA and auxin, PLA-responsive root growth was assessed in the auxin-signaling mutant *tir1-1 afb2*. The TIR1/AFB F-box proteins act as auxin receptors, and the mutants are resistant to auxin (Dharmasiri et al. 2005). Neither D-PLA nor L-PLA had an auxin-like effect on root growth in *tir1-1 afb2* mutants (Figure 1A, B, C).

Auxin has been reported to increase the level of expression of *IAA19*, a member of the AUX/IAA gene family (Tatematsu et al. 2004). Analysis of the level of expression of the *IAA19* gene in roots using an *IAA19* promoter:*β-glucuronidase* (*pIAA19:GUS*) fusion reporter showed that the addition of PLA to growth medium enhanced the expression of the *GUS* reporter gene in roots (Figure 1D), suggesting that PLA regulates Arabidopsis root growth via an auxin signaling pathway.

PLA partly rescued root growth in an IAA biosynthesis mutant

To further understand the relationship between PLA and auxin response pathways, the effect of D-PLA was tested in the *wei8-1 tar2-1* mutant, a double mutant of *TAA1* (*WEI8*) and its homolog *TAR2*, both of which are involved in IAA biosynthesis (Stepanova et al. 2008). Root meristematic activity has been reported reduced in this mutant due to decreased endogenous auxin levels. Seed with the genotype *WEI8-1/wei8-1 tar2-1/tar2-1*, which were heterozygous for *TAA1*, were sown on normal MS medium. Of these seedlings, 19% showed severe phenotypes with no primary root formation and genotyping by PCR showed that all these seedlings were homozygous for *wei8-1* (Figure 1E). When grown on medium containing 200 μM D-PLA, however, all of these seedlings formed primary roots, including those homozygous for *wei8-1* (Figure 1E), suggesting that PLA plays a role in compensating for the decrease in endogenous auxin.

PLA does not promote the degradation of IAA2 in a yeast heterologous reconstitution system

To evaluate whether PLA itself functions as an auxin, PLA-dependent degradation of Aux/IAAs was analyzed using a yeast heterologous reconstitution system (Shimizu-Mitao and Kakimoto 2014). This system allows evaluation of the role of compounds that act as a molecular glue between Aux/IAA and TIR1/AFBs (i.e. their auxin activity), by assessing the effects of these compound on the decay of luminescence in yeast expressing luciferase-bound Aux/IAA and TIR1/AFBs. The results showed that 10 μM IAA promoted the degradation of Aux/IAA2 when coexpressed with TIR1 or AFB2 in 30 min as reported previously (Shimizu-Mitao and Kakimoto 2014; Figure 2A), whereas, at concentrations of 1 mM, neither D-PLA nor L-PLA promoted the degradation in 2 h (Figure 2B, C).

