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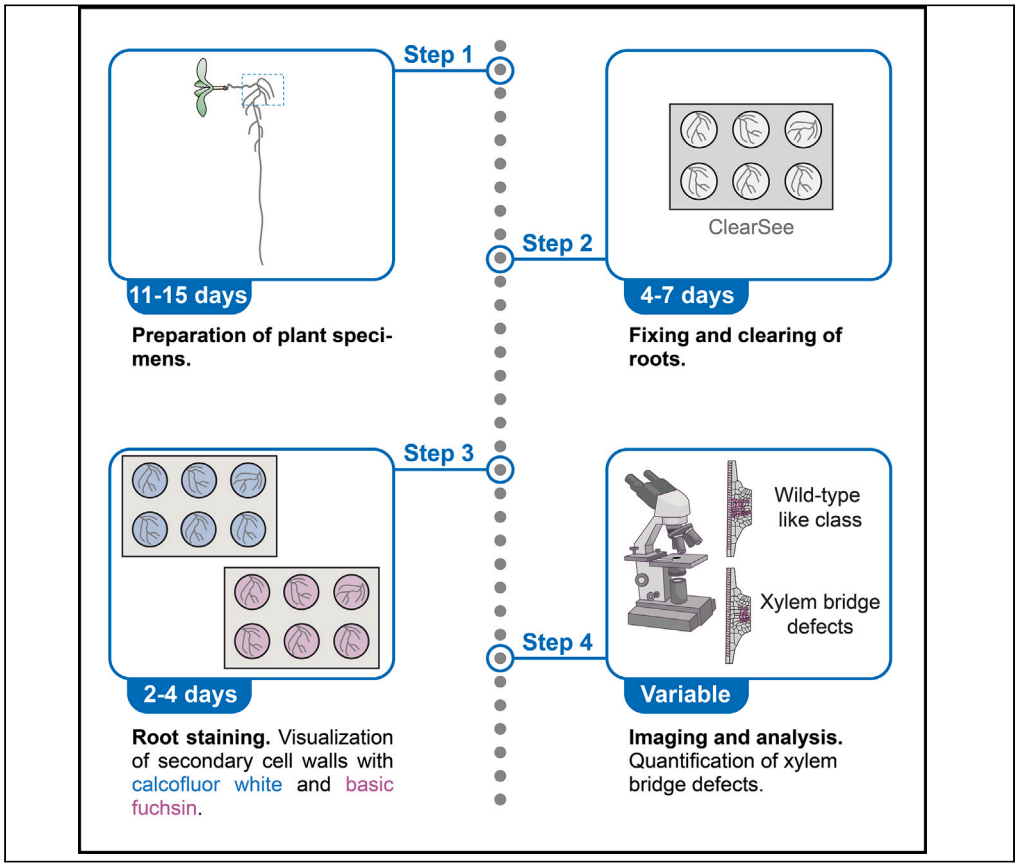
Quantification of xylem connection defects during lateral root development in Arabidopsis



The cellular and molecular mechanisms underlying the establishment of vascular connections between primary and lateral roots have recently gained significant attention. Here, I present a protocol to visualize and quantify xylem connection defects during lateral root development. I describe steps for employing stains to infer whether the defects observed in xylem bridges are associated with alterations in the xylem differentiation program, including programmed cell death and secondary cell wall deposition.

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

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Highlights

Visualization of xylem connections between primary and lateral roots

Quantitative evaluation of xylem connection defects during LR development

Protocol applies to a variety of plant species

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Quantification of xylem connection defects during lateral root development in Arabidopsis

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SUMMARY

The cellular and molecular mechanisms underlying the establishment of vascular connections between primary and lateral roots have recently gained significant attention. Here, I present a protocol to visualize and quantify xylem connection defects during lateral root development. I describe steps for employing stains to infer whether the defects observed in xylem bridges are associated with alterations in the xylem differentiation program, including programmed cell death and secondary cell wall deposition.

