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Pipecolic Acid Quantification Using Gas Chromatography-coupled Mass Spectrometry

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Abstract

Pipecolic acid (Pip), a non-proteinacious product of lysine catabolism, is an important regulator of immunity in plants and humans alike. For instance, Pip accumulation is associated with the genetic disorder Zellweger syndrome, chronic liver diseases, and pyridoxine-dependent epilepsy in humans. In plants, Pip accumulates upon pathogen infection and is required for plant defense. The aminotransferase ALD1 catalyzes biosynthesis of Pip precursor piperideine-2-carboxylic acid, which is converted to Pip via ornithine cyclodeaminase. A variety of methods are used to quantify Pip, and some of these involve use of expensive amino acid analysis kits. Here, we describe a simplified procedure for quantitative analysis of Pip from plant tissues. Pipecolic acid was extracted from leaf tissues along with an internal standard norvaline, derivatized with propyl chloroformate and analyzed by gas chromatography-coupled mass spectrometry using selective ion mode. This procedure is simple, economical, and efficient and does not involve isotopic internal standards or multiple-step derivatizations.

Keywords: Pipecolic acid, GC-MS, *Arabidopsis thaliana*, Plant defense, Propyl chloroformate

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Background

Pip, a non-protein amino acid derivative of lysine, accumulates in local and distal tissue of pathogen infected plants and in body fluids of patients with peroxisomal disorders (Schutgens *et al.*, 1986; Yoon and An, 2010; Návarová *et al.*, 2012; Wang *et al.*, 2018). In plants, Pip is synthesized from lysine via ALD1 (AGD2 like defense response protein) encoded aminotransferase (Návarová *et al.*, 2012). ALD1 localizes to chloroplast and converts lysine to ε-amino-α-keto caproic acid, which then cyclizes to form Δ1-piperideine-2 carboxylic acid (P2C) (Ding *et al.*, 2016; Hartmann *et al.*, 2017). The P2C intermediate is subsequently converted to Pip by ornathine cyclodeaminase (encoded by SARD4) (Ding *et al.*, 2016; Hartmann *et al.*, 2017). Pip accumulation upon pathogen infection is associated with the induction of systemic acquired resistance (SAR), a form of broad-spectrum defense that protects uninfected parts of the plant against secondary infections. Pip confers SAR by increasing levels of the free radicals, nitric oxide (NO) and reactive oxygen species (ROS), which act upstream of glycerol-3-phosphate (G3P). Thus, a linear pathway comprising Pip⇒NO⇔ROS⇒AzA⇒G3P functions in parallel with salicylic acid-derived signaling, and that both pathways are essential for the induction of SAR (Chanda *et al.*, 2011; Yu *et al.*, 2013; Gao *et al.*, 2014; Wang *et al.*, 2014; Bernsdorff *et al.*, 2016; Lim *et al.*, 2016; Wenig *et al.*, 2019). Pip and G3P have also been suggested to operate in a positive feedback loop that functions upstream of volatile pinenes (Wenig *et al.*, 2019). Notably, *de novo* synthesis of Pip in distal tissues is dependent on both SA and G3P (Wang *et al.*, 2018). These results suggest that metabolites in a signaling cascade can stimulate biosynthesis of each other depending on their relative levels and their site of action. Notably, ALD1-derived factors (such as Pip) also contribute to SAR-associated transcriptional reprograming in the systemic tissue since pathogen-responsive transcriptional changes in the distal tissue are almost completely absent in *ald1* mutant plants (Gruner *et al.*, 2013). Exogenous application of pipecolic acid also enhances disease resistance against bacterial and viral pathogens in *Nicotiana* plants (Vogel-Adghough *et al.*, 2013; Wang *et al.*, 2019). Recent work has shown that Pip levels are regulated by calmodulin-binding transcription factors (Kim *et al.*, 2020; Sun *et al.*, 2020), calcium-dependent protein kinase 5 (Guerra *et al.*, 2020), and Jumonji domain containing K3K4 demethylase (Li *et al.*, 2019). Thus, determination of pipecolic acid in the plant tissues is an integral part of research on plant defense.