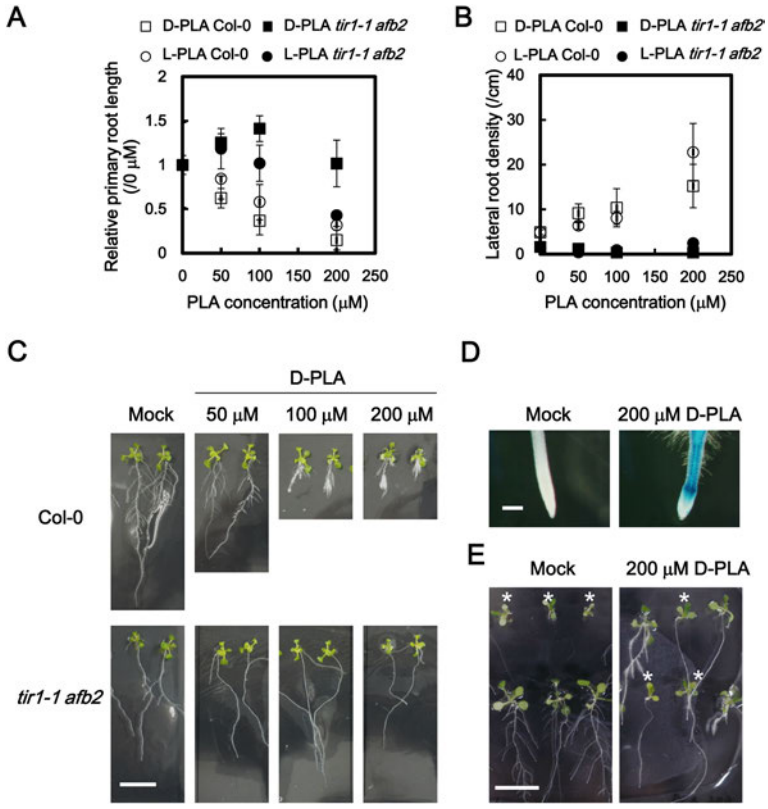

Figure 1. PLA affects root growth via auxin signaling pathway in Arabidopsis. (A) Relative primary root length and (B) lateral root density of Col-0 and *tir1-1 afb2* seedlings grown on 1/2 MS medium supplemented with 0, 50, 100, or 200 μM D-PLA or L-PLA for 14 days. Primary root length is reported relative to each control. Error bars indicate standard deviation (SD) (n=4). (C) Photographs of the seedlings grown for 14 days on D-PLA-containing medium. Bar: 20 mm. (D) GUS staining of primary root tips of *pIAA19:GUS* reporter seedlings grown on 1/2 MS medium in the presence or absence of exogenous 200 μM D-PLA for 14 days. Bar: 0.1 mm. (E) Growth recovery analysis of the IAA biosynthesis mutant *wei8-1 tar2-1* grown on 1/2 MS medium in the presence or absence of exogenous 200 μM D-PLA for 14 days. Asterisks indicate *wei8-1 tar2-1* doubly homogenous mutant seedlings. Bar: 20 mm.

Coexpression with the other AFB proteins (AFB1, 3, 4, 5) also did not promote the degradation of the Aux/IAA2 under the PLA treatment (Figure 2B, C). These findings indicate that PLA is not a ligand of auxin receptors.

PLA treatment increases the amount of PAA in adzuki cuttings

PAA is a compound with a phenyl group, similar to PLA, and is biosynthesized in plants, where it functions as a natural auxin (Simon and Petrášek 2011). Application of exogenous PLA had no effect on the level of endogenous IAA in adzuki cuttings (Maki et al. 2021), but the effect of exogenous PLA on endogenous PAA was not determined. Therefore, endogenous PAA levels in adzuki cuttings treated with PLA for 24 h were measured by LC-MS/MS. PAA levels were increased depending on the concentration of exogenous PLA (Figure 3A).

Similar to PLA, indole-3-lactic acid (ILA), a metabolite of lactic acid bacteria, has been reported to have auxin-like activity (Gibson et al. 1972; Hilbert et al. 2012). ILA also shows adventitious root-inducing activity in adzuki cuttings (Supplementary Figure S1). Because the biosynthetic pathways of PAA from phenylalanine and IAA from tryptophan are parallel in plants (Sugawara et al. 2015), it is implicated that ILA may increase IAA in plants (Figure 3B). As expected, addition of exogenous ILA increased the amount of IAA in adzuki cuttings (Figure 3C).

PLA can be converted to PAA in plants

The PLA-induced increase in PAA suggested the existence of a hitherto unknown conversion pathway from PLA to PAA in plants. To determine whether PLA is converted to PAA in plants, plants were fed stable isotope labeled PLA. Labeled D8 PLA was synthesized from labeled D8 phenylalanine (Supplementary Figure S2), with LC-MS (ESI-) analysis of the synthesized D8 PLA showing that its molecular weight was 174 Da (PLA: 166 Da), with product ions detected at m/z 154, 110, and 126 (PLA: m/z 147, 103, and 119), confirming synthesis of the target compound (Supplementary Figure S2). Adzuki cuttings were fed D8 PLA for 24 h, followed by dipping in distilled water for 48 h. LC-MS analysis using SIM mode identified peaks at m/z 140 and 141, not present in control, with the same retention times as PAA standard (Figure 4A, Supplementary Figure S3).