For complete details on the use and execution of this protocol, please refer to Blanco-Touriñán et al. (2023) and Ursache et al. (2018).[1,2]

BEFORE YOU BEGIN

The protocol below describes the specific steps and experimental conditions to visualize the xylem bridges established between the xylem strands of the primary root and of the lateral roots in *Arabidopsis thaliana* (Arabidopsis) and *Solanum lycopersicum* (tomato).[1,3] Additionally, I present the criterion to classify xylem connection defects during lateral root development. This criterion was developed using the HD-ZIP III quadruple mutant *phb-13 phv-11 cna-2 athb8-11* as a model due to its severe defects in xylem bridge formation.[1] Finally, I include a step-by-step protocol for determining if xylem bridge defects are caused by altered differentiation processes.

Preparation of $^1/_2$ MS plates and surface sterilization of Arabidopsis and tomato seeds

Timing: 2–4 h

1. Prepare $^1/_2$ MS agar plates for Arabidopsis and tomato growth:
   a. Dissolve 2.2 *g* of Murashige & Skoog (MS) medium and 3 *g* of sucrose in 700–800 mL of distilled water (ddH_2O). Adjust pH to 5.7 using 1 M KOH. Add 8 *g* of agar; fill up to 1 L with ddH_2O and autoclave.
   b. In a sterile hood, pour 50–60 mL of $^1/_2$ MS agar into 120 × 120 × 15 mm square plates and let the medium solidify.
   c. Close the plates. Plates can be stored for 1–2 weeks at 4°C.
2. Surface sterilization of Arabidopsis seeds:
   a. Put the required number of seeds in a 1.5 mL tube and add 1 mL of 70% ethanol (with 0.01% Triton X-100). Rotate the tubes for 10 min.
   b. Spin briefly, remove the 70% ethanol, and add 95% ethanol. Rotate the tubes for 5 min.
   c. Spin briefly and remove the ethanol in a sterile hood. Wash with 1 mL of sterile water three times, for 1 min each.

Reagent	Final concentration	Amount
Murashige & Skoog medium (+vitamins and MES buffer)	2.2 g /L	2.2 g
Sucrose	3 g /L	3 g
Agar	8 g /L	8 g
ddH_2O	N/A	fill up to 1 L

d. Add water and stratify seeds at 4°C and in darkness for 2–4 days to synchronize germination.

Note: Avoid extended incubation of the seeds with ethanol due its negative impact on seed germination, and ensure that the seeds are not forming clumps. Other protocols used to sterilize Arabidopsis seeds (e.g., chlorine gas sterilization) are also compatible with this protocol.

3. Surface sterilization of tomato seeds.
   a. Surface-sterilize the required number of tomato seeds in a 50-mL centrifuge tube by immersing them in 60% bleach and a few drops of Tween 20 detergent for 20–30 min with gently swirling.
   b. In a sterile hood, remove the seed sterilization solution and wash with sterile water four times, for 10 min each.

Note: Tomato seeds do not require stratification.

Preparation of solutions for clearing and staining

Timing: 2–3 h

4. 10x Phosphate Buffered saline (PBS) solution.
   a. Dissolve all the reagents in 700–800 mL ddH_2O, adjust pH to 7.2–7.4, and fill with ddH_2O up to 1 L.

Reagent	Final concentration	Amount
$Na_2HPO_4 \cdot 2H_2O$	70 mM	12,46 g
$NaH_2PO_4 \cdot 2H_2O$	30 mM	4,68 g
KCl	27 mM	2 g
NaCl	1.37 M	80 g

   b. Autoclave.

Note: To prepare 1x PBS, dilute 1 volume of 10x PBS in 9 volumes of ddH_2O. Store at room temperature (20°C–22°C).

5. Paraformaldehyde (PFA) 4% solution.
   a. Weigh 4 g of PFA powder and dissolve in 70–80 mL of 1x PBS in a fume hood.
   b. Transfer the mixture to a stirrer and heat gently (max. 60°C) while stirring.
   c. Gradually adjust the pH by adding drops of 1 N NaOH with a pipette until the solution becomes clear.
   d. Adjust the pH with HCl to approximately 6.9. Fill up to 100 mL with 1x PBS.
   e. Cool down the 4% PFA solution.

