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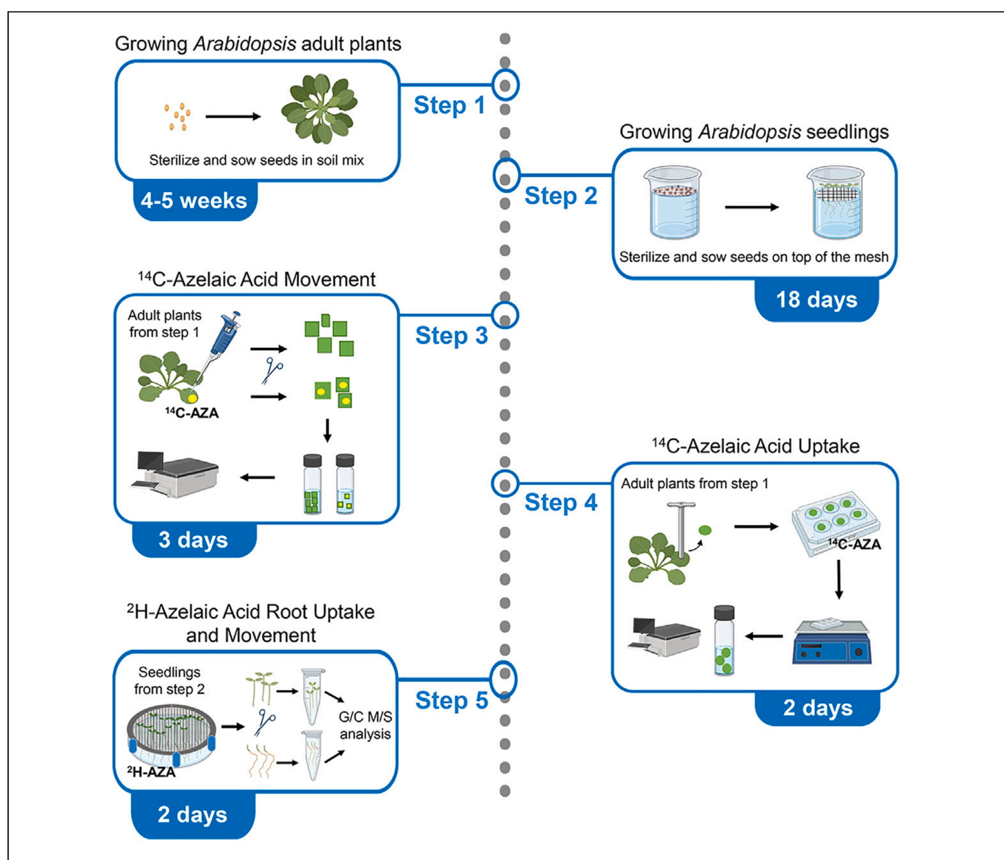
Protocol for analyzing the movement and uptake of isotopically labeled signaling molecule azelaic acid in *Arabidopsis*



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Highlights

Analysis of the movement of the defense signal AZA from leaf to distal tissues

Quantification of the uptake of AZA into leaf discs

Method to investigate AZA capacity for root-to-shoot movement

Understanding the generation, movement, uptake, and perception of mobile defense signals is key for unraveling the systemic resistance programs in flowering plants against pathogens. Here, we present a protocol for analyzing the movement and uptake of isotopically labeled signaling molecule azelaic acid (AZA) in *Arabidopsis thaliana*. We describe steps to assess ^{14}C-AZA uptake into leaf discs and its movement from local to systemic tissues. We also detail the assay for uptake and movement of ^{2}H-AZA from roots to the shoot.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol for analyzing the movement and uptake of isotopically labeled signaling molecule azelaic acid in *Arabidopsis*

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SUMMARY

Understanding the generation, movement, uptake, and perception of mobile defense signals is key for unraveling the systemic resistance programs in flowering plants against pathogens. Here, we present a protocol for analyzing the movement and uptake of isotopically labeled signaling molecule azelaic acid (AZA) in *Arabidopsis thaliana*. We describe steps to assess ^{14}C-AZA uptake into leaf discs and its movement from local to systemic tissues. We also detail the assay for uptake and movement of ^{2}H-AZA from roots to the shoot.