In the present method, we used propyl chloroformate based one-step derivatization procedure for quantification of Pip from *Arabidopsis* leaves. Norvaline was used as an internal standard and the derivatized products were analysed by gas chromatography (GC)-mass spectrometry (MS) using selective ion monitoring (SIM) mode. Our method is based on two earlier procedures that either used methyl chloroformate to derivatize amino acids (Villas-Bôas *et al.*, 2003) or propyl chloroformate based derivatization of amino acids which were extracted using a commercial kit (EZ:faast free amino acid analysis kit, Phenomenex) (Kugler *et al.*, 2006). One other GC-MS based method for Pip quantification from human plasma involved trimethylsilyl- and trifluoroacyl-derivatizations (Yoon and An, 2010). This method is laborious and moreover uses [$2H^9$]-Pip as an internal standard, which is ~1,000 times more expensive compared to norvaline. The reaction scheme for internal standard norvaline (Figure 1A) and the analyte pipecolic acid (Figure 1B) are shown in Figure 1. The method reported here is simple, cost effective, and can be used to process 100-200 samples in a day.

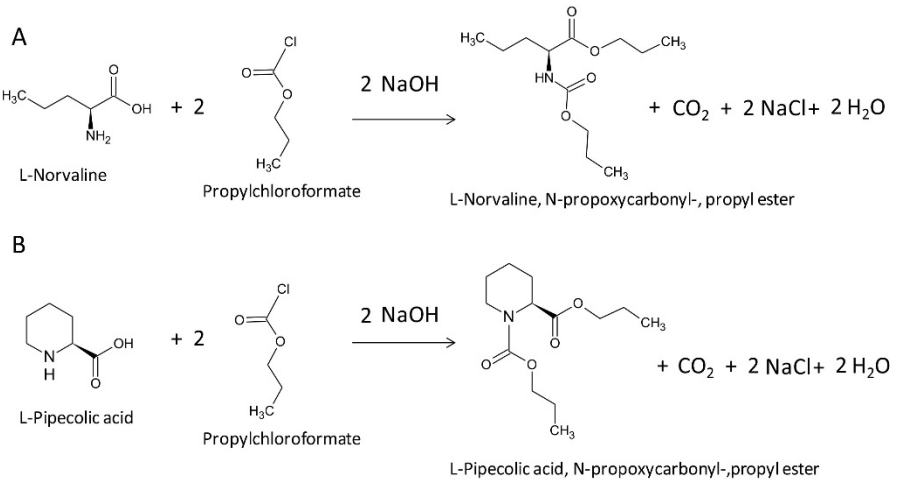

Figure 1. Derivatization reactions for internal standard L-norvaline (A) and analyte L-pipecolic acid (B) with propyl chloroformate.
Propyl chloroformate reacts with both carboxyl and amino groups in basic conditions releasing HCl for respective condensation reactions. The HCl is neutralized by NaOH in the reaction mixture to produce H_2O and NaCl. The mixed anhydride formed from the carboxyl group and propyl chloroformate is lost as CO_2.

Materials and Reagents

1. BD™ Tuberculin syringe, 1 ml (Becton Dickinson, catalog number: 309659)
2. Kimwipes, 1-Ply (Fisher, catalog number: 06-666)
3. Microfuge tubes, 1.5 ml (Sarstedt, catalog number: 72.690.300)
4. Disposable test tubes, 13 x 100 mm (VWR, catalog number: 47729-572)
5. GC vials, 2 ml (Thermo Scientific, catalog number: C4000-1)
6. Screw caps (Agilent; catalog number: 5182-0717)
7. Pasteur pipets (Fisher, catalog numbers: 13-678-20A and 13-678-20C)
8. Micro capillary pipets (Kimble, catalog number: 71900-20)
9. *Pseudomonas syringae* expressing *avrRpt2* (a gift from Dr. Barbara Kunkel, Washington University in St. Louis)
10. *Arabidopsis thaliana* ecotype Col-0 and mutant *ald1* plants
11. Liquid nitrogen (American Welding & Gas, 22 Psi)
12. Magnesium chloride hexahydrate (Fisher, catalog number: BP214-500)
13. Acetonitrile (ACROS, catalog number: 26827-0040)
14. Hydrochloric acid (36.5-38% w/w, approximately 12 N) (Fisher, catalog number: A144-212)
    ! CAUTION: The reagent is corrosive and volatile; wear protective gloves and handle it in a fume hood.
15. L-Norvaline (ACROS, catalog number: 6600-40-4)
16. L-Pipecolic acid (TCI, catalog number: P1404)
17. Sodium hydroxide (Fisher, catalog number: BP359-500)
18. Methanol (Fisher, catalog number: A452-4)
19. Pyridine (EMD Millipore, PX2020-6)
20. Propyl chloroformate (Beantown Chemical, catalog number: 133200-100G)
    CAUTION: This reagent is extremely toxic, volatile, flammable and corrosive and should be handled in a fume hood. Store at 4 °C in a closed jar containing some calcium sulfate desiccant (DRIERITE).
21. Anhydrous calcium sulfate desiccant (W. A. Hammond DRIERITE Company LTD, catalog number: 13005), which is used for storage of propyl chloroformate
22. Chloroform (EMD Millipore, catalog number: CX1058-1)