Note: It is not required to use fresh PFA, so once prepared, it can be aliquoted and stored at −20°C for several months.

⚠ CRITICAL: PFA severely irritates the eyes, skin and respiratory tract. Use a mask and gloves and work in a fume hood.

6. ClearSee.[4]

Reagent	Final concentration	Amount
Urea	25% (w/v)	250 *g*
Xylitol	10% (w/v)	100 *g*
Sodium deoxycholate	15% (w/v)	150 *g*
ddH_2O	N/A	fill up to 1 L
Total	N/A	1 L

Note: Mix the solution on a magnetic stirrer for at least 1 hour to ensure complete dissolution. ClearSee can be stored at room temperature (20°C–22°C) for several months.

7. Basic Fuchsin (0.2%) and Calcofluor white (0.1%) stainings.
   a. Basic Fuchsin solution: Weigh Basic Fuchsin powder and dissolve in ClearSee by mixing on the magnetic stirrer.
   b. Calcofluor white solution: Dilute the stock solution (25%) in ClearSee to a final concentration of 0.1% (v/v).
   c. Mix the solutions in a magnetic stirrer before each use.

Note: Store the solutions protected from the light and at room temperature (20°C–22°C; Calcofluor white) or at four degrees (Basic Fuchsin). Both solutions can be stored for several weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige and Skoog medium (including vitamins and MES buffer)	Duchefa Biochemie	MO255.0050
Sucrose	Merck	CAS-No: 57-50-1
Phytoagar	Duchefa Biochemie	CAS-No: 9002-18-0
Corning Gosselin square Petri dish 120 × 15 mm	Corning	CAS-No: BP124-05
Absolute alcohol	Merck	CAS-No: 64-17-5
Triton X-100	Merck	CAS-No: 9036-19-5
Tween 20	Merck	CAS-No: 9005-64-5
Bleach (sodium hypochlorite)		CAS-No: 231-668-3
50 mL centrifuge tubes	Merck	CAS-No: CLS430828
3M Micropore surgical tape	3M	CAS-No: 1530-1
$Na_2HPO_4 \cdot 2H_2O$	Merck	CAS-No: 10028-24-7
$NaH_2PO_4 \cdot 2H_2O$	Merck	CAS-No: 13472-35-0
KCl	PanReac AppliChem	CAS-No: 7447-40-7
NaCl	Sigma-Aldrich	CAS-No: 7647-14-5
Paraformaldehyde	Sigma-Aldrich	CAS-No: 30525-89-4
Xylitol	Sigma-Aldrich	CAS-No: 87-99-0
Sodium deoxycholate	Sigma-Aldrich	CAS-No: 302-95-4
Urea	Sigma-Aldrich	CAS-No: 57-13-6
Fluorescent Brightener 28 disodium salt solution (calcofluor)	Sigma-Aldrich	CAS-No: 4193-55-9
Basic Fuchsin	Sigma-Aldrich	CAS-No: 58969-01-0
DAPI	Sigma-Aldrich	CAS-No: 28718-90-3

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Solanum lycopersicum cv. M82		N/A
Arabidopsis thaliana: Columbia erecta-2 (Col er-2)	Widely distributed	N/A
Arabidopsis thaliana: *phb-13 phv-11 cna-2 athb8-11*	Refer to Prigge et al.[5]	N/A
Arabidopsis thaliana: *pMYB46::GFP*	Refer to Lee et al.[6]	N/A
Software and algorithms		
GraphPad Prism 9.0	GraphPad Software	https://www.graphpad.com
Fiji	N/A	https://imagej.nih.gov/ij/
Other		
Leica Stellaris 8		N/A
Zeiss LSM 880		N/A

Note: Alternative reagents and tools or equipment could be used but were not tested as part of this protocol.