For complete details on the use and execution of this protocol, please refer to Cecchini et al.[1,2]

BEFORE YOU BEGIN

Plants have a complex immune system that relies on local responses triggered by microbial recognition and on non-autonomous systemic resistance programs, key for a broad-spectrum and long-lasting resistance to future infections.[3] These systemic defense responses usually involve a low-cost "primed state", enabling faster and/or stronger defense responses upon recurrent infections.[4] One essential step for the induction of systemic resistance is the local generation of signals that can move to distal tissues, away from the site of infection, and induce priming. Although multiple mobile signals have been characterized in systemic defense pathways, mechanisms regulating their tissue-specific movement from the site of synthesis to their perception at the systemic sites of action, have been largely unexplored.[5–12]

The use of radio- and heavy- isotope labeled compounds to monitor small molecule distribution in whole plants and/or uptake by different tissues is typically a standard experimental choice due to the high sensitivity and low background signal of these compounds. Based on previously established methods, we have recently shown that the priming signaling molecule azelaic acid (AZA) is capable of systemic movement when locally applied to leaves.[1,13–15] AZA is a plastid lipid-related oxylipin that induces a systemic primed state when locally applied to aerial shoot or underground root tissues. Moreover, using isotopically labeled AZA we showed that systemic resistance programs signaling component proteins, AZELAIC ACID INDUCED 1 (AZI1) and EARLY ARABIDOPSIS ALUMINUM INDUCED 1 (EARLI1), are required for its movement and uptake in *Arabidopsis*.[1,2,8]

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Here, we outline the methods for analyzing the distribution and transport of [^{14}C]azelaic acid (^{14}C-AZA) in whole *Arabidopsis thaliana* adult plants from a single leaf (the site of application) to distal systemic tissues (aerial stem/leaves and roots), as well as its uptake into leaf discs.[1] The AZA movement assay accounts for the fate of the total pool of label applied to plants by using a small ^{14}C-AZA drop, instead of a large volume of infiltrated radiolabel. This allows the detection of low amounts of transported AZA and, moreover, the estimation of AZA transported to the roots. The experiment includes a control analysis of the movement of ^{14}C-sucrose; this permits the experimenter to distinguish between an AZA-specific or general defect in phloematic fluxes. Finally, using commercially available deuterated-azelaic acid (^{2}H-AZA), we also describe a method to investigate if AZA has the capacity for root-to-shoot movement in *Arabidopsis* seedlings.[2] Used in combination with GC/MS techniques, ^{2}H-AZA has significant potential to augment or replace the synthesized ^{14}C-AZA in movement/uptake assays. Similar to previous studies,[16,17] we propose that these methods can be adapted, optimized and applied to other plant species and other potentially mobile small molecules.

Institutional permissions

Handling of all the radioactive material and methods utilizing radioactive materials must be carried out in established areas following appropriate institutional training and permissions.

Growing *Arabidopsis* adult plants

Timing: 4–5 weeks

1. Surface-sterilize *Arabidopsis* seeds in a 1.5 mL tube by adding 1 mL 70% ethanol for 2 min followed by 5 min with 1 mL of 25% bleach supplemented with 0.1% Triton X-100 and three washes with sterile distilled water. Resuspend the seeds in 1 mL of a sterile solution of 0.1% agar (see recipe).
2. Using a plastic transfer pipette, sow 6 seeds in each 8x cells tray filled with soil mix and stratify at 4°C for 2–3 days for synchronized germination.
3. Transfer the trays to a growth chamber and grow the *Arabidopsis* plants for 4 weeks. Grow the plants under 12/12 h light/night conditions at 20°C, ~135–145 μmol s^{-1} m^{-2} of mix 50/50 of sodium and metal halide light at rosette level and 50%–70% relative humidity. Alternatively, 75–120 μmol s^{-1} m^{-2} of cool white fluorescent light can be used. Troubleshooting 1.

Growing *Arabidopsis* seedlings

Timing: 18 days

4. Prepare 60 mL ½ MS medium supplemented with 1% sucrose and 0.35% agarose (see recipe) in a small 80 mL beaker in sterile conditions. Place a sterile disk mesh with a diameter of ~60 mm diameter (or the diameter of the beaker used) with a pore size of 0.375 mm (Figure 3A).
5. Sterilize *Arabidopsis* seeds as described in **Step 1** (Growing Arabidopsis adult plants) and sow ~50 seeds with a P-1000 micropipette homogenously distributed on top of the mesh (Figure 3A). Cover the beaker with a layer of parafilm.

Note: The 0.35% ½ MS-agar medium allows for easy collection of the seedlings with entire roots. However, if some MS-agar medium remains stuck to the roots before transferring the mesh to AZA solution, the seedling roots can be washed in 5 mM MES buffer (pH 5.6).

6. Stratify, germinate and grow the seedlings for 12 days under 12/12 h light/night conditions at 20°C, ~100–120 μmol s^{-1} m^{-2} of cool white fluorescent light in a growth chamber or incubator with 50%–70% relative humidity. Troubleshooting 1.