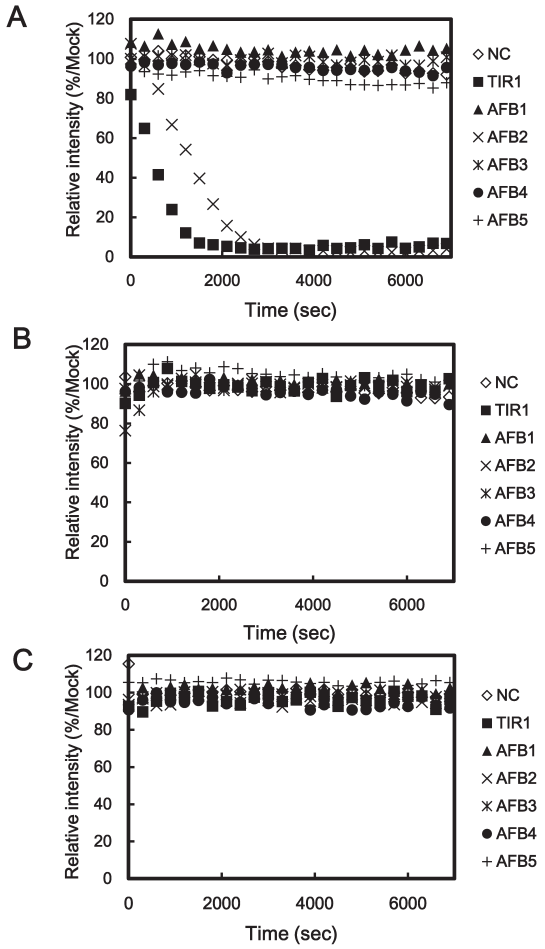

Figure 2. Evaluation of auxin-dependent degradation of Aux/IAA2 using a yeast heterologous reconstitution system. Evaluation of the decay of luminescence derived from luciferase-fused Aux/IAA2 in yeast in medium containing (A) 10 μM IAA, (B) 1 mM D-PLA, and (C) 1 mM L-PLA. Luciferase signal intensity was calculated relative to the values obtained in absence of exogenous compound.