STEP-BY-STEP METHOD DETAILS

Preparation of plant specimens

Timing: 11–15 days

1. Stratify Arabidopsis seeds:
   a. As mentioned in the "before you begin" section, stratify Arabidopsis seeds at 4°C in darkness for 2–4 days after sterilization.
2. Sowing the seeds:
   a. In a sterile hood, use a 1 mL pipette tip to carefully release the seeds drop by drop onto the plate.
   b. Close the lid and seal the plate with Micropore tape.
   c. Place the plates vertically in the plant growth chamber under continuous light (50–60 $\mu mol\ m^{-2} \cdot s^{-1}$) at 22°C.

Note: Place the seeds in the plates at a sufficient distance (>0.5 cm) to minimize excessive contact between lateral roots. Troubleshooting 2.

3. Growth conditions (Figure 1):
   a. OPTION A (standard vertical growth): Let the seedlings grow vertically for 9–11 days.
   b. OPTION B (gravitropic growth): Let the seedlings grow vertically for 3–4 days. Then, rotate 90 degrees the plates and grow the seedlings for 6–7 additional days before proceeding with the fixation/clearing (see Figure 1). Troubleshooting 5.

Note: Gravitropic stimulation of lateral root formation facilitates the synchronization of their development, ensuring that they are all at the same stage of development. While this treatment is not essential, as comparable results can be obtained through standard seedling growth (see Figure 2), it is recommended to compare the outcomes under both growth conditions for each mutant of interest to ensure reproducibility.

Fixing and clearing of roots

Timing: 2–4 h + 4–7 days clearing

The following steps enable the visualization and quantification of defects in the formation of the xylem bridges. By employing specific stains, we can also assess whether these anomalies arise from