CAUTION: The reagent is toxic and volatile and should be handled in a fume hood.

23. Sodium bicarbonate (Fisher, catalog number: BP328-1)
24. Anhydrous sodium sulfate (Fisher, catalog number: S-421)
25. 10 mM Magnesium chloride (see Recipes)
26. Extraction buffer (see Recipes)
27. Internal standard (see Recipes)
28. 1 M Sodium hydroxide (see Recipes)
29. 50 mM Sodium bicarbonate (see Recipes)

Equipment

1. Plastic pestle (Axygen Scientific, catalog number: PES15BSI)
2. Milli-Q Advantage A10 Water purification system (EMD Millipore, model: Z00Q0V0WW)
3. Autoclave (Consolidated Stills & Sterilizers, model: SSR3APB)
4. Scissors (Harbor Freight Tools, catalog number: 62458)
5. Balance (METTLER TOLEDO, model: AT261)
6. Benchtop Dewar Flask (Thermo Scientific, catalog number: 10-194-100B)
7. Vortexer with a Styroform platform (Labnet, model: S0100)
8. Table top centrifuge (Eppendorf, model: 5417C), which is used for centrifuging microfuge tubes
9. Parker Source TriGas Generator (Parker Hannifin, model: LCMS-5000NA, compressor model: SF120872PH)
10. Table-top swing-arm centrifuge (Clay Adams, model: Dynac 0101), used for centrifuging disposable test tubes
11. Latex bulbs, 2 ml (VWR, catalog number: 82024-554)
12. Gas chromatograph-mass spectrometer (GC-MS) (Agilent, model: 7890A-5977)
13. GC column, HP-5MS (Agilent, catalog number: 19091J-413)
14. Computer for GC-MS (Dell, model: 3430)
15. Syringe for manual GC injection, 5 µl (Hamilton, catalog number: 87993)

Software

1. MassHunter Workstation for qualitative and quantitative analysis (Agilent, software version: 10.0)
2. Enhanced MassHunter Workstation for data acquisition (Agilent, software version: 10.0)
3. MS spectral library (NIST)

Procedure

A. Equipment setup

1. Gas chromatography (GC) set up
   Chromatography: perform chromatography with a 30-m capillary HP-5MS column. Use helium as the carrier gas at a constant pressure of 14 psi. Set the injection port temperature to 240 °C and the transfer line temperature to 280 °C. Samples are run in splitless mode. Use the following temperature program for chromatography as shown in Table 1.

Table 1. GC temperature used for profiling norvaline and pipecolic acid

Time interval	Temperature gradient
0-1 min	70 °C
1-19 min	70-260 °C (10 °C/min ramp)
19-21 min	260 °C

2. Mass spectrometer (MS) set up
   Automatically tune the instrument according to the manufacturer's instructions.

B. Sample preparation and analysis

1. For pathogen assays, the largest three leaves of ~4 weeks old plant are infiltrated with either 10 mM $MgCl_2$ or the pathogen *Pseudomonas syringae avrRpt2* using a needleless syringe from the abaxial side of the leaves. After leaf infiltration, remove excess droplets of 10 mM $MgCl_2$ or pathogen suspension with Kimwipes. Figure 2 shows a pot of *Arabidopsis thaliana* ecotype Columbia-0 plants just after pathogen infiltration (Figure 2).

Figure 2. A picture of *Arabidopsis thaliana* ecotype Col-0 plants after infiltration with *Pseudomonos syringae* expressing *avrRpt2*. Infiltrated leaves were marked with a white marker.