Note: The main purpose of using the mesh is to provide a physical barrier for the easy isolation of root and shoot tissues. Thus, in practice, any suitable plastic mesh that has a pore size greater than the thickness of the *Arabidopsis* root can be utilized for this purpose.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ethanol	Decon Labs Inc.	cat #: 2701
Agar	Fisher Scientific	cat #: BP1423
Bleach	Clorox concentrated germicidal bleach	N/A
Triton X-100	Sigma-Aldrich	cat #: 9036-19-5
2-[N-morpholino]ethane-sulfonic acid (MES)	Sigma-Aldrich	cat #: M3671
Scintillation cocktail for ^{14}C	Sigma-Aldrich	cat #L8286
Synthesized ^{14}C-azelaic acid solution (^{14}C-AZA; specific activity 16 mCi/mmol).[13]	N/A[13]	N/A[13]
^{14}C-sucrose (sucrose [$^{14}C(U)$], 435 mCi $mmol^{-1}$)	PerkinElmer	cat #: NEC100
Azelaic acid (AZA; $C_9H_{16}O_4$, MW 188.22)	Sigma-Aldrich	cat #: 246379
Sucrose	Fisher Scientific	cat #: 11487417
Murashige & Skoog (MS) salts including B5 vitamins	Duchefa Biochemie	cat #: M0231
Phytoagar	Duchefa Biochemie	cat #: P1003
^{2}H-azelaic acid (^{2}H-AZA; MW 202.31)	Medical Isotopes Inc.	D4123
Experimental models: Organisms/strains		
Arabidopsis thaliana (L. Heyhn.) ecotype Col-0	Arabidopsis Biological Resource Centre	N/A
Other		
Soil mixture (Potting Supreme No 2)	John Innes	SKU 241
Tweezers and scissors	N/A	N/A
Soil trays (8x cells trays, 10 cm square cells and corresponding plastic lids)	Hummert International	cat #: 11-33480 and 11-33010
3 mL plastic transfer pipette	N/A	N/A
Delicate task wipers (Kimwipes)	Fisher Scientific	cat #: 06-666A
Cork borer (4 mm diameter)	N/A	N/A
Orbital rotator	N/A	N/A
80 mL glass beakers	N/A	N/A
6-multiwell plates	Corning, Costar	N/A
1.5 mL microfuge tubes	Midsci	cat #: AVSS1700
Cork borer 4 mm diameter	N/A	N/A
7 mL scintillation vials	Dot Scientific Inc.	cat #: #7600-B
Fume hood for radioactive safety use	N/A	N/A
Sterile 0.375 mm polyester plastic mesh discs	McMaster-Carr	cat #: 93185T22
50 × 9 mm round sterile Petri dishes	N/A	N/A
Magic tape	Scotch	N/A
Humid chamber	N/A	N/A
Nitrogen gas stream	N/A	N/A
Liquid scintillation counter	Beckman Coulter	LS 6000 IC

MATERIALS AND EQUIPMENT

½ MS-agar medium		
Reagent	Final concentration	Amount
Murashige & Skoog (MS) with B5 vitamins	0.22%	2.2 *g*
MES	0.05%	0.5 *g*
Sucrose	1%	10 *g*

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Phytoagar	0.35%	3.5 *g*
ddH_2O	N/A	Up to 1 L
Total	**N/A**	1 L

To prepare 1 L of MS-agar medium, dissolve 4.4 *g* MS with B5 vitamins, 0.5 *g* MES, and 10 *g* of sucrose in 500–700 mL of distilled water, adjust the pH to 5.6 with KOH, add 3.5 *g* Phytoagar and distilled water to 1 L, and autoclave at 121°C for 20 min. Store at 20°C–25°C for up to 1–2 month.

^{14}C-azelaic acid working solution

Reagent	Final concentration	Amount
Synthesized ^{14}C-azelaic acid solution	0.025 µCi/µL	N/A
MES	5 mM	N/A
triton X-100	0.1%	N/A
ddH_2O	N/A	Up to 20 µL
Total	**N/A**	**20 µL**

To prepare ^{14}C-azelaic acid working solution for one plant/leaf, evaporate 0.5 µCi of the synthesized ^{14}C-AZA solution (^{14}C-AZA; specific activity 16 mCi mmol^{-1})[13] to complete dryness under a stream of nitrogen gas (to remove the acetonitrile and formic acid solvent). Then, dissolve the dried precipitate with a solution of 5 mM MES (pH 5.6) supplemented with 0.1% triton X-100 detergent to a final concentration of 0.025 µCi/µL (1.56 mM ^{14}C-AZA). Use freshly prepared ^{14}C-azelaic acid working solution. Do not store ^{14}C-azelaic acid working solution.