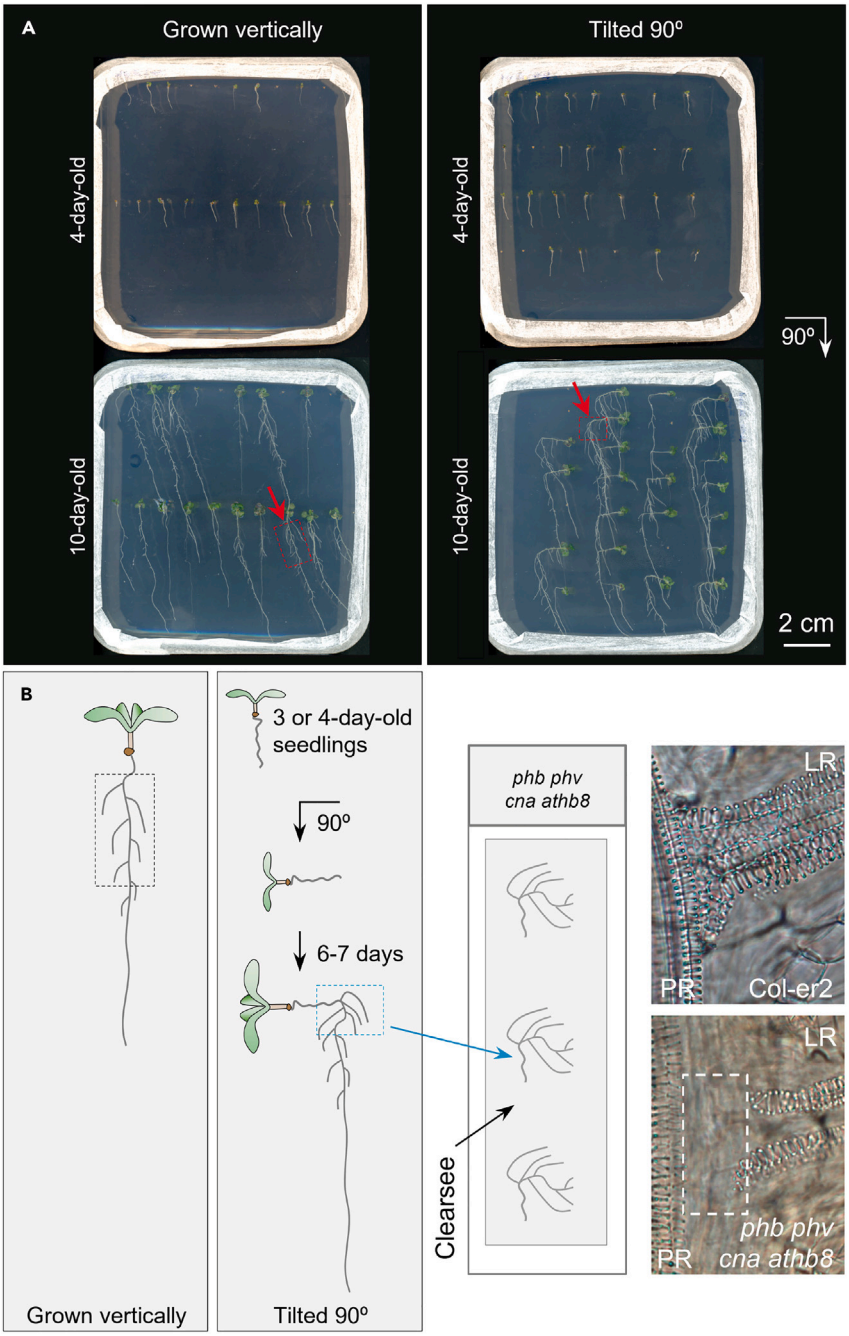

Figure 1. Preparation of samples to examine defects in xylem bridge formation during lateral root development

(A) The analysis of xylem connection phenotypes can be conducted using 9 to 11-day-old seedlings grown vertically on agar plates or seedlings gravistimulated by 90° rotation to synchronize lateral root formation. The displayed seedlings belong to the *phb phv cna athb8* mutant. Red arrows and dashed squares indicate the regions to be analyzed. Scale bar, 2 cm.

(B) Scheme of the setup showed in (A). Root sections are directly incubated in ClearSee without prior fixation. Bright-field images of representative images obtained in this way are shown. The dashed square indicates the absence of xylem bridge cells. PR, primary root; LR, lateral root.

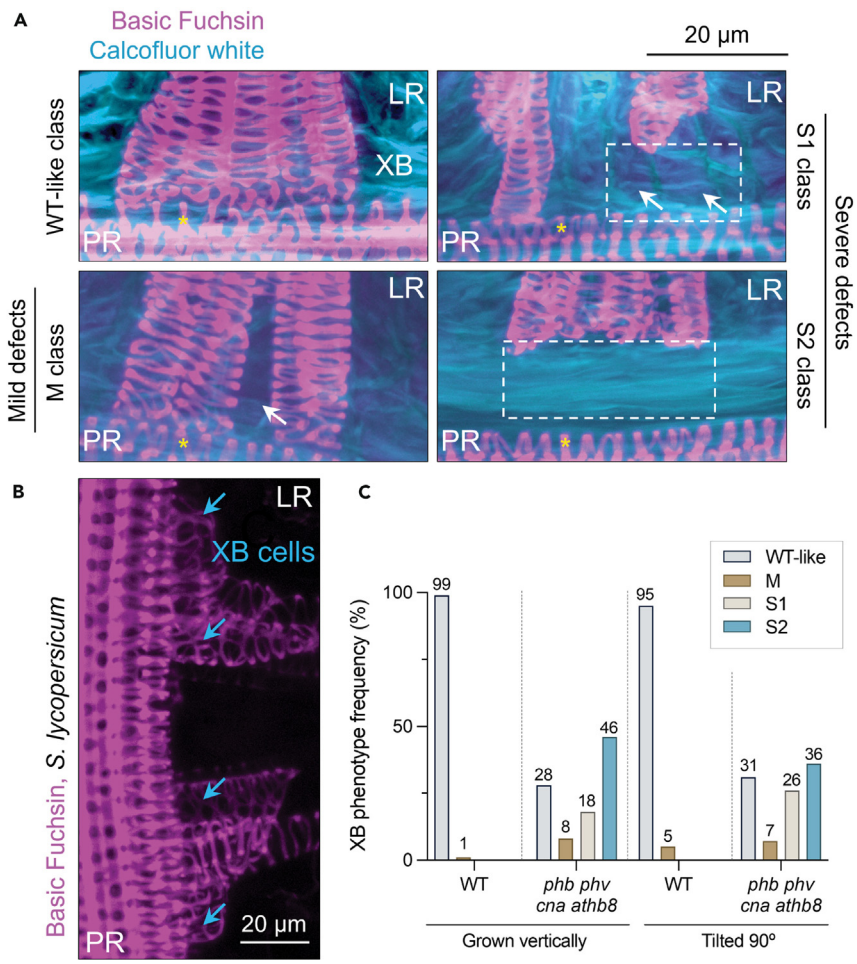

Figure 2. Characterization of xylem bridge defects during lateral root development