2. About 24 h later, collect ~100 mg infiltrated leaf tissue. The entire leaf was sampled using a pair of scissors.
3. Weigh the tissue with a balance that is accurate to the 1 mg digit. Record the weight of each sample. ΔCRITICAL STEP
4. Place the tissue in a microfuge tube, cap the tube, and drop it into a bucket of liquid nitrogen. Frozen tissues can be stored at -80 °C indefinitely. •PAUSE POINT
5. Grind the tissue with a plastic pestle.
6. Continue to the next step before the tissue is thawed. ΔCRITICAL STEP
7. Add 400 µl of extraction buffer.
8. Add 20 µl of internal standard.
9. Vigorously shake for 15 min on a vortexer at room temperature.
10. Centrifuge for 5 min at 18,000 *x g*.
11. Supernatant is transferred to a 13 x 100 mm glass test tube. Extract can be stored at -80 °C indefinitely. •PAUSE POINT
12. Dry the supernatant with a stream of nitrogen gas. Dried extract can be stored at -80 °C indefinitely. •PAUSE POINT

13. Add 200 µl of 1 M sodium hydroxide.
14. Add 167 µl of methanol and 34 µl of pyridine.
15. Vortex vigorously and break up any pellets with a Pasteur pipet.
16. Add 20 µl of propyl chloroformate and vigorously vortex for 30 s. Use a glass micro capillary pipette instead of a plastic pipette tip to avoid contamination of plasticizers. ΔCRITICAL STEP
17. Add another 20 µl of propyl chloroformate and vigorously vortex for 30 s.
18. Add 400 µl of chloroform and vortex vigorously for 10 s.
19. Add 400 µl of sodium bicarbonate (50 mM) and vortex vigorously for 10 s.
20. Centrifuge for 2 min with a table-top swing-arm centrifuge at about 80% speed (approximately 1,250 *x g*).
21. The lower layer is transferred to a new 13 x 100 mm glass test tube with a Pasteur pipet. The lower phase obtained after the transfer is approximately 0.375 ml.
22. Dry the chloroform extract by adding approximately 40 µl of anhydrous sodium sulfate and vortex until clear.
23. Centrifuge for 1 min with a table-top swing-arm centrifuge at about 80% speed (approximately 1,250 *x g*).
24. Transfer chloroform extract to a GC vial with a Pasteur pipet.
25. Inject 0.5-1 µl to GC/MS for analysis using selective ion monitoring mode (SIM) using quantifier ions 158 and 170 and qualifier ions 72 and 128.

C. GC-MS data from standard compound analysis

1. Figure 3 shows the total ion chromatogram (Figure 3A) and corresponding MS of norvaline (Figure 3B left panel) and Pip (Figure 3B right panel) standards run in scan mode.