^{14}C-sucrose working solution

Reagent	Final concentration	Amount
^{14}C-sucrose	0.025 µCi/µL	N/A
MES	5 mM	N/A
triton X-100	0.1%	N/A
ddH_2O	N/A	Up to 20 µL
Total	**N/A**	**20 µL**

To prepare ^{14}C-sucrose working solution, dilute the ^{14}C-sucrose stock solution (sucrose [^{14}C(U)], 435 mCi mmol^{-1}) in 5 mM MES (pH 5.6) supplemented with 0.1% triton X-100 detergent to a final concentration of 0.025 µCi/µL. Use freshly prepared ^{14}C-sucrose working solution. Do not store ^{14}C-sucrose working solution.

^{14}C-azelaic acid bathing medium

Reagent	Final concentration	Amount
Synthesized ^{14}C-azelaic acid solution	0.248×10^{-3} µCi/mL	N/A
Azelaic acid	1 mM	0.56 mg
MES	5 mM	N/A
ddH_2O	N/A	Up to 3 mL
Total	**N/A**	**3 mL**

For this, use the synthesized solution of ^{14}C-AZA to prepare a 15.5 µM ^{14}C-AZA (0.744×10^{-3} µCi) in 3 mL of 5 mM MES (pH 5.6) (without Triton X-100 supplementation), and add 0.56 mg of cold (unlabeled) AZA to a concentration of 1 mM. Use freshly prepared ^{14}C-azelaic acid working solution. Do not store ^{14}C-azelaic acid working solution.

^{14}C-sucrose bathing medium

Reagent	Final concentration	Amount
^{14}C-sucrose	0.09×10^{-3} µCi/mL	N/A
Sucrose	1 mM	0.1 mg
MES	5 mM	N/A
ddH_2O	N/A	Up to 3 mL
Total	**N/A**	**3 mL**

For this, dilute the stock solution to prepare a 0.2 µM ^{14}C-sucrose (0.268×10^{-3} µCi) in 3 mL of 5 mM MES (pH 5.6) (without Triton X-100 supplementation), and add 0.1 mg of cold (unlabeled) sucrose to a concentration of 1 mM. Use freshly prepared ^{14}C-sucrose working solution. Do not store ^{14}C-sucrose working solution.

^{2}H-azelaic acid working solution		
Reagent	Final concentration	Amount
^{2}H-azelaic acid	1 mM	2.02 mg
MES	5 mM	N/A
ddH_2O	N/A	Up to 10 mL
Total	**N/A**	**10 mL**

To prepare 1 mM ^{2}H-labeled azelaic acid working solution, dissolve 2.02 mg of ^{2}H-AZA in 10 mL solution of 5 mM MES buffer (pH 5.6). Use freshly prepared ^{2}H-labeled azelaic acid working solution. Do not store ^{2}H-labeled azelaic acid.

- 70% ethanol: 73.7 mL of 95% ethanol in 100 mL distilled water.

Store at ambient 20°C–25°C for up to 60 days.

- 25% bleach supplemented with 0.1% Triton X-100: 25 mL of bleach in 74.9 mL distilled water and 100 μL of Triton X-100.

Prepare freshly, store at ambient or 20°C–25°C for up to 24 h.

- 0.5 M MES stock solution (pH 5.7): 97.62 *g* in 1 L distilled water. Adjust the pH to 5.8 with KOH.

Filter sterilize or autoclave. Store at 4°C for up to 30 days.

- 5 mM MES (pH 5.7): Add 5 mL of 0.5 M MES stock solution and 995 mL of distilled water.

Filter sterilize or autoclave. Store at 4°C for up to 30 days.

- 5 mM MES (pH 5.7) supplemented with 0.1% triton X-100 detergent: Add 5 mL of 0.5 M MES stock solution and 994 mL of distilled water and 1 mL of 100% triton X-100.

Filter sterilize or autoclave. Store at 4°C for up to 30 days.

- 0.1% agar: 0.1 *g* agar in 100 mL sterile distilled water by autoclaving, swirl the solution while it cools.

Store unopened bottles at ambient or 20°C–25°C for up to 30 days. Once opened, use within 3- days.

⚠ CRITICAL: The concentration of synthesized ^{14}C-AZA may differ a little bit from its ordered concentration and needs to be accurately determined after synthesis.[13] Handling of all the radioactive material and methods utilizing radioactive materials must be carried out in established areas for radioactive handling safely following appropriate institutional training.

Alternatives: For growing *Arabidopsis* plants in soil, we typically utilized cell trays containing 10 cm square cells for individual plants. However, any sized cell trays, containers, or plant pots suitable for growing *Arabidopsis* could be utilized for these experiments. For growing *Arabidopsis* seedlings, we used $^1/_2$ MS salts with B5 vitamins supplemented with Phytoagar. Nevertheless, other media suitable for plant tissue culture (e.g., *Arabidopsis thaliana* salts [ATS]) or any other plant agar would be suitable. As a phloematic flux and movement/uptake control, we utilized radiolabeled sucrose (viz., sucrose [^{14}C(U)], 435 mCi mmol^{-1}, Perkin Elmer). However, depending on availability, different brands of uniform ^{14}C radiolabeled sucrose can be used. For AZA root movement assays, we utilized McMaster-Carr #93185T22, but any other autoclavable mesh with the same characteristics could be used. Suitable alternative sources for several common laboratory materials and reagents, including delicate task wipers, ethanol, domestic bleach, and sucrose, can be utilized.