(A) Z-stack confocal images showing the xylem bridge (XB) phenotypes found in 9- to 11-day-old seedlings grown vertically on agar plates (see Figure 1A). Roots were stained with Calcofluor white (cyan) and Basic Fuchsin (magenta). Arrows and dashed squares indicate the absence of xylem bridge cells. Yellow asterisks mark the primary root protoxylem strand that connects to the lateral root xylem. PR, primary root; LR, lateral root.

(B) Z-stack confocal image showing the morphology of xylem bridge cells in a *S. lycopersicum* lateral root. The seedling was stained with Basic Fuchsin (magenta). The blue arrows point to different xylem bridge cells.

(C) The xylem bridge defects in the *phb phv cna athb8* quadruple mutant are observed in seedlings grown vertically or gravistimulated by 90° rotation. The graph was generated with GraphPad Prism 9.0. n = 100 for each genotype and condition.

impaired differentiation programs. These steps allow visualizing the activity of fluorescent reporters in xylem bridge cells as well.[1]

4. Excise the root section where gravitropism induced the formation of lateral roots. If the plates were not rotated, proceed to cut the section of the root with emerged lateral roots (see Figure 1).

   Note: When using seedlings that have not been gravistimulated, try to include lateral roots of a similar developmental stage in the analysis, as in very short lateral roots, only some of the xylem bridge cells have been formed.

5. Fix the samples with 4% PFA in a 6- or 12-well plate for 30 min – 1 h at room temperature (20°C–22°C) with gentle agitation. Add enough PFA solution to ensure that all the roots are in contact with the solution.

Note: Vacuum is not required.

6. Wash twice with 1x PBS for 1–2 min with gentle agitation.
7. Remove the 1x PBS and add ClearSee solution. Incubate the plates with gentle agitation. Troubleshooting 3.

⚠ CRITICAL: Clearing takes approximately 4–7 days in Arabidopsis samples, but from three weeks to one month in tomato roots. It is essential to refresh the ClearSee solution every other day to achieve optimal visualization of the xylem bridge.

Note: Although recommended, it is not essential to fix the samples for the assessment of defects in the formation of xylem bridges. The images shown in Figure 1 were obtained from samples not subjected to fixation in 4% PFA. To acquire these images, excised root tissues were directly placed onto slides with ClearSee or chloral hydrate solution and incubated at room temperature (20°C–22°C) for 1 or 2 days prior to imaging, as illustrated in Figure 1. Do not exceed 4–5 days incubation since ClearSee might begin to crystallize in the slides. Troubleshooting 1.

Root staining

Timing: 2–4 days

Following differentiation, xylem cells undergo programmed cell death and initiate the deposition of secondary cell walls composed of cellulose and impregnated with lignin.[7] These processes can be visualized by staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; for nuclei), Basic Fuchsin (for lignin) and Calcofluor white (for cellulose).[2] By using these different stains, we can determine if defects in xylem bridge formation are a consequence of defects in the cell death program or problems in the deposition of secondary cell walls.[1,8]

8. Stain the roots with 0.2% Basic Fuchsin (in ClearSee) for 9–12 h in Arabidopsis or 1–2 days in tomato, with gentle agitation. Cover the samples with aluminum foil.
9. Remove the Fuchsin solution and wash two times with 1x PBS (10–15 min each).
10. Wash for 30 min – 1 h in ClearSee with gentle agitation.
11. Refresh the ClearSee solution and incubate the samples for 1–2 days with gentle agitation.
12. Stain with 0.1% Calcofluor white (in ClearSee) for 1 h. Cover the samples with aluminum foil.
13. Remove the Calcofluor white solution and rinse two times with 1x PBS (10–15 min each).