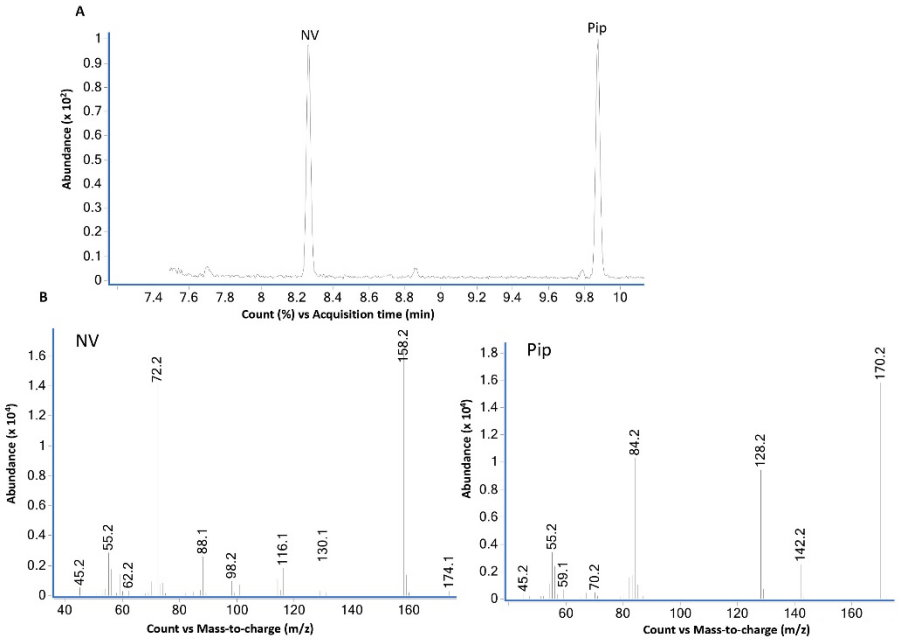

Figure 3. GC-MS analysis of norvaline (NV) and pipecolic acid (Pip) using scan mode.
(**A**) GC-MS total ion chromatogram of equal weight (2 µg each) mixture of L-norvaline (NV) and L-pipecolic acid (Pip) derivatized with propyl chloroformate as described in B using scan mode. L-norvaline, N-propoxycarbonyl, propyl ester (at 8.2 min) and L-pipecolic acid, N-propoxycarbonyl, propyl ester (at 9.9 min) are the two major peaks. The bottom panel (**B**) shows mass spectra of NV- and Pip-N-propoxycarbonyl propyl esters. The loss of propoxycarbonyl group produces major ions 158 from NV and 170 from Pip derivatives.

2. Figure 4 shows the GC-MS data from standard compound analysis using selective ion monitoring (SIM) mode. Based on the mass spectra shown in Figure 1, two major ions for each compound were used to monitor NV (158 and 72) and Pip (170, 128) in SIM mode.