STEP-BY-STEP METHOD DETAILS

In this protocol we outline the steps for analyzing the transport of ^{14}C-AZA from a single leaf to distal systemic tissues (steps 1 to 9), the uptake of ^{14}C-AZA into leaf discs (steps 10 to 16), and the uptake and movement of ^{2}H-AZA from roots to the aerial tissue in seedlings (steps 17 to 23).

Assessing ^{14}C-azelaic acid movement

Timing: 3 days

1. Prepare the working solution of ^{14}C-AZA for the Azelaic Acid Movement Assay (materials and equipment section).
2. Using a micropipette, apply a 20 μL drop of the ^{14}C-AZA working solution to the abaxial side of an *Arabidopsis* adult plant fully expanded leaf covering approx. 3–5 mm^2 of the tip area (Figure 1A).
3. Cover the tray with a plastic lid and tape it around the sides to ensure maintenance of humidity.
   a. Leave the tray for 24 h in a fume hood with the cool white fluorescent lights continuously on.
4. Using a small pair of scissors, carefully cut out the treated leaf.
   a. Collect the rest of the aerial tissue by cutting the entire rosette from the base (Figure 1B).
   b. Separately dry the leaf and the rest of the plant between several layers of Kimwipes absorbent paper for 24 h.
5. Cut the treated leaf and the rest of the dried rosette tissue into small pieces (approximately 5–7 mm^2 squares) and place the samples in scintillation vials (Figure 1C).
6. Add ~5 mL scintillation cocktail to cover all the tissue and wait for at least 1 h before radioactivity quantification (Figure 1D).
7. Quantify the radioactivity (CPM) in the vials with the treated leaf and the rest of the plant in a liquid scintillation counter (see quantification and statistical analysis section). Troubleshooting 2.
8. To estimate the ^{14}C-AZA moved to the roots from shoots, the total amount of radiolabel must be considered. For this, determine the CPM applied in the initial drop.
   a. Treat three leaves with ^{14}C-AZA to the abaxial side and immediately take the sample.
   b. Dry for 24 h and measure the CPM (see quantification and statistical analysis section).
9. For the phloematic flux and movement control, follow the same steps using the working solution of ^{14}C-sucrose (see materials and equipment).

⚠ CRITICAL: Use freshly prepared ^{14}C-AZA bathing medium using MES buffer that has not exceeded its shelf life. Make sure that the isolated plant tissue is completely dry for accurate detection of radioactivity using a scintillation counter. Treat a minimum of three different plants for each experiment/plant background to minimize experimental bias.

Optional: The radioactive leaves and plants can also be imaged using autoradiographic images to visualize the radioactive signal. The advantage of imaging is that it gives spatial information. It is also possible to quantify the level of radioactivity from the images using suitable imaging analysis software. However, in our experience, quantitative radioactivity measurements were found to be more sensitive and accurate using a scintillation counter.

Assessing ^{14}C-azelaic acid uptake

Timing: 2 days

10. Prepare the ^{14}C-azelaic acid bathing medium using the stock solution as described in materials and equipment section.
11. Collect 15 leaf discs (4 mm diameter) from at least 5 different *Arabidopsis* adult plant per replicate using a 4 mm cork borer (Figure 2A).