Note: Alternatively, the Calcofluor white staining (step 12) can be replaced by DAPI staining to visualize nuclei instead of cellulose deposition. Use a solution of 20 μg/mL DAPI dissolved in water and 0.5% (v/v) Triton X-100 and incubate for 30 min – 1 h. Then, continue on step 13.

14. Wash at least for 1–2 h in ClearSee.

Confocal imaging

Timing: 10–20 min per sample if a z stack is performed

15. For confocal imaging, mount the seedlings on slides with ClearSee solution and delicately place a coverslip over the seedlings.
16. Acquire images on a Leica Stellaris 8 or Zeiss LSM880 confocal with a 63x objective. Calcofluor white or DAPI (405 nm excitation, 425–475 nm detection), Basic Fuchsin (561 nm excitation, 600–650 nm detection), green fluorescent proteins (488 nm excitation, 500–550 nm). Troubleshooting 4.

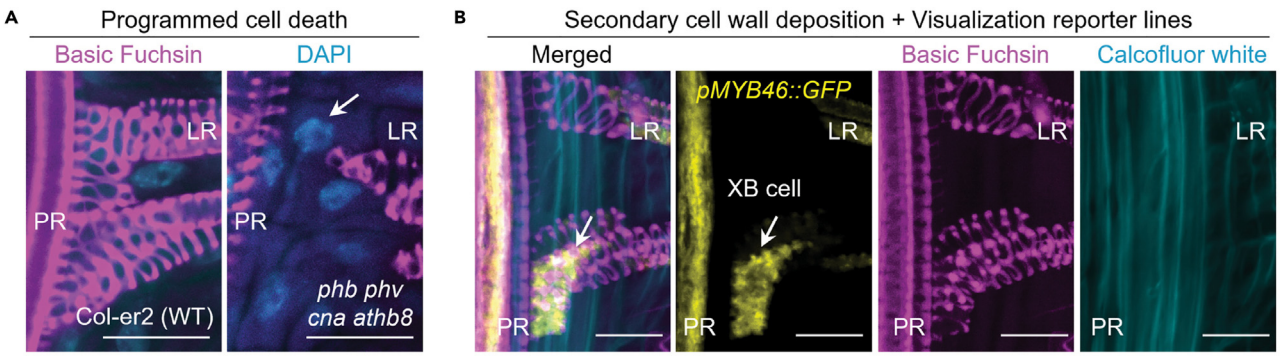

Figure 3. Determining if xylem bridge defects are caused by altered differentiation processes

(A) Analysis of nuclear persistence in roots stained with DAPI (cyan) and Fuchsin (magenta). The impairment of enucleation in cells in the xylem bridge position in the *phb phv cna athb8* quadruple mutant is shown.

(B) Cellulose and lignin depositions in roots stained with Calcofluor white (cyan) and Fuchsin (magenta). The compatibility of the protocol with the visualization of reporter lines (in this case, *pMYB46::GFP*[6]) is shown. The absence of cellulose in xylem bridge cells indicates that these cells are close to being fully differentiated. PR, primary root; LR, lateral root; XB cell, xylem bridge cell. The arrow in (A) points to one nucleus in the XB position, while in (B) marks the expression of *MYB46::GFP*. Scale bar, 20 μm.

Note: To obtain high-quality 3D reconstructions of xylem bridges in *A. thaliana* and *S. lycopersicum*, the z-stacks can be scanned with a step size of 0.36–0.42 μm (AU = 1.00). In the Leica Stellaris 8 confocal, the scan parameters include 1024 × 1024 8-bit resolution, ScanSpeed 200 Hz, LineAverage 1, FrameAverage 2, Bidirectional scanning, and pixel dwell time of 3.84 μs.

Image analysis and phenotypic characterization

Timing: variable, depending on the number of samples

17. Analyze the images and/or generate 3D renderings from the z-stacks using Fiji or Imaris Viewer. Example of images analyzed with Fiji is provided in the "expected outcomes" section (Figures 2 and 3).