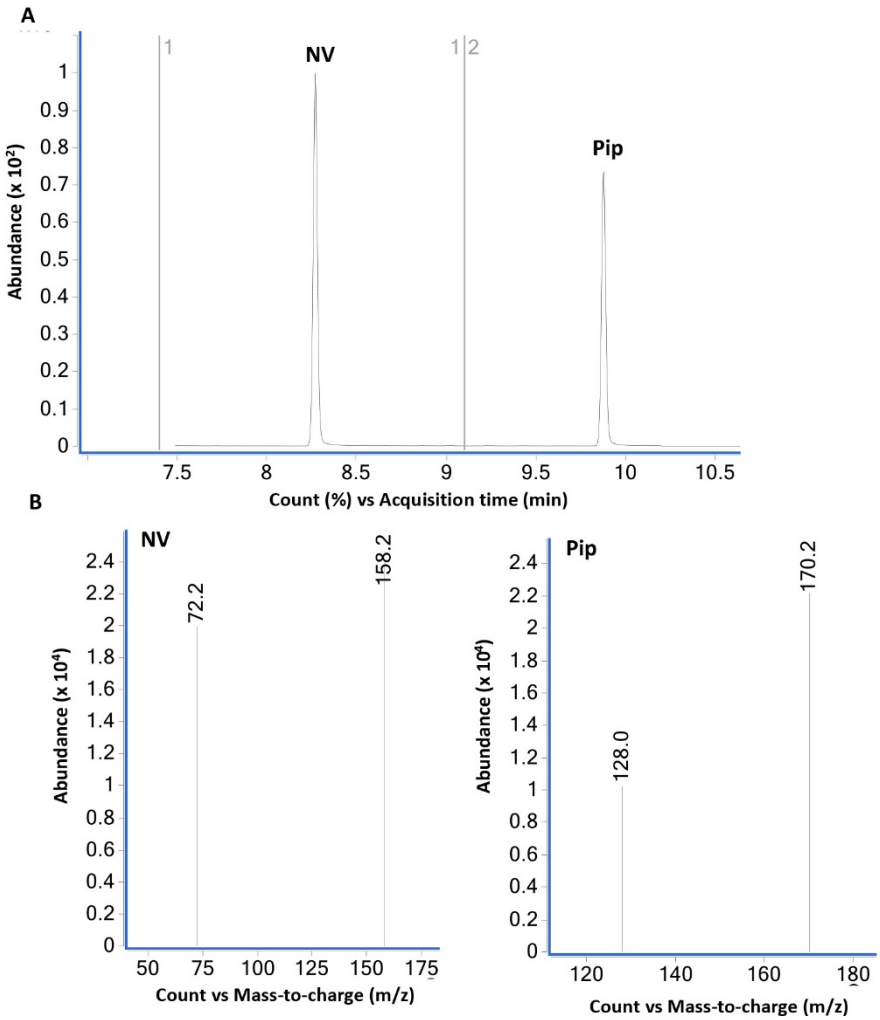

Figure 4. GC-MS analysis of norvaline (NV) and pipecolic acid (Pip) using selective ion monitoring (SIM) mode.
(**A**) GC-MS total ion chromatogram of equal weight (2 µg each) mixture of NV and Pip derivatized with propyl chloroformate as described in B using SIM mode. NV- (at 8.2 min) and Pip-N-propoxycarbonyl propyl ester (at 9.9 min) are the two major peaks. The bottom panel (**B**) show mass spectra of NV- and Pip-N-propoxycarbonyl propyl esters in SIM mode selected for the major ions 158.2 and 72.2 (NV) and 172.2 and 128.0 (Pip). The dwell time for each ion was set at 25 ms. The SIM mode was set to monitor ions 158 and 72 in the time interval 7.4-9.1 min and ions 170 and 128 from 9.1-10.5 min. The relative abundance of major and qualifier ions monitored in SIM and scan modes was similar.

D. Pip levels in *Arabidopsis* leaf tissue

1. GC-MS data for pipecolic acid analysis from *Arabidopsis thaliana* leaf tissue samples using scan (Figure 5) and SIM (Figure 6) modes.

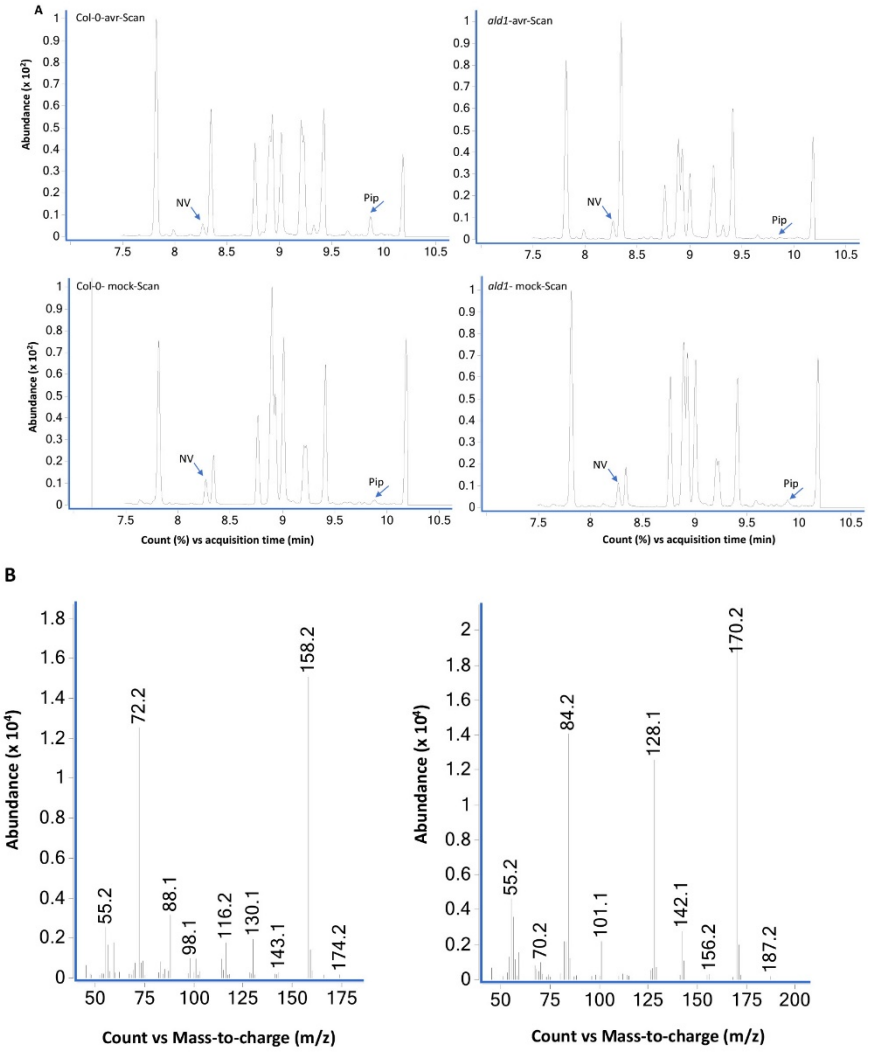

Figure 5. GC-MS analysis of extracts prepared from Col-0 and *ald1* plants analyzed using scan mode. (**A**) Total ion chromatograms of mock (10 mM $MgCl_2$)- and pathogen (*Pseudomonas syringae* expressing *avrRpt2*) inoculated wild-type Col-0 and mutant *ald1* plants. The leaf tissues were sampled at 24 h post treatment and NV (2 µg) was used as an internal standard as described in Procedure B. The *ald1* plants did not accumulate Pip after pathogen infection. The bottom panels (**B**) show MS spectra of NV- and Pip-N-propoxycarbonyl propyl esters.