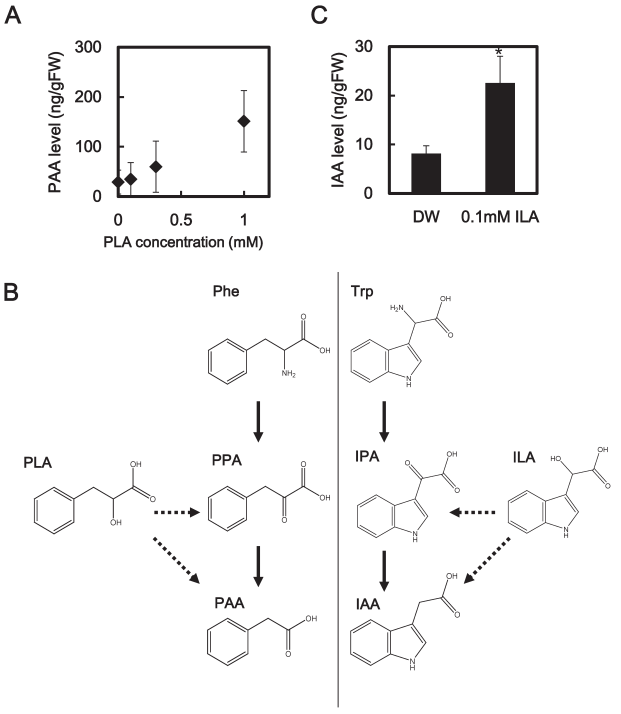

Figure 3. Conversion of PLA to PAA in adzuki cuttings. (A) LC-MS/MS (ESI) determined concentrations of endogenous PAA in adzuki cuttings treated with PLA for 24 h. Results reported as means±SD (n=3). (B) Schematic diagram of the metabolic pathways of PAA and IAA biosynthesis in plants. Black arrows indicate pathways reported in plants. Dashed arrows indicate the predicted pathway catalyzing the conversion of PLA and ILA to PAA and IAA, respectively, in plants. Abbreviations: Phe, phenylalanine; Trp, tryptophan; PPA, phenylpyruvic acid; IPA, indole-3-pyruvic acid. (C) LC-MS/MS (ESI) determined concentrations of endogenous IAA in adzuki cuttings treated with ILA for 24 h. Results reported as means±SD (n=3). * $p<0.05$ compared with distilled water treatment by Student's t-test.

Fragmentation of the peak at m/z 140 and scanning in product ion scan mode resulted in peaks at m/z 140 and 96 were detected. In comparison, fragmentation of the PAA standard (m/z 135) yielded peaks at m/z 135 and 91 (Figure 4B), indicating that D5 PAA had been produced from D8 PLA in these cuttings.

Because the test using adzuki cuttings was conducted under non-sterile conditions, there was a possibility of microbial conversion. Thus, the same test using Arabidopsis seedlings was performed under sterile conditions. D5 PAA was detected in Arabidopsis grown on medium containing D8 PLA under sterile conditions (Figure 4C), thus indicating that PLA can be converted to PAA in plants.

Discussion

Many microorganisms, called plant growth promoting bacteria (PGPB), have been reported to produce plant hormone-like substances. PLA derived from the fermented broth of LAB has been shown to promote adventitious root growth in root cuttings of adzuki plants (Maki et al. 2021). Although exogenous PLA has been reported to have bioactivity in rice and adzuki (Adachi et al. 2010; Maki et al. 2021; Mikami et al. 1970; Tamura and Chang 1965), the mode of action in plants has not been clarified. The present study showed that found PLA promoted the auxin signaling pathway and affected root growth in Arabidopsis. PLA increased lateral root density while inhibiting primary root growth in Arabidopsis, and enhanced the expression of the auxin response marker gene *IAA19* in roots. The auxin-like activity of PLA was clearly diminished in the auxin signaling mutant, *tir1-1 afb2*, providing further evidence that PLA regulates root growth via the auxin signaling pathway.

PLA, however, did not promote the degradation of the AUX/IAA IAA2 in a yeast heterologous reconstitution system, indicating that PLA itself does not function as an auxin in plants. Intriguingly, PLA could be converted to PAA in both adzuki cuttings and Arabidopsis seedlings. Feeding of these plants with labeled PLA resulted in the detection of labeled PAA, indicating that exogenous