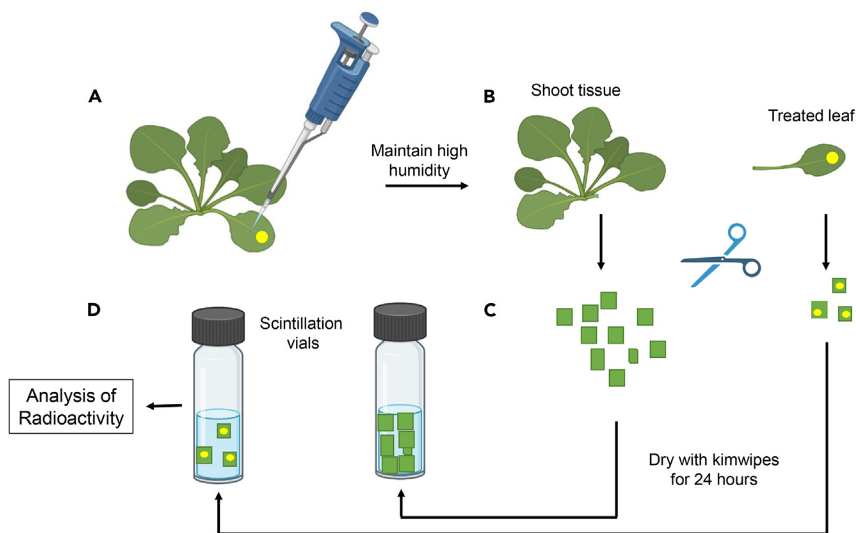

Figure 1. ^{14}C-azelaic acid movement assay

(A) A single leaf of a 4-week-old Arabidopsis plant is treated with radiolabeled AZA solution using a micropipette. Humidity is maintained for 24 h.

(B) Following this, the treated leaf and the rest of the untreated aerial tissue is isolated separately and dried for 24 h using Kimwipes or similar absorbent paper.

(C) The dried tissues are then cut into small pieces using scissors. The treated leaf and untreated shoot tissue pieces are then placed into separate liquid scintillation cocktail vials followed by quantification of radioactivity (D). With this method, radioactivity in the roots is calculated based on how much radioactivity is applied and recovered from the aerial tissue.

12. In a 6-well multi-well plate add 3 mL bathing medium (0.744×10^{-3} μCi of ^{14}C-AZA) and the 15 leaf discs per well (Figure 2B).
    a. Incubate for 3 h in an orbital rotator at minimum shaking speed (avoiding spilling the medium).
13. Wash at least three times with only 5 mM MES (pH 5.6) for a total of 1 h each incubating in an orbital rotator (Figure 2C).

Note: Use a pro-pipette to replace the ^{14}C-AZA and washing solutions while avoiding touching the leaf discs. Safely discard the medium and washing solutions.

14. After the last wash, dry the discs completely in the well for 24 h. Use Kimwipes absorbent paper to carefully help mop the excess of washing solution in the discs.
15. Collect the discs in a scintillation vial using tweezers and macerate them in scintillation cocktail for 1 h before radioactivity quantification (Figure 2D).
16. Quantify the radioactivity (CPM) in a liquid scintillation counter.

⚠ CRITICAL: Use freshly prepared ^{14}C-AZA bathing medium using MES buffer that has not exceeded its shelf life. Make sure that the leaf discs are completely dry for accurate detection of radioactivity using a scintillation counter.

Optional: The radioactive leaf discs can also be imaged using autoradiographic images to visualize the radioactive signal. It is also possible to quantify the level of radioactivity from the images using suitable imaging analysis software. However, in our experience, radioactivity measurements were found to be more sensitive and accurate using a scintillation counter.

Assessing ^{2}H-azelaic acid root uptake and movement

Timing: 2 days

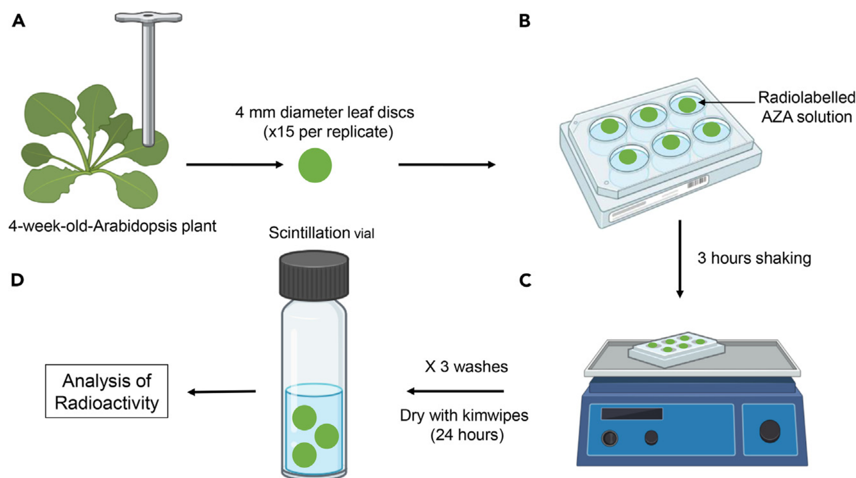

Figure 2. ^{14}C-azelaic acid uptake assay

(A) Approximately 15 4-mm-diameter leaf discs are isolated from the leaves of 4-week-old Arabidopsis plants per replicate.
(B) These discs are placed into 6-well-multiwell plates and bathed in AZA solution.
(C) Subsequently placed on a low-speed orbital shaker for 3 h. The leaf discs are then washed three times and dried for 24 h using Kimwipes or absorbent paper.
(D) Finally, the leaf discs are transferred to scintillation cocktail in scintillation vials followed by quantification of radioactivity.