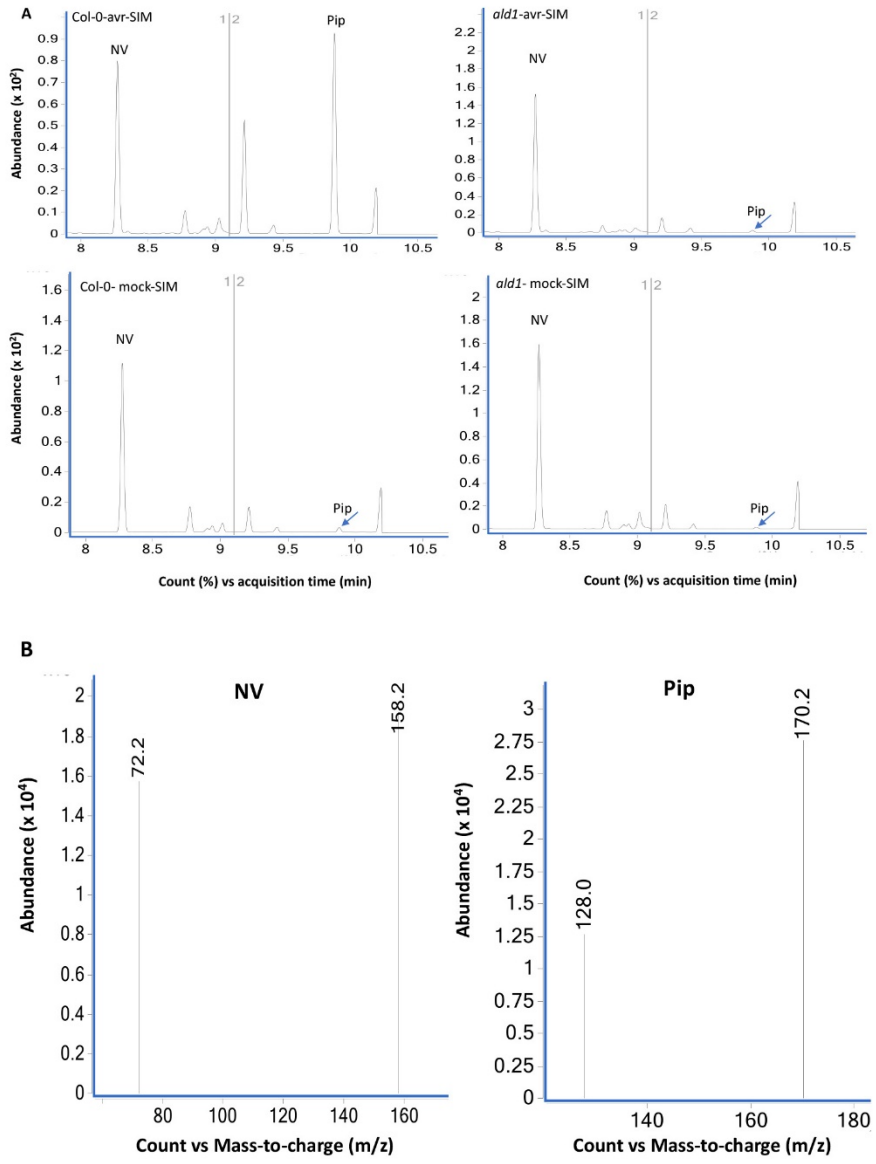

Figure 6. GC-MS analysis of extracts prepared from Col-0 and *ald1* plants analyzed using SIM mode. **(A)** Chromatograms of mock (10 mM $MgCl_2$)- and pathogen (*Pseudomonas syringae* expressing *avrRpt2*) inoculated wild-type Col-0 and mutant *ald1* plants. The leaf tissues were sampled at 24 h post treatment and NV (2 µg) was used as an internal standard as described in Procedure B. The *ald1* plants did not accumulate Pip after pathogen infection. The bottom panels (**B**) show MS spectra of NV- and Pip-N-propoxycarbonyl propyl esters in SIM mode selected for the major ions 158.2 and 72.2 (NV) and 172.2 and 128.0 (Pip).