Note: To obtain a 3D reconstruction in Fiji, you can use Image > Stacks > 3D project and choose the projection method (such as brightest point), then click interpolate. Alternatively, you can use Image > Stacks > Z project and choose the projection type (such as max intensity). Once you have selected the section of interest with the rectangle tool, crop it by going to Image > Crop. You can save the image in Tiff, PNG or JPEG format by going to File > Save as. When working with images performed with multiple stains (or reporters), a merged image can be created by going to Image > Color > Make Composite. Add the scale bar in Analyze > Tools > Scale bar.

Note: To characterize xylem bridge defects, you can omit the fixing and staining procedures mentioned in the "fixing and clearing of roots" section. Instead, capture bright-field images after clearing and use the criterion shown in Figure 2A. Mild defects (M class) are considered when only one xylem strand of the lateral root is not connected to the primary root xylem (i.e., most likely only one xylem bridge cell is missing). Severe defects are considered when several (S1 class) or all (S2 class) the lateral root xylem strands are not connected to the protoxylem strand of the primary root. To ensure reproducibility, it is recommended to analyze no less than 50–100 lateral roots.

EXPECTED OUTCOMES

Figure 1B presents representative images of xylem bridges obtained without fixing or staining, which are of sufficient quality to quantify the xylem bridge defects during lateral root development.

Representative images of the morphology of the xylem bridge and defects in the xylem connection during lateral root development are shown in Figures 2 and 3. It is important to note that the roots included in Figures 2 and 3 were fixed with 4% PFA and stained with Basic Fuchsin and Calcofluor white (or DAPI), as described above. In Figure 2A (S2 class), the absence of lignin and cellulose following a xylem-like cell pattern suggests that secondary cell wall deposition is affected, which is not the case in a WT-like class (Figure 2A). Figure 3A shows that the cell death program is altered in the *phb-13 phv-11 cna-2 athb8-11* quadruple mutant, as evidenced by the observation of nuclei in the xylem bridge position, which is not observed in differentiated xylem bridge cells in WT lateral roots. In addition to the xylem bridge morphology, this protocol allows the simultaneous visualization of reporter lines, as shown in Figure 3B.

LIMITATIONS

The primary challenge in visualizing xylem bridges using this protocol lies in the clearing process of the roots. It is likely that, when working with plant species with bigger and more opaque roots, some adjustments to the clearing steps will be necessary for optimization.

TROUBLESHOOTING

Problem 1

Impossibility of quantifying xylem bridge phenotypes due to (i) evaporation or (ii) crystallization of the ClearSee solution in the slides.

Potential solution

- To prevent ClearSee solution crystallization, reduce the incubation time for root clearing.
- To avoid the mounting medium from evaporating, it is recommended to conduct the incubation in a wet humidity chamber, such as a sealable plastic box lined with water-saturated tissue paper.

Problem 2

No lateral roots emergence in the loss-of-function mutant of interest.

Potential solution

- Treat with 1-naphthylacetic acid (NAA)[9] to induce the formation of lateral roots.
- Use knock-down instead of knock-out mutant plants.

Problem 3

While the ClearSee solution is effective for visualizing xylem bridges in Arabidopsis and tomato, it may not provide adequate results for other plant species.

Potential solution

- In such cases, it might be necessary to apply vacuum to ensure proper clearing of deep tissues, or to extend incubation times to clear more opaque tissues.
- Alternatively, the use of ClearSeeAlpha[10] may improve xylem bridge visualization, as it appears to be effective across a broader range of plant species and tissues.

Problem 4

Low or absence of signal of the reporter gene in the confocal.

Potential solution

- Performing immunostaining of these specific transgenic lines may enhance the signal due to its higher sensitivity.

Problem 5
Contamination on plates.

Potential solution

- It might be necessary to sterilize seeds for a longer duration, prepare new media and/or re-sterilize all the material required to work in the sterile hood.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Noel Blanco-Touriñán (noel.blancotourinan@unil.ch).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
N.B.-T. performed the experiments and conceptualized and wrote the article.

DECLARATION OF INTERESTS
The author declares no competing interests.

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