17. Prepare a working solution of ^{2}H-labeled azelaic acid (^{2}H-AZA) (see materials and equipment section).
18. After 12 days post germination (see Section 1: Growing Arabidopsis seedlings; Figure 3A), carefully transfer the mesh together with the seedlings using tweezers to 9 mm plates containing 10 mL solution of 1 mM ^{2}H-AZA in 5 mM MES buffer (Figure 3B).
19. Tightly affix the mesh to the plate borders with tape permitting the roots, but not the aerial tissues to be in contact with the solution (Figure 3C).

Note: The mesh containing the *Arabidopsis* seedlings must be tightly affixed to the plate edge such that there is no "sagging" and only the *Arabidopsis* roots are permitted to come into contact with the labeled AZA solution. We used magic tape for this process, but in practice, any tape that is broadly water resistant can be utilized for this purpose.

20. Place the plates inside a humid chamber and after 24 h separately collect roots and aerial tissues cutting with scissors along the mesh (Figure 3D). Wash the roots in 5 mM MES buffer (pH 5.6) and carefully dry the excess of liquid with Kimwipes paper.
21. Weigh the roots and aerial tissues in 1.5 mL Eppendorf tubes before snap freezing the samples in liquid nitrogen for the metabolite extraction (Figure 3E).

Note: Each sample should be around 20 seedlings to get the minimum ~100 mg required per replicate.

    a. Repeat the experiment at least three independent times.
22. Extract the metabolites from the leaves.

Note: For a detailed protocol for the extraction see Mölders et al.[16]

23. Derivatize the extracts and subject them to GC/MS analysis to determine the concentration of labeled ^{2}H-azelaic acid and unlabeled azelaic acid in root and aerial tissue.[2,8,18] Troubleshooting 3.

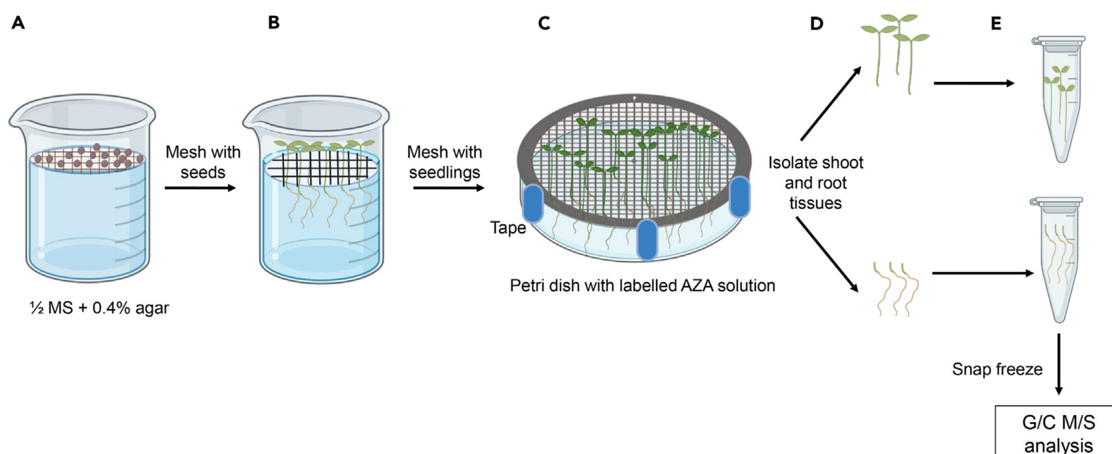

Figure 3. ^{2}H-azelaic acid root uptake and movement assay

(A and B) Arabidopsis seedlings are on sterile mesh discs grown in sterile beakers containing $^1/_2$ MS media supplemented with 0.3%–0.4% agar for 12 days, or until seedling roots are clearly elongated.

(C) The mesh containing the seedlings is then carefully transferred to a 9 mm Petri dish containing 10 mL of heavy isotope-labeled AZA solution. The mesh is tightly affixed to the sides of the Petri dish with tape, such that only the roots are allowed to contact the AZA solution.

(D and E) 24 h later, root and shoot tissue are isolated separately using clean scalpel blades. The root and shoot tissue are transferred to Eppendorf (or similar) tubes and snap frozen in liquid nitrogen. These samples can then be subjected to metabolite extraction and GC/MS analysis following derivatization.

Note: The process of metabolite extraction is likely to depend on the derivatization and GC/MS system used for AZA determination. Thus, the extraction method can be optimized and adapted according to the amount of AZA in each sample.

Alternatives: Labeled ^{2}H-azelaic acid and this method of extraction, derivatization and GC/MS analysis of labeled AZA, can also be used for AZA movement and uptake assays to avoid using radioactive materials and the scintillation counter.