2. Relative Pip levels in Col-0 and *ald1* plants.
   Figure 7 shows the relative Pip levels in Col-0 and *ald1* plants inoculated with mock or pathogen. The pathogen inoculated wild-type plants accumulated ~18-fold higher Pip levels compared to mock inoculated plants. In contrast to wild-type plants, the *ald1* accumulate reduced basal levels of Pip and did not show any increase in Pip levels after pathogen infection.

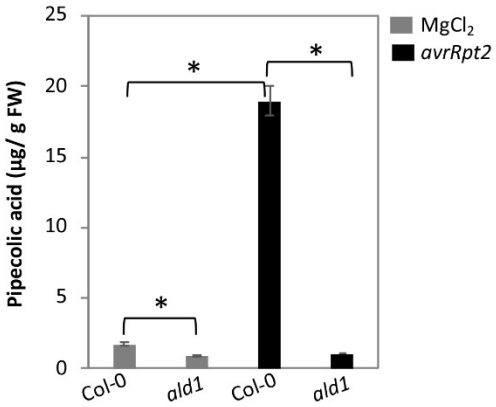

Figure 7. Pip levels in *Arabidopsis* wild-type Col-0 and Pip deficient *ald1* plants post 24 h of inoculation with mock (10 mM $MgCl_2$) or pathogen (*P. syringae* expressing *avrRpt2*).
Plants were inoculated with 10^5 CFU ml^{-1} in 10 mM $MgCl_2$. Error bars indicate standard deviations (n = 3) and astetisks indicate statistical significane ($P > 0.001$).

Data analysis

1. MassHunter Workstation 10.0 was used for qualitative and quantitative data analysis. The ion ratios between quantitative and qualifier ions were used as an additional measure to access proper identification of the analytes using SIM mode. Pipecolic acid levels were calculated based on the standard curves generated using varying concentration of NV and Pip standards.
2. Statistical analysis was performed using Student *t*-test. For pathogen assays, ~16 plants/ genotype/treatment were analyzed in a single experiment. At least 4 technical replicates/genotype/treatment were plated. For metabolite quantification, ~12 plants/genotype/treatment were analyzed in each experiment. Experiments were repeated at least two-three times with a different set of plants. Unless otherwise mentioned error bars indicate SD.

Notes

The protocol has shown excellent reproducibility and been used to analyze numerous extractions reported in our recent studies (Wang *et al.*, 2018; Lim *et al.*, 2020).

Recipes

1. 10 mM Magnesium chloride

a. Weigh 20.33 g of magnesium chloride hexahydrate and dissolve it in Milli-Q water to a final volume of 100 ml to obtain 1 M $MgCl_2$
b. Autoclave 1 M $MgCl_2$ along with 1 liter of Milli-Q water at 121 °C for 30 min using a liquid cycle
c. After the solutions have cooled down to room temperature, take 1 ml of the 1 M $MgCl_2$ and add it to 99 ml of the autoclaved Milli-Q water, mix well to obtain 10 mM $MgCl_2$

2. Extraction buffer

a. Add 8.33 ml of concentrated HCl to 91.67 ml of Milli-Q water and mix well to obtain 1 N HCl

b. Take 1 ml of the 1 N HCl, add it to 99 ml of Milli-Q water, mix well to obtain 0.01 N HCl
c. Mix 25 ml of acetonitrile with 75 ml of the 0.01 N HCl and use this solution as extraction buffer

3. Internal standard
Prepare 100 ng/µl norvaline (wt/vol) in Milli-Q water

4. 1 N Sodium hydroxide
Weigh 40 g of NaOH pellets and dissolve it in Milli-Q water to a final volume of 100 ml to obtain 10 N NaOH, then dilute 10-fold with Milli-Q water

5. 50 mM Sodium bicarbonate
Weigh 420 mg $NaHCO_3$ and dissolve it in Milli-Q water to a final volume of 100 ml

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Competing interests

The authors declare no conflict of interest.

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