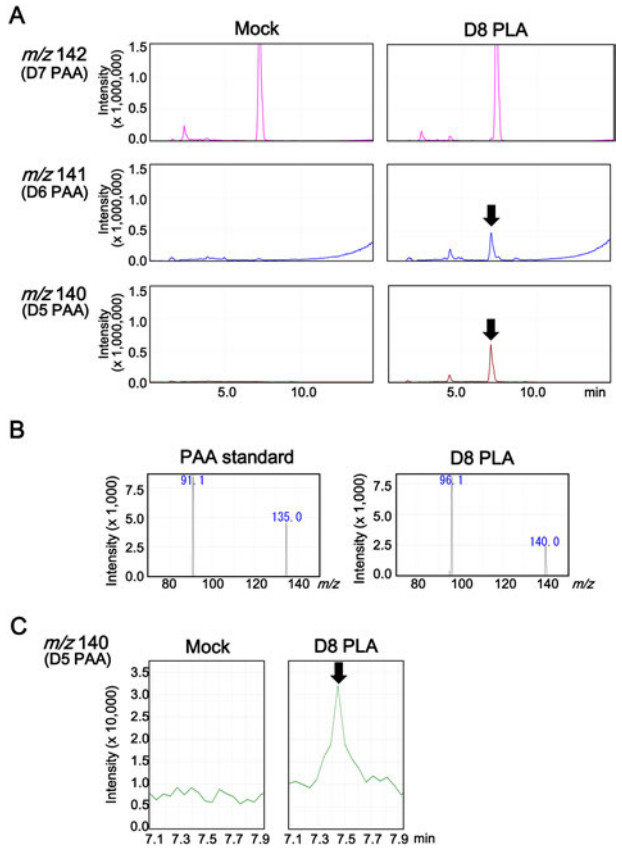

Figure 4. Confirmation of conversion from PLA (D8) to PAA (D5 and D6) in adzuki cuttings. (A) Detection of labeled PAA in adzuki cuttings treated for 24 h with 1 mM PLA (D8 PLA) followed by dipping in distilled water for 48 h. Specific peaks of labeled PAA were detected by LC-MS (SIM; selected ion monitoring) at *m/z* 141 (D6 PAA), and 140 (D5 PAA) after D8 PLA treatment. The arrows indicate specific peaks associated with D8 PLA treatment. (B) MS spectra of peaks detected by product ion scan mode in adzuki cuttings treated with PLA (D8 PLA), including at *m/z* 135 for PAA standards (left) and at *m/z* 140 for the peak in PLA (D8 PLA) treated Arabidopsis (right). (C) Detection of labeled PAA (D5 PAA) in Arabidopsis grown on medium containing 200 μM PLA (D8 PLA) for 14 days by SIM at *m/z* 140. The arrow indicates a specific peak associated with D8 PLA treatment.

PLA is metabolized to PAA in plants. PAA, which functions as an endogenous auxin (Koepfli et al. 1938), is recognized by TIR1/AFBs, induces the expression of auxin responsive genes and affects root growth, as similar to the effects of IAA in Arabidopsis, although the activity of PAA was lower than that of IAA (Cook 2019; Sugawara et al. 2015). PAA is partly biosynthesized by TAA and YUCCA and metabolized by GH3, similar to IAA (Sugawara et al. 2015), but the details of PAA biosynthesis pathways remain uncertain. Consistently with conversion of labeled PLA to PAA, application of exogenous PLA increased PAA amounts in adzuki seedlings. Taken together, these results indicate that PLA promotes auxin signaling by increasing PAA amounts, thus regulating root growth. Exogenous D8 PLA was converted mainly to D5 PAA (*m/z* 140), not D7 PAA (*m/z* 142). This may have been due to isotope exchange by the keto-enol tautomerism of pyruvate (Knox and Pitt 1957), suggesting a possible metabolic pathway by which PLA is converted to PAA via PPA.

This study also found that exogenous application of ILA increased IAA amounts in adzuki seedlings. ILA, a metabolite of LAB, may have auxin-like activity in plants (Gibson et al. 1972; Hilbert et al. 2012), although the detailed molecular basis of the ILA activity remains unclear. The results of the present study suggest that exogenous ILA may be converted to IAA and activate auxin signaling in plants. The metabolic pathway by which aromatic lactic acid is converted to aromatic acetic acid in plants has not been determined, and further studies are required.

Plants form symbiotic relationships with microorganisms, such as rhizobia and mycorrhizal fungi. Establishment of symbiosis is partly mediated by signaling molecules produced by these microorganisms. It is of great interest to determine whether PLA is involved in communications between plants and microorganisms. PLA has not been reported to be an endogenous metabolite of plants, but is produced by many species of LAB. LAB has also been reported to industrially produce as much as 5.2 mM PLA (Gerez et al. 2010). However, recent studies have reported that only a small number of LAB are found in the leaves and rhizosphere of plants, and their symbiosis establishment rates are low (Miller et al. 2019). Further research is needed to evaluate the physiological functions of PLA in regulating plant root growth in nature.

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