⚠ CRITICAL: Make sure that the mesh containing the *Arabidopsis* seedlings does not come into contact with the AZA solution (step 19, Figure 4) to ascertain that only the root tissue has contact with the solution.

EXPECTED OUTCOMES

These protocols allow for the quantification of the transport of AZA in whole *Arabidopsis thaliana* adult plants from a single leaf (the site of application) to distal systemic tissues (aerial stem/leaves and roots) as well as its uptake into leaf discs,[1] and investigate if AZA has the capacity for root-to-shoot movement in *Arabidopsis* seedlings.[2] They permit the detection of low amounts of transported AZA (at least ~450 CPM - ~0.25% of the applied CPM -)[1] and, moreover, estimate the amount of AZA transported to the roots from aerial tissues. Furthermore, as noted above, another advantage of using radio-labeled AZA, is that it is possible to utilize autoradiography to check the distribution of radiolabeled AZA *in situ.*[1]

The methods can be used to compare AZA movement and transport between WT Arabidopsis plants and different systemic resistance-deficient mutants, overexpressors of presumed factors affecting this priming signal movement, or even to screen a collection of suspected systemic immunity mutants or ecotypes for a GWAS study, among others.

QUANTIFICATION AND STATISTICAL ANALYSIS

Repeat each experiment at least three independent times.

Figure 4. Representative images of the mesh setup to prevent aerial tissues contact with the ^{2}H-AZA solution in the root uptake and movement assay
(A) Mesh with *Arabidopsis* seedlings overlaying the Petri dish containing AZA solution.
(B) Bottom view of the Petri dish containing AZA solution overlaid by the mesh containing *Arabidopsis* seedlings.
(C) Washing of the mesh containing seedling roots with MES buffer after AZA treatment.

To estimate the ^{14}C-AZA moved to the total systemic tissues or to the roots from the treated leaf, it must be determined and calculated the following:

CPM_A: CPM applied; drop-treat with ^{14}C-AZA three leaves, immediately dried, measure and average the CPM.

CPM_L: measure the CPM in each of the treated leaf after 24 h.

CPM_S: measure the CPM in the above ground part of each of the plants (minus the cut treated leaf).

$CPM_T = CPM_A - CPM_L$: estimation of the CPM moved to the total systemic tissues.

$CPM_R = CPM_A - (CPM_L + CPM_S)$: estimation of the CPM moved to the roots.

To determine if differences between measurements are statistically significant, perform an analysis of variance (ANOVA) followed by a post hoc test such Tukey's HSD test or Newman-Keuls (SNK), among others. If only two conditions are compared, a Student's *t*-test can also be used.

LIMITATIONS

It is possible that some of the radio-labeled AZA may be converted into another molecule or AZA-derivative.[1] Scintillation counting only monitors the label and cannot give information about whether the radiolabel is still attached to the AZA; however, depending on the small molecule of interest, a simple extraction procedure and subsequent chromatography can be carried out to assess this.[19] On the other hand, the GC/MS analysis of heavy isotopes has the advantage that it can directly identify and quantify the labeled ^{2}H-AZA; however, specialized extraction methods and expertise is needed to do the derivatization and specialized equipment that not all labs have access to is required.

TROUBLESHOOTING

Problem 1

Arabidopsis seeds have a low germination rate leading to insufficient numbers of plants for experiments (Before you begin, steps 3 and 6).

Potential solution

Use fresh seed stocks for experiments, and stratify the seeds for 2–3 days prior to placing them in growth chambers.

Problem 2

Excessive variation in the amount of estimated labeled AZA in drop-treated leaves and AZA moved to systemic tissues due to a high degree of variation of humidity conditions (step 7).

Potential solution

Ensure that humidity is maintained uniformly by keeping the tray lid tightly closed.

Problem 3

Inaccurate estimation of root-to-shoot movement of labeled ^{2}H-AZA, due to seedling shoot tissues coming into contact with the medium (step 23).

Potential solution

Ascertain that the mesh is tightly affixed to the sides of the beaker to prevent 'sagging' in the middle. Using smaller beakers is recommended.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jean T. Greenberg (jgreenbe@uchicago.edu).

Technical contact

Further information about the technical specifics of performing the protocol should be directed to and will be fulfilled by the technical contacts, Suruchi Roychoudhry (s.roychoudhry@leeds.ac.uk) and Nicolás M. Cecchini (ncecchini@unc.edu.ar).

Materials availability

This study did not generate new unique reagents. Non-commercial material (^{14}C-AZA) described in this study can be synthesized according to an established protocol.[13]

Data and code availability

This study did not generate/analyze datasets.

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AUTHOR CONTRIBUTIONS

S.R., J.T.G., and N.M.C. co-wrote and edited the manuscript. S.R. and N.M.C. curated the tables and